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In vitro synthesis and oligomerization of the mechanosensitive channel of large conductance, MscL, into a functional ion channel

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ABSTRACT

Elucidation of high-resolution structures of integral membrane proteins is drastically lagging behind that of cytoplasmic proteins. In vitro synthesis and insertion of membrane proteins into synthetic membranes could circumvent bottlenecks associated with the overexpression of membrane proteins, producing sufficient membrane-inserted, correctly folded protein for structural studies. Using the mechanosensitive channel of large conductance, MscL, as a model protein we show that in vitro synthesized MscL inserts into YidC-containing proteoliposomes and oligomerizes to form a homopentamer. Using planar membrane bilayers, we show that MscL forms functional ion channels capable of ion transport. These data demonstrate that membrane insertion of MscL is YidC mediated, whereas subsequent oligomerization into a functional homopentamer is a spontaneous event.

1. Introduction

Bacterial cells are surrounded by a lipid bilayer membrane, which serves as a scaffold for membrane proteins that perform many essential functions in the cell such as ion transport, stimulus transduction and energy transduction. Elucidation of the high-resolution structures of integral membrane proteins is lagging behind that of cytoplasmic proteins. There are currently only 250 unique high-resolution structures of membrane proteins present in the protein data base (curated data base of membrane proteins of known structure at http://www.blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Bottlenecks in the elucidation of membrane protein structures include overexpression, which can be toxic to the cell, targeting of the membrane protein for insertion, and correct folding and oligomerization of the protein in question.

In bacteria, membrane proteins are synthesized in the cytoplasm and are targeted to and inserted into the cytoplasmic membrane. Most of these membrane proteins are inserted co-translationally via the general secretory pathway otherwise known as the Sec system. Herein, ribosome-bound nascent chains are targeted by the bacterial signal recognition particle (SRP) to the SecYEG translocase via the SRP receptor FtsY (reviewed in [1]). Associated with the Sec translocase is the accessory protein YidC which is thought to play an important role in assisting the insertion and folding of transmembrane segments (TMSs) at the SecYEG channel [2]. YidC can also act independently of the Sec translocase where it functions as an insertase for a small subset of integral membrane proteins. In *Escherichia coli* it has been shown that YidC is essential for the insertion of subunit a and c of the F_1F_0 ATP synthase (F_oa and F_oc) [3], subunit a of the cytochrome *o* oxidase (CyoA) [4,5] and the folding of various membrane transporters. The mechanosensitive channel of large conductance, MscL, from *E. coli* was recently identified as using the YidC-only insertion pathway [6]. MscL acts as an emergency relief valve during osmotic downshock [7]. It forms a homopentamer in the crystal structure of the *Mycobacterium tuberculosis* homologue [8]. The activity of MscL can be measured by electrophysiological techniques, [9,10] making it a good model protein to investigate whether in vitro synthesized proteins can be inserted into synthetic membranes, oligomerize and form functional protein complexes.

Here, we have determined the minimal insertion requirements of in vitro synthesized MscL with the aim to produce fully functional MscL homopentamers in a cell-free system. We followed the activity of MscL in planar bilayer membranes in order not to manipulate the size of in vitro synthesized proteoliposomes, which are too small for patch clamp techniques. MscL insertion is strongly

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stimulated by YidC. MscL was observed to oligomerize to form homopentamers in vitro, which were fully functional as ion channels. Oligomerization of MscL is however inherent in the primary sequence and not chaperone mediated.

2. Materials and methods

2.1. Bacterial strains and plasmids

See S1 Materials and methods for bacterial strains used. The plasmids pET20MscL and pETMscLG22C were constructed for in vitro expression of MscL and MscLG22C, respectively. For details see S1 Materials and methods.

2.2. In vitro synthesis and insertion reactions

Synthesis reactions were carried out essentially as described in [11]. Briefly, reactions were carried out at 37 °C using T7 polymerase (Fermentas) and Easytag express protein labeling mix (Perkin Elmer) in the presence of inner membrane vesicles (IMVs) or proteo(liposomes). A small sample of the reaction was removed as a synthesis control and the remainder was spun through a 20% (w/v) sucrose cushion. Isolated membranes were resuspended and treated with proteinase K on ice. Samples were TCA-precipitated and analyzed by SDS–PAGE and phospho-imaging. To study the oligomerization of in vitro MscL, synthesis reactions were performed as described above except that proteinase K was added directly after synthesis. Membranes were collected through a sucrose cushion containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and solubilized in 0.1% dodecyl maltoside (DDM). Blue Native PAGE (BN-PAGE) analysis was performed on 5–15% gradient gels as described [12].

2.3. MscL activity measurements in planar bilayer membranes

MscLG22C was synthesized in vitro in the presence of empty or YidC-containing *E. coli* lipid:ergosterol (molar ratio 4:1) (proteo)liposomes. Insertion reactions were performed as described above except that the isolated membranes were resuspended in 50 mM Tris–HCl, pH 8. Nystatin (Sigma–Aldrich) was stored as powder at −20 °C and 2.5 mg/ml stocks in dry methanol were prepared fresh before every use. The stock was added to the proteoliposome suspension to a final concentration of 150 μg/ml, vortexed for 5 min and stored on ice until use.

The planar lipid bilayer workstation from Warner Instrument (Harvard Apparatus, Holliston, MA) was used for single-channel recordings. The bilayer set-up consists of two chambers connected by a 150 μm aperture through a Delrin cup. Painted planar bilayers were formed in the aperture from a 10 mg/ml *n*-decane solution of PE:PC (7:3, w/w) as described [13,14]. The cup was placed in a black Delrin chamber and filled with 1 ml bilayer buffer (150 mM NaCl, 8 mM HEPES, pH 7.5). Membranes with capacitances of 60 pF or above were used for the experiments.

The chamber to which the proteoliposomes were added is referred to as "*cis*" and the other as "*trans*". The proteoliposomes were added into the *cis* chamber and their fusion to the bilayer were induced by forming an osmotic gradient of 720:150 mM NaCl (*cis*/*trans*) across the bilayer. Signals were recorded with a Bilayer Clamp Amplifier (BC-535) (Warner Instrument), and data were analyzed by pCLAMP10 (Axon Instruments, Union City, CA). Signals were sampled at 50 kHz and filtered at 5 kHz. Experiments were conducted at a room temperature of 18 ± 2 °C.

Liposome fusions were usually detected immediately as sudden transient increases in bilayer conductance, resulting from the incorporation of nystatin channels via the fusion of proteoliposomes. MscL channels were activated by the addition of MTSET to a final concentration of 1 mM to the *trans* chamber.

2.4. Lipids

E. coli phospholipids (*E. coli* Total Lipid Extract), L-α-phosphatidylcholine (Brain PC) and L-α-phosphatidylethanolamine (Brain PE) were purchased from Avanti Polar Lipids and ergosterol (45480) was purchased from Sigma–Aldrich.

2.5. Miscellaneous

YidC [15] and SecYEG [16] were purified and reconstituted in *E. coli* phospholipids at protein/lipid ratios indicated. Signal capture and quantification were performed using FUJIFILM LAS-4000 luminescent image analyzer. IMVs were isolated as described previously [17].

3. Results

3.1. YidC alone is sufficient to catalyze MscL insertion

MscL is a 15 kDa protein containing two TMSs separated by a periplasmic-facing polar loop. A C-terminal helix protrudes into the cytoplasm (Fig. 1A). Insertion of in vitro synthesized MscL was investigated using proteoliposomes so that the minimal requirements of MscL insertion could be defined (Fig. 1B). YidC was reconstituted into *E. coli* lipids with a YidC/lipid ratio (w/w) of 0.03 and 0.06. MscL was cloned into the expression vector pET20b for in vitro synthesis from the T7 promoter and synthesized using an *E. coli* BL21 (DE2) Rosetta™ lysate and [^{35}S]methionine. This resulted in the production of a ~16 kDa protein visualized on SDS–PAGE (Fig. 1B, lane 1). When in vitro synthesis was performed in the presence of (proteo)liposomes, proteinase K treatment of MscL resulted in the protection of full length MscL as well as two other fragments of ~15.5 and ~12 kDa (Fig. 1B, lanes 5–7). The larger fragment most likely represents digestion of a few C-terminal residues while the smaller fragment represents the two TMSs of MscL (ΔC-MscL). An increase in the YidC content of the proteoliposomes led to an increase in MscL insertion (Fig. 1B compare lanes 6 and 7). The presence of empty liposomes also led to proteinase K protected MscL (Fig. 1B compare lanes 5 and 8). This could be due to non-specific hydrophobic interactions between the liposomes and the TMSs of MscL or there could be a low level of MscL insertion in the absence of YidC. The results of three independent experiments were quantified and expressed as the percentage of in vitro synthesized MscL inserted (Fig. 1C). SecYEG proteoliposomes did not support MscL insertion (Supplementary Fig. 1). YidC alone is therefore sufficient to catalyze MscL insertion into proteoliposomes.

3.2. MscL forms a pentamer in vitro

Since the MscL ion channel is a homopentamer, the oligomeric state of in vitro synthesized MscL was investigated by BN-PAGE (Fig. 2). Following synthesis in the absence or presence of (proteo)liposomes, samples were incubated with proteinase K, and the membranes were collected through a sucrose cushion, solubilized in DDM and subjected to BN-PAGE. When MscL was synthesized in the absence of (proteo)liposomes and proteinase K, a mixed population of MscL was observed (Fig. 2, lane 1). The dominant species was observed below 66 kDa. In general membrane proteins migrate more slowly on BN-PAGE than globular proteins. Approximation of the size of the dominant species was not possible owing to its small size but since it was the smallest species