Cytosolic Phosphorylation of Calnexin Controls Intracellular Ca\(^{2+}\) Oscillations via an Interaction with SERCA 2b

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Abstract. Calreticulin (CRT) and calnexin (CLNX) are lectin chaperones that participate in protein folding in the endoplasmic reticulum (ER). CRT is a soluble ER luminal protein, whereas CLNX is a transmembrane protein with a cytosolic domain that contains two consensus motifs for protein kinase (PK) C/proline-directed kinase (PDK) phosphorylation. Using confocal Ca\(^{2+}\) imaging in Xenopus oocytes, we report here that coexpression of CLNX with sarco endoplasmic reticulum calcium ATPase (SERCA 2b) results in inhibition of intracellular Ca\(^{2+}\) oscillations, suggesting a functional inhibition of the pump. By site-directed mutagenesis, we demonstrate that this interaction is regulated by a COOH-terminal serine residue (S562) in CLNX. Furthermore, inositol 1,4,5-trisphosphate–mediated Ca\(^{2+}\) release results in a dephosphorylation of this residue. We also demonstrate by coimmunoprecipitation that CLNX physically interacts with the COOH terminus of SERCA 2b and that after dephosphorylation treatment, this interaction is significantly reduced. Together, our results suggest that CRT is uniquely regulated by ER luminal conditions, whereas CLNX is, in addition, regulated by the phosphorylation status of its cytosolic domain. The S562 residue in CLNX acts as a molecular switch that regulates the interaction of the chaperone with SERCA 2b, thereby affecting Ca\(^{2+}\) signaling and controlling Ca\(^{2+}\)-sensitive chaperone functions in the ER.

Key words: phosphorylation • calnexin • ER lectin chaperones • Ca\(^{2+}\) ATPases • Ca\(^{2+}\) signaling

Introduction

Multiple cellular processes, including the control of gene transcription (Dolmetsch et al., 1998; Li et al., 1998), growth cone formation in neurons (Gomez and Spitzer, 1999), and activation of mitochondrial respiration (Robb-Gaspers et al., 1998) are encoded by spatial–temporal aspects of intracellular Ca\(^{2+}\) oscillations. Repetitive cytosolic Ca\(^{2+}\) transients in Xenopus oocytes are under the control of two major opposing factors: Ca\(^{2+}\) release through the inositol 1,4,5-trisphosphate receptor channel (IP\(_3\)R) (Iino, 1990; Parker and Iorva, 1990; Bezpovavny et al., 1991; Finch et al., 1991) and Ca\(^{2+}\) reuptake into the ER Ca\(^{2+}\) store by the Ca\(^{2+}\) ATPases (Camacho and Lechleiter, 1993). Sarco endoplasmic reticulum calcium ATPase (SERCA)–type ATPases control the frequency of IP\(_3\)-induced Ca\(^{2+}\) oscillations in Xenopus oocytes (Camacho and Lechleiter, 1993). We have demonstrated previously that calreticulin (CRT), a lectin chaperone located in the ER, inhibits these oscillations and specifically targets SERCA 2b (Camacho and Lechleiter, 1995a; John et al., 1998). The COOH terminus of SERCA 2b, unlike that of SERCA 2a, faces the ER lumen and contains an asparagine residue that forms part of a consensus site for N-linked glycosylation (N1036) (Gunteski-Hamblin et al., 1988) that we demonstrated is required for interaction with CRT (John et al., 1998). From these data we concluded that a functional interaction between CRT and SERCA 2b may be required for inhibition of these oscillations.

CRT is a soluble luminal ER protein, whereas calnexin (CLNX) and its testis-specific isoform, calmegin (CLMG), are type I transmembrane (TM) proteins (Michalak et al., 1992; Bergeron et al., 1994; Ohsako et al., 1994; Watanabe et al., 1994). This family of molecular chaperones is characterized by a luminal domain that contains a motif of 17 amino acids repeated three times in CRT and four in CLNX and CLMG (Fliegel et al., 1989; Michalak et al., 1992; Ohsako et al., 1994; Watanabe et al., 1994). This do-
main is responsible for interaction of the chaperone with the monoglycosylated form of N-linked glycoproteins during protein folding in the ER (Vassilakos et al., 1998). The presence of this conserved domain led us to test whether CLNX, like CRT, also inhibits Ca\textsuperscript{2+} oscillations. Here, we demonstrate that CLNX inhibits Ca\textsuperscript{2+} oscillations in a manner consistent with inhibition of SERCA 2b. Furthermore, as was the case for CRT, repetitive Ca\textsuperscript{2+} waves were unaffected by coexpression of CLNX with a SERCA 2b mutant lacking the luminal asparagine (SERCA 2b-N1036A).

In contrast to CRT, CLNX has in its cytosolic domain consensus sites for phosphorylation by protein kinase (PK) C, casein kinase II, PKA, and proline-directed kinase (PDK) (Tjoelker et al., 1994; Wong et al., 1998). Recently, serine 562 located close to the COOH terminus of CLNX has been shown to support phosphorylation by the PDK, extracellular-signal-regulated kinase 1 (ERK-1) (Wong et al., 1998), and was implicated in regulating an interaction of CLNX with ribosomal proteins (Chevet et al., 1999). The second residue, S485, faces the cytosol in proximity to the single TM segment. However, phosphorylation of this residue has not eluded detection (Wong et al., 1998). Since activation of the IP\textsubscript{3} signaling pathway results in the production of both IP\textsubscript{3} and diacylglycerol (DAG), which is a known activator of PKC, we have also tested whether the two consensus motifs for PKC/PDK phosphorylation in CLNX play a role in the modulation of Ca\textsuperscript{2+} oscillations. Here, we demonstrate that when the S562 is mutated to an unreactive alanine (S562A), Ca\textsuperscript{2+} oscillations are no longer inhibited, suggesting that S562 in CLNX plays a critical role in the regulation of Ca\textsuperscript{2+} oscillations. The presence of the S485 was required for regulation by S562. By immuno précipitation of CLNX from \textsuperscript{32P}-labeled oocytes, we demonstrate that CLNX becomes de-phosphorylated in S562 after mobilization of intracellular Ca\textsuperscript{2+} by IP\textsubscript{3}. Furthermore, we demonstrate by coimmunoprecipitation that CLNX physically interacts with the COOH terminus of SERCA 2b after phosphoacceptor sites in the serine kinase (A537 to the COOH terminus). This primer had sequence 5'CTGGAGATCATGGCTGAAGAAGATGGTGG-3'.

**Materials and Methods**

**Construction of Expression Vectors**

A II cDNAs used in this study were subcloned between the 5' and 3' untranslated regions of X enusop laevis \(\beta\)-globin into a vector (pH N) as described previously (Camacho and Lechleiter, 1995a,b). The cDNA encoding rat CLNX (Tjoelker et al., 1994) was excised from the pH-CMV vector by BamHI and XbaI (restriction enzymes from GIBCO BRL) digestion and subcloned between the corresponding sites in a variant vector of pH N containing additional restriction sites in the multiple cloning site (pHN-CLNX). Mutations in the consensus sites for PKC/PDK in CLNX were generated using the Quick-Change™ mutagenesis kit (Stratagene). The introduction of the desired mutation(s) in the plasmid recombinants was determined by automatic sequencing by the UTHSCA core facility. A II oligonucleotides used to generate mutations were purchased from Operon Technologies. The CLNX-5485A mutant was generated using the forward primer with sequence 5'-CTGGTGAATCCTGCCCTGGTGCGTGCGTGAAGAAGATGGTGG-3' and reverse primer with sequence 5'-GGAAGGCTGGTTTCCCTCCAGCACAGCAGAAGAGGATCACAAGG-3', and using as template pH N-CLNX. The CLNX-S562A mutant was generated using the forward primer with sequence 5'-GGAGGATCCATGCCCCCCTGGGAGAACATCTGGGCTG-3' and incorporated a BamHI site at its 5' end. The antisense oligonucleotide had sequence 5'-ACTGGGATCCATGCCCCCCTGGGAGAACATCTGGGCTG-3', which is complementary to the 3' untranslated region of X enusop \(\beta\)-globin. The product of this PCR amplification was digested with BamHI and XbaI and after purification was subcloned into vector pH N and similarly digested (plasmid pH N-CLNX) for sequencing. The CLNX cytosolic peptide was generated by exchanging the RsaI to NheI fragment containing the sequence 5'-ACTGGGATCCATGCCCCCCTGGGAGAACATCTGGGCTG-3' incorporating a BamHI site and as the reverse primer 5'-GCTTAGAGACTCCATTC-3', which is complementary to the 3' untranslated region of X enusop \(\beta\)-globin present in the template (pHN-SERCA 2b). The PCR products were digested with BamHI and HindIII and ligated to the similarly digested X enusop expression vector, pH N. The final constructs encode the COOH terminus of SERCA 2b and SERCA 2b-N1036A starting at the methionine residue 924, which is located just before TM 9 (Bayle et al., 1995). The plasmids were named pH N-SERCA 2b/TM 9-11 and pH N-SERCA 2b-N1036A/TM 9-11. A similar strategy was followed to generate the SERCA 2a/TM 9-10 using as PCR template plasmid pH N-SERCA 2a (J ohn et al., 1998). All vectors generated were fully sequenced automatically by the core facility at the UTHSCA.

**In Vitro Transcription and Oocyte Protocols**

Synthetic mRNA was prepared as described previously (Camacho and Lechleiter, 1995a). Plasmids were linearized with NotI, except for plasmid pH N-CLNX, which was linearized by NheI digestion. Transcription initiated at the T7 promoter was performed using the M egascript™ high yield transcription kit and capped with m7G(5')ppp(5') (both from Ambion). A II synthetic mRNA s were resuspended at a concentration of 1.5–2.0 \(\mu\)g/\(\mu\)l and stored in aliquots of 3 \(\mu\)l at \(-80^\circ\)C. Stage VI defolliculated oocytes were injected with a bolus of 30 nl of mRNA using a standard positive pressure injector (Nanoject; Drummond Scientific). A fer tRNA injection concentration was cultured for 5–7 d until Ca\textsuperscript{2+} oscillations were unobserved. In Ca\textsuperscript{2+}-sensitive man-
In each group were injected with 1P3 (−300 nM final; Calbiochem). Oocytes were instantly frozen on dry ice 6 min after the 1P3 injection. During these treatments, oocytes were maintained in ND96 buffer, which contained 1 mM EGTA, 96 mM NaCl, 2 mM KCl, 2 mM MgCl2, 5 mM Hepes, pH 7.5. Membrane extracts were prepared as follows: oocytes were homogenized in lysis buffer (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 2 mM EDTA, 0.5 mM EGTA) supplemented with phosphatase inhibitors (10 mM NaF, 0.4 mM Na3VO4, 0.1 mM ZnCl2, 1 mM Na3P04) and the protease inhibitors 15 gM PMSF, 20 gM leupeptin, and 250 gM pepstatin A final). The oocytes were extracted twice in volumes of 300 l, with the particulate fraction being removed by centrifugation at 10,000 g for 15 s between each extraction. The membrane fraction was isolated by centrifugation for 1 h at 100,000 g. Membrane proteins were extracted at room temperature in solubilization buffer (10 g/100 g oocyte) consisting of lysis buffer containing 1% IGEPAL (Sigma Chemical Co.) and 3% SDS for 20 min. The soluble fraction was then isolated by centrifugation at 10,000 g for 5 min. A filter a fivefold dilution in lysis buffer, 10 g/100 g of a 50% suspension of protein A–Sepharose (pretreated by incubation 3 × 20 min in 5% BSA) was added to the membrane fraction. A filter over-end rotation at room temperature for 2 h, the protein A–Sepharose was removed by centrifugation. The CLN X antibody was added at a dilution of 1:250 and incubated with over-end rotation at 4°C overnight. To the cell lysate, 8 g/100 g of the 50% suspension of pretreated protein A–Sepharose was added and incubated with over-end rotation for 1 h at room temperature. The immune complexes were washed three times with lysis buffer containing 1% IGEPAL and harvested by centrifugation and eluted from the protein A–Sepharose by boiling in 4 min in Laemmli SD S sample buffer. The proteins were separated by SD S-PAGE through an 8% gel. A filter the gel was fixed and dried, the proteins were visualized by autoradiography. 

Confocal Imaging of Intracellular Ca2+ 

Ca2+ activity was imaged with the fluorescent Ca2+ indicator, Oregon green I (12.5 mM final concentration assuming an oocyte volume of 1 µl) (Molecular Probes, Inc.). With exception to experiments using pyruvate malate-treated oocytes, confocal imaging was performed using a NORA N OZ confocal laser scanning microscope at zoom 0.7 attached to a Nikon 100 E microscope inverted microscope. A 20× (0.75 NA) water immersion Nikon objective was used and images were collected at 0.5-s intervals. The confocal slit was set at 50 µm. Confocal imaging of pyruvate malate-treated oocytes was performed using a Zeiss confocal laser scanning microscope (LSM 310) at zoom 2 attached to a Zeiss plan apochromat 100× objective. Images were analyzed using ANALYZE software (M d@ Foundation) on a Silicon Graphics O2 workstation. Ca2+ waves were analyzed using a TLA 45 rotor (Oliver et al., 1996). 


calcium 2+ oscillations

To determine whether the COOH terminus of SERCA 2b was glycosylated, microsomes were isolated from in vitro translation reactions programmed with appropriate mRNAs as described above. The isolated microsomes were solubilized by boiling in endo H denaturing buffer (New England Biolabs, Inc.) for 10 min. To half of each reaction, one tenth volume of 2 mM and incubated for 5 min at 30°C. Nascent chains were released from ribosomes by treatment with puromycin (Fisher Scientific), which was added to a concentration of 1 mM and incubated for 5 min at 30°C. To inhibit ongoing translation, emetine (Sigma Chemical Co.) was added to a final concentration of 2 mM and incubated on ice for 5 min. Finally, the micromolar fraction was isolated by ultracentrifugation at 125,000 g for 12 min through a high salt/sucrose cushion and processed for coimmunoprecipitation. 

Results

Overexpression of CLNX in Xenopus Oocytes Inhibits Intracellular Ca2+ Oscillations

Although CRT has been shown to modulate different aspects of Ca2+ signaling (Bastianutto et al., 1995; M ery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; J ohn et al., 1998; Roderick et al., 1998; M esaeti et al., 1999), most of these effects were attributed to CRT’s ability to bind Ca2+ with high capacity (Baksh and Michalak, 1991; Bastianutto et al., 1995; M ery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; Roderick et al., 1998; M esaeti et al., 1999). However, we demonstrated by deletion mutagenesis that the inhibition of Ca2+ waves observed when CRT is overexpressed in Xenopus oocytes survives deletion of the high capacity Ca2+ binding C-domain (Camacho and Lechleiter, 1995a; J ohn et al., 1998). Since CLNX shares homology to CRT on the P-domain and both have been characterized as lectin chaperones, we tested whether CLNX would also modulate Ca2+ oscillations. CLNX–overexpressing oocytes injected with 1P3 (300 nM final) were imaged confocally 5 s after mRN A injection. Very few of these oocytes exhibited Ca2+ oscillations (12%; n = 17). These oscillations are rarely observed in control, nonoverexpressing oocytes after 6 d of culture (17%; n = 18). Thus, we tested the effects of CLNX overexpression under conditions that reliably elicit robust Ca2+ oscillations (i.e., overexpression of SERCA pumps or energization of mitochondria with pyruvate/malate) (Camacho and Lechleiter, 1993, 1995a; J ohn et al., 1998;
Jouaville et al., 1995). Compared with oocytes overexpressing SERCA2b alone, oocytes coexpressing CLNX + SERCA2b displayed inhibition of high frequency Ca\(^{2+}\) oscillations (Fig. 1a). A large percentage of oocytes overexpressing SERCA2b alone exhibited high-frequency repetitive Ca\(^{2+}\) waves (88%; \(n = 52\)), whereas this percentage was significantly lower in CLNX + SERCA2b oocytes (30%; \(n = 63\)) (Fig. 1b, left, and Table I). In addition, in comparison to SERCA2b-overexpressing oocytes, CLNX + SERCA2b coexpressing oocytes appear to have a more rapid initial rise in cytosolic Ca\(^{2+}\), suggesting that in these
Table I. Detailed Analysis of Ca\textsuperscript{2+} Wave Activity in Oocytes that Exhibited Repetitive Ca\textsuperscript{2+} Waves

| Oocytes per group | Oocytes displaying oscillations | Wave Period | Decay Time |
|-------------------|--------------------------------|-------------|------------|
| n                 | %                             | \(t\)       | \(s\)      |
| SERCA2b           | 52                            | 88.0        | 2.49 ± 0.01| 0.62 ± 0.01|
| CLNX + SERCA2b    | 63                            | 30.0\(a\)   | 3.13 ± 0.02| 0.93 ± 0.01\(a\) |
| CLNX-S562A + SERCA2b | 36                        | 92.0\(b\)   | 3.06 ± 0.02| 0.73 ± 0.01\(b\) |
| CLNX-S485A + SERCA2b | 27                       | 15.0        | 3.45 ± 0.04| 1.05 ± 0.02|
| CLNX-S562A/S485A + SERCA2b | 34                 | 26.5        | 3.42 ± 0.02| 1.07 ± 0.01\(b\) |
| CLNX\(\textsubscript{S562A} + CLNX + SERCA2b | 18                           | 72.0\(d\)   | 2.97 ± 0.03| 0.79 ± 0.01\(d\) |
| SERCA2b-N1036A   | 20                            | 100         | 1.88 ± 0.01\(e\)| 0.35 ± 0.02|
| CLNX + SERCA2b-N1036A | 19                       | 100         | 2.10 ± 0.03| 0.46 ± 0.007**|

Mean ± SEM. \(n\), total number of oocytes per category. Percentage is the percent of \(n\). The statistical significance was analyzed with a Chi-square test for comparisons related to the percentage of oocytes displaying oscillations \((P < 0.005)\). All other comparisons were tested for statistical significance using \(t\) test, and \(P\) values are indicated.

\(a\)CLNX + SERCA2b is not equivalent to SERCA2b \((P < 0.005)\).
\(b\)CLNX-S562A + SERCA2b is not equivalent to CLNX + SERCA2b \((P < 0.01)\).
\(c\)CLNX-S562A/S485A + SERCA2b is not equivalent to CLNX + SERCA2b \((P < 0.05)\).
\(d\)CLNX\(\textsubscript{S562A} + CLNX + SERCA2b is not equivalent to CLNX + SERCA2b \((P < 0.05)\).
\(e\)SERCA2b-N1036A is not equivalent to SERCA2b \((P < 0.05)\).

**CLNX is not equivalent to control \((P < 0.005)\).**

Oocytes, rapid amplification of Ca\textsuperscript{2+} release occurs because the Ca\textsuperscript{2+} ATPase is inhibited. Therefore, we performed measurements of interwave period and decay time of individual waves \((t_{1/2})\), which reflect the Ca\textsuperscript{2+} uptake process (John et al., 1998). Compared with control oocytes overexpressing SERCA2b alone, oocytes coexpressing CLNX + SERCA2b had statistically significantly longer interwave periods and longer decay times for individual waves (Fig. 2 b, middle and right, and Table I).

Energization of mitochondria by injection of pyruvate malate (10 mM final) results in a Ca\textsuperscript{2+} tide followed by robust, low-frequency Ca\textsuperscript{2+} waves (Fig. 2 a). Overyexpression of CLNX alone in oocytes injected with pyruvate malate results in inhibition of Ca\textsuperscript{2+} oscillations. Specifically, the percentage of oocytes displaying oscillations was significantly reduced by CLNX overexpression in relation to control nonoverexpressing oocytes (Fig. 2 b, left, and Table II). More importantly, interwave periods and decay times for individual waves were significantly increased in CLNX-overexpressing oocytes (Fig. 2 b, middle and right, and Table II).

The functional interaction of CRT with SERCA2b requires the presence of a luminal asparagine in the pump (John et al., 1998). To determine whether the mechanism of inhibition by CLNX also involves this asparagine, we overexpressed SERCA2b-N1036A and compared these oocytes to oocytes coexpressing CLNX + SERCA2b-N1036A (Fig. 3). High-frequency repetitive Ca\textsuperscript{2+} waves without a preceding tide were observed in all of these oocytes (Table I). As was the case with CRT (John et al., 1998), CLNX did not inhibit Ca\textsuperscript{2+} oscillations when coexpressed with SERCA2b-N1036A. Thus, the percentage of oocytes exhibiting repetitive Ca\textsuperscript{2+} wave activity was unchanged in oocytes coexpressing CLNX \((n = 19; 100\%\), relative to control oocytes \((n = 20; 100\%)\) (Table I). In addition, there is no statistical difference between SERCA2b-N1036A and CLNX + SERCA2b-N1036A overexpressing oocytes in interwave periods (Table I). Thus, both members of this family of chaperones appear to functionally interact with SERCA2b, and mutagenesis of the luminal asparagine interferes with the manifestation of this inhibitory effect.

**CLNX Physically Interacts with the COOH Terminus of SERCA2b**

In the preceding experiments we demonstrated a functional interaction between CLNX and SERCA2b but not with SERCA2b-N1036A. In the modulation of protein folding, CLNX has been shown to interact with substrates by two mechanisms, either by a lectin interaction with the monoglucosylated N-linked glycans, or by a more classical chaperone protein–protein interaction (Helenius et al., 1997; Iharra et al., 1999). Thus, our results with the N1036A mutant could be attributed either to the removal of the N-linked glycan or to a change in charge, which undermines the interaction of the Ca\textsuperscript{2+} ATPase with CLNX. To characterize this interaction, we first determined whether SERCA2b is glycosylated. We generated constructs encoding the COOH terminus of SERCA2b and its N1036A mutant starting at TM9 (i.e., SERCA2b/TM9-11 and SERCA2b-N1036A/TM9-11), as well as of SERCA2a/TM9-10 to use as control (Gunterski-Hamblin et al., 1988). Correct polytopic insertion of the translation products in the ER membrane has been demonstrated previously for similar constructs in microsomes (Baylor et al., 1995). A 5′ mRNA synthesis, translation was performed in rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. All constructs were efficiently translated and run at the correct predicted molecular masses (SERCA2b and SERCA2b-N1036A/TM9-11, ~13.2 kD; and SERCA2a ~7.2 kD). Posttranslational processing was observed on a
protein glycosylated on three asparagine residues that served as positive control (glycosylated S. cerevisiae α-factor, ∼30 kD). SERCA2b and the N1036A mutant migrate at a similar rate, indicating that SERCA2b might not be glycosylated (Fig. 4 a). To corroborate this, we treated in vitro translated SERCA2b/TM9-11 and SERCA2b-N1036A/TM9-11 products with endoglycosidase H (endo H). Under the experimental conditions, S. cerevisiae α-factor underwent de-glycosylation by endo H (Fig. 4 b). However, endo H treatment did not alter the mobility of SERCA2b/TM9-11 or of SERCA2b-N1036A/TM9-11, indicating that SERCA2b is not glycosylated.

Since CLNX has been shown to interact also via protein–protein interactions regardless of whether the substrate is glycosylated (Ihara et al., 1999), the possibility remained that CLNX would interact with the COOH terminus of SERCA2b. To test this hypothesis, we performed coimmunoprecipitations in which endogenous CLNX present in the pancreatic microsomes was immunoprecipitated with a CLNX-specific antibody. Coimmunoprecipitated proteins were subsequently detected by fluorography. SERCA2b, and to a significantly lower extent, SERCA2a-N1036A were coimmunoprecipitated (0.61 ± 0.02, P < 0.005, n = 5) (Fig. 4 c). More importantly, no interaction with the COOH terminus of SERCA2a was detected.

Figure 3. CLNX does not inhibit Ca\textsuperscript{2+} oscillations induced by overexpression of the SERCA2b-N1036A mutant. Confocal Ca\textsuperscript{2+} imaging in an oocyte overexpressing SERCA2b-N1036A after an injection of IP\textsubscript{3} (∼300 nM final) shows high-frequency Ca\textsuperscript{2+} oscillations (top). These oscillations are not inhibited in oocytes coexpressing CLNX + SERCA2b-N1036A (bottom). Bar, 50 μm.

Figure 4. CLNX coimmunoprecipitates with the COOH terminus of SERCA2b. In vitro translations of synthetic mRNAs were performed in rabbit reticulocyte lysate supplemented with canine pancreatic microsomes and L-[\textsuperscript{35}S]methionine. Samples were subjected to 15% SDS-PAGE and detected by fluorography. (a) Membrane fractions isolated from in vitro translation reactions were loaded as follows: S. cerevisiae α-factor (lane 1), negative control without RNA (lane 2), SERCA2a/TM9-10 (lane 3), SERCA2b/TM9-11 (lane 4), and SERCA2b-N1036A/TM9-11 (lane 5). (b) Endo H treatment demonstrates that the COOH terminus of SERCA2b is not glycosylated. Samples were loaded as follows: negative control without mRNA (lane 1), paired samples were loaded ± endo H as follows: S. cerevisiae α-factor (second set of lanes), SERCA2b/TM9-11 (third set of lanes), SERCA2b-N1036A/TM9-11 (fourth set of lanes), and SERCA2a/TM9-10 (fifth set of lanes). (c) Coimmunoprecipitations of CLNX with SERCA COOH terminus constructs demonstrates an interaction of CLNX with SERCA2b, a reduced interaction with the SERCA2b-N1036A mutant, but no interaction with SERCA2a. Lanes were loaded as follows: negative control without RNA in the translation (lane 1), S. cerevisiae α-factor (lane 2), SERCA2a/TM9-10 (lane 3), SERCA2b/TM9-11 (lane 4), and SERCA2b-N1036A/TM9-11 (lane 5).
Finally, the S. cerevisiae α factor was efficiently coimmunoprecipitated, indicating that a lectin interaction with CLNX may be at play. These results demonstrate that CLNX specifically interacts with SERCA2b and that mutagenesis of N1036 in SERCA2b drastically interferes with this interaction, possibly by changing the charge at the COOH terminus of the Ca^{2+}ATPase.

**Phosphorylation in the Cytosolic Domain of CLNX Regulates the Inhibition of Ca^{2+} Oscillations**

Stimulation of the IP_3-mediated Ca^{2+} signaling cascade activates PKC, which subsequently phosphorylates downstream targets (Berridge and Irvine, 1989; Berridge, 1993; Newton, 1995). The presence of consensus sites for phosphorylation by PKC/PDK in the cytosolic domain of CLNX raises the possibility that their state of phosphorylation modulates ER lumenal interactions with SERCA2b. To test this hypothesis, we first generated mutants of CLNX in which PKC/PDK consensus phosphorylation sites were changed to unreactive alanines (Fig. 5). We then confocally imaged IP_3-induced Ca^{2+} release in oocytes that were coexpressing these CLNX mutants with SERCA2b, and compared their activity to oocytes coexpressing wild-type CLNX + SERCA2b. Interestingly, the CLNX-S562A mutant did not inhibit Ca^{2+} oscillations (Fig. 6a). The percentage of oocytes overexpressing CLNX-S562A + SERCA2b that exhibited Ca^{2+} oscillations was indistinguishable from that of control oocytes overexpressing SERCA2b alone, and more importantly, was significantly greater than that of oocytes coexpressing wild-type CLNX + SERCA2b (Fig. 6b and Table I). These results suggest that phosphorylation of the distal conserved PKC phosphorylation site regulates the inhibition of Ca^{2+} oscillations from the cytosol.

To determine whether the second PKC phosphorylation consensus site is functional, we overexpressed CLNX 485A + SERCA2b in oocytes. In contrast to what was observed with the CLNX-S562A mutant, repetitive Ca^{2+} waves were inhibited in oocytes overexpressing CLNX-S485A + SERCA2b (n = 27, 85%) (Fig. 6 and Table I). This percentage does not differ significantly from that obtained for oocytes overexpressing CLNX + SERCA2b. This observation is consistent with two hypothetical scenarios. The S485 residue is not functional and the inhibition of Ca^{2+} oscillations can be attributed solely to the distal phosphorylated S562, or S485 is required for the regulation of Ca^{2+} oscillations by S562. To determine which of these two hypotheses was correct, we generated a double mutant in which both serine residues were mutated to alanines (mutant CLNX-S485A/S562A) (Fig. 5). Ca^{2+} wave activity was imaged in oocytes either coexpressing CLNX-S485A/S562A + SERCA2b or overexpressing SERCA2b by itself (Fig. 6 and Table I). Ca^{2+} oscillations were inhibited in the CLNX-S485A/S562A + SERCA2b coexpressing oocytes, suggesting that S485 enables regulation of repetitive Ca^{2+} waves by the S562 residue. Furthermore, the data are consistent with the modulation of Ca^{2+} oscillations by CRT reported previously (Camacho and Lechleiter, 1995a), in that the double CLNX mutant displays constitutive channel properties that are not regulated by cytosolic phosphorylation. Taken together, our results reveal the presence of a phosphorylation-dependent regulatory switch in the S562 residue of CLNX.

**A Cytosolic Domain Peptide of CLNX Removes the Inhibition of Ca^{2+} Oscillations**

A dominant-negative experimental approach was adopted to further implicate an endogenous kinase/phosphatase operating on S562. We hypothesized that coexpression of CLNX with a peptide encompassing the distal PKC/PDK consensus site would compete with the responsible kinase/phosphatase to abrogate the inhibitory effect of CLNX on
Ca^{2+} oscillations. Thus, we engineered a *Xenopus* vector for expression of a cytosolic CLNX peptide, encompassing amino acids 534–571 of mature rat CLNX (CLNX_{cyt}). In these experiments, oocytes were injected with mRNA encoding wild-type CLNX + SERCA2b. 18 h before imaging of Ca^{2+} oscillations, the percentage of oocytes exhibiting repetitive Ca^{2+} oscillation increased from 30% in CLNX + SERCA2b to 72% in oocytes that, in addition, coexpressed CLNX cyt (n = 18) (Fig. 7 b and Table I). Thus, the cytosolic peptide successfully competes for the endogenous cytosolic kinase/phosphatase that determines the phosphorylation status of S562 and the regulation of Ca^{2+} oscillations.
**IP₃-mediated Ca²⁺ Mobilization Causes Dephosphorylation of the Cytosolic Domain of CLNX**

Having established the unique contribution of CLNX residue S562 in the inhibition of Ca²⁺ oscillations, we determined the state of phosphorylation of wild-type and CLNX-S562 before and after mobilization of Ca²⁺ by IP₃. This was accomplished by first injecting [γ-³²P]ATP into oocytes that were coexpressing CLNX or its S562A mutant, with SERCA2b. Half of the oocytes for each experimental condition was subsequently injected with IP₃ (~300 nM final). Immunoprecipitations with a CLNX-specific antibody were then carried out. Similar measurements were also performed in oocytes coexpressing the cytosolic peptide CLNXcyt with wild-type CLNX (Fig. 8 a). In control oocytes overexpressing SERCA2b alone, CLNX was not detected. Interestingly, CLNX was phosphorylated under resting conditions and was dephosphorylated by IP₃-mediated mobilization of Ca²⁺. Basal phosphorylation of the S562A mutant was less than half of wild-type CLNX and was not changed by IP₃ injection. These observations suggest first that the S562 residue is phosphorylated, and second that the Ca²⁺-sensitive dephosphorylation of CLNX is specific for this residue. Under resting conditions, phosphorylation of CLNX was slightly reduced by coexpression with the CLNXcyt peptide, and after IP₃ injection, there was a further reduction in the level of phosphorylation seen. This suggests that the function of the CLNXcyt peptide was to compete with the phosphatase activity responsible for CLNX dephosphorylation. Data from oocytes expressing the S485A and S485A/S562A mutants of CLNX are not shown, since the level of phosphate label detected was too low to reliably demonstrate the phosphorylation status of either mutant, consistent with the report by Wong et al. (1998).

Western blot analysis with an anti-CLNX antibody was performed on oocyte fractions coexpressing CLNX or its PKC/PDK phosphorylation mutants, with SERCA2b (Fig. 8 b). A single band migrating at ~90 kD was observed in membrane extracts prepared from oocytes overexpressing CLNX or its mutants, consistent with the reported molecular weight for CLNX (Wada et al., 1991). Thus, the differences in intensity of phosphorylated CLNX and its mutants cannot be attributed to differences in overexpression levels. In control oocyte extracts overexpressing SERCA2b alone, no detectable CLNX immunoreactivity was observed. To determine whether SERCA2b was coexpressed with CLNX, we probed oocyte extracts with an anti-rat SERCA2b antibody (John et al., 1998). SERCA2b overexpression was demonstrated in all extracts (Fig. 8 c). We observed no significant differences in SERCA2b immunoreactivity. When small variations did occur, they did not correlate with the presence or absence of Ca²⁺ oscillations. In particular, notice that the SERCA2b immunoreactivity detected in extracts from CLNX-S562A + SERCA2b overexpressing oocytes was slightly lower, but these levels of SERCA2b expression did not correlate with a decrease in the frequency of Ca²⁺ oscillations.

**The Interaction of CLNX with SERCA2b Is Reduced by Dephosphorylation Treatment**

To determine whether cytosolic dephosphorylation of...
CLNX affects its interaction with SERCA 2b, we immunoprecipitated endogenous CLNX from microsomes treated with alkaline phosphatase and detected proteins bound to CLNX by fluorography (Fig. 9). Calf intestinal phosphatase removes phosphate groups from phosphoserine and phosphothreonine residues, which accounts for >97% of phosphate bound to eukaryotic cells. The microsomes were isolated from translation reactions programmed to express the COOH terminus of SERCA 2b (TM 9-11) and S. cerevisiae α factor as a positive control. Interestingly, alkaline phosphatase treatment significantly reduced the immunoprecipitation of both substrates with CLNX, suggesting that in the dephosphorylated state CLNX no longer associates with the SERCA 2b substrate.

Discussion

In this study, we demonstrated that CLNX overexpression in Xenopus oocytes modulates IP$_3$-mediated Ca$^{2+}$ oscillations. As observed for CRT, modulation of Ca$^{2+}$ release is manifested by an inhibition of repetitive Ca$^{2+}$ waves in a manner consistent with inhibition of SERCA 2b activity (Camacho and Lechleiter, 1995a; John et al., 1998). More importantly, unlike the modulation by CRT, the inhibition of Ca$^{2+}$ oscillations by CLNX is subject to regulation by the phosphorylation status of its cytosolic domain.

Store refilling by SERCA pumps is the predominant factor that controls the frequency of Ca$^{2+}$ oscillations (Camacho and Lechleiter, 1993). Consistent with this, we find that overexpression of SERCA 2b alone results in ~90% of oocytes exhibiting high-frequency Ca$^{2+}$ waves, whereas coexpression of CLNX + SERCA 2b reduces this number to 30%. Detailed analysis of Ca$^{2+}$ wave activity in CLNX + SERCA 2b overexpressing oocytes that did not show complete inhibition also revealed an effect consistent with inhibition of the Ca$^{2+}$ ATPase. Specifically, we found statistically significant increases in wave periods and the $t_{1/2}$ decay of individual waves when CLNX was coexpressed with SERCA 2b. Both of these parameters are indicators of the refilling process. Furthermore, the inhibition of Ca$^{2+}$ oscillations was equally manifested regardless of whether the oscillations were generated by overexpression of SERCA 2b pumps or whether mitochondria were energized.

We have shown previously that CRT inhibition of Ca$^{2+}$ oscillations is dependent on the presence of a lumenal asparagine in SERCA 2b (N1036), which appeared to be targeted by the chaperone (John et al., 1998). Here we found that in oocytes coexpressing CLNX + SERCA 2b-N1036, Ca$^{2+}$ oscillations were not inhibited, suggesting that both members of this family of proteins inhibit Ca$^{2+}$ oscillations by similar mechanisms. We found that there is a physical interaction of CLNX with the COOH terminus of SERCA 2b which is reduced by mutagenesis on N1036A and is not at all existent with SERCA 2a. This reduction in the interaction may be due to a change in charge induced by mutagenesis of SERCA 2b-N1036A, since we found no evidence that SERCA 2b was glycosylated on N1036. Together, these results raise the possibility that CLNX inhibition of Ca$^{2+}$ oscillations is due to a protein–protein interaction with the COOH terminus of SERCA 2b. Recently, Ihara et al. reported that CLNX is a classical molecular chaperone in that it can operate independently of the state of glycosylation of target proteins (Ihara et al., 1999). The authors suggest that CLNX recognizes exposed hydrophobic pockets in substrate proteins maintaining them in a folding-competent state. It is then possible that proteins residing in the ER might benefit from the chaperone activity of CLNX to allow them to function in the oxidizing/gel matrix of the ER.

CLNX was first characterized as a phosphoprotein in the ER (Wada et al., 1991) and more recently, efforts have been directed to identify the specific residues that support phosphorylation (Wong et al., 1998). In particular, these authors described three phosphorylatable residues in the cytosolic domain of dog CLNX, including the homologous residue to S562 in rat CLNX. They demonstrate that phosphorylation of S562 modulates the interaction of CLNX with the ribosome. Here, we discovered by site-directed mutagenesis that the S562 in CLNX supports phosphorylation with functional consequences for the control of Ca$^{2+}$ oscillations. Specifically, unlike the inhibition of repetitive Ca$^{2+}$ waves observed in oocytes overexpressing CLNX + SERCA 2b, the frequency of repetitive waves observed in oocytes overexpressing CLNX-S562A + SERCA 2b is similar to that of the Ca$^{2+}$ ATPase expressed...
alone. In addition, dominant-negative expression of a peptide encompassing this PKC/PDK phosphorylation site of CLNX relieved the inhibition of Ca\(^{2+}\) oscillations imparted by the wild-type chaperone. From these results, we conclude that S562 in the cytosolic domain of CLNX is a molecular switch that controls Ca\(^{2+}\) oscillations. Our results suggest that dephosphorylation of S562 removes the functional interaction of the chaperone with SERCA 2b, resulting in a pump that is no longer inhibited. CLNX has a second consensus site for phosphorylation by PKC (S485), although it is not known whether it is functional. Coexpression of the S485A mutant with SERCA 2b decreased the number of oocytes that displayed repetitive waves to 15%, suggesting stronger inhibition of the pump. Two interpretations are possible for this finding. Either S485 has opposite effects to S562, or it is nonfunctional. We resolved this issue by coexpressing the double mutant CLNX-S562A/S485A with SERCA 2b, which also resulted in inhibition of Ca\(^{2+}\) oscillations. This result suggests that the S485 residue is required for the regulation of the Ca\(^{2+}\) oscillations upon dephosphorylation of the distal S562 residue. Interestingly, this proposed mechanism of action is reminiscent of the relief from inhibition of SERCA 2a after store depletion in cardiac muscle (Bhogal and Colyer, 1998). In this tissue, phosphorylation of phospholamban is responsible for relief of inhibition of the pump, although a chaperone interaction is not involved (Jackson and Colyer, 1996; MacLennan and Toyofuku, 1996). Thus, CLNX appears to be the functional equivalent of phospholamban in nonmuscle cells, where it provides phosphorylation-

![Diagram](image-url)

Figure 10. Model depicting the functional consequences of cytosolic phosphorylation of CLNX. (1) Under resting conditions, CLNX is phosphorylated on S562 located in the cytosolic domain of CLNX as shown. In this state, we suggest that CLNX is free to interact with the COOH terminus of SERCA 2b. CLNX may also interact with the ribosome. The Ca\(^{2+}\) ATP-ase is inhibited, the Ca\(^{2+}\) stores are expected to be full (dark gray) and in an optimal condition for protein folding. (2) Mobilization of the Ca\(^{2+}\) stores by IP\(_3\) (light gray) results in a Ca\(^{2+}\)-dependent dephosphorylation of S562 in CLNX. In the dephosphorylated state, inhibition of repetitive Ca\(^{2+}\) waves by CLNX is not observed, suggesting a loss of interaction with the pump resulting in Ca\(^{2+}\) store refilling. This phosphorylation switch implies that cytosolic Ca\(^{2+}\) regulates interactions of CLNX with luminal proteins (e.g., SERCA 2b), resulting in control of Ca\(^{2+}\) uptake and also modulation of protein folding in the ER lumen. In contrast, a bi-directional arrow is shown to indicate that the interaction of CRT with targets is determined by luminal conditions. (See Discussion for further details).
dependent regulation of Ca$^{2+}$ uptake by SERCA 2b. Furthermore, the mutants S485A and S485A/S562A behaved like CRT, which lacks cytosolic regulation and is consistent with the observation that a soluble ER luminal domain of CLNX ($\Delta$TM C), which promotes refolding of ribonuclease B, acts like CRT (Zapun et al., 1997).

The serine residues characterized here conform to a consensus motif for PKC/PDK phosphorylation (Wong et al., 1998). From a signaling perspective, activation of the IP$_3$ pathway would be expected, via PKC, to provide Ca$^{2+}$-mediated feedback to regulate downstream phosphorylatable targets such as CLNX. To test this hypothesis, immunoprecipitations of CLNX and CLNX-S562A mutant were performed from membrane fractions of [32P]ATP-labeled oocytes in the presence or absence of IP$_3$. These studies revealed that wild-type CLNX was phosphorylated under resting conditions and dephosphorylated after IP$_3$ injection. Furthermore, the detected phosphate label is on S562, since CLNX-S562A has a corresponding decrease in the amount of phosphate at rest, and more significantly, there is no further decrease upon stimulation by IP$_3$. We find that treatment with calf intestinal alkaline phosphatase decreases the interaction of CLNX with the COOH terminus of SERCA 2b. This is consistent with the possibility that dephosphorylation of S562 is transduced to the ER lumen, causing dissociation of CLNX from SERCA 2b. The phosphatase responsible for the Ca$^{2+}$-dependent dephosphorylation of CLNX remains to be identified. A candidate for this role may be the Ca$^{2+}$-dependent phosphatase, calcineurin (Klee et al., 1998).

Recently, Chevet et al. demonstrated that the equivalent residue in dog CLNX is phosphorylated by extracellular-signal regulated kinase 1 (ERK-1), and interestingly, phos-
phorylation by ERK-1 and CKII increased CLNX association with ribosomes in pancreatic microsomes (Chevet et al., 1999). The kinase responsible for phosphorylation of S562 remains to be identified in the oocyte.

One of the major implications of our study is that CRT should be viewed as a chaperone that is strictly controlled by ER luminal conditions such as Ca$^{2+}$ and interactions with other proteins (Corbett et al., 1999). In contrast, CLNX, via phosphorylation of its cytosolic domain, introduces an additional level of complexity to the signaling that controls protein processing. In Fig. 10, we present a model describing the Ca$^{2+}$-dependent regulation via cyto-
solic phosphorylation of the interaction of CLNX with SERCA 2b. In this model, we also attempt to incorporate the recent findings of Chevet et al. (1999) regarding the phosphorylation-dependent interaction of CLNX with the ribosome. We suggest that in the resting state (1), CLNX is phosphorylated on S562 and the Ca$^{2+}$ stores. Dephosphorylation of CLNX also causes its disso-
ciation from the ribosome (Chevet et al., 1999). Since pro-
tein translation is inhibited after store depletion, an inter-
action between CLNX and the ribosome is no longer required (Reilly et al., 1998). Restoration of ER luminal Ca$^{2+}$ has interesting consequences for the maintenance of ER protein folding. Luminal Ca$^{2+}$ is required for association of the lectin chaperones with misfolded targets, and more importantly, it is required for the action of the UDP-Glc glycoprotein glucosyl transferase (Trombetta and Par-
odi, 1992). Furthermore, depletion of Ca$^{2+}$ from inter-
nal stores causes accumulation of misfolded proteins in the ER (Lodish and Kong, 1990; Lodish et al., 1992; Choudhury et al., 1997) and upregulation of CRT expres-
sion at the mRNA and protein levels within a few hours (Llewellyn et al., 1996; Waser et al., 1997). Likewise, CLNX isoforms in yeast are induced by ER stress ([an-
natopur and Rokeach, 1995). Our data are consistent with the idea that dephosphorylation of CLNX is an acute re-
sponse (minutes) to Ca$^{2+}$ store depletion, and removal of pump inhibition then ensures optimal ER luminal condi-
tions for protein folding. We suggest that folding events in the ER lumen are under the control of the IP$_3$-mediated Ca$^{2+}$ signaling system. Since these chaperones in turn modulate cytosolic Ca$^{2+}$ by virtue of their putative luminal interactions with the SERCA 2b, this system represents a novel bi-directional mode of signaling between the ER and the cytosol.

We wish to thank E. Nasi and D. Castle for critical reading of the manus-
cript. We are grateful to the following individuals for kindly providing re-
agents: L. Tjoeker (rat calnexin cDNA), J. Bergeron (anti-calnexin anti-
body), and J. Lytton (anti-SERCA 2 antibody). We also thank Clarise Rivera and Marta Serbala for their technical support.

This work was funded the National Institutes of Health grant R01 GM 55372 to P. Camacho.

Submitted: 26 A pril 2000
Acepted: 2 M ay 2000

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