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Lanthanum ions inhibit the mammalian Sec61 complex in its channel dynamics and protein transport activity

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ABSTRACT

Previous electrophysiological experiments characterized the Sec61 complex, which provides the aqueous path for entry of newly-synthesized polypeptides into the mammalian endoplasmic reticulum, as a highly dynamic channel that, once activated by precursor proteins, fluctuates between main open states with mean conductances of 220 and 550 pS. Millimolar concentrations of lanthanum ions simultaneously restricted the dynamics of the Sec61 channel and inhibited translocation of polypeptides. Molecular modeling indicates that lanthanum binding sites cluster at the putative lateral gate of the Sec61 complex and suggests that structural flexibility of the lateral gate is essential for channel and protein transport activities of the Sec61 complex.

1. Introduction

Transport into the endoplasmic reticulum (ER) is the decisive step in the biogenesis of many polypeptides, including secretory and plasma membrane proteins. Transport into the ER is mediated by the Sec61 complex, which comprises α-, β-, and γ-subunits in mammals [1,2]. Analyses from electron microscopy (EM), as well as Cryo-EM, suggested the active Sec61 complex in the microsomal membrane comprises two to four heterotrimeric Sec61 subcomplexes [3–7] and its overall structure changes upon activation by precursor polypeptides [6]. This dynamic behavior of the Sec61 complex was confirmed by our previous electrophysiological analysis of the canine Sec61 complex [8]. Based on the crystal structure of the inactive archaean SecY complex, as well as recent cryo-EM studies with the same SecY complex bound to a non-translating ribosome, it was postulated that a single heterotrimer constitutes the protein conducting channel [9,10]. In addition, the α-subunit was characterized as comprising two linked halves clamped together by the γ-subunit. A so-called lateral gate was suggested on the opposite side that would allow signal peptides of precursor polypeptides to intercalate between transmembrane helices 2 and 7 of the α-subunit, thereby, opening the centrally located aqueous channel [9].

2. Materials and methods

2.1. Materials

CHAPS and deoxy big CHAP were purchased from Calbiochem. Purified L-α phosphatidylcholine (egg) was obtained from Larodan Fine Chemicals. Rabbit reticulocyte lysate (nuclease treated) was obtained from Promega. [^{35}S]methionine was from Perkin–Elmer. A plasmid that codes for mutated preprocecropin (ppcec) A was constructed using the QuickChange site directed mutagenesis kit (Stratagene). The mutated ppcec A protein contained two methionine residues substituted for isoleucines at positions 45 and 46, i.e. in the procecropin region.

2.2. Electrophysiological experiments

Rough microsomes (RM) were prepared from dog pancreas. Proteoliposomes were made from purified components as previously

Abbreviations: EM, electron microscopy; ER, endoplasmic reticulum; ppcec, preprocecropin; ppl, preprolactin; RM, rough microsomes

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described [8]. Vesicles for planar bilayer experiments were prepared by mixing (3:2, v/v) of the Sec61-containing vesicles with preformed liposomes (egg L-α phosphatidylcholine, 10 mg/ml) in 50 mM KCl, 10 mM Mops/Tris, pH 7.0. Mega-9 (nonanoyl-*N*-methylglucamide) was added to 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 a buffer (5 l) containing 50 mM KCl, 10 mM Mops/Tris, pH 7.0. Typically, 10 μl aliquots (10 mg/ml protein, lipid/protein 2:1 [w/w]) of the proteoliposomes derived from RM vesicles were incubated with 200 μM puromycin plus 2 mM GTP and 250–500 mM KCl for 15–30 min on ice. After 15 min, 1 μl aliquots underwent bilayer fusion. Proteoliposomes containing the purified Sec61 complex were subjected to bilayer fusion directly from dialysis.

Planar lipid bilayers were produced by the painting technique, as previously described [11]. The resulting bilayers had a typical capacitance of 0.5 μF/cm^2 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 previously described [8]. Voltage ramps were conducted at a rate of 6 mV/s. All electrolyte solutions were buffered with 10 mM Mops/Tris to pH 7.0.

2.3. Protein transport experiments

Nascent preprolactin (ppl), mutant preprocropin A, and Ubc6 were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine for 20 min at 30 °C. For ppcec A and Ubc6, translation was terminated by the addition of cycloheximide and RNaseA. The translation reaction was supplemented with buffer or RM as indicated. Following translation, the translation reactions were divided into two aliquots. The samples were treated with buffer, or proteinase K (Protease), or proteinase K plus 0.2% Triton X-100 for 60 min at 0 °C. All samples were subjected to SDS–PAGE and phosphor imaging. Alternatively, the microsomes were reisolated by centrifugation and subjected to chemical cross linking or carbonate extraction. For cross linking, the microsomes were resuspended in crosslinking buffer (50 mM triethanolamine, pH 7.4, 200 mM sucrose, 50 mM potassium acetate, 5 mM magnesium acetate) and further incubated further for 20 min at 30 °C in the presence or absence of puromycin and GTP (1.25 mM). Subsequently, the samples were incubated with SMCC (335 μM) (succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, Pierce) or its more polar relative Sulfo-SMCC for 30 min at 0 °C. For carbonate extraction, the microsomal pellets were resuspended in 100 mM Na_2CO_3 and incubated for 60 min on ice. After a second centrifugation, the pellets contained the membrane proteins.

2.4. Molecular modeling

The amino acid sequence of human Sec61 complex and its sequence alignment with archaean SecY complex were used [9]. Ten homology models were generated for the human Sec61 complex by the program MODELLER 9.5 using an all-hydrogen model [12]. Each model was optimized with the variable target function method, followed by the default refinement step using molecular dynamics with simulated annealing (equilibration for 0.8 ps each at 150, 250, 400, 700, and 1000 K; sampling for 2.4 ps each at 1000, 800, 500, 400, and 300 K). The whole optimization procedure was repeated three times and the best model was selected according to the molecular probability density function score. The homology model of the Sec61 complex was scanned for favorable ion binding sites using a grid with a 3 Å edge length. This scanning procedure was implemented in C++ using the BALL library [13] and energies between ion and protein model were calculated using the AMBER 96 force field [14]. At each grid point, a La^{3+} ion was placed and its position was energetically minimized using 500 steps of steepest descent while the protein atoms were held fixed. The potential energy of the most favorable position was used to define a threshold and all positions were removed with a potential energy higher than 60% of this value. The remaining ion positions were clustered so that binding sites separated by less than 1 Å were merged and sorted by ascending potential energy. As these binding sites may exclude each other, a second set of favorable positions was identified by iteratively placing La^{3+} ions at the sites sorted by ascending potential energy. In this setup, we included the solvation free energy by solving the Poisson–Boltzmann equation computed using the BALL implementation with a cubic grid of 0.6 Å spacing, atomic charges and radii from the AMBER 96 force field, 0 mM ionic strength, an internal dielectric constant of 2 and a solvent dielectric constant of 78, and setting the boundary potential at 8 Å distance to zero. Then we kept only those positions with a potential energy of less than 0 kJ/mol. The La^{3+} ions were modeled with Lennard–Jones parameters of Ca^{2+} ions with an atomic charge of +3e.

3. Results

3.1. Lanthanum ions arrest the Sec61 channel in the open state

We examined if trivalent cations such as lanthanum ions affect the Sec61 complex. In the first series of experiments, Sec61 complexes, present in either intact RM or proteoliposomes comprised of purified Sec61 complexes and defined phospholipids, were electrophysiologically characterized under our established conditions [8].

In the presence of 1 mM La^{3+}, we observed drastic changes in current recordings from the Sec61 channel. Mean conductances of Λ_{small} = 146 ± 3 pS and Λ_{large} = 316 ± 2 pS for RM vesicles and Λ_{small} = 103 ± 2 pS for Sec61 proteoliposomes were obtained in the presence of La^{3+}. Mean conductance values were decreased and their relative occurrence was reduced (Fig. 1a and b) compared to those obtained in the absence of La^{3+}, especially for the purified Sec61 complex, where nearly all single channel gating amplitudes corresponding to conductances >200 pS were inhibited (inserts in Fig. 1a and b). Furthermore, channel selectivity and its voltage dependent open probability were changed. The Sec61 channel remained in a voltage independent open state after addition of La^{3+} (Fig. 1c and d). Moreover, the reversal potential changed from V_{rev} = −5 mV ($P_{Cl^-}/P_{K^+} = 1.3:1$) to V_{rev} = −44 mV ($P_{Cl^-}/P_{K^+} = 10.4:1$) corresponding to an eightfold increase in anion-selectivity (Fig. 1e and f). These results indicate binding of La^{3+} to negatively charged groups facilitates the transport of anions through the pore, presumably by decreasing the net negative surface charge density of the pore. Moreover, negative charges seem to be an essential part of the voltage sensing gating moiety. Upon binding of La^{3+}, voltage dependent gating disappeared and the Sec61 channel remained in the fully open state. Thus, La^{3+} changes the open probability and the selectivity of the channel. Other trivalent ions such as Al^{3+} had similar effects (data not shown).

3.2. Lanthanum ions inhibit the Sec61 complex with respect to protein transport

Lanthanum ions were shown to restrict the dynamic behavior of the Sec61 channel. Therefore, we addressed whether or not La^{3+} affects the Sec61-mediated translocation of polypeptides into intact microsomes. First, nascent preprolactin (ppl-86mer) was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine. The translation reaction was supplemented with RM and divided