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

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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. © 2010 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

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). Bottleeckes 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 o F 1 ATP synthase (F o,a and F o,c) [3], subunit a of the cytochrome c 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
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

MscL-G22C 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 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 7.3). Membranes with capacitances of 60 pF or above were used for the experiments.

The membrane 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 [35S]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 (AC-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...
observed, it likely represents the MscL monomer. Also, when the sample was heat treated, most of the signal could be attributed to the small species (Fig. 2, lane 2). A broad “fuzzy” signal was observed above this small species possibly corresponding to differently folded monomers as observed for Lactococcus lactis MscL [18]. A third species was present at approximately 120 kDa. The MscL homopentamer would have a molecular mass of 75 kDa. The migration of membrane proteins by BN-PAGE has been investigated and a correction factor of 1.8 has been suggested to take into account the contribution of coomassie brilliant blue dye binding and bound detergent [19]. Using this correction factor, the MscL pentamer should migrate at 135 kDa. The species at 120 kDa therefore most likely represents the MscL homopentamer. When (proteo)liposomes were present, only the MscL pentameric species was observed after proteinase K treatment (Fig. 2, lanes 4 and 5). This does not represent a proteinase K resistant pentamer in solution, since no MscL was observed when proteinase K was added to MscL synthesized in the absence of liposomes (Fig. 2, lane 3). The observations suggest that YidC is not essential for MscL pentamer formation as these were found in solution as well as inserted into empty liposomes, albeit at low levels.

3.3. In vitro synthesized MscL inserted into proteoliposomes is functional

The observation that pentameric MscL was present in (proteo)liposomes did not reveal whether it formed functional ion channels in vitro. To address this question, the ion channel activity of in vitro synthesized/inserted MscL was investigated with electrophysiology using planar bilayer membranes. To date MscL has been studied with patch clamp techniques, which are performed on giant proteoliposomes or giant spheroplasts, which are at least 5–10 μm in diameter. In order not to manipulate the proteoliposomes by increasing their size, we decided to use planar bilayer measurements. The method is based on fusion of liposomes doped with a polye- ray antibiotic, nystatin, with planar bilayers in the presence of an osmotic gradient [20]. It relies on the ability of nystatin to form channels in the presence of the membrane sterol, ergosterol and dissociate in the absence of it [21–24]. Fusion of liposomes to the bilayer can be detected since this results in the simultaneous insertion of many nystatin channels. If the planar bilayer is devoid of ergosterol, nystatin channels are inactivated within a few seconds following insertion, whereas any other co-reconstituted channel remains active in the bilayer.

This procedure was adapted for incorporation of MscL from liposomes to the planar bilayer. Herein, a G22C mutant of MscL was used [25]. Briefly, MscLG22C-containing E. coli lipid-ergosterol liposomes were doped with nystatin and fused to the bilayer in the presence of an osmotic gradient. MscL activity was followed by exploiting the charge-induced activation of MscLG22C [25,26]. MscLG22C can be activated by labeling the cysteine at position 22 with the charged molecule [2-(trimethylammonium)ethyl] methane thiosulfonate bromide (MTSET). This covalent modification results in the opening and closing of the channel in the absence of its native trigger, i.e. membrane tension. Covalent modification of MscL produces channels with flickery openings and pronounced sub-states [27].

MscLG22C was cloned into pET20b to create pETMscLG22C to allow in vitro synthesis. The dependence of MscLG22C on YidC for insertion was tested using inner membrane vesicles (IMVs) isolated from YidC− and YidC+ strains [28] (Fig. 3A). YidC levels were monitored by Western blot to confirm YidC depletion (data not shown). When in vitro synthesis was performed in the presence of IMVs, MscLG22C (Fig. 3A, upper panel) insertion was greatly reduced in the YidC− strain when compared to YidC+ as observed for...
the wild type Mscl (Fig. 3A, lower panel). Solubilization of the IMVs with Triton X100 resulted in complete degradation of full length MsclG22C and Mscl with a small residual protein band of 11–12 kDa remaining possibly resulting from the formation of a detergent micelle around the hydrophobic segments of Mscl (Fig. 3A, upper and lower panels, lanes 3 and 6). Also, YidC was reconstituted into liposomes containing E. coli lipids and ergosterol, and these were also able to catalyze Mscl insertion (Fig. 3B).

To measure the activity of in vitro-synthesized and inserted Mscl, synthesis reactions were performed in the presence or absence of plasmid pETMsclG22C in the presence of empty or YidC-containing E. coli lipid:ergosterol (proteo)liposomes. Reactions were treated as for the oligomerization studies except that the membranes were resuspended in 50 mM Tris–HCl, pH 8. In Fig. 4, single channel activity measurements of Mscl in planar lipid bilayers are shown. Synthesis reactions were performed with pETMsclG22C in the presence of YidC-containing proteoliposomes and the isolated liposomes were fused to the lipid bilayer (Fig. 4A). Upon addition of MTSET Mscl channel activity was observed. Similarly, when MsclG22C was synthesized in vitro in the presence of empty liposomes, fusion of the isolated liposomes to the lipid bilayer, also resulted in channel activity upon addition of MTSET (Fig. 4B). As a control, purified MsclG22C was reconstituted into E. coli lipid:ergosterol liposomes and fused to the lipid bilayer. Upon addition of MTSET, a high level of channel activity could be observed (Fig. 4C). When the synthesis reactions were performed in the absence of plasmid pETMsclG22C in presence of YidC-containing proteoliposomes (Fig. 4D) or empty liposomes (Fig. 4E), no activity was measured. The channel activity measured in Fig. 4A and B is therefore due to the presence of in vitro synthesized Mscl and not compromised membrane integrity.

Together with the results obtained from BN-PAGE analysis of in vitro synthesis assays, this also indicates that although YidC stimulates Mscl insertion, oligomerization is inherent in the primary sequence of Mscl.

![Fig. 3. MsclG22C insertion is YidC dependent. (A) Insertion assays with YidC+ (lanes 1–3) and YidC− (lanes 4–6) IMVs were performed as described in the legend to Fig. 1 with plasmids pETMsclG22C (upper panel) and pETMscl (lower panel) as templates. (B) MsclG22C was synthesized in the presence or absence (lanes 3 and 6) of (proteo)liposomes containing E. coli lipids and ergosterol at a molar ratio of 4:1 and either no protein (lanes 1 and 4) or YidC (lanes 2 and 5). For the reconstitutions, YidC was used at protein/lipid concentrations indicated. Standards of 10% of the synthesis reactions are shown (lanes 1–3).](image1)

![Fig. 4. In vitro-synthesized Mscl forms functional ion channels independently of YidC. MsclG22C was synthesized in vitro as described in the legend to Fig. 2. The isolated (proteo)liposomes were reconstituted in 50 mM Tris–HCl, pH 8 prior to doping with nystatin. Once the bilayer was formed, a salt gradient was generated following which proteoliposomes were fused to the bilayer. Mscl channels were activated by adding MTSET to a final concentration of 1 mM. Single channel conductance of Mscl in planar lipid bilayers are shown. Recordings of 20 s are shown in the left panel while an expansion of the segments indicated by an asterisk is shown in the right panel. (A) MsclG22C was synthesized in the presence of YidC-containing proteoliposomes. (B) MsclG22C was synthesized in the presence of empty liposomes. (C) As a control, purified MsclG22C was reconstituted into empty liposomes prior to doping the liposomes with nystatin and activity was measured. (D) Synthesis reactions were performed in the without plasmid pETMsclG22C in the presence of YidC-containing proteoliposomes. (E) Synthesis reactions were performed in the absence of plasmid pETMsclG22C in the presence of empty liposomes.](image2)
4. Discussion

Here we demonstrate that MscL synthesized in vitro is inserted into the membrane by YidC yielding a functionally active homotetramer. Our data confirm a previous in vivo study that indicated that MscL is a substrate of the YidC-only insertion pathway [6], but oppose the view that YidC is required only for the oligomerization and/or stability of MscL in the membrane rather than insertion itself [29]. The reconstitution data indicates that in the absence of YidC, only a low level of ‘spontaneous’ insertion is observed. Using F_{C}C mutants defective in oligomerization it has been tested whether YidC functions only as a chaperone, stabilizing “spontaneously” inserted F_{C}C and mediating its oligomerization into decameric rings, or whether it is involved in the actual insertion of this protein [30]. The study showed that YidC does indeed function as an inserter, which is now further supported by the data presented on MscL.

We used a newly developed method to determine the activity of MscL inserted into (proteo)liposomes. This allowed us to determine whether in vitro synthesized MscL could form active ion channels. Channel activity was observed with MscL inserted both in YidC-containing and empty (proteo)liposomes. However, the proteinase-protection assays show that reconstituted YidC significantly increases the amount of membrane-inserted MscL in vitro. We thus conclude that YidC catalyzes insertion of MscL, while the formation of pentameric ion channels is inherent in the primary structure of the protein.

Spontaneous insertion in in vitro systems utilizing liposomes is an inherent problem associated with the use of purified E. coli lipids [31] and has been well-documented for the phase coat proteins M13 and PF3 [32,33]. Originally these proteins were thought to spontaneously insert in vivo but it was later shown that YidC stimulates the integration of PF3 in proteoliposomes [34]. The inclusion of diacylglycerol (DAG) into liposomes blocks spontaneous insertion in vitro and has been utilized to more accurately define the roles of proteinaceous factors in membrane insertion [35]. DAG is present in E. coli MC4100 at a concentration of 1.5% and is therefore likely sufficient to prevent any spontaneous insertion in vivo [35]. A recent study demonstrates that in vitro synthesized MscL can insert spontaneously into E. coli liposomes yielding function channels [36]. However, unlike this study, the effect of YidC on the insertion efficiency or kinetics was not examined (Ref. [38] is cited in Supplementary material).

The use of in vitro systems to express large quantities of proteins coupled to insertion reactions as those described in this study could circumvent bottlenecks associated with the overexpression of membrane proteins. As shown here with MscL, it is possible to synthesize a membrane protein in vitro and, if the insertion and oligomerization requirements are known, isolate functional protein complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febsi.let.2010.11.057.


