Calmodulin regulation of the calcium-leak channel Sec61 is unique to vertebrates

Anke Harsman, Annika Kopp, Richard Wagner, Richard Zimmermann and Martin Jung

1Medical Biochemistry and Molecular Biology; Saarland University; Homburg; 2Biophysics; Osnabrueck University; Osnabrueck, Germany
3Current address: Biophysik; Molekulare Infektiologie; Forschungszentrum Borstel; Borstel, Germany

In eukaryotes, protein transport into the endoplasmic reticulum (ER) is facilitated by a protein-conducting channel, the Sec61 complex. The presence of large, water-filled pores with uncontrolled ion permeability, such as those formed by Sec61 complexes in the ER membrane would interfere with the regulated release of calcium from the ER lumen into the cytosol, an essential mechanism of intracellular signaling. We identified a calmodulin (CaM) binding motif in the cytosolic N-terminus of Sec61α from Canis familiaris that binds CaM, but not Ca2+-free apo-CaM, with nanomolar affinity and sequence specificity. In single channel lipid bilayer measurements, CaM potently mediated Sec61-channel closure in a Ca2+-dependent manner. No functional CaM binding motif was identified in the corresponding region of Sec61p from Saccharomyces cerevisiae, and no channel closure occurred in the presence of CaM and Ca2+. Therefore, CaM binding to the cytosolic N-terminus of Sec61α is involved in limiting Ca2+-leakage from the ER in C. familiaris but not S. cerevisiae.

Key words: calcium homeostasis, endoplasmic reticulum, ER calcium leakage, Sec61 complex, yeast Saccharomyces cerevisiae

Abbreviations: CaM, calmodulin; ER, endoplasmic reticulum; SERCA, sarcoplasmic endoplasmic reticulum calcium ATPase; SOCE, store-operated calcium entry; CDI, calcium-dependent inactivation
In order to determine whether CaM is still able to bind and act as a modulator of Sec61-induced ion permeability, despite this weak conservation of the putative CaM binding motif in the yeast Sec61α-subunit, we investigated the binding characteristics of CaM to the yeast Sec61 complex using peptide dot-blot and flotation experiments. We also used electrophysiological measurements to investigate the possible regulation of the yeast Sec61 channel by CaM.

Results

Using the planar lipid bilayer technique, the ionic current through the mammalian Sec61 channel was shown to be drastically reduced in the presence of Ca\textsuperscript{2+}-CaM.\textsuperscript{2} To validate the specificity of this interaction, we utilized the CaM antagonist ophiobolin A, which was previously demonstrated to enhance Sec61-dependent Ca\textsuperscript{2+} leakage from the ER in live cell calcium imaging experiments.\textsuperscript{2} The single channel current through Sec61 was nearly completely blocked by the addition of 0.5 μM CaM in the presence of calcium (Fig. 2A and B). The subsequent addition of 100 μM ophiobolin A resulted in a full recovery of the single channel current and frequent

Figure 1. Sec61α IQ motif from Canis lupus familiaris (C.f.) and corresponding sequence from Saccharomyces cerevisiae (S.c.) aligned with the IQ consensus sequence logo (logo was created using http://weblogo.berkeley.edu/logo.cgi).\textsuperscript{15} The data for this logo consisted of 3,740 sequences from the full Pfam\textsuperscript{16} alignment of this family (Accession no. PF00612). Amino acids are colored according to their chemical properties: green, polar amino acids (G, S, T, Y, C, Q, N); blue, basic (K, R, H); red, acidic (D, E); and black, hydrophobic (A, V, L, I, P, W, F).

Figure 2. The calmodulin antagonist ophiobolin A interferes with calmodulin binding to Sec61 from C. familiaris. (A) Single channel current in the absence of calmodulin (left). The corresponding current histogram demonstrates the high overall current level (right). Buffer conditions: 100 mM KCl, 10 mM CaCl\textsubscript{2}, 10 mM Mops/Tris, pH 7.0. (B) Addition of 0.5 μM calmodulin led to a reduction in the single channel current level. (C) Subsequent addition of 100 μM ophiobolin A restores the single channel characteristics in (A) with regard to the high current level and appearance of dynamic gating transitions. Holding potential was +30 mV.
An alignment of the N-terminal sequences of Sec61α subunits from C. familiaris and S. cerevisiae revealed only a partially conserved IQ motif in the yeast sequence (Fig. 1). To test whether a calcium-dependent CaM interaction occurs with the Sec61α-IQ-motifs, we synthesized mammalian IQ-peptide spots and the yeast N-terminal cytosolic peptide domain (33 amino acids; shift of two) on a cellulose-membrane and carried out CaM-binding assays in the presence and absence of Ca²⁺ ions (Fig. 4). Conserved amino acids in the N-terminus of the mammalian Sec61α-IQ-motif were exchanged for alanines as negative controls.

The wild type IQ-motif (mammalian) bound to yeast and rat GST-CaM in the presence, but not the absence, of free calcium (Fig. 4, spots 1 and 4). Changing the conserved residues at positions 7 and 11 to alanine drastically reduced the CaM binding efficiency (Fig. 4, spots 2 and 3). In contrast the peptides of the yeast Sec61α N-terminus did not bind CaM. No binding was observed when only 14C-labeled GST was subjected to the peptides (data not shown).

We also measured the single channel currents of yeast Sec61p from rough microsomal vesicles after puromycin/KCl treatment in the absence and presence of Ca²⁺-CaM (Fig. 3A). The addition of Ca²⁺-CaM had no effect on the current amplitudes or gating behavior of the Sec61p channel (Fig. 3A and B). Statistical evaluation of the open probability of different single channels (n = 3) revealed comparable open probabilities in the presence and absence of Ca²⁺-CaM at all applied positive or negative membrane potentials (Fig. 3C).
Sec61α (Fig. 5). GST-CaM (rat/yeast) floated with canine-RM in the presence of calcium (Fig. 5A and B), but no co-flotation of GST-CaM (yeast) with yeast-RM was observed, not even in the presence of calcium (Fig. 5C and D). Therefore, CaM is not able to bind native yeast Sec61 complexes in vitro. We note that the blots were sequentially incubated with the antibodies against Sec61α and GST (Fig. 5C and D). Therefore Sec61α showed up in the GST blots.

Discussion

The release of free calcium from the ER lumen to the cell cytosol is one of the key factors in the regulation of physiological processes in mammals, such as muscle contraction, exocytosis and apoptosis. This action is spatially and temporally well coordinated, and the regulatory mechanisms involve combinations of positive (SOCE) and negative feedback (CDI) controls by cytosolic calcium. Uncontrolled permeability of the large, aqueous Sec61 pore to calcium ions would interfere with the role of the ER membrane in maintaining calcium gradients of 3–4 orders of magnitude ([Ca²⁺]cyt = 0.05–0.1 μM; [Ca²⁺]ER = 100–800 μM) and the tight control of calcium release with regard to its spatial and temporal patterns. Similar to mammalian cells, S. cerevisiae and other budding yeasts possess the ability to dynamically control the free cytosolic [Ca²⁺] and downstream signaling pathways. However, yeast cells use slow calcineurin-dependent gene expression control pathways to regulate their long-term calcium homeostasis. Even in the presence of high extracellular calcium, yeast cells only respond with a slow calcium shock reaction. The diffusion of calcium within the cytosol is limited by the combination of high protein content and small cell size. Furthermore, ER resident IP3 or ryanodine receptors are not known to be present in the budding yeast and neither S. cerevisiae, nor other budding yeasts do retain a SERCA pump. The lack of such a specialized Ca²⁺ pump might account for the low level of free Ca²⁺ in the ER lumen (<10 μM), limiting its impact on intracellular Ca²⁺ signaling.

Therefore, the yeast vacuole serves as the main storage site for calcium and other divalent cations and acts as a buffering system to maintain the calcium concentration (0.05–0.2 μM) within the cytosol. Thus, a calcium leak in the ER membrane, even if it increases to a local steep calcium gradient or calcium microdomain, would not activate downstream pathways. In summary, in Saccharomyces cerevisiae no obvious need exists for a fast ER calcium efflux response to prevent erroneous calcium signaling; therefore,
as we have shown here, the yeast Sec61 complex is not regulated by CaM in a calcium-dependent manner.

**Materials and Methods**

**Single channel recordings.** Vesicles were prepared for planar bilayer experiments by mixing (3:2, v/v) the different Sec61-containing vesicles with pre-formed liposomes (e.g., L-α phosphatidylincholine, 10 mg/ml) in 50 mM KCl and 10 mM Mops/Tris (pH 7.0). Mega-9 (nonanoyl-N-ethylglucamide) was added at a final concentration of 80 mM. After mixing, the sample was dialyzed for 4 h at room temperature, and then overnight at 4°C against 50 mM KCl and 10 mM Mops/Tris (pH 7.0). Aliquots (10 μl) of the proteoliposomes derived from RM vesicles were incubated with 200 mM puromycin and 250–500 mM KCl for 15–30 min on ice. Planar lipid bilayers were produced using the painting technique. An osmotic gradient was used for vesicle fusion. Membrane potentials refer to the trans compartment. Data recording and analysis were performed as described previously in reference 13. Voltage ramps were achieved at a rate of 5 mV/s.

**Peptide spot binding assays.** IQ-peptide constructs and the first 33 cytosolic N-terminal amino acids of yeast Sec61p were synthesized on acid-hardened cellulose membranes, derivatized with a polyethylene glycol spacer as described previously in reference 7. Membranes were equilibrated for 2 h at 4°C in binding buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.5, 0.1% Triton X-100 and 1 mM CaCl₂ or 4 mM EGTA). 14C-labeled GST-calmodulin (rat/yeast) was added and incubated at 4°C overnight. The membrane was washed three times for 10 minutes with binding buffer, dried at room temperature and subjected to phosphorimaging using a Typhoon imaging device (GE Healthcare).

**Sucrose gradient flotation.** 10 μl of canine-RM or 15 μl of yeast-RM in 100 ml of 150 mM KCl, 50 mM Hepes/KOH (pH 7.4), 5 mM MgCl₂, 10% sucrose and 0.05 mg/ml BSA were incubated with 50 pmol GST-CaM (rat/yeast) and either 2 mM CaCl₂ or 4 mM EGTA for 60 min at 0°C. The samples were adjusted to 77% sucrose, 150 mM KCl, 50 mM Hepes/KOH (pH 7.4), 5 mM MgCl₂, and 0.05 mg/ml BSA (900 ml) and covered with a 62% (800 ml) and 8.5% (500 ml) sucrose cushion in 150 mM KCl, 50 mM Hepes/KOH (pH 7.4), 5 mM MgCl₂, 0.05 mg/ml BSA and 2 mM CaCl₂, or 4 mM EGTA, respectively. The step gradients were centrifuged for 18 h at 116,000 g in a SW55 rotor at 2°C and fractionated into 10 aliquots representing 12–70% sucrose densities. The protein content of the fractions was precipitated according to Wessel and Fluegge (1984). Samples were dissolved in SDS sample buffer, incubated for 10 min at 56°C and analyzed by SDS-PAGE and subsequent Western blotting on PVDF membranes. Blots were incubated with antibodies against GST and Sec61β (C.f.) or GST and

---

Figure 5. Coflotation experiments in sucrose gradients of canine pancreatic rough microsomes (RM) with GST-CaM (rat) (A) or GST-CaM (yeast) (B) in the presence of calcium. Coflotation experiments in sucrose gradients of yeast RM with GST-CaM (yeast) in the presence (C) or absence (D) of calcium.
Sec61α (S.c). Bound antibodies were visualized using peroxidase (POD)-coupled anti-rabbit antibodies and ECL™, and detected with a Lumi-Imager F1 (Roche).

Acknowledgments
We gratefully acknowledge the excellent technical assistance of S. Amann (Homburg). Anke Harsman was supported by a grant from the Deutsche Forschungsgemeinschaft (FOR 967). This work was supported by grants from the Deutsche Forschungsgemeinschaft to Richard Zimmermann (SFB 530/C1 & FOR 967) and Richard Wagner (FOR 967).

References
1. Erdmann F, Jung M, Maurer P, Harsman A, Zimmermann R, Wagner R. The mammalian and yeast translocon complexes comprise a characteristic Sec61 channel. Biochem Biophys Res Commun 2010; 396:714-20.
2. Erdmann F, Schäuble N, Lang S, Jung M, Honigmann A, Ahmad M, et al. Interaction of calmodulin with Sec61alpha limits Ca2+ leakage from the endoplasmic reticulum. EMBO J 2011; 30:17-31.
3. Davis TN, Thorner J. Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable. Proc Natl Acad Sci USA 1989; 86:7909-13.
4. Luan Y, Matsuura I, Yazawa M, Nakamura T, Yagi K. Yeast calmodulin: structural and functional differences compared with vertebrate calmodulin. J Biochem 1987; 102:1531-7.
5. Cyert MS. Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. Annu Rev Genet 2001; 35:647-72.
6. Bähler M, Rhoads A. Calmodulin signaling via the IQ motif. FEBS Lett 2002; 513:107-13.
7. Clapham DE. Calcium signaling. Cell 2007; 131:1047-58.
8. Cunningham KW. Acidic calcium stores of Saccharomyces cerevisiae. Cell Calcium 2011; 50:129-138.
10. Cui J, Kaandorp JA, Shook PM, Lloyd CM, Filatov MV. Calcium homeostasis and signaling in yeast cells and cardiac myocytes. FEMS Yeast Res 2009; 9:1137-47.
11. Strayle J, Pozzan T, Rudolph HK. Steady-state free Ca2+ in the yeast endoplasmic reticulum reaches only 10 microM and is mainly controlled by the secretory pathway pump pmr1. EMBO J 1999; 18:4733-43.
12. Müller P, Rudin DO, Tien HT, Wescott WC. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. Nature 1962; 194:779-80.
13. Wirth A, Jung M, Bies C, Frien M, Tyedmers J, Zimmermann R, Wagner R. The Sec61p complex is a dynamic precursor activated channel. Mol Cell 2003; 12:261-8.
14. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 1984; 138:141-3.
15. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res 2004; 14:1188-90.
16. Finn RD, Mistry J, Tate J, Coggill P, Heger A, Pollington JE, et al. The Pfam protein families database. Nucleic Acids Res 2010; 38:211-22.