Tissue-specific Alternative Splicing of Ascidian Troponin I Isoforms

REDESIGN OF A PROTEIN ISOFORM-GENERATING MECHANISM DURING CHORDATE EVOLUTION

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In vertebrates, troponin I (TnI) exists as shorter and longer isoforms encoded by distinct genes expressed in skeletal and cardiac muscle, respectively. We report that the protochordate ascidian Ciona intestinalis expresses a homologous set of shorter and longer TnI isoforms in body wall muscle and heart, respectively. The heart-specific segment of the ascidian longer TnI isoform shares several sequence features with vertebrate cardiac TnI but lacks the protein kinase A phosphorylation sites implicated in sympathetic control of cardiac function. In contrast with vertebrates, the ascidian longer and shorter TnI isoforms are produced from a single gene by tissue-specific alternative RNA splicing; remarkably, the molecular mechanism of TnI isoform generation has been entirely reworked during ascidian/vertebrate evolution. Because alternative splicing is the more probable chordate ancestral condition, the long/cardiac versus short/somatic muscle pattern of TnI isoforms likely existed before the occurrence of the gene duplication events that created the vertebrate TnI gene family. Thus, gene duplication was apparently not the primary engine of isoform diversity in this aspect of TnI gene family evolution; rather, it simply provided an alternative (transcriptional) means of maintaining a previously established system of isoform diversity and tissue specificity based on alternative RNA splicing.

Many organismal functions are carried out by families of related protein isoforms. The most important molecular genetic mechanisms for generating protein isoform variants are alternative RNA splicing from a single gene (1) and transcription from distinct genes in a multigene family (2, 3). TnI is a subunit of the troponin complex which, along with tropomyosin, constitutes the Ca\(^{2+}\)-sensitive trigger mechanism that controls contraction in vertebrate sarcomeric muscles (4). Vertebrates express three distinct TnI isoforms from a family of three unlinked genes (5, 6) that are differentially expressed through tissue-specific transcriptional mechanisms (7–10) and are not known to undergo alternative RNA splicing. The TnIfast and TnIslow isoforms are expressed in skeletal muscle, in fast and slow fibers, respectively (11), and also, transiently, in the developing heart (12–15). The TnIcardiac gene is expressed exclusively in the heart (15–17). The three TnI isoforms align well, except that whereas the skeletal muscle TnI isoforms are -180–185 residues in length, TnIcardiac is 208–244 residues long in various vertebrate species due to a 25–55 residue insertion very near the N terminus (5, 16–18). In the mouse TnIcardiac gene, the extra 29-residue near N-terminal sequence is encoded by an internal exon that has no counterpart in the TnIfast and TnIslow genes (9). Features of the TnIcardiac insertion sequence have been conserved across vertebrate species (16, 18), suggesting functional importance perhaps related to the interaction of the TnI N terminus with the Ca\(^{2+}\)-binding troponin subunit TnC (4, 5). ß-Adrenergic stimulation of the mammalian heart leads to protein kinase A (PKA) phosphorylation of conserved adjacent Ser residues in the insertion sequence, and this is associated with a reduced Ca\(^{2+}\) sensitivity of the contractile apparatus, which may facilitate relaxation in the epinephrine-stimulated heart (4, 19).

Ascidians are a protochordate group that may resemble vertebrate ancestors (20). The body plan of the swimming larva shows numerous points of homology with the vertebrate body plan (21), although the postmetamorphic adult shows little similarity. Three major muscle types are known: sarcomeric muscle cells flanking the larval tail, sarcomeric muscle cells in the heart, and the nonsarcomeric (but troponin-regulated) muscle of the adult body wall (22, 23). Despite outward differences, adult body wall muscle has important molecular similarities to vertebrate skeletal muscle (22–24), including expression of MyoD-like transcription factors (25, 26).

We report studies of ascidian TnI isoforms that show a marked overall parallel with vertebrates; a 182-residue TnI similar to vertebrate skeletal muscle TnI isoforms is expressed in ascidian body wall muscle, and a longer TnI isoform with a near N-terminal insertion resembling that of vertebrate TnIcardiac but lacking the PKA phosphorylation sites is expressed in the heart. However, whereas vertebrates produce the long (heart) and short (somatic muscle) TnI isoforms from specialized genes, we found that ascidians produce both isoforms from a single gene by a tissue-specific alternative splicing mechanism. Thus, the molecular mechanism generating TnI isoform diversity has been entirely reworked in one of these organismal lineages while maintaining intact the structural relationships between the isoforms and their tissue-specific expression patterns. We present a likely scenario for these evolutionary events and consider the functional implications of the similarities and differences between ascidian and vertebrate heart TnI isoforms.

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† The abbreviations used are: Tn, troponin I; PKA, protein kinase A; bp, base pair(s); PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Animals and cDNA Cloning—Animal collection/maintenance, RNA/DNA isolation, and blot hybridization were done as described previously (24). The *Ciona* TnI cDNA clone pCpT2 was isolated from an oligo(dT)-primed Agt10 cDNA library of adult body wall muscle poly(A) + mRNA (24) by low stringency plaque hybridization with mouse TnIfast cDNA clone cM113 (11). The EcoRI insert was subcloned into pBlue- script KS (+) (Stratagene) and sequenced throughout on both strands using Sequenase from U. S. Biochemical Corp./Amersham Life Science, Inc. pCpT2 contained 62 bp of 5'-untranslated mRNA sequence, an open reading frame encoding a 182-amino acid residue TnI isoform, and 189 bp of 3'-untranslated sequence, including the stop codon.

PCR-based Methods—PCR amplification of heart and body wall muscle TnI mRNAs and TnI genomic (sperm) DNA sequences was based on rightward (5'-GCTTAGCAACTGCAAAAAATAC-3') and leftward (5'-GC- AACATTGCAAAAGAAAAATAC-3') primers corresponding to 5'- and 3'-untranslated mRNA sequences (the leftward primer also served as the reverse transcription primer in reverse transcription-PCR). The resulting 874-bp heart TnI mRNA product was sequenced on both strands by cycle sequencing (Cortec, Queen's University, Kingston, Ontario). The 2.1-kb PCR product amplified from genomic DNA was cloned using the pCScript Amp SK(+) cloning system (Stratagene) and sequenced on both strands by supercoil sequencing (Sheldon Biotechnology Center, McGill University). The CLONTECH 5'-Amplifier rapid amplification of cDNA ends kit was used to generate additional mRNA 5'-sequence information using 5'-TCCGACGAGATCCTAGTA-3' (complementary to codons 110–115 in Fig. 2) as the reverse transcriptase primer and 5'-AGTGGATCCGCTGAGTGGCTCAAGTCGT-3' (complementary to codons 93–101 with an added BamHI site) as the gene-specific amplification primer. By leftward cycle sequencing of 2'-rapid amplification of cDNA ends products, heart and body wall TnI mRNAs were found to contain an additional and identical 18-bp mRNA sequence 5' to that shown in Fig. 2.

RESULTS

Ascidian Heart and Body Wall Muscle TnI Isoforms—We isolated TnI cDNA clone pCpT2 from a body wall muscle cDNA library from the ascidian *Ciona intestinalis*. pCpT2 encoded a 182-residue TnI protein sequence that aligned well (55% identity) with the ~180–185-residue TnIfast and TnIslow isoforms of vertebrate skeletal muscle. Phylogenetic analysis (Fig. 1) indicated that the *Ciona* TnI gene diverged from the vertebrate TnI gene before the latter underwent the gene duplication events that established the TnIfast/TnIslow/TnIcardiac gene family.

In Northern blots probed with pCpT2 (Fig. 2a), hybridizing RNA species were detected in heart (~1,050 nucleotides) as well as in body wall muscle RNA (~900 nucleotides). Reverse transcription-PCR/sequence analysis showed that the heart TnI mRNA was similar to the pCpT2 body wall muscle TnI mRNA, except that it contained a 141-nucleotide (47-codon) insertion following codon 4 (bold sequence in Fig. 2b), which accounts for its greater length. As deduced from the mRNA sequences, the body wall muscle and heart TnI proteins are identical, except that the latter contains a 47-residue near N-terminal insertion encoded by the 141-nucleotide insertion.

The 47-residue insertion of *Ciona* heart TnI clearly resembles vertebrate TnIcardiac near N-terminal insertion sequences in length (47 versus ~25–55 amino acids), and location, and in particular features (Fig. 3) including 1) a Glu-rich segment at the upstream end (also prominent in *Xenopus* but not in bird or mammal, TnIcardiac), 2) a central Pro-rich/hydrophobic/basic motif, and 3) an AXEXH motif near the downstream end. These points of similarity argue strongly for homology of the heart-specific insertion sequences of vertebrate and *Ciona* TnI. The *Ciona* sequence lacks the dual PKA phosphorylation site conserved among vertebrate TnIcardiac sequences (16, 18).

Alternative Splicing of Ascidian TnI Isoforms—Three lines of evidence showed that *Ciona* heart and body wall muscle TnI mRNAs are produced from the same gene by an alternative splicing mechanism. Except for the 141-nucleotide insertion, the two TnI mRNA sequences were virtually identical. In the 736 nucleotides that could be compared (Fig. 2b), there were only three single-base differences: synonymous differences in codons 125 and 131 and a single base change in the 3'-untranslated sequence. This near identity of corresponding sequences, including only one base difference in 187 nucleotides of 5' and 3'-untranslated sequence, strongly suggests that the heart and body wall muscle TnI mRNAs are produced from a single gene by an alternative splicing mechanism rather than from independent genes. The codon 125 difference apparently corresponds to a *HinClI* site allelic polymorphism (see below), and it is likely that allelic polymorphism could also account for the other two single-base differences.

Making use of allelic polymorphisms, we were able to demonstrate genetically that both heart and body wall muscle TnI mRNAs are derived from the same gene. In reverse transcription-PCR products from the heart and body wall muscle of individual animals we identified polymorphisms affecting *ClaI* and *HinClI* restriction sites at codons 121/122 and 124/125. Each of 4 animals examined had a different allelic profile at these sites (Fig. 4). In the case of animals 2 and 3, the body wall muscle TnI PCR products were entirely cleaved by *ClaI* into the expected products (Fig. 4a, lanes 4 and 5), indicating that in these animals, both maternal and paternal TnI gene alleles contained the *ClaI* site. However, for animal 1, about one-half of the body wall muscle product was not cut (lane 3), and animal 4's body wall muscle product was *ClaI*-unrestricted. Identical results (not shown) were obtained using three times as much *ClaI* enzyme, so these were likely not partial, digests. Presumably one of animal 1's TnI gene alleles contained, and one lacked, the *ClaI* site, whereas both of animal 4's alleles lacked the site. By like reasoning, the *HinClI* site was present in one allele of animal 3's body wall muscle products (Fig. 4a, lane 13) but was absent from both alleles in animals 1, 2, and 4 (lanes 11, 12, and 14). Although all four animals had
distinct allelic profiles for body wall muscle TnI, in each case a precisely matching profile of ClaI and HinCII sites was found in the heart TnI products (Fig. 4a, lanes 7–10 and 15–18). Size differences in the reverse transcription-PCR products due to the heart-specific insertion sequence preclude the possibility that the matching allelic profiles could have been due to cross-contamination of the tissues during dissection. Corresponding patterns of allelic variation in heart and body wall muscle TnI transcripts is compelling evidence that these transcripts are derived from the same gene.

The intron/exon structure of the Ciona TnI gene was found to be entirely consistent with alternative splicing. Sequence analysis of PCR-amplified genomic DNA showed that the heart-specific 141-nucleotide mRNA sequence block corresponded precisely to two exons in the Ciona TnI gene (Fig. 5). Moreover, all introns lie between codons, so that inclusion of the two heart-specific exons during mRNA maturation in the heart and their exclusion in body wall muscle would not affect the reading frame of the remainder of the TnI polypeptide. The first of the two heart-specific exons encodes the Glu-rich domain that is also present in amphibian, but not bird or mammal, TnIcardiac. The second encodes the remainder of the heart-specific sequence including the broadly conserved Pro-rich/hydrophobic/basic and AXExH motifs (see Fig. 6).

**DISCUSSION**

**TnI Structure/Function**—Our results show that ascidians, like vertebrates, express a 180–185-residue TnI in somatic muscle and a longer TnI isoform with a characteristic near N-terminal insertion sequence in the heart. Presumably, the TnI near N-terminal insertion sequence has an ancient functional role in the chordate heart.

At present, the only known function of the heart-specific insertion sequence is that, in vertebrates, it is a target for PKA phosphorylation in response to sympatho-adrenal β-adrenergic stimulation. Epinephrine strengthens the heart beat and increases the heart rate, and concomitant phosphorylation of the TnI heart-specific insertion sequence apparently permits a more rapid relaxation of the contractile apparatus that a high heart rate requires (4, 19). Ascidians are unlikely to share this cardiac regulatory mechanism. There is no known nerve supply to the ascidian contractile heart (29), and we know of no endocrine source of circulating catecholamines to play the role of the vertebrate adrenal medulla. Moreover, our results show that the ascidian TnI heart-specific near N-terminal insertion sequence does not contain PKA phosphorylation sites. Either PKA phosphorylation has been lost in the ascidian lineage, or it arose de novo in the vertebrate lineage, possibly in concert with the evolution of sympatho-adrenal control of heart rate. As
A Glu-rich domain similar to that seen in a PKA phosphorylation target. This additional function and the sequence, despite the absence of PKA target sites, suggests that expression of specialized longer and shorter TnI isoforms, the former containing a Pro-rich near N-terminal domain, may be an ancient metazoan character.

Homologous Isoforms Produced by Nonhomologous Mechanisms—Although ascidians and vertebrates undoubtedly inherited the system of heart versus somatic muscle TnI isofrom specialization from a common early chordate ancestor, these two lineages now use entirely different molecular genetic mechanisms to produce the homologous tissue-specific TnI isoforms.

Whereas vertebrates employ distinct genes and tissue-specific transcriptional control mechanisms, ascidians use a single gene and a tissue-specific alternative RNA splicing mechanism (5'-rapid amplification of cDNA ends analysis (not shown) indicated that both Ciona heart and body wall muscle TnI mRNAs initiate from the same promoter, so that transcriptional alternatives do not appear to play any role in the alternative splicing mechanism.) Thus, there has been in one lineage or the other a remarkable evolutionary reworking of the fundamental mechanism of isofrom generation while maintaining intact the structural specificities of the different isoforms and their tissue-specific expression.

Different molecular genetic mechanisms have been reported to produce heterogeneity of immunoglobulin variable regions (34) and serpin serine protease inhibitor active centers (35) in different organismal lineages. However, the present report is to our knowledge the first to document the production of homologous differentially expressed tissue-specific protein isoforms by nonhomologous molecular genetic mechanisms.

TnI Gene Family Evolution—Because vertebrate gene families appear generally to have arisen after the divergence of ascidians from the cephalochordate/vertebrate lineage (36, 37), alternative splicing is a more probable ancestral chordate TnI isofrom-generating mechanism than is the multigene family mechanism. The existence of TnI near N-terminal alternative splicing in a distantly related phylum, the arthropods (32–33), and the phylogenetic placement of Ciona TnI outside of the vertebrate TnI fast/TnI slow/TnI cardiac sequence group (Fig. 1) are also consistent with this view. Given a scenario in which early chordate ancestors contained a single TnI gene expressed in heart and somatic muscle and undergoing tissue-specific alternative splicing of near N-terminal exon(s), a plausible transformation to the current situation seen in vertebrates would involve 1) gene duplication, 2) evolution of heart versus somatic muscle isofrom transcriptional specificity in the duplicate TnI genes (the transient expression of skeletal muscle TnI genes during vertebrate heart development (12–15) suggests the evolution of secondary gene repression mechanisms), 3) loss of heart-specific exons from the somatic muscle TnI gene by deletion or sequence drift, 4) duplication of the somatic muscle TnI gene to give rise to the TnI fast and TnI slow skeletal muscle genes of vertebrates.

Gene Duplication: Engine of Isoform Diversity?—Gene duplication is generally considered the primary engine of isofrom diversity in the evolution of multigene families (2). However, a different dynamic holds in the scenario just discussed, where the heart versus somatic muscle TnI isofrom specificity and differential expression were already established before the gene duplication events that gave rise to separate heart and somatic muscle TnI genes. Thus, gene duplication did not play a primary, creative role in this aspect of TnI isofrom diversity but merely provided an alternative, i.e., transcriptional means to maintain a pre-existing system of isofrom diversity based on
gene. Apart from the presence of introns, the genomic DNA differs from the heart TnI mRNA sequence in Fig. 2 with the consensus gt..ag boundary sequences bolded or in directing heart-specific alternative splicing in other genes. In utilization of the heart-specific exon of the TnIcardiac gene whether these may play some role in living vertebrates either alternative splicing mechanism survive in the vertebrates and whether the vertebrate mechanism arose by neutral drift in a transcriptional control mechanism in the vertebrate lineage or information from an alternative splicing mechanism to a multigene family in which corresponding alternatively spliced protein isoforms could give rise in the vertebrates and corresponding isoforms are now encoded by distinct genes.

One question raised by the proposed scenario is whether there was any selective advantage in the postulated transformation from an alternative splicing mechanism to a multigene transcriptional control mechanism in the vertebrate lineage or whether the vertebrate mechanism arose by neutral drift in a setting that was permissive for gene duplication and/or genome expansion. Another is whether any components of the ancestral alternative splicing mechanism survive in the vertebrates and whether these may play some role in living vertebrates either in utilization of the heart-specific exon of the TnIcardiac gene or in directing heart-specific alternative splicing in other genes.

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