Conserved regulation of cardiac calcium uptake by peptides encoded in small open reading frames

Magny et al

Cardiac contraction requires continuous cycles of calcium release and reuptake between the sarcoplasm and sarcoplasmic reticulum. In vertebrate cardiomyocytes, resequestration of calcium to the sarcoplasmic reticulum is accomplished by the SERCA whose activity is dampened by interaction with the small integral membrane proteins, phospholamban and sarcolipin. In a recent report published in Science, Magny et al identify 2 small peptides in Drosophila encoded in a putative long noncoding RNA that buffers calcium reuptake by sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a in a similar manner to sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a regulation by phospholamban and sarcolipin. These findings demonstrate that regulation of Ca^{2+}-ATPases by small transmembrane peptides is a conserved and ancient strategy. Furthermore, this study highlights the possibility that there may be many undiscovered small peptides encoded within putative long noncoding RNAs that regulate important biological pathways.

Regulation of calcium signaling is vitally important to normal heart function, and dysregulation of calcium handling is a common feature of many models of cardiovascular disease and progressive heart failure. The importance of calcium regulation in cardiomyocytes is multifactorial, but stems from its direct role in regulation of the sarcomeric contraction machinery as well as roles in gene regulation and other processes including cell death. During each cycle of contraction and relaxation, calcium is released from the sarcoplasmic reticulum (SR) and binds myofilaments to induce sarcomere shortening. After contraction, the SR calcium pump, SERCA, replenishes the cardiac calcium store by recycling calcium back to the SR from the sarcoplasm (Figure). Clearing calcium from the sarcoplasm allows the sarcomere to relax and the cardiac chambers to refill with blood.

The activity of SERCA is modulated by several soluble factors such as histidine-rich calcium-binding protein, calreticulin, and S100A. SERCA is also governed by 2 small transmembrane proteins, phospholamban (PLN) and sarcolipin (SLN). PLN acts to inhibit SERCA, but on phosphorylation, inhibition is released, thus allowing SERCA to pump calcium into the SR at a higher rate. SLN similarly inhibits the activity of SERCA and recently was shown to uncouple ATP hydrolysis by SERCA from calcium transport, creating a futile cycle that is important for the thermogenic properties of skeletal muscle. Modest overexpression of SERCA in mice has been shown to increase cardiac SR calcium reuptake, contractility, and relaxation. Thus, strategies that modulate expression or activity of SERCA or its regulators are interesting therapeutic possibilities. Currently, clinical trials aimed at elevation of SERCA2a expression through adeno-associated viral delivery are underway and have reported promising results thus far.

PLN and SLN, the small transmembrane regulators of SERCA in higher organisms, were first identified as small peptides associated with SERCA in the SR of myocytes. Because of its ability to form a highly stable pentamer, PLN was originally perceived to be 22 kDa in mass. However, subsequent cloning of the transcript revealed that PLN is encoded by a small open reading frame (ORF) within a single exon of a large, spliced transcript, that gives rise to a single 52 amino acid transmembrane protein with a mass of only 6 kDa. SLN is similarly encoded within a single exon of a highly conserved, spliced transcript and encodes a 31 amino acid protein—currently the second smallest ORF annotated in mice.

Recent focus on long noncoding RNAs has largely overlooked the possibility that many putatively noncoding transcripts may contain small ORFs that encode functional peptides—it is also worth noting that if not for meticulous characterization of SR protein composition in the 1970s, PLN and SLN might still be considered noncoding transcripts. Small ORFs are highly under-represented in current genome annotations, which may reflect either genuine rarity in nature or the difficulty of identifying bona fide small ORFs among the large number of false ORFs that occur purely by chance. In practice, many transcripts are annotated as noncoding solely because they lack an ORF of appreciable length, as determined by an arbitrary or statistically determined threshold. Several studies have aimed to refine the process of short ORF annotation through the use of comparative genomics, relying on the differential evolutionary patterning of coding and noncoding regions.

From a comparative genomics screen of hypothetical small ORFs in Drosophila, Magny et al identified 2 highly similar small peptides encoded within a single putative noncoding transcript, pncr003:2L (Figure). In situ hybridization showed that this transcript is expressed in somatic muscle of embryos and larvae and in the hearts of larvae and adults. These peptides were
predicted to form single-pass transmembrane helices, and localization studies using both fluorescent and epitope tags showed that the peptides localize to the SR, suggesting a possible role in myocyte calcium signaling. From structural prediction and localization assays, the authors noted similarity with the vertebrate small peptides, SLN and PLN, and therefore dubbed these peptides sarcolamban A and B. However, this similarity is only weakly conserved in their amino acid sequences and is perhaps why this relationship was not observed sooner.

The authors generated loss- and gain-of-function mutants to study the role of these peptides in *Drosophila*. Loss-of-function flies were designed to carry 2 overlapping deficiencies (transheterozygotes) across the *pncr003:2L* locus. Together, these alleles ablate the region containing the gene of interest as well as 2 neighboring genes. Transheterozygous flies exhibited no overt behavioral or ultrastructural defects in somatic or cardiac muscle. However, visual examination of the beating hearts of these negative flies revealed apparent amplitude perturbations in cardiac rhythm, which were further corroborated by electrophysiological measurement of action potentials. Using a genetically encoded calcium indicator, GCaMP3, the investigators observed increased calcium transient amplitudes and steepened decay rates, although contractility seemed largely unaffected as inferred from fractional shortening.

To demonstrate that loss of the peptides encoded in *pncr003:2L* was responsible for the observed phenotype, the authors generated several alleles to attempt mutant rescue. They found that the calcium handling defects of transheterozygous flies could be rescued by expression of constructs encoding either small ORF, but not by frame shift constructs, indicating that lack of *pncr003:2L* was the cause of this phenotype and that translation of the small ORFs was required for normal heart function. Furthermore, a dominant negative allele of Ca-P60A, the fly ortholog of SERCA, which inhibits calcium uptake into the SR, rescued the arrhythmic and transient decay rate phenotypes, suggesting that the defect stemmed from unbridled SERCA activity. Although ectopic expression of human PLN or SLN did not rescue arrhythmia, PLN did restore the transient decay rates to normal levels. Interestingly, forced overexpression of either sarcolamban peptide, PLN, or SLN also increased cardiac arrhythmia and dampened calcium transient amplitude and decay rate, suggesting that the human peptides may have synergism with the endogenous sarcolamban peptides despite that the human peptides alone were unable to compensate for loss of *pncr003:2L*.

To explore interactions between these peptides and Ca-P60A, immunoprecipitation experiments were performed with epitope tagged constructs of the sarcolamban peptides, as well as PLN and SLN. All of these small transmembrane peptides coimmunoprecipitated with Ca-P60A, consistent with their functional influence on calcium handling.

This study raises several interesting questions that warrant future investigation. First, there are thousands of long noncoding RNAs encoded by metazoan genomes, but much remains to be learned about the functions of these transcripts. This study suggests that some may encode previously unrecognized peptides with important functions. Identification and functional characterization of such peptides represents a significant challenge, particularly if they are not conserved at the primary amino acid level, as will likely be the case with many functional small ORFs. Detection of such peptides and determination of their functions are also significant challenges, but for highly conserved peptides, this may be overcome by strategies that use comparative genomics.

This study also expands the evolutionary context of small ORFs with respect to myocyte calcium handling, suggesting a common ancient origin. Alternatively, it is plausible that these peptides represent a polyphyletic grouping arising from convergence such that the strict constraints of transmembrane helices drive selective pressure toward similar secondary structures. Unfortunately, the small nature and vast divergence of these peptides severely limit the extent to which evolutionary relationships can be inferred reliably, but demonstration of a common mechanism of action with vertebrate homologs is strongly supportive of a common ancestor.

The nature of the *pncr003:2L* transcript and the 2 encoded small peptides is intriguing and unlike most other protein-coding transcripts. Questions remain as to how the 2 small ORFs in *pncr003:2L* are regulated: (1) Are both ORFs translated concurrently or might there be translational regulation that could result in differential expression of each peptide? and (2) Does alternative splicing allow for discrete expression of each peptide, since there are currently 3 annotated transcripts for *pncr003:2L*, 1 of which (NR_001661.2) would produce only sarcolamban B?

Recently, thousands of long noncoding RNAs have been discovered in metazoan genomes through transcriptome sequencing. This study raises the possibility that a subset of these novel transcripts may encode small functional peptides. Notably, this study also demonstrates the power of bioinformatics to identify transcripts with coding potential through the use of comparative genomics. Annotated small ORFs that encode functional peptides in the genome are extremely rare in mammals—in mice there is only 1 annotated ORF smaller than 30 codons. This may be attributable to biological constraints for producing functional peptides of such short length, or may be attributable...
to the difficulty of identifying bona fide small ORFs among the many that exist by chance. Using bioinformatics-based strategies, bolstered by the recent explosion of comparative genome and transcriptome sequencing, it should now be possible to identify evolutionarily conserved small ORFs.

Finally, PLN and SLN are classic examples of functional small ORFs in vertebrates, but these small ORFs are still viewed as rare exceptions more than they are considered archetypes of small functional peptides. Foreseeably, the small size of these peptides may be critical to their functional niche, fulfilling roles that may be problematic for larger proteins. Small peptides might also be particularly well suited for all kinds of biological roles possibly including regulation of enzyme activity, intracellular signal transduction, shuttling between cellular compartments, and cell surface signaling. Supposing that small peptides may be more evolutionarily labile, it is also conceivable that there may be less conserved or unconserved small peptides that serve to fine-tune cellular machinery in a species-specific fashion. Just as microRNAs were long overlooked, it is tempting to speculate that there remains a world of tiny biologically active peptides yet to be explored. Although many questions remain concerning the scope and nature of small peptide-encoding ORFs, this study makes clear the fact that we cannot ignore this potentially important and exciting area of biology.

Disclosures

None.

References


Small Open Reading Frames Pack a Big Punch in Cardiac Calcium Regulation
Benjamin R. Nelson, Douglas M. Anderson and Eric N. Olson

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