Interaction of *Pseudomonas aeruginosa* Exotoxin A with the human Sec61 complex suppresses passive calcium efflux from the endoplasmic reticulum

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According to live-cell calcium-imaging experiments, the Sec61 complex is a passive calcium-leak channel in the human endoplasmic reticulum (ER) membrane that is regulated by ER luminal immunoglobulin heavy chain binding protein (BiP) and cytosolic Ca\(^{2+}\)-calmodulin. In single channel measurements, the open Sec61 complex is Ca\(^{2+}\) permeable. It can be closed not only by interaction with BiP or Ca\(^{2+}\)-calmodulin, but also with *Pseudomonas aeruginosa* Exotoxin A which can enter human cells by retrograde transport. Exotoxin A has been shown to interact with the Sec61 complex and, thereby, inhibit ER export of immunogenic peptides into the cytosol. Here, we show that Exotoxin A also inhibits passive Ca\(^{2+}\) leakage from the ER in human cells, and we characterized the N-terminus of the Sec61 α-subunit as the relevant binding site for Exotoxin A.

**Introduction**

Transport of precursor polypeptides into and calcium ions (Ca\(^{2+}\)) out of the endoplasmic reticulum (ER) is mediated by the so-called translocon and it must be regulated in mammalian cells with high fidelity to prevent fatal signaling. Therefore, the translocon, which represents a dynamic entrance into the lumen of the ER is regulated by proteins that modulate the structure of the central Sec61 channel from the open to the closed state, and vice versa. The Sec61 complex is a passive calcium-leak channel. On the cytosolic side, it is closed by calcium-calmodulin (Ca\(^{2+}\)-CaM) which acts near an IQ motif in the N-terminus, and on the luminal side, it is closed by immunoglobulin heavy chain binding protein (BiP), which acts on loop 7 of the Sec61 α-subunit (Fig. 1).\(^1\)\(^3\) BiP activity is mediated by ER-resident Hsp40s via their J-domains, which show high affinity for the ATP-bound form of BiP and focus the ATPase cycle and subsequent substrate binding capability near the luminal exit site of the translocon. A further mediator of the closed state is the ER membrane protein Sec62, which interacts with the N terminus of the pore-forming Sec61 α-subunit.\(^4\) All interactions prevent calcium efflux through the ion-conducting Sec61 channel. Interestingly, all interactions occur near the connections of transmembrane segments 2/3 and 7/8 of Sec61α which are believed to form the lateral gate of the pore into the lipid bilayer of the ER-membrane. Mammalian Sec61 complexes have also been described as the ER-export sites for immunogenic peptides that were originally imported into the ER via the transporter associated with antigen presentation (TAP), and for certain bacterial toxins, such as Exotoxin A and Cholera toxin, which are bacterially secreted ADP-ribosyltransferases that enter the eukaryotic cytosol by retrograde exocytosis.\(^5\)\(^7\) We previously observed in planar lipid bilayer experiments that after
Sec61 complexes are activated by a precursor polypeptide, *Pseudomonas aeruginosa* Exotoxin A modulates the gating properties of the open Sec61 channels in nanomolar concentrations and with a preference for the luminal side of the complex.8

Here, we used live-cell calcium-imaging to characterize the interaction of Exotoxin A with the translocon. We observed that a sublethal dose of Exotoxin A lowers the passive Ca\(^{2+}\) efflux through the Sec61 complex. We also determined the Exotoxin A binding site on the Sec61\(\alpha\)-subunit by using a variety of biochemical approaches. Based on our results, we propose that Exotoxin A acts on the Sec61\(\alpha\)-subunit in a manner similar to that of the physiological regulator Ca\(^{2+}\)-CaM.

### Results

Live-cell calcium-imaging was previously established to verify the respective effects of modulatory substances and proteins on ER-calcium leakage via the Sec61 complex.9,10 We used this approach to investigate the effect of low concentrations of Exotoxin A on the ER-calcium leakage from the ER of HeLa cells. When the cells were incubated for 10 min with Exotoxin A (30 nM) at 37°C and ER-calcium leakage was unmasked by thapsigargin the amount of calcium efflux was lower compared with the control cells (Fig. 2A). To verify that this effect was not due to decreased Ca\(^{2+}\) loading of the ER, ionomycin was applied. Exotoxin A appeared to have a very small stimulatory effect on ER Ca\(^{2+}\) loading (Fig. 2B). Thus, Exotoxin A showed a direct inhibitory effect on the ER Ca\(^{2+}\) leakage in HeLa cells, most likely by direct interaction.

The proposed interaction between the Sec61\(\alpha\)-subunit and Exotoxin A was confirmed by coimmunoprecipitation. After incubation of \(^{14}\)C-labeled Exotoxin A (20 µg/ml) with HeLa cell lysate, an antibody directed against the Sec61\(\beta\)-subunit precipitated the Sec61-complex and radioactive Exotoxin A from the lysate (Fig. 3A).5

Since we had observed a direct interaction between Exotoxin A and the Sec61 complex in the coimmunoprecipitation experiments, we wondered if we could map the interaction sites in the Sec61\(\alpha\)-subunit by screening a peptide library with \(^{14}\)C-labeled Exotoxin A. This assay identified the N-terminal region as a...
potential interaction site (data not shown).

To test whether there is indeed an Exotoxin A interaction at the N-terminus of the Sec61 α-subunit, we synthesized the mammalian Sec61 α-subunit N-terminal domain as peptide spots (12 amino acid residues; shifted by 2) on a cellulose membrane and performed Exotoxin A binding assays. As shown in Figure 3B, 14C-labeled Exotoxin A was bound to the N-terminal domain at amino acid residues 19–30; this represents the same region at which the Ca2+-CaM interaction site was identified and it is near the region where the Sec62 interaction with Sec61 α-subunit occurs in the absence of calcium.4

To further validate the binding properties of Exotoxin A around the IQ motif, we performed competition experiments with radioactively labeled and nonlabeled Ca2+-CaM as well as Exotoxin A. Ca2+-CaM bound much more efficiently to the IQ motif and effectively competed for Exotoxin A binding (Fig. 3C). In contrast, Exotoxin A bound less efficiently and was unable to compete with the binding of Ca2+-CaM. Thus, the affinity of Exotoxin A for the IQ motif in the N-terminal region of the Sec61 α-subunit appeared to be lower than that of Ca2+-CaM. This result was further substantiated by surface plasmon resonance (SPR) experiments, in which biotinylated peptides representing the N-terminal IQ peptide of Sec61 α-subunit or a region of the ER luminal part of loop 7 (negative control) were bound to a streptavidin-coated sensor chip and binding affinities were recorded. Ca2+-CaM bound with a high affinity, i.e., K_D of 136 nM, to the IQ peptide and was released quickly after the Ca2+ was removed (Fig. 4A). This K_D was consistent with the K_D that had previously been determined by fluorescence correlation spectroscopy (121 nM).1 By comparison, the Exotoxin A association was low and, in contrast to Ca2+-CaM,
Figure 3. Exotoxin A directly interacts with the N-terminus of the Sec61 α-subunit. (A) Antibody against Sec61β subunit co-precipitated Sec61 complex and radioactively labeled Exotoxin A added to the cell lysate, GAPDH antibody was used as negative control. Upper panel: autoradiogram; lower panel: detection of Sec61 α-subunit with antibody against the C-terminus of the Sec61 α-subunit. (B) The first 60 cytosolic N-terminal amino acids of the mammalian Sec61 α-subunit were synthesized as 12mers on cellulose membranes. 14C-labeled Exotoxin A was added overnight and binding was mapped on two membranes by radiography. The corresponding peptide sequences are depicted above the spots (IQ motif in red) and the known interaction regions with Sec62 and CaM are indicated by blue brackets. (C) 14C-Exotoxin A and GST-CaM or Exotoxin A and 14C-GST-CaM were mixed prior to addition to the membrane spotted with peptide corresponding to the Sec61α IQ motif (n = 6). The normalized radioactivity is shown.
Figure 4. In contrast to Exotoxin A, Ca\(^{2+}\)-CaM binds to the IQ motif of the Sec61 α-subunit with high affinity. (A) Calmodulin association and dissociation was measured by surface plasmon resonance at different concentrations in the presence of Ca\(^{2+}\). The dissociation constant was calculated as 136 nM. (B) Association and dissociation kinetics of Exotoxin A and Ca\(^{2+}\)-CaM were compared under similar conditions.
the toxin could not be released from the chip surface by a low ionic strength in the buffer (Fig. 4B). Exotoxin A was released from the immobilized peptide only with higher salt and detergent concentrations.

**Discussion**

Our results demonstrate that Exotoxin A interacts with the Sec61 α-subunit and suppresses Ca\(^{2+}\) leakage from the ER in human cells; this confirms the previous conclusion that the Sec61 complex is a major ER Ca\(^{2+}\) leak channel.\(^1,3,9\) Furthermore, we identified the IQ motif in the N-terminus of Sec61 α-subunit as an Exotoxin A binding site, which most likely explains the inhibitory effect of Exotoxin A on Ca\(^{2+}\) leakage from the ER. This motif was previously shown to provide the binding site for Ca\(^{2+}\)-CaM.\(^1\) We suggest that in both cases ligand binding to the IQ motif in the Sec61 α-subunit shifts the equilibrium between open and closed states of the Sec61 channel to the closed conformation. This is also consistent with the inhibitory effect of Exotoxin A on ER export of immunogenic peptides to the cytosol.\(^5\) We note that the IQ motif is close to the Sec62 binding site, which also contributes to Sec61 channel gating (Fig. 1).\(^6\)

Compared with the effects of BiP and Ca\(^{2+}\)-CaM, the physiological suppressors of Ca\(^{2+}\) leakage from the ER, the effect of Exotoxin A was not dramatic. We attribute this to the following facts: (1) the toxin has a lower affinity for the Sec61 complex as compared with Ca\(^{2+}\)-CaM; (2) Ca\(^{2+}\)-CaM has a much higher cytosolic concentration (6 µM) compared with the toxin (30 nM);\(^11\) and (3) due to its size the toxin can only be expected to act on ribosome-free Sec61 complexes (Fig. 5).\(^1,12\)

In principle, the inhibitory activity of Exotoxin A could contribute to its toxicity in HeLa cells.\(^13\) However, the low affinity of Exotoxin A for the IQ motif and its competition with Ca\(^{2+}\)-CaM argue...
Materials and Methods

Live-cell calcium imaging

Live-cell calcium imaging for cytosolic Ca\(^{2+}\) was performed with Fura-2, as previously described.\(^9\) The cytosolic Ca\(^{2+}\) concentration was deduced from ratio measurements by an established calibration method.\(^9\) Data were analyzed with Excel 2007. During the experiment, cells were treated with Exotoxin A (30 nM; Calbiochem) or the respective buffer, respectively, and after 10 min with either thapsigargin (1 µM) to unmask the ER calcium leakage or iomycin (5 µM) to release the total Ca\(^{2+}\) from the intracellular pools. Data were collected on the iMIC microscope and the polychromator V (Till Photonics) by alternating excitation at 340 and 380 nm and measurement of the emitted fluorescence at 510 nm. Images containing 50–55 cells/frame were sampled every 3 s.

Coimmunoprecipitation

Coimmunoprecipitation was performed with lysate of HeLa cells grown in 60 mm dishes at 37 °C and 5% CO\(_2\) to a confluence of 80–90%. Cells were washed twice with PBS (Gibco) and lysed in 1 ml of ice-cold Celllytic-M lysis buffer with protease inhibitor cocktail (Sigma). Supernatant was collected following centrifugation at 16,000 x g and 4 °C for 10 min and preclarified for 1 h with protein G- and A-sepharose (Amersham Biosciences). After removing the beads the preclarified lysate was incubated with \(^{14}\)C-Exotoxin A (20 µg/ml) for another 5 h at 4 °C. For input, 100 µl of the sample was collected. To precipitate the Sec61 complex, affinity purified antibody against Sec61β (rabbit; 1 µg/ml) or GAPDH (Santa Cruz, rabbit; 1 µg/ml) as a negative control was added to equal amounts of the \(^{14}\)C-Exotoxin A-treated lysate and incubated overnight at 4 °C. Precipitation of antibodies was performed by addition of 100 µl of protein G- and A-sepharose for 8 h at 4 °C. The beads were washed 3 times with 1 ml of NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 1% Nonider-P40). Precipitated protein as well as input were analyzed by SDS-PAGE and western blotting. For detection, a Sec61 α-subunit specific rabbit polyclonal antibody was used as well as a secondary peroxidase (POD)-coupled anti-rabbit antibody (Sigma) and ECL\(^\text{TM}\), visualized with a Lumi-ImagerF1 (Roche). Detection of \(^{14}\)C-Exotoxin A was performed by phosphorimaging and after visualization with the Typhoon-Trio imaging system (GE Healthcare).

Peptide spot-binding assays

IQ-peptide constructs (length 12 amino acid residues) or the first 60 cytosolic N-terminal amino acids (spots: length 12 amino acid residues, shifted by 2) of the mammalian Sec61 α-subunit were synthesized on acid-hardened cellulose membranes, derivatized with a polyethylene glycol spacer, as described previously.\(^1,2,15\) 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 CaCl\(_2\)). \(^{14}\)C-labeled proteins (GST, GST-CaM, and Exotoxin A) were added and incubated at 4 °C overnight. For competition experiments, \(^{14}\)C-Exotoxin A (62.5 nM) was preincubated with GST-CaM (625 nM) or \(^{14}\)C-GST-CaM (62.5 nM) with Exotoxin A (625 nM) prior to its addition to the membrane. The membrane was washed 3 times for 10 min with binding buffer, dried at room temperature, and subjected to phosphorimaging with a Typhoon imaging device (GE Healthcare).

SPR measurements

IQ peptide and a control peptide 325–339 were synthesized with biotinylated dipeptide KG at the C-terminus and the first peptide immobilized in the measuring cell of the Biacore avidin sensor chip SA.\(^3\) GST-CaM and Exotoxin A binding to IQ peptide were analyzed at analyte concentrations of 0.0625 to 1 µM (GST-CaM) and 5 µM (Exotoxin A) in comparison to peptide 325–339 in the reference cell. The running buffer was 10 mM HEPES/KOH pH 7.4, 150 mM NaCl, 6.4 mM KC1, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 0.005% Surfactant P2. Measurements were performed on a Biacore X instrument.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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