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Widespread control of calcium signaling by a family of SERCA-inhibiting micropeptides

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Abstract

Micropeptides function as master regulators of calcium-dependent signaling in muscle. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), the membrane pump that promotes muscle relaxation by taking up Ca^{2+} into the sarcoplasmic reticulum, is directly inhibited by three muscle-specific micropeptides: myoregulin (MLN), phospholamban (PLN), and sarcolipin (SLN). The widespread and essential function of SERCA across diverse cell types has raised questions as to how SERCA is regulated in cells that lack MLN, PLN, and SLN. We identified two transmembrane micropeptides, endoregulin (ELN) and another-regulin (ALN), that share key amino acids with their muscle-specific counterparts and function as direct inhibitors of SERCA pump activity. The distribution of transcripts encoding ELN and ALN mirrored that of SERCA isoform-encoding transcripts in nonmuscle cell types. Our findings identify additional members of the SERCA-inhibitory micropeptide family, revealing a conserved mechanism for the control of intracellular Ca^{2+} dynamics in both muscle and nonmuscle cell types.

INTRODUCTION

Precise regulation of intracellular Ca^{2+} is essential for various cellular functions, including cell motility, fertilization, platelet cell activation, cardiac hypertrophy, vascular tone, neuronal transmission, synaptic plasticity, and muscle contraction (1–4). The distribution and concentration of intracellular Ca^{2+} is controlled by a network of Ca^{2+}-binding proteins, channels, and pumps that maintain resting cytosolic Ca^{2+} concentrations ~10,000 times

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lower than those in the sarco/endoplasmic reticulum (S/ER), the main store of intracellular calcium (5). Although Ca^{2+} release from the S/ER occurs through various distinct channels and through passive leak, reuptake of Ca^{2+} into the S/ER occurs exclusively through the S/ER Ca^{2+} ATPase (adenosine triphosphatase) (SERCA) pump (5).

In vertebrates, SERCA is encoded by three genes (*SERCA1* to *SERCA3*) that give rise to multiple splice isoforms expressed in distinct cell types (6). The expression of SERCA1 is restricted to fast- and slow-type skeletal muscles, and an isoform of *SERCA2* (SERCA2a) is specific to cardiac and slow-type skeletal muscles. The dominant SERCA isoform in smooth muscle is SERCA2b, which is also broadly distributed in many nonmuscle cell types. *SERCA3* transcripts are absent from muscle but are highly abundant in the endothelial and epithelial cells lining the vascular and visceral organs, platelets, and pancreatic β cells (7–9).

In cardiac and skeletal muscle, the activity of SERCA is inhibited by the binding of small transmembrane micropeptides, which lower the affinity of SERCA for Ca^{2+} and decrease the rate of Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) (10, 11). In the heart, phospholamban (PLN) and sarcolipin (SLN) inhibit the activity of SERCA2a and function as important regulators of cardiac contractility and disease (12–14). We have identified two micropeptides that directly bind SERCA in muscle, myoregulin (MLN) and dwarf open reading frame (DWORF), which were concealed within RNA transcripts misannotated as long noncoding RNAs (15, 16). MLN shares sequence and structural similarity with PLN and SLN and functions as the predominant micropeptide inhibitor of SERCA activity in fast-type skeletal muscle (15). Among the micropeptide regulators of SERCA pump activity, MLN, PLN, and SLN share a conserved hydrophobic motif within their transmembrane helices that forms their interaction face with SERCA in the SR membrane. This motif is also present in sarcolamban (SCL), the invertebrate SERCA-inhibiting micropeptide found in the cardiac and somatic muscle of *Drosophila melanogaster* (17). Despite the widespread and essential function of SERCA in non-muscle cell types, the regulation of SERCA by micropeptides appeared to be restricted to muscle cells because of the muscle-specific distribution of MLN, PLN, SLN, and DWORF. Here, we detail the discovery and functional characterization of two SERCA-inhibitory micropeptides that codistributed with SERCA isoforms in nonmuscle cell types. These data demonstrate that micropeptide control of Ca^{2+} signaling is conserved across diverse cell types and have important implications for our understanding of the many cellular functions governed by Ca^{2+} signaling.

RESULTS

To determine whether vertebrate genomes harbor additional micropeptides that could bind and regulate SERCA, we used a bioinformatics approach to screen the mouse genome for potential open reading frames containing the SERCA binding motif of MLN, PLN, and SLN. We identified two genes encoding uncharacterized transmembrane micropeptides that share the SERCA binding motif, which we named endoregulin (ELN) and another-regulin (ALN) (1110017F19Rik/SMIM6 and 1810037I17Rik, respectively) (Fig. 1A).

The *ELN* and *ALN* genes each consist of two exons that span 1.9 and 1.8 kb, respectively (fig. S1A). The ALN micropeptide is the largest member of the family, with 65 amino acids,

compared with ELN, which contains 56 amino acids, and both are conserved in mammals (fig. S1B). Similar to what we demonstrated for the *MLN* RNA (15), in vitro transcription and translation of the full-length *ELN* and *ALN* RNAs produced micropeptides of their predicted molecular weights (fig. S1C). Furthermore, the addition of frameshift mutations within their open reading frames abolished the expression of the MLN, ELN, and ALN micropeptides (fig. S1C).

Both ALN and ELN contain C-terminal transmembrane helices that are predicted to insert into membranes similarly to MLN, PLN, and SLN, with the residues in common aligned along a single helical face (Fig. 1B, green- and blue-highlighted residues). To examine their subcellular distribution, we coexpressed ALN or ELN as N-terminal FLAG-fusion proteins with mCherry-tagged SERCA2b in COS-7 cells. Similar to MLN, PLN, and SLN, ALN and ELN perfectly colocalized with mCherry-SERCA2b in a pattern consistent with the reticulated membranes of the ER (Fig. 1C). To determine whether ALN or ELN could form a stable complex with SERCA, we performed coimmunoprecipitation experiments using hemagglutinin (HA)–tagged micropeptides with Myc-tagged SERCA2b transiently expressed in COS-7 cells. All of the micropeptides formed a stable complex with Myc-SERCA2b, but not with the Myc-tag alone (Fig. 2A).

Previous crystallography studies have shown that PLN and SLN bind to a pocket formed by the M2, M6, and M9 helices of SERCA within the membrane of the SR (18–20). To determine whether ALN or ELN binds to the same region of SERCA as PLN, we tested whether PLN could compete with ALN or ELN for SERCA binding. To test this, we expressed equal amounts of either HA-tagged ALN or ELN with Myc-SERCA2b and an increasing amount of a green fluorescent protein (GFP)–tagged PLN fusion protein (GFP-PLN). We found that increasing the expression of GFP-PLN reduced the amount of HA-ALN or HA-ELN coprecipitated by SERCA, suggesting that these micropeptides bound competitively for the same region of SERCA (Fig. 2B).

Apart from their distinct cell-type specific distribution patterns, SERCA isoforms display unique intrinsic Ca^{2+} pump kinetics that may accommodate or impart cell-specific requirements for Ca^{2+} handling (21). To compare the Ca^{2+} transport (V_{max}) and Ca^{2+} dependence of Ca^{2+} transport activity (K_{Ca}) of the major mouse isoforms of SERCA, we performed oxalate-dependent Ca^{2+} uptake assays using whole-cell homogenates prepared from transiently transfected COS-7 cells (fig. S2, A and B) (22). Consistent with previous reports, we found that mouse SERCA1 and 2 showed significantly higher affinities for Ca^{2+} than mouse SERCA3 (fig. S2, A and B).

Because ALN and ELN formed a stable interaction with SERCA in the membrane of the ER, we next assessed their ability to regulate different SERCA isoforms using oxalate-supported Ca^{2+}-dependent Ca^{2+} uptake assays. Similar to the effect of the SERCA-inhibitory micropeptides, coexpression of ALN or ELN caused a significant reduction in the apparent affinity for Ca^{2+} of SERCA2b and SERCA3a, respectively (Fig. 2, C and D, and fig. S2, C and D). None of the micropeptides altered the maximal rate of Ca^{2+} uptake (V_{max}). Together, these findings reveal that vertebrates encode five structurally related transmembrane micropeptides that inhibit Ca^{2+} uptake by diverse SERCA isoforms.

To determine the spatial and temporal distribution of ALN and ELN, we examined the relative expression of the mRNAs encoding these micropeptides and major SERCA isoforms during developmental and adult time points in the mouse (Fig. 3, A to C, and fig. S3, A and B). As we previously reported, *MLN* was expressed in a pattern corresponding with that of *SERCA1* in both fast- and slow-type skeletal muscles. *PLN* was specifically expressed in both the atria and ventricles of the heart and bladder, whereas *SLN* expression was specific to the atria of the heart and embryonic slow-type skeletal muscles. In adulthood, these patterns remained consistent, with the notable exception that the embryonic skeletal muscle–specific expression of *SERCA2a* and *SLN* became restricted to only a few slow-type skeletal muscles in the adult. These patterns were consistent with mouse cardiac (HL-1) and skeletal muscle (C2C12) cell lines, which robustly expressed *PLN* and *MLN*, respectively (Fig. 3C).

Strikingly, *ALN* was expressed in a pattern similar to *SERCA2b*, with enrichment in the heart, epidermal epithelium, salivary gland, brown fat, intestinal epithelium, and urothelium of the bladder (Fig. 3, A and B, and fig. S3A). The expression of *ELN* was distinct from that of the other micropeptides in that it was largely expressed in nonmuscle tissues, with the exception of weak expression in body wall muscles at embryonic day 14.5 (E14.5) in the developing mouse and in differentiated C2C12 myotubes. *ELN* expression showed a large degree of overlap with that of SERCA3 in the epithelial cells of the trachea and bronchus, lung, intestine, pancreas, and liver (Fig. 3, A and B, and fig. S3B). These findings reveal that the expression of *ALN* and *ELN* overlaps with the major SERCA isoforms expressed in nonmuscle cell types that lack PLN, MLN, and SLN.

DISCUSSION

The importance of Ca^{2+} signaling is highlighted by the precise nature by which Ca^{2+} ions are extensively regulated within cells. The widespread regulation of SERCA activity by activating and inhibiting transmembrane micropeptides represents a conserved and ancient mechanism to control Ca^{2+} handling in muscles of vertebrates and invertebrates (15–17). In vertebrates, the SERCA family has expanded to encode three distinct genes that give rise to multiple splice isoforms expressed in muscle and nonmuscle tissues (6). Here, we showed that the distribution of SERCA isoforms in nonmuscle cell types overlapped with that of two trans-membrane micropeptides, ELN and ALN, which shared structural and functional similarity with their muscle-specific counterparts, MLN, PLN, and SLN. The complex and dynamic regulation of SERCA in these tissues underscores the importance for SERCA activity and the role of Ca^{2+} signaling in multiple aspects of cellular biology, including muscle contraction, metabolism, cell growth, and cell death pathways. Considering the importance of intra-cellular Ca^{2+} dynamics for countless cellular processes, it is likely that ELN and ALN play key roles in the physiology and pathology in the tissues in which they are expressed.

The reciprocal expansion of SERCA and micropeptide family members suggests that these two families of partner proteins have coevolved as an effective and general mechanism for the control of Ca^{2+} handling in a diverse range of cell types. We note a remarkable overlap in the distribution of individual micropeptides with major SERCA isoforms (SERCA1 with MLN, SERCA2a with PLN, SERCA2b with ALN, and SERCA3 with ELN) (Fig. 4).

However, partial overlap in the expression of muscle-specific isoforms of SERCA and micropeptides, such as in atrial cardiomyocytes or slow skeletal muscle, suggests that there may be functional redundancy among these factors in regulating muscle contractility. Furthermore, the broad distribution of SERCA2b and ALN suggests that a degree of functional redundancy may exist in part among these factors in muscle and nonmuscle cell types. In this regard, ALN may be functionally redundant with PLN or SLN in the heart and/or with ELN in the intestinal epithelium. Future loss-of-function studies will be required to understand the extent to which individual micropeptides and SERCA isoforms can compensate for one another in the tissues in which they are codistributed.

The dynamic nature of calcium signaling in response to extra-cellular signals is partially mediated by the relief of SERCA inhibition through phosphorylation of conserved N-terminal residues in PLN and SLN (23–25). ALN and ELN contain N-terminal domains exposed to the cytosol that contain conserved serine and threonine residues. Similar to PLN, we note that ALN contains a protein kinase A (PKA) (RxxS) phosphorylation motif in its N-terminal domain that has been identified by mass spectrometry as phosphorylated (Ser^{19}) in liver, pancreas, and heart tissues (26, 27). Although potentially redundant, this suggests that ALN and PLN may share a conserved mechanism to regulate their ability to control SERCA activity in response to extracellular signaling, such as β-adrenergic stimulation (28).

SERCA belongs to a family of P-type ATPases represented by 40-membrane–bound ion pumps essential for the active transport of Ca^{2+}, Mg^{2+}, H^+, Na^+, and K^+ (29, 30). Considering the conservation of micropeptides in the control of SERCA pump function and Ca^{2+} handling, similar mechanisms may exist to regulate the intracellular transport of other ions by related P-type ATPases and concealed micropeptides. In this regard, the uncharacterized transmembrane micropeptides present in mammalian genomes may be a potential reservoir of essential regulators of P-type ATPases for the control of ion handling in diverse cell types.

MATERIALS AND METHODS

Mouse strains and cell lines

All experimental procedures involving animals in this study were reviewed and approved by the University of Texas Southwestern Medical Center’s Institutional Animal Care and Use Committee. The sexes of embryos used in the gene expression studies were not determined. Adult mouse tissues were harvested from 8-week-old male C57Bl/6 mice and processed for RNA using TRIzol (Life Technologies). The C2C12 skeletal muscle cell line, COS-7 cells, and 10T1/2 mouse fibroblasts were obtained from American Type Culture Collection, and the HL-1 cardiomyocyte cell line was donated by W. Claycomb (Louisiana State University, New Orleans, LA). Cell lines were verified by morphology, and no testing for mycoplasma contamination was performed.

Real-time PCR

Total RNA was prepared from whole muscles using TRIzol (Life Technologies) and treated with de-oxyribonuclease before reverse transcription by SuperScript III (Life Technologies).

Real-time RT-PCR was performed using SYBR Green, as previously described (15). Primer sequences are listed in table S1.

Radioisotopic in situ hybridization

In situ hybridizations were performed, as previously described (31). Primer sequences used to clone micropeptide and SERCA complementary DNA (cDNA) templates used to generate radiolabeled antisense RNA probes are listed in table S1.

Coimmunoprecipitations

Coimmunoprecipitations were performed, as previously described (15). Briefly, COS-7 cells were transfected with expression plasmids encoding HA-PLN, HA-SLN, HA-MLN, HA-ALN, or HA-ELN and Myc-SER-CA2b or Myc alone. For competition experiments, COS-7 cells were also cotransfected with varying amounts of GFP or GFP-PLN expression plasmids. Whole-cell lysates were prepared in coimmunoprecipitation buffer [20 mM $NaPO_4$, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM dithiothreitol (DTT), and cOmplete protease inhibitor (Roche)]. Immunoprecipitations were carried out using 1 mg of mouse monoclonal anti-Myc antibody (Life Technologies, mouse monoclonal) and collected with Dynabeads (Life Technologies). Standard Western blot procedures were performed on immunoprecipitated fractions using mouse anti-HA (Life Technologies, mouse monoclonal) or horseradish peroxidase–conjugated GFP (Pierce, GF28R) antibodies.

Oxalate-supported Ca^{2+} uptake measurements

Oxalate-supported Ca^{2+} uptake in cardiac homogenates was measured by a modified Millipore filtration technique (15, 16, 22). COS-7 cells were transfected with equal amounts of an expression plasmid encoding mCherry-SERCA1, mCherry-SERCA2a, mCherry-SERCA2b, mCherry-SERCA3a or mCherry-SERCA3b and untagged ALN, ELN, PLN, MLN, or SLN. About 36 hours after transfection, COS-7 cells were homogenized in 50 mM phosphate buffer (pH 7.0) containing 10 mM NaF, 1 mM EDTA, 0.3 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT. Ca^{2+} uptake was measured in reaction solution containing 40 mM imidazole (pH 7.0), 95 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM K^+ oxalate, 1 μM ruthenium red, and various concentrations of $CaCl_2$ to yield 0.02 to 5 μM free Ca^{2+}. Homogenates were incubated at 37°C for 2 min in the above reaction buffer, and the reaction was initiated by the addition of ATP (final concentration of 5 mM). The data were analyzed by nonlinear regression using Prism 6 (GraphPad), and the K_{Ca} values were calculated using an equation for a general cooperative model for substrate activation. The values for maximal SERCA activity were taken directly from the experimental data and normalized for total protein concentration (μmol/mg protein per minute), and mCherry fluorescence was measured on a FLUOstar/POLARstar OPTIMA plate reader (BMG Labtech) with an excitation wavelength of 580 nm and an emission wavelength of 610 nm.

In vitro transcription and translation of micropeptides

The full-length *MLN*, *ALN*, and *ELN* RNAs were subcloned into pcDNA3.1(+) (Invitrogen) containing the T7 RNA polymerase promoter. Frameshift mutations were introduced immediately after the endogenous ATG start codon of each transcript using standard mutagenesis to disrupt the open reading frames of the micropeptides. Coupled in vitro transcription and translation assays were performed using radiolabeled [^{35}S]methionine and [^{35}S]cysteine (PerkinElmer) and the TNT T7 Coupled Wheat Germ Extract System (Promega), as per the manufacturer's protocols. Products were resolved on 18% SDS–polyacrylamide gels, dried, and exposed to film for 48 hours at room temperature.

Statistical analyses

All statistical analyses were performed using Prism 6 (GraphPad). Statistical analyses were performed using an unpaired *t* test, and data are presented as means ± SEM. Values were considered significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. Relative expression data in Fig. 3C are shown from a single representative experiment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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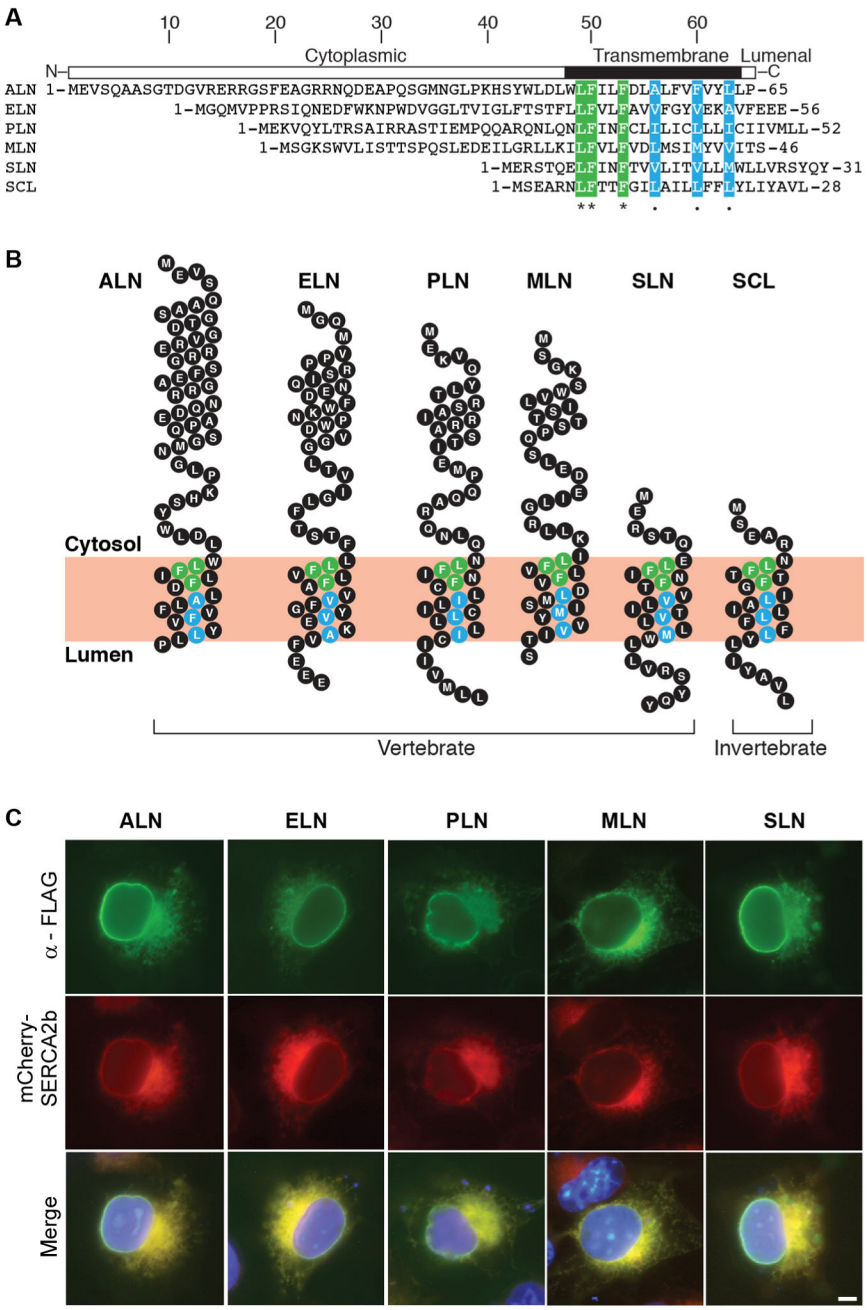

Fig. 1. Discovery of transmembrane micropeptides related to MLN, PLN, and SLN

(**A**) Sequence alignment of the vertebrate micropeptides ALN, ELN, PLN, MLN, and SLN from mouse and SCL from *D. melanogaster* highlights the residues that they share in common in their transmembrane helices. "*" denotes identically conserved residues, and "." denotes weakly similar residues. (**B**) ALN and ELN encode single C-terminal transmembrane α helices and are predicted to insert into intracellular membranes similar to MLN, PLN, and SLN. The residues that they share in common, which mediate SERCA binding, are arranged along a single helical face. (**C**) Colocalization of FLAG-tagged

micropeptides and mCherry-SERCA2b in the ER of COS-7 cells. Images are representative of two independent experiments. Scale bar, 5 μm.

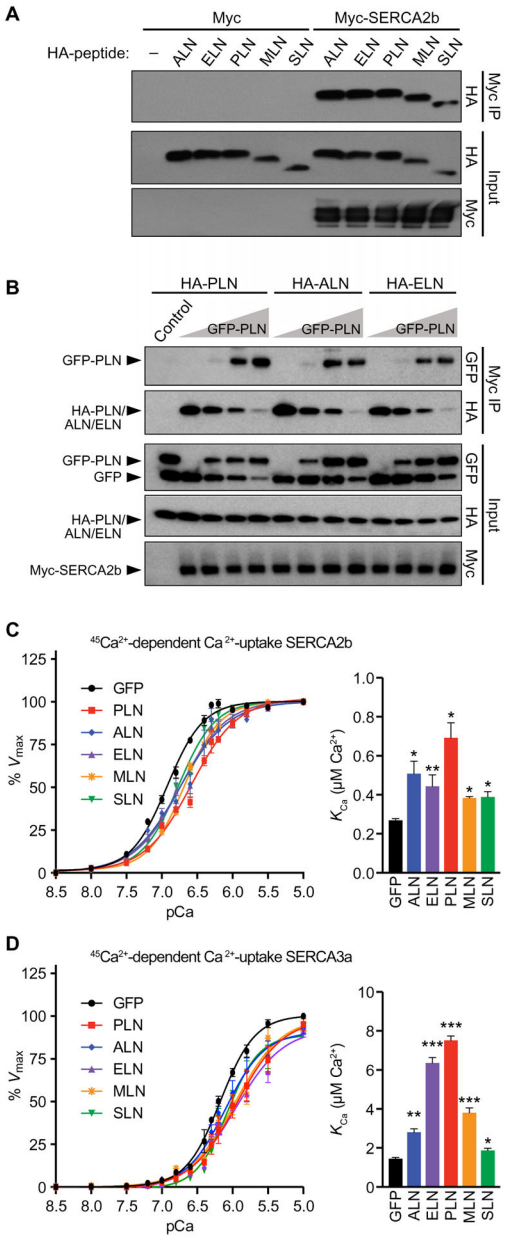

Fig. 2. ALN and ELN bind directly to and inhibit SERCA pump activity

(**A** and **B**) Coimmunoprecipitation experiments with HA-tagged micropeptides and a Myc-tagged SERCA2b using transient cotransfections in COS-7 cells in the absence (A) or presence (B) of competing GFP-tagged PLN. Immunoblots are representative of three independent experiments. (**C** and **D**) Oxalate-supported Ca^{2+}-dependent Ca^{2+} uptake assays demonstrating the inhibitory function of micropeptide expression on SERCA2b (C) and SERCA3a (D). V_{max} and K_{Ca} values were calculated from 12 independent measurements for each sample using an unpaired *t* test. Bar graphs depict the calculated average K_{Ca} ± SEM (six independent experiments). *$P < 0.05$; **$P < 0.01$.

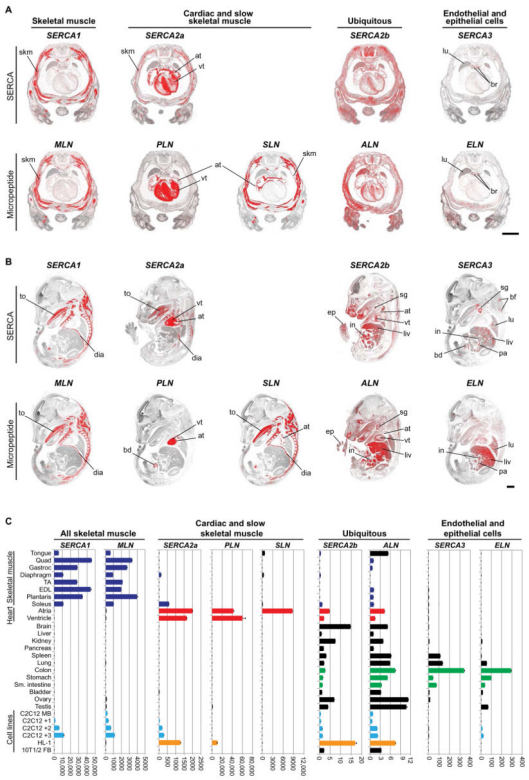

Fig. 3. Tissue and cell-type specific expression of micropeptides and *SERCA* isoforms in the developing and adult mouse

(**A** and **B**) In situ hybridization of antisense RNA probes specific for major *SERCA* isoforms and micropeptides during mouse embryonic day E14.5 transverse (A) and E15.5 midsagittal (B). skm, skeletal muscle; at, atria; vt, ventricle; lu, lung; br, bronchus; to, tongue; dia, diaphragm; sg, salivary gland; bf, brown fat; liv, liver; pa, pancreas; in, intestine; bd, bladder; ep, epidermal epithelium. Scale bar, 1 mm. Images are representative of two independent experiments. (**C**) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showing relative expression of major *SERCA* isoforms and micropeptides across adult mouse tissues. Quad, quadriceps; TA, tibi-alis anterior; EDL, extensor digitorum longus; FB, fibroblasts; MB, myoblasts; C2C12 +1, +2, +3, C2C12 differentiation day 1, day 2, and day 3. Bar graphs depict means ± SEM from a single experiment (representative of three independent experiments).

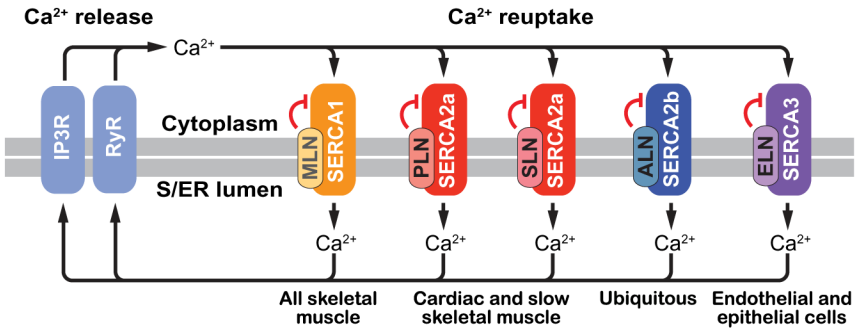

Fig. 4. A family of SERCA-inhibiting micropeptides

Model depicting the expression patterns of the predominant SERCA and micropeptide inhibitors across different muscle and nonmuscle tissues in vertebrates. The discovery of ELN and ALN suggests that the regulation of SERCA activity by SERCA-inhibiting micropeptides represents a general mechanism to control calcium handling across diverse cell types.