Sec61 complexes form ubiquitous ER Ca\(^{2+}\) leak channels

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In mammalian cells, the endoplasmic reticulum (ER) plays a key role in protein biogenesis and in calcium signalling. The heterotrimeric Sec61 complex in the ER membrane provides an aqueous pathway for transporting newly synthesized polypeptides into the ER lumen and may also allow calcium leakage from the ER into the cytosol. In this study, planar lipid bilayer experiments demonstrated that the Sec61 complex is permeable to calcium ions. We also investigated whether silencing the SEC61A1 gene affected calcium leakage from the ER. Silencing the SEC61A1 gene using two different siRNAs in HeLa cells for 96 hours had little effect on cell growth and viability. However, calcium leakage from the ER was greatly decreased in the SEC61A1-silenced cells. Thus, the Sec61 complexes that are present in the ER membrane of nucleated cells form calcium leak channels that may play a crucial role in calcium homeostasis.

Introduction

In mammalian cells, the endoplasmic reticulum (ER) plays a key role in the synthesis, folding and sorting of proteins and is also involved in calcium signalling. The drug thapsigargin inhibits SERCAs and, thereby, reveals the calcium leak pathway. Leakage of Ca\(^{2+}\) from the ER can be visualized as a reduction of Ca\(^{2+}\) concentration in the ER by employing an ER calcium indicator, such as MagFURA-2, in digitonin-permeabilized cells. Alternatively, it can be visualized as an increase in the cytosolic Ca\(^{2+}\) concentration by employing a cytosolic calcium indicator, such as FURA-2, in intact cells in the presence or absence of extracellular Ca\(^{2+}\). Four types of membrane proteins are supposed to be involved in the ER calcium leak pathway, Bcl-2, pannexin 1, presenilins and TRPC1. Indirect evidence from various studies suggests that the major entry site of newly synthesized polypeptides in the ER membrane, the Sec61 complex (also called translocon or protein translocase) contributes to the Ca\(^{2+}\) leak. The indirect evidence is as follows. First, puromycin-induced release of nascent polypeptides from ribosomes leads to a drop in ER lumenal Ca\(^{2+}\) that cannot necessarily be seen as a concomitant increase in cytosolic Ca\(^{2+}\) due to PMCA action, in turn, the drop in ER lumenal Ca\(^{2+}\) leads to a reduced thapsigargin-induced increase in cytosolic Ca\(^{2+}\) that is thought to prevent ER calcium overload and thus, to allow cytosolic calcium signalling. Therefore, calcium leakage from the ER is essential for calcium signalling.
subsequent store operated calcium entry (SOCE) in the presence of extracellular calcium. Third, the inhibition of elongation by emetine or anisomycin prevents the action of puromycin.

Here, we directly addressed the question of whether the Sec61 complex contributes to passive calcium efflux from the ER by two lines of experiments. We show in planar lipid bilayer experiments that the open Sec61 complex is permeable to Ca\(^{2+}\) and in live cell Ca\(^{2+}\) imaging experiments (employing puromycin and thapsigargin) that silencing of the \(SEC61A1\) gene leads to a significant drop in calcium efflux from the ER in the presence of thapsigargin and the complete elimination of the effect of puromycin on calcium efflux. Therefore, Sec61 complexes represent ubiquitous calcium leak channels. These data support our previous notion, that Ca\(^{2+}\)-calmodulin limits Ca\(^{2+}\)-leakage from the ER at the level of the Sec61 complex.

**Results**

Recent studies suggest that the Sec61 complex may be a major contributor to Ca\(^{2+}\) leakage currents at the ER membrane. We, therefore, analyzed the electrophysiological properties of the Sec61 channel in planar lipid bilayer experiments as described previously in reference 13, except that these experiments were conducted in the presence of Ca\(^{2+}\)-instead of K\(^{+}\). The Sec61 complexes were present in either canine pancreatic microsomes or in proteoliposomes after purification from canine pancreatic microsomes. Single-channel current recordings in CaCl\(_2\) buffer at isovisco-strength to the standard KCl buffer displayed similar gating characteristics (Fig. 1A and B). The variability of open channel states was also similar in CaCl\(_2\) buffer and in KCl buffer (Fig. 1C and D). Experiments using the two buffers showed that under symmetric electrolyte conditions, the probability that the Sec61 complex was closed or in one of its open states was reduced with increasing holding potentials. In both buffers, the channel was nearly completely open at potentials of ±20 mV, and the open probability symmetrically decreased to about 50% at ±50 mV. Thus, neither a reduction of the open probability nor a change in the voltage dependence was observed in the presence of Ca\(^{2+}\) compared to K\(^{+}\). Even when we performed a detailed analysis of conductances from experiments performed under symmetric CaCl\(_2\) conditions, the conductance distribution histograms were only slightly different from those obtained in KCl buffers (Fig. 1E and F). Under KCl electrolyte conditions, the main conductance level of the Sec61 complex was 151 ± 1 pS and the subconductance state was 480 ± 19 pS. Under CaCl\(_2\) conditions, the most frequent conductance level of the Sec61 complex was 165 ± 10 pS and the subconductance state was 733 ± 16 pS. Thus, the Sec61 complex is permeable to calcium ions.

In order to demonstrate that the Sec61 complex was involved in calcium leakage from the ER in cells, an efficient protocol for silencing of the \(SEC61A1\) gene had to be developed. HeLa cells were treated with candidate siRNAs for various incubation times. Western blot analysis showed that two different siRNAs that were directed against the coding \((SEC61A1)\) and untranslated \((SEC61A1-UTR)\) regions of the \(SEC61A1\) gene were the most efficient at silencing the gene when used individually at a concentration of 20 nM or used in combination at 10 nM each. Whether used alone or in combination, there was maximum silencing (about 89%) after 96 h of incubation. At that time, the growth and viability of the silenced cells only began to be affected more than that of cells that had been transfected with a negative control siRNA.

These experiments allowed us to analyze the effect of \(SEC61A1\) silencing on calcium leakage from the ER. Calcium leakage was monitored in the cytosol employing the ratiometric calcium indicator FURA-2 as described previously in references 14 and 15. Using established procedures, calcium leakage was studied directly after thapsigargin addition as well as indirectly as a reduction in thapsigargin-induced leakage following puromycin pre-treatment. Both analyses were carried out using untreated cells, cells treated with either one or both of the two \(SEC61A1\)-specific siRNAs, and with cells treated with the negative control siRNA. Compared to untreated cells and to cells treated with the negative control siRNA (Fig. 2A, B and K), cells with the \(SEC61A1\) gene silenced using either one or both of the two \(SEC61A1\)-specific siRNAs had significantly reduced thapsigargin-inducible calcium leakage, and the effect of puromycin pre-treatment on the response to thapsigargin was eliminated completely (Fig. 2C–E and K). Thus, silencing of the \(SEC61A1\) gene inhibits calcium leakage from the ER. In order to further substantiate this conclusion we attempted expression of the \(SEC61A1\) cDNA, lacking the UTR of the \(SEC61\) gene, in the presence of the \(SEC61A1\)-UTR siRNA and observed that it indeed rescues the phenotype of \(SEC61A1\) silencing, i.e., in contrast to the vector control it partially restored the effects of puromycin and thapsigargin (Fig. 2F, G and K). Based on western blot analysis the complementation efficiency of the \(SEC61A1\) expression plasmid was around 65% (Fig. 2J).

As a control, we asked if inhibition of elongation prevented the action of puromycin in our experimental system as observed previously in references 3 and 7. Indeed, the elongation inhibitor emetine prevented the effect of puromycin (Fig. 2H). Additional control experiments were carried out to rule out indirect effects of the \(SEC61A1\) gene silencing on calcium leakage from the ER. In a first experiment, the levels of SERCAs and presenilin were evaluated by western blot after silencing of the \(SEC61A1\) gene (data not shown). \(SEC61A1\) silencing did not reduce SERCA and presenilin levels, i.e., did not affect the refilling of the Ca\(^{2+}\) store or function of an established Ca\(^{2+}\) leak channel. Next, we evaluated the effect of silencing of the \(ERJ1\) gene, which encodes a type I ER membrane protein.\(^{16,17}\) Silencing the \(ERJ1\) gene did not affect calcium leakage from the ER after addition of puromycin or thapsigargin (Fig. 2I and K). Thus, these control experiments demonstrated that silencing of the \(SEC61A1\) gene directly and specifically inhibited calcium leakage from the ER.

**Discussion**

In mammalian cells, the ER plays a key role in protein biogenesis and in calcium homeostasis. The heterotrimeric Sec61 complex in the ER membrane provides...
Figure 1. Calcium permeability of the Sec61 channel. (A) Representative single-channel current recording in KCl buffer of a bilayer containing the purified Sec61 complex. The holding potential was clamped to 55 mV. An expanded view of the time scale corresponding to the shaded part of the upper trace is shown below. (B) Representative single-channel current deduction in CaCl$_2$ buffer of a bilayer containing the purified Sec61 complex at a membrane potential of 40 mV. A zoom plot of the shaded part of the upper trace is shown below. (C) Voltage-dependent open probabilities for the purified Sec61 channel in KCl buffer for the given membrane potentials (n = 6 independent bilayers). (D) Voltage-dependent open probabilities for the purified Sec61 complex in CaCl$_2$ buffer (n = 5 independent bilayers). (E) Conductance histogram obtained for n = 2,203 gating events of the Sec61 complex in microsomes in KCl buffer. (F) Conductance distributions for n = 738 single channel gating events of the Sec61 complex in CaCl$_2$ buffer. All experiments were conducted under symmetric electrolyte conditions. (A, C and E) 250 mM KCl, 10 mM MOPS/Tris pH 7. (B, D and F) 184 mM CaCl$_2$, 10 mM MOPS/Tris pH 7.
Figure 2. Effect of SEC61A1 gene silencing on calcium leakage from the ER. HeLa cells were left untreated (A and H), treated with siRNAs directed against the SEC61A1 (C–G) or ERJ1 genes (I) or treated with a negative control siRNA (B) for 96 h as indicated. (F) Cells were co-transfected with the respective vector control. The cells were then loaded with the calcium indicator FURA-2 AM and imaging experiments were carried out in a Ca²⁺ free buffer containing 0.5 mM EGTA. Ca²⁺ release was initiated by the addition of thapsigargin or by sequential application of puromycin (Puro) and thapsigargin. Average values are given, error bars represent standard errors of the means (SEM). Effect of SEC61A1 gene silencing on calcium leakage from the ER. (G) Cells were co-transfected with SEC61A1 expression plasmid. The cells were then loaded with the calcium indicator FURA-2 AM and imaging experiments were carried out in a Ca²⁺ free buffer containing 0.5 mM EGTA. Ca²⁺ release was initiated by the addition of thapsigargin or by sequential application of puromycin (Puro) and thapsigargin. Average values are given, error bars represent standard errors of the means (SEM). (H) HeLa cells were treated simultaneously with emetine (100 μM) and FURA-2 AM. Then imaging experiments were carried out in a Ca²⁺ free buffer as in (A). (K) Statistical analysis of the changes in the cytosolic Ca²⁺ concentration after addition of thapsigargin in the experiments in (A–G) and (I). Average values are given, error bars represent standard errors of the means (SEM). p values < 0.001 were defined as significant by unpaired t tests and are indicated by three asterisks (***) n.s., not significant. The numbers of cells that were analyzed are indicated. (J) Silencing was evaluated by western-blot analysis using Sec61α- or ERJ1-specific antibodies and the anti-β-actin antibody as a control. The average values are shown with error bars that represent the standard errors of the means (SEM). The numbers of experiments that were analyzed are indicated.
Figure 2. For figure legend, see opposite page.
a similar function was attributed to cytosolic Ca²⁺-calmodulin.12 Notably, the ER calcium leakage described here and elsewhere occurred in the presence of ER and SOCE, 4,6,7 and (iii) inhibition of elongation prevents the action of puromycin.1,6 Furthermore, these data are consistent with the results of a global RNAi screen for genes that are involved in thapsigargin-induced SOCE in Drosophila cells, which identified the STIM, ORAI and SEC61alpha genes.18 Stim1 and Orai1 were subsequently shown to provide the link between ER luminal calcium concentration and SOCE in insect as well as mammalian cells. The identification of Sec61α in this screen was dismissed as an indirect result, possibly due to the effect of Sec61a on the biogenesis of proteins such as Stim1 and Orai1. This interpretation should be reconsidered in light of the results presented here.

Previous work identified Bcl-2,8 presenilins,9 pannexin 1,10 and TRPC1,11 as ER calcium leak channels, but these were not identified in the Drosophila screen mentioned above.18 However, for Bcl-2, TRPC1 and pannexin 1, the calcium leak activity was observed when the genes were overexpressed, whereas TRPC1 and pannexin 1 are plasma membrane proteins under normal conditions (Fig. 3). A double knock-out strategy in mouse embryonic fibroblasts was employed to identify presenilins 1 and 2, with the authors concluding that the presenilins account for approximately 80% of the leak channels. In the present study, we estimated that Sec61 complexes account for about 60% of the leak channels in HeLa cells. Thus, the contributions of Sec61 complexes and presenilins to ER Ca²⁺ leakage in particular cells may vary according to the expression levels of the PSN and SEC61A1 genes.

The question arises: How is Ca²⁺ efflux from the ER through the Sec61 complex limited? Previous work suggests that different ligands can prevent ion flux through this channel.19-22 For example, it was proposed that ribosomes with nascent polypeptide chains that are in transit into the ER lumen seal the Sec61 complex from the cytosolic side.19 However, 3D reconstructions using cryo-electron microscopy of ribosome-Sec61 complexes show a gap between the Sec61 complex and the ribosome, challenging this proposal.23,24 BiP in the ER lumen contributes to sealing of the Sec61 complex during translocation of polypeptides as well as in the absence of translocation (Fig. 3). A double knock-out strategy in mouse embryonic fibroblasts was employed to identify presenilins 1 and 2, with the authors concluding that the presenilins account for approximately 80% of the leak channels. In the present study, we estimated that Sec61 complexes account for about 60% of the leak channels in HeLa cells. Thus, the contributions of Sec61 complexes and presenilins to ER Ca²⁺ leakage in particular cells may vary according to the expression levels of the PSN and SEC61A1 genes.
these regulatory mechanisms. Therefore, calcium leakage from the ER via the Sec61 complex may be even more pronounced, and may even reach toxic levels under cellular conditions such as protein misfolding in the ER or glucose starvation that result in BiP occupation or inactivation, respectively. This should be kept in mind when interpreting functional studies of the SEC61A1 gene in model organisms. Specifically, β-cell apoptosis was described recently in a mouse model of diabetes in which there is a mutation in the SEC61A1 gene, and overexpression of the SEC61A1 gene in Drosophila is associated with neurotoxicity.25,26

Materials and Methods

Single channel recordings. Rough microsomes were prepared from dog pancreas. Proteoliposomes were made from purified components as described previously in reference 13. Vesicles for planar bilayer experiments were prepared by mixing (3:2, v/v) the different Sec61-containing vesicles with pre-formed liposomes (egg L-α-phosphatidylcholine, 10 mg/ml) in 50 mM KCl, 10 M Mops/Tris (pH 7.0). Mega-9 (nonanoyl-N-methylglucamide) was added to a final concentration of 80 mM. After mixing, the sample was dialysed for 4 h at room temperature and then overnight at 4°C against buffer (5 l) containing 50 mM KCl, 10 mM Mops/Tris (pH 7.0). Aliquots of the proteoliposomes derived from the RM vesicles (10 μl; typically 10 mg/ml protein, lipid/protein 2:1 [w/w]) were incubated with 200 μM puromycin and 250–500 mM KCl for 15–30 min on ice. After 15 min, 1 μl aliquots were used for bilayer fusion. Proteoliposomes containing the purified Sec61 complex were used for bilayer fusion directly from dialysis. Planar lipid bilayers were produced by the painting technique, as described previously.13 The resulting bilayers typically had a capacitance of 0.5 μF/cm² and a resistance of >100 GΩ. The noise was 3 pA (r.m.s.) at 5 kHz bandwidth. An osmotic gradient was used for vesicle fusion. Membrane potentials refer to the trans compartment. Recording and analysis of the data were performed as described previously in reference 13. All electrolyte solutions were buffered with 10 mM Mops/Tris to pH 7.0. KCl, and CaCl₂, and MgCl₂ concentrations were calculated to represent comparable ionic activities (250 mM KCl ≈ 184 mM CaCl₂ and 20 mM KCl ≈ 10.7 mM CaCl₂).

Silencing of gene expression by siRNA. HeLa cell (ATCC no. CCL-2) were cultured in DMEM-medium (Gibco) containing 10% FBS (Biochrom) and 1% penicillin/streptomycin (PAA) in a humidified environment with 5% CO₂ at 37°C. Typically, cells were grown in 6 cm culture dishes on 25 mm coverslips, pre-treated with poly-lysine (1 mg/ml) for 1 h. Growth rates and viability were determined by employing the Countess® Automated Cell Counter (Invitrogen). Cell viability was also evaluated employing the Nuclear-ID™ Blue/Green cell viability reagent (Enzo Life Sciences) according to the manufacturers instructions.

For gene silencing, 5.2 x 10⁴ HeLa cells were seeded per 6 cm culture dish in normal culture. The cells were transfected with SEC61A1 siRNA (GGG AUA UGC CUG CUA AUC Att, Ambion), SEC61A1-UTR siRNA (CAC UGA AAU GUC UAC GUU Utc, Ambion), ERJ1 siRNA (CCU CAA UAU UUC UAC GUC Att, Ambion) or control siRNA (AllStars Negative Control siRNA, Qigene) at final concentration of 20 nM using HiPerFect Reagent (Qiagen) according to the manufacturer’s instructions. After 24 h, the medium was changed and the cells were transfected a second time. Silencing was evaluated by RT-PCR and western blot analysis.

Complementation analysis. In order to rescue the phenotype of SEC61A1 silencing, the SEC61A1 cDNA was inserted into the multiple cloning site (MCS) of a pCDNA3-internal ribosomal entry site (IRES)-GFP vector that contained the cytomegalovirus (CMV) promoter, the MCS, the IRES, plus the green fluorescent protein (GFP) coding sequence. Cells were treated with SEC61A1-UTR siRNA as described above for 48 h. Subsequently, the cells were transfected with either vector or SEC61A1 expression plasmid using Fugene HD (Roche). According to GFP fluorescence the transfection efficiency was around 80%.

Live cell calcium imaging. Live cell calcium imaging was carried out as described previously in references 14 and 15. Briefly, cells were loaded with 4 μM FURA-2 AM in DMEM for 45 min at 25°C. Cells were washed twice and incubated in a calcium-free buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 0.5 mM EGTA, 10 mM glucose in 10 mM HEPES-KOH (pH 7.35)). During the experiment, cells were treated with puromycin (500 μM) in calcium-free buffer or with the same buffer for 2 min. After ratiometric measurements were carried out for 3 min, thapsigargin (1 μM) was added and the measurements resumed. Data were collected on an iMIC microscope and the polychromator V by alternately exciting at 340 nm and 380 nm and measuring the emitted fluorescence at 510 nm. Images containing 20–50 cells/ frame were sampled every 3 s. FURA-2 signals were recorded as the F340/F380 ratios, where F340 and F380 correspond to the background-subtracted fluorescence intensity at 340 and 380 nm, respectively. Cytosolic [Ca²⁺] was estimated from ratio measurements by an established calibration method1 and data were analyzed using Excel 2007, Origin 6.1 and Visio 2007. Where indicated, HeLa cells were treated with siRNA directed against the SEC61A1 gene or with a negative control siRNA for 96 h prior to calcium imaging. Under these conditions, puromycin and emetine efficiently inhibited protein synthesis within 1 min of addition according to pulse-chase experiments (data not shown).

Statistical analysis. p values <0.001 were defined as significant using an unpaired t test and are indicated by three asterisks (***)). The imaging experiments in combination with gene silencing were carried out using four different batches of HeLa cells over a period of four months and two coverslips with at least 20 cells on each were analyzed for each condition for each single experiment.

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