Transmembrane Helix 11 Is a Genuine Regulator of the Endoplasmic Reticulum Ca\(^{2+}\) Pump and Acts as a Functional Parallel of \(\beta\)-Subunit on \(\alpha\)-Na\(^+\),K\(^+\)-ATPase*§

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Background: The ubiquitous sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase SERCA2b has a C-terminal extension that increases the Ca\(^{2+}\) affinity. It consists of a transmembrane helix (TM11) and a luminal extension.

Results: Both parts control Ca\(^{2+}\) affinity independently.

Conclusion: TM11 is an independent and highly conserved functional region of SERCA2b.

Significance: This study shows that TM11 acts as a genuine regulator of the Ca\(^{2+}\) pump.

The housekeeping sarco(endoplasmic reticulum Ca\(^{2+}\) ATPase SERCA2b transports Ca\(^{2+}\) across the endoplasmic reticulum membrane maintaining a vital Ca\(^{2+}\) gradient. Compared with the muscle-specific isoforms SERCA2a and SERCA1a, SERCA2b houses an 11th transmembrane segment (TM11) and a short luminal extension (LE) at its C terminus (2b-tail). The 2b-tail imposes a 2-fold higher apparent Ca\(^{2+}\) affinity and lower \(V_{\text{max}}\). Previously, we assumed that LE is the sole functional region of the 2b-tail and that TM11 is a passive element providing an additional membrane passage. However, here we show that peptides corresponding to the TM11 region specifically modulate the activity of the homologous SERCA1a in co-reconstituted proteoliposomes and mimic the 2b-tail effect (i.e. lower \(V_{\text{max}}\) and higher Ca\(^{2+}\) affinity). Using truncated 2b-tail variants we document that TM11 regulates SERCA1a independently from LE, confirming that TM11 is a second, previously unrecognized functional region of the 2b-tail. A phylogenetic analysis further indicates that TM11 is the oldest and most conserved feature of the 2b-tail, found in the SERCA pump of all Bilateria, whereas LE is only present in Nematoda and vertebrates. Considering remarkable similarities with the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-\(\beta\) interaction, we now propose a model for interaction of TM11 with TM7 and TM10 in the anchoring subdomain of the Ca\(^{2+}\) pump. This model involves a TM11-induced helix bending of TM7. In conclusion, more than just a passive structural feature, TM11 acts as a genuine regulator of Ca\(^{2+}\) transport through interaction with the pump.

The sarco(endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) generates and maintains a 1000-fold Ca\(^{2+}\) gradient over the endo/sarcoplasmic reticulum membrane, ensuring a high luminal Ca\(^{2+}\) concentration. This is vital to control cellular activities such as contraction, secretion, growth, proliferation, differentiation, and cell death (1).

Atomic resolution structures of the fast twitch skeletal muscle SERCA1a isoform show that the SERCA Ca\(^{2+}\) ATPases cycle between an E1 conformation with high affinity Ca\(^{2+}\)-binding sites facing the cytosol and an E2 conformation with low affinity Ca\(^{2+}\)-binding sites facing the lumen of the endoplasmic reticulum (2, 3). SERCA consists of three cytosolic domains for ATP hydrolysis and one transmembrane (TM) domain for Ca\(^{2+}\) binding and transport. ATP hydrolysis in the cytosolic domain drives the conformational changes in the TM region that lead to the opening or closing of the gates to the Ca\(^{2+}\)-binding sites. The TM region is divided into a highly mobile part (TM1–6) that controls the access to the Ca\(^{2+}\)-binding sites, and a relatively immobile part (TM7–10) that is thought to serve as the anchoring region of the pump in the lipid bilayer (2, 3).

Humans evolved three SERCA genes (SERCA1–3, human gene nomenclature ATP2A1–3) (4). Although their exon-intron layout is nearly entirely conserved, the 3’ ends of the primary gene transcripts are subjected to alternative processing. This generates a plethora of SERCA splice variants with a specific tissue distribution and activity range (5, 6). With the additional aid of tissue-specific SERCA regulators, the SERCA
activity is tightly adjusted to the physiological needs of each specific cell type (6). However, how these extrinsic regulatory proteins or the intrinsic C-terminal variations alter the enzymatic properties of the Ca\(^{2+}\) ATPase remains poorly understood.

Here, we focus on the regulation of SERCA2, the oldest and most widespread isoform, which is alternatively processed to form the muscle-specific SERCA2a (cardiac, slow twitch skeletal and smooth muscle) or the housekeeping SERCA2b (7). In the heart, SERCA2a is regulated by a small TM protein called phospholamban (PLB), which reduces its apparent affinity for Ca\(^{2+}\) (8). PLB occupies a hydrophobic groove in between the mobile and anchoring part of the TM domain of SERCA2a (TM2, 4, 6, and 9), leading to a stabilization of the low Ca\(^{2+}\) affinity E2 conformation (9). This interaction is relieved by phosphorylation of PLB during β-adrenergic stimulation (8). SERCA2b, on the other hand, uniquely differs from the other SERCA1–3 splice variants by housing a 49-amino acid long C terminus (2b-tail) that imposes the unusual enzymatic properties on this pump, i.e. a 2-fold higher apparent affinity for Ca\(^{2+}\) (K\(_{c_{a}}\)) and lower catalytic turnover rate (V\(_{\text{max}}\)) compared with the muscle isoforms SERCA1a or SERCA2a (7, 10). The 2b-tail increases the apparent Ca\(^{2+}\) affinity of the pump, whereas SERCA2b decreases it through a different mechanism (11, 12). The 2b-tail consists of a cytosolic part (19 amino acids, Gly\(^{994}\)–Asp\(^{1012}\)), a transmembrane segment (TM11, 18 amino acids, Gly\(^{1013}\)–Tyr\(^{1039}\)), and a luminal extension (12 amino acids, Ser\(^{1031}\)–Ser\(^{1042}\)) (see Fig. 1A). Earlier work pointed to the luminal extension as the sole functional region of the 2b-tail (13). TM11 would interact with the anchoring part of the pump at the periphery of TM7 and TM10 (12). The short luminal extension would then follow a path from this remote site, running between luminal loops L5–6 and L7–8, toward a luminal binding site for the last four residues (Thr\(^{1032}\)) of the C-terminal tetrapeptide stretch. This functional C-terminal tetrapeptide stretch would here interact with a pocket formed in the E1 state by the upstream luminal loops. The interaction therefore stabilizes the E1 conformation, which at least partially accounts for the increased apparent Ca\(^{2+}\) affinity of SERCA2b (12).

However, two observations indicate that the interaction of LE with its luminal interaction site does not fully explain the 2b-tail effect and that TM11 plays a more active role than simply providing a passive membrane passage. First, there remains a clear functional effect of the 2b-tail upon truncation of its last 11 residues (Thr\(^{1032}\)). But removal of one additional residue (Ser\(^{1031}\)) completely abolishes the effect of the 2b-tail (12, 13). It is thus clear that Ser\(^{1031}\) takes a key position in the function of the 2b-tail. Second, an affinity-increasing effect is also seen in the SERCA1a context, i.e. when the 2b-tail is coupled to SERCA1a (SERCA1a2b chimera) (12). Because the SERCA1a luminal domain does not present the right interface for interaction with the last four residues of LE, the affinity modulation must by elimination be attributed to a more upstream region of the 2b-tail, presumably Ser\(^{1031}\) or TM11. Using peptides corresponding to TM11, we will here convincingly demonstrate that TM11 is the second functional region of the 2b-tail acting independently from Ser\(^{1031}\) and LE.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptide Handling**—Synthetic peptides corresponding to parts of the 2b-tail were purchased from Biomatik, Wilmington, DE (95% purity grade, HPLC and MS verified). Unless otherwise specified, all synthetic peptides are acetylated at the N terminus and amidated at the C terminus to closely mimic the peptide backbone of the endogenous 2b-tail. Peptides were solubilized in 3:1 chloroform/trifluoroethanol at a concentration of 0.5–4 mg/ml. The peptide concentration was verified by quantitative amino acid analysis.

**Co-reconstitution of SERCA1a and Synthetic TM11 Peptides**—Sarcoplasmic reticulum membranes of rabbit hind limb skeletal muscle were prepared as described previously (14). The membranes were then solubilized in octaethylene glycol monododecyl ether (C\(_{12}\)E\(_{10}\); Barnet Products), and SERCA1a was purified by affinity chromatography as in Ref. 14. Functional co-reconstitution of SERCA1a with TM11 peptides has been performed essentially as described before for SERCA1a/PLB co-reconstitution (15).

Briefly, the required amount of pTM11 was mixed with lipids (360 μg of egg yolk phosphatidylycholine and 40 μg of egg yolk phosphatidic acid; Avanti Polar Lipids) and dried to a thin film. The peptide/lipid film was rehydrated in water, and detergent (800 μg of C\(_{12}\)E\(_{8}\)) was added. Buffer (20 mM imidazole, pH 7.0, 100 mM KCl, 3 mM MgCl\(_{2}\), and 0.02% NaN\(_{3}\)) and SERCA1a (300 μg) were added to achieve final molar stoichiometries of 1:0.5–15 SERCA1a:peptide and 1:120 SERCA1a:phospholipids. The detergent was extracted via gradual administration of SM-2 Biobeads (Bio-Rad). Finally, the SERCA1a-containing vesicles were purified via sucrose step-gradient centrifugation.

To detect the peptides in the proteoliposomes, 7.5 μl of the vesicles were loaded on Tricine-SDS-PAGE as described in Ref. 16. Proteins and peptides were visualized in the gel by silver staining.

**Orientation of TM11 in Co-reconstituted Proteoliposomes via Biotinylation**—The orientation of pTM11 in our co-reconstituted proteoliposomes was determined using a biotin surface labeling assay as in Ref. 15. Co-reconstituted vesicles (5 μg of protein) were mixed in labeling buffer (20 mM borate-KOH, pH 9.0) with 5 mM EZLink Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate; Thermo Scientific) in the absence or presence of 0.5% n-octyl-β-D-glucopyranoside (Anatrace). The mixture was incubated overnight at 4 °C followed by quenching with an equal volume of SDS-PAGE sample buffer. The amount of biotin labeling was quantified on a Western blot using IRDye 800CW streptavidin conjugate and an Odyssey Infrared Imaging System (LI-COR Bioscience).

**Ca\(^{2+}\)-dependent ATPase Activity Assays**—Ca\(^{2+}\)-dependent ATPase activities of the co-reconstituted proteoliposomes were measured at 25 °C by an enzyme-coupled assay (as in Ref. 15) or at 37 °C by an end point colorimetric measurement (as in Ref. 12). SERCA1a activity was followed in the presence and absence of 0.5–4 mg/ml. The peptide concentration was verified by quantitative amino acid analysis.

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SERCA2b mutations were introduced by site-directed mutagenesis (QuikChange). Ca^{2+}-dependent ATPase activities of the overexpressed SERCA variants in COS cells were measured using an end point colorimetric assay (12). The contribution of the endogenous SERCA2b in COS cells to the measured Ca^{2+}-dependent ATPase activity of the overexpressed SERCA variants is negligible. The $K_Ca$ (Ca^{2+} concentration at half-maximal activity), the $V_{max}$ (maximal activity), and the $n_H$ (Hill coefficient) were calculated via nonlinear least squares fitting of the activity data to the Hill equation using Sigma Plot (SPSS Inc.).

Statistics—Results are presented as average ± S.E. of $n$ (number) independent experiments ($n$ is provided in the legends). Multiple comparison statistical analysis was carried out by one-way ANOVA followed by a post hoc Fisher least squares difference test (Statistica 8). $p < 0.05$ was considered statistically significant.

RESULTS

Peptides Corresponding to TM11 Region of 2b-tail Modulate SERCA1a Activity—We showed previously that when the 2b-tail is coupled to the SERCA1a isoform, it increases the apparent Ca^{2+} affinity of the pump (12). We therefore used purified SERCA1a reconstituted in proteoliposomes as a model system (15) to address the role of TM11 and Ser^{1031}.

First, SERCA1a was reconstituted into proteoliposomes in the presence or absence of pTM11, i.e. a peptide that corresponds to the predicted TM11 region (Thr^{1011}–Tyr^{1030}) of the 2b-tail, but which also includes the first three residues of the LE (Ser^{1031}–Asp^{1033}) (Fig. 1C). Several reconstitutions were carried out with increasing molar ratios of pTM11 (pTM11/SERCA1a molar ratios 0.15–4.5). $E$, normalized Ca^{2+}-dependent ATPase activity of SERCA1a in the absence (CT) or presence of increasing amounts of pTM11 (pTM11/SERCA1a molar ratios 0.15–4.5). $n = 3$–10 independent reconstitutions. Error bars, S.E.
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To exclude possible side effects of the peptide, we tested two peptide variants of pTM11 with alanine substitution at sites Pro1017 and Trp1028 (Fig. 2A). The specificity of the reconstitution system is further corroborated by the opposite effect of adding pTM11 or PLB on the apparent Ca²⁺ affinity. pTM11 peptides increase the apparent Ca²⁺ affinity, whereas addition of PLB lowers it. This is in line with their respective expected physiological performance (15, 17).

Therefore, we can conclude that in our reconstituted system, pTM11 is a specific regulator of SERCA1b that recapitulates the apparent Ca²⁺ affinity of the pump.

To rule out that the pTM11 effect on pump activity is not merely due to an aspecific hydrophobic interaction with the Ca²⁺ pump, we also tested the effect of a randomized pTM11 peptide (pTM11rand, supplemental Fig. 2). pTM11rand is predicted to be a TM-spanning peptide (predicted by the TMHMM software). Different molar ratios of pTM11rand to SERCA1a were tested (0.5–10, supplemental Fig. 2, B–D), i.e., in the same range as for the WT TM11 peptide. Although we confirmed that pTM11rand is incorporated into the proteoliposomes (supplemental Fig. 2B), we found in contrast to pTM11 no significant effect of pTM11rand on the maximal SERCA1a activity (supplemental Fig. 2C) and a modest, but not significant increase in $K_{Ca}$ (supplemental Fig. 2D).

The specificity of the reconstitution system is thus corroborated by the opposite effect of adding pTM11 or PLB on the SERCA1a apparent Ca²⁺ affinity.
Ser\textsuperscript{1031} occupies a key position in the 2b-tail. We knew that removing the last 12 amino acids (Ser\textsuperscript{1031}+) results in a full loss of the 2b-tail effect rendering a pump with SERCA2a-like properties (12, 13), whereas truncation at position Thr\textsuperscript{1032} displays a Ca\textsuperscript{2+} affinity between that of SERCA2a and SERCA2b (12).

Here, we wanted to recapitulate the findings of the SERCA2b truncation mutant Ser\textsuperscript{1031}+ by comparing the maximal effect of shorter pTM11 peptides in our reconstituted system. First, we tested the amidated peptides pS1031-CN and pT1032-CN, which, respectively, correspond to fragments of the 2b-tail that are found in the SERCA2b Ser\textsuperscript{1031}+ and Thr\textsuperscript{1032}+ mutants (Fig. 3A). Unexpectedly, the pS1031-CN variant altered the 

$K_{\text{Ca}}$ and $V_{\text{max}}$ to the same extent as the longer variants pTM11 and pT1032-CN (Fig. 3, C and D). This demonstrates that pTM11, and therefore also the corresponding TM11 stretch of the 2b-tail, represents a second, previously unrecognized functional region that acts independently from Ser\textsuperscript{1031} and the LE. However, this contrasts with our earlier conclusion that LE would be the only functionally important element in the 2b-tail (13).

Note that the SERCA2b truncation mutant Ser\textsuperscript{1031}+ contains a free C terminus, whereas with the peptide approach we used the peptide pS1031-CN that is amidated at the C terminus to better mimic the original peptide backbone. We therefore hypothesized that the presence of the free C terminus might lead to the complete loss of 2b-tail function in the SERCA2b truncation mutant Ser\textsuperscript{1031}+. To test this, we also determined the lead to the complete loss of 2b-tail function in the SERCA2b. SERCAa and SERCAb splice forms were indeed reported for Artemia franciscana (18) and Caenorhabditis elegans (19). At least in C. elegans, these variants showed structural and functional conservation to the vertebrate SERCA2a/b proteins (19).

In Fig. 4B, we attempted to reconstruct the phylogeny of the 2b-tail to pinpoint the most conserved regions of TM11. At least three different forms of the 2b-tail can be recognized in Bilateria (supplemental Fig. 3, A–C): a very short 2b-tail in Protostomia consisting of mainly a predicted TM11 region (supplemental Fig. 3, A–C); an intermediate long 2b-tail in vertebrates (Fig. 4B), and a much longer 2b-tail in proteins (19).
Nematoda with the most extended luminal and cytosolic parts (supplemental Fig. 3C). Thus, a LE, which, as we documented earlier, is a functional region in the vertebrate 2b-tail, is only found in vertebrates and Nematoda and is absent in Protostomia and lower Deuterostomia. This suggests that it is not an essential feature of the 2b-tail and that it evolved independently in Nematoda and Chordata. The pronounced difference in the peptide sequence and length of the LE supports this.

In contrast, the 11th TM helix forms the essential and ancestral part of the 2b-tail (Fig. 4B). Despite remarkable sequence differences, TM11 seems to have originated from a common ancestral sequence, since the TM11 sequences of Strongylocentrotus purpuratus (Echinodermata) and Saccoglossus kowalevskii (Hemichordata) resemble TM11 of the Protostomia better than TM11 of the vertebrates, whereas TM11 of Branchiostoma floridae (Cephalochordata) takes an intermediate position (Fig. 4B). Note that according to a helical wheel plot analysis, the most conserved residues are mainly found on one side of the helix, suggesting that these might be important for the interaction of the 2b-tail with the pump (supplemental Fig. 3D).

Among the Deuterostomia, the most conserved residues in TM11 are Ile1014, Leu1021, Leu1025, Val1026, Val1029, and Tyr1030 (hSERCA2b numbering, supplemental Fig. 3B, arrows), of which Ile1014, Leu1021, Leu1025, and Val1029 are grouped along one side of the helical wheel plot (supplemental Fig. 3D). The wheel plot further shows that the highly conserved side of the human TM11 helix contains residues Ile1014, Ser1015, Phe1018, Leu1021, Leu1025, and Trp1028, and Val1029. This overlaps well with the interface area of the published SERCA2b model consisting of the residues Pro1017, Phe1018, Leu1021, Leu1025, Trp1028, Val1029, and Tyr1030 (overlap underlined) (12).

Functional evidence further highlights the central role of some of these residues. We already reported that P1017A, W1028A, and Y1030A substitutions reduced the apparent affinity of SERCA2b (12). In contrast, F1018A and L1025A had no detectable effect (12), although these residues seem to be extremely well conserved and positioned on the conserved side of the helix for interaction (Fig. 4B and supplemental Fig. 2D). Our failure to detect a functional effect might however be due to the conservative nature of the substitutions. To test this, we now substituted Leu1025 and Phe1018 in the full-length SERCA2b by a polar glutamine. The F1018Q and L1025Q mutants were overexpressed in COS cells. The substitutions significantly increased the $K_{Ca}$ value of Ca$^{2+}$/H$^{+}$-dependent ATPase activity, which is in better agreement with the model and the conservation (Fig. 5).

Note that within TM11 the C-terminal part is most conserved across all species. It contains a number of conserved aromatic and/or hydrophobic residues (arrows in supplemental Fig. 3, A–C). This part of TM11 may thus hold a conserved functional feature. This is underscored by the functional effect of the W1028A and Y1030A mutations in the human SERCA2b (12). Furthermore, introduction of a free C terminus by truncation after Tyr1030 leads to a complete disruption of the 2b-tail effect.
In the vertebrate TM11, the N terminus is also remarkably well conserved (from the primitive Myxinidae Eptatretus burgeri to Homo sapiens in Fig. 4B), but it significantly differs from TM11 sequences in nonvertebrates and even early Deuterostomia. We previously showed that the P1017A mutation had an effect on the apparent Ca\(^{2+}\) affinity of SERCA2b (12), and we now show that the D1012A and G1013A mutations in SERCA2b drastically reduce the apparent Ca\(^{2+}\) affinity of SERCA2b (Fig. 5). These data point also to a crucial role of the TM11 conserved N-terminal part.

Together, TM11 is the essential and oldest part of the 2b-tail. We were able to point out TM11 residues that are highly conserved, positioned at the same side of the \(\alpha\)-helix, predicted to be in the interface area, and that are functionally important.

**DISCUSSION**

**TM11, the Oldest Functional Region of 2b-tail**—The current study demonstrates that TM11 acts as a previously unrecognized functional region of the 2b-tail. TM11 significantly increases the apparent affinity of SERCA for cytosolic Ca\(^{2+}\), but nearly halves the \(V_{\text{max}}\) of the pump. We showed that TM11 works independently from the LE, the other, earlier discovered and better described functional region of the 2b-tail. Phylogenetic analysis suggests that TM11, which is found in SERCA of most Bilateria, in fact represents the more primitive element of the 2b-tail and that its action is secondarily reinforced by a LE only in Nematoda and Chordata.

The 2b-tail increases the apparent Ca\(^{2+}\) affinity and reduces the \(V_{\text{max}}\) of the Ca\(^{2+}\) pump, explaining the kinetic differences between the housekeeping pump SERCA2b and the muscle variant SERCA2a (7, 10, 13). Detailed kinetic comparisons of SERCA2b and SERCA2a already showed that the 2b-tail reduces the off-rate for Ca\(^{2+}\) from the pump transport sites and slows down the E1P to E2P and E2P to E2 conversions (10). TM11 and LE may each have a different kinetic effect, but when combined they lead to the observed kinetic differences imposed by the 2b-tail. For instance, we described how LE might interact with upstream regions in the luminal domain of the pump. Modeling studies suggest that this holds the pump in the high Ca\(^{2+}\) affinity conformation E1 contributing to an increased apparent Ca\(^{2+}\) affinity with little effect on \(V_{\text{max}}\) (12).

Here, we described the functional effect of the 18-residue-long TM11 via co-reconstitution with SERCA1a in vitro. Because of the strong \(V_{\text{max}}\) effect of TM11, it is likely that TM11 specifically affects the E1P to E2P and E2P to E2 conversions because modeling studies indicate that these slower conformational changes may significantly reduce the \(V_{\text{max}}\) (12). Future detailed kinetic measurements on SERCA2 with or without TM11 will be required to verify this by comparing the kinetic effects of LE versus TM11.

We further show that both the N- and C-terminal parts of TM11 are important. A more puzzling, but key observation was that the presence of a free carboxyl group after residue Tyr\(^{1030}\) in the SERCA2b-Ser\(^{1031}\) mutant or in the pS1031-CO peptide abolishes the functional effect of TM11. However, the same peptide with an amidated carboxyl group (pS1031-CN) remains active and confers partial SERCA2b-like properties. Although pS1031-CO clearly associates with the membrane, we speculate that the additional negative charge of the free carboxyl group might prevent the approach of pS1031-CO to its binding site, e.g. as a result of charge repulsion by the phospho-head groups of the lipids. Alternatively, the C-terminal part of TM11 is extremely well conserved among different species, pointing to a critical functional role. A C-terminal carboxyl group might therefore interfere with the action of TM11.

**Comparison of Ca\(^{2+}\) Affinity Regulation by TM11 and PLB**—The best described affinity regulator of the Ca\(^{2+}\) pump is PLB. In contrast to TM11, the primary effect of PLB is to reduce the apparent Ca\(^{2+}\) affinity 2-fold. Earlier findings of our group showed that the presence of PLB increases the \(K_{\text{Ca}}\) of SERCA2b and SERCA2a to the same extent (i.e. in the presence or absence of the 2b-tail) (11, 12). The opposite and independent effect of PLB and TM11 on the pump therefore strongly indicates that the 2b-tail and PLB have different binding sites and different modes of action, making it unlikely that PLB has an effect on TM11 function. The opposite effect on the apparent affinity is in part related to the fact that PLB stabilizes the E2 conformation (9), whereas the 2b-tail stabilizes E1 (11, 12). An independent working mechanism of PLB and TM11 is also in agreement with our structural model of SERCA2b, postulating that TM11 interacts at site TM7, 10 (11, 12), whereas the TM region of PLB presumably interacts at TM2, 4, 6, 9 (9). Note that in addition to a TM domain, PLB contains a cytosolic domain that is regulated by phosphorylation, whereas the 2b-tail contains TM11 and a LE, which are not regulated by phosphorylation.

Different from PLB, TM11 forms an intrinsic element of the SERCA2b pump, but here we show that it preserves its function when it is added in trans, i.e. uncoupled from the pump. The analysis of SERCA1a2b chimera already pointed out that the interaction site of TM11 is highly conserved in SERCA1a (12), which makes the SERCA1a proteoliposome system extremely suitable to study TM11. The maximal effect of TM11 was observed at molar ratio 4.5, which is in the same molar range of PLB inhibition (effective SERCA:PLB molar ratio is 1:5) (15), pointing to a high affinity interaction with the pump. Note that the maximal effect occurs at a higher molar ratio than in the endogenous situation of SERCA2b where the 2b-tail is coupled to the pump in a 1:1 ratio. Several factors might explain this higher ratio. The ratio 4.5 describes the amount of peptide and pump added to the system, but the actual ratio reconstituted in the proteoliposomes is difficult to estimate. Also, as our bioti-
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It is difficult to overlook the striking parallel in the mode of regulation of $\text{Na}^+\text{,K}^+\text{-ATPase}$ and SERCA by their respective TM interactors (i.e. the $\beta$- and $\gamma$-subunits of the $\text{Na}^+\text{,K}^+\text{-ATPase}$ versus the $2b$-tail and PLB in SERCA) (12). In all of these cases, single TM helix interacts with the anchoring region of the pump, which is critical for ion affinity regulation (12, 23). Thus, like in $\text{Na}^+\text{,K}^+\text{-ATPase}$ the anchoring region of the $\text{Ca}^{2+}\text{-ATPase}$ might represent a previously unrecognized regulatory site.

**Mechanism of TM11 Regulation in the Anchoring Region of SERCA $\text{Ca}^{2+}\text{-Pump}$**—Because the structure of the overall TM domain is remarkably conserved between $\text{Na}^+\text{,K}^+\text{-ATPase}$ and SERCA (12), we compared the published structure of TM7 of $\text{Na}^+\text{,K}^+\text{-ATPase}$ in the presence of the $\beta$-subunit (Fig. 6D) with that of SERCA1a in the absence of TM11 (Fig. 6C). Differences in the position of TM7 between the SERCA1a and Na$^+\text{,K}^+\text{-ATPase}$ the anchoring region of the $\text{Ca}^{2+}\text{-ATPase}$ might represent a previously unrecognized regulatory site.

**Proposed mechanism of TM11.** A, sequence alignment of TM7 of human and chicken SERCA1a, SERCA2b, and Na$^+\text{,K}^+\text{-ATPase}$. Numbers on top refer to sequence numbering of the glycines in hSERCA2. B, proposed model of TM11 mechanism. Left, in SERCA1a, TM7 undergoes a tilt between the E1 and E2 conformation. Center, in Na$^+\text{,K}^+\text{-ATPase}$ in the presence of the $\beta$-subunit, the upper part of TM7 remains in the E1 conformation, whereas the lower part can shift to E2. The binding of TM7 might be supported by conserved glycines in TM7. Right, conserved glycines in TM7 of SERCA2b might also support the binding of TM7 in the presence of TM11. This might prevent the repositioning of TM7 during the E1–E2 conversions. C–E, schematics of published SERCA1a structure (C; E2, 1WPG) (Ref. 27), $\alpha\beta\gamma\text{Na}^+\text{,K}^+\text{-ATPase}$ (D; E2) (Refs. 20, 21), and the SERCA2b structural model (E; E1) (Ref. 12). TM7 is shown in blue for SERCA1a and SERCA2b, and in green for Na$^+\text{,K}^+\text{-ATPase}$. The conserved glycines are shown in red.

nylation results indicate, half of pTM11 is inserted in the opposite orientation. Together, a higher concentration is required for a maximal effect than in the SERCA2b pump where a high local concentration of TM11 is guaranteed by the fusion of the $2b$-tail to the pump.

**Similarities between SERCA/TM11 and $\alpha\beta\text{Na}^+\text{,K}^+\text{-ATPase}$.** Interactions Point to Anchoring Region as Emerging Site of Regulation—Our structural model of SERCA2b predicts that TM11 occupies a binding site formed by TM7 and TM10, i.e. a remote and relatively immobile position in the anchoring region of the pump (12). This is somewhat difficult to reconcile with the strong functional effect of pTM11 on $V_{\text{max}}$ and $K_{\text{Ca}}$. In the absence of structural information of SERCA2b, it is difficult to understand how TM11 regulates the enzymatic properties of the pump at the anchoring domain.

At least in Na$^+\text{,K}^+\text{-ATPase}$ the anchoring region emerges as a site of regulation. Crystal structures of $\alpha\beta\gamma$-Na$^+\text{,K}^+\text{-ATPase}$ illustrate that the $\gamma$- (on TM9), $\beta$- (on TM7), and auto-inhibitory C terminus (between TM7 and TM8) all interact at the anchoring region (20–22). Thus, besides a structural feature for stabilizing the pump in the lipid bilayer, the helices TM7–TM10 appear to have a dynamic role in Na$^+\text{,K}^+\text{-ATPase}$ regulation. Indeed, the E2–E1 transition of Na$^+\text{,K}^+\text{-ATPase}$ is accompanied by the straightening of TM5, which pushes TM7 toward TM10. Because of an extensive hydrogen-bonding network, this induces large scale structural changes involving parts of the phosphorylation domain (P1), TM3, and the C terminus of the $\alpha$-subunit, as well as the $\beta$-subunit (21). This network might explain why mutations in the TM of the $\beta$-subunit alter the apparent K$^+$ affinity of the Na$^+\text{,K}^+\text{-ATPase}$ (23).
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We hypothesize that in SERCA2b, TM7 might undergo a similar bending because three extremely conserved glycine residues are found (G841, G842, and Gly850) (Fig. 6A). However, in the SERCA1a crystal structures, i.e. in the absence of TM11, TM7 is always a straight helix, which slightly shifts position between E1 (closer to TM5 as also pointed out in the E1 SERCA2b model in Fig. 6E) and E2 (further from TM5) (Fig. 6C). Note that the position of the N-terminal part of TM7 in \( \text{Na}^+ \)-ATPase (Fig. 6D) corresponds well with the E1 position of TM7 in SERCA1a (Fig. 6C). We therefore hypothesize that Gly841 or Gly845 in SERCA2b facilitates the bending of TM7, which would be imposed by the presence of TM11. TM11 might keep the N-terminal part of TM7 in the E1 position, whereas the lower part of TM7 might be allowed to shift between an E1 and E2 position (Fig. 6B). Thus, TM11 might restrict the movement of TM7, which would reduce the maximal turnover rate and increase the apparent Ca\(^{2+}\) affinity.

TM7 bending could for instance reposition the cytosolic loops L6–7 and/or L8–9, which tightly connect the TM and phosphorylation domains via an extensive hydrogen bond network (24). Because these loops mediate the communication between both domains, their repositioning could have an impact on the kinetics of phosphorylation and dephosphorylation (24), which would be in line with the strong effect of the 2b-tail on the E1P to E2P, and E2P to E2 conversions (10). At least some mutations in L6/7 are known to affect the Ca\(^{2+}\) affinity of the pump pointing to L6/7 as a possible mediator for TM11 function (25).

Alternatively, TM11 might directly alter the packing of the other TM helices closer to the Ca\(^{2+}\)-binding sites affecting the Ca\(^{2+}\) affinity. Another possibility is that TM11 might promote the unwinding of TM7 in SERCA2b at the conserved glycines Gly841 and/or Gly845. Indeed, in the \( \text{Na}^+\cdot\text{K}^+\cdot\text{ATPase} \) the \( \beta \)-subunit promotes unwinding of TM7 at position Gly855, which is of central importance for \( \text{K}^+ \) binding (21). Note that the glycines on TM7 of the Ca\(^{2+}\)-pump are also part of a classical GXXG helix-helix interaction motif, mediating the interaction with TM5 (supplemental Fig. 4B). The GXXG sequence in TM7 of \( \text{Na}^+\cdot\text{K}^+\cdot\text{ATPase} \) is, however, not a typical helix-helix interaction motif (supplemental Fig. 4, A and C), and in the PMR1 and PMCA Ca\(^{2+}\)-ATPases a much lower number of glycines in TM7 is found (supplemental Fig. 4A).

Together, the conserved glycines in TM7 of the Ca\(^{2+}\)-pump and Na\(^+\),K\(^+\)-ATPase might support important structural features as helix unwinding, bending, and TM interaction in the presence of respectively TM11 and the \( \beta \)-subunit. These similarities between TM11 and the \( \beta \)-subunit point to an interesting example of convergent evolution (12). A SERCA2b crystal structure will be required to get more detailed insight into the structural changes that accompany the TM11 interaction, but so far, purification of SERCA2b for subsequent crystallization is hindered by its low tissue expression levels (26).

We conclude that TM11 is a previously unrecognized, but highly conserved functional region of the SERCA2b Ca\(^{2+}\) pump. TM11 acts independently from PLB, presumably by restricting the movement of TM7, which could explain why TM11 exerts a strong effect on the maximal turnover rate and apparent Ca\(^{2+}\) affinity.

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