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# Coupled cell-free synthesis and lipid vesicle insertion of a functional oligomeric channel MscL
# MscL does not need the insertase YidC for insertion *in vitro*

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ABSTRACT

The mechanosensitive channel MscL of the plasma membrane of bacteria is a homopentamer involved in the protection of cells during osmotic downshock. The MscL protein, a polypeptide of 136 residues, was recently shown to require YidC to be inserted in the inner membrane of *E. coli*. The insertase YidC is a component of an insertion pathway conserved in bacteria, mitochondria and chloroplasts. MscL insertion was independent of the Sec translocon. Here, we report sucrose gradient centrifugation and freeze-etching microscopy experiments showing that MscL produced in a cell-free system complemented with preformed liposomes is able to insert directly in a pure lipid bilayer. Patch-clamp experiments performed with the resulting proteoliposomes showed that the protein was highly active. *In vitro* cell-free synthesis targeting to liposomes is a new promising expression system for membrane proteins, including those that might require an insertion machinery *in vivo*. Our results also question the real role of insertases such as YidC for membrane protein insertion *in vivo*.



## 1. Introduction

The *in vitro* synthesis of membrane proteins represents a new and powerful system for their large scale production. Cell-free (CF) synthesis systems are by nature open and allow the addition of any compound that might be necessary for protein folding. It has been shown recently that several membrane proteins, including transporters, channels and G protein-coupled receptors, can be produced in cell extracts supplemented with detergents or non-detergent surfactants [1–7], for a review see [8]. In several cases, the proteins were shown to oligomerize in detergent [1,2] and were functional when reconstituted in a lipid bilayer [1,2,4,6]. A wide range of detergents can be used for *in vitro* studies.

While cell-free synthesis of membrane proteins in the presence of detergent is now an established procedure, a few studies have more recently started to address the possibility of producing membrane proteins *in vitro* in the absence of detergent and in the presence of liposomes. The bacterial potassium channel KcsA can insert and tetramerize in a pure phospholipid bilayer when it is produced *in vitro* [9]. The *Escherichia coli* mannitol permease, the M13 procoat and Pf3 procoat also insert spontaneously in liposomes [10,11]. When bacteriorhodopsin was produced by CF expression in the presence of added liposomes, it was shown by protease protection experiments to be targeted and inserted in the liposomes. The resulting proteoliposomes were able to pump protons when appropriately illuminated [12]. The transmembrane subunit of human NADH oxidase (gp91) was amenable to cellular delivery when it was produced *in vitro* in proteoliposomes [13]. Moreover, the *in vitro* co-production of the mitochondrial porin VDAC (Voltage Dependant Anionic Channel) and the pro-apoptotic protein Bak in the presence of lipid vesicles resulted in the formation of mix proteoliposomes [14,15]. The secretin PulD from the outer membrane of *Klebsiella oxytoca* was only synthesized as monomers when produced in the presence of detergent. However, when the detergent was replaced by liposomes, PulD formed typical secretin rings composed of 12 monomers [16]. AqpZ, the aquaporin from *E. coli*, was also reported to insert in liposomes after cell-free synthesis. Stopped flow light scattering experiments performed on the resulting proteoliposomes were indicative of water transport [17]. Finally, nanolipoprotein particles have recently been used in combination with cell-free synthesis for the production of membrane

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proteins [18]. Nanolipoprotein particles are discoidal particles comprising amphipathic helical membrane proteins that wrap themselves around a piece of planar lipid bilayer.

Coupled CF synthesis and targeting to liposomes or nanolipoprotein particles might thus become an interesting new system for membrane protein production, allowing one to bypass protein purification and reconstitution for functional studies. However, it is unclear how these observations can be reconciled with the accepted view that most membrane proteins, eukaryotic or prokaryotic, require membrane insertases for their correct membrane insertion and/or assembly. Whereas the Sec translocation pathway is found in the endoplasmic reticulum of eukaryotic cells and in the plasma membrane of prokaryotic cells, the recently discovered YidC/OXA1 membrane insertase is present only in prokaryotic and organellar membranes [19,20]. Since KcsA, Bacteriorhodopsin, gp91, PulD or AqpZ are not known to require specific factors for membrane insertion, one cannot yet draw clear conclusions on the general applicability of CF synthesis and targeting to liposomes. However, the *E. coli* mannitol permease, normally uses SecYEG for insertion and the M13 and Pf3 procoats use YidC insertase *in vivo*.

The double spanning MscL protein [21], a mechanosensitive channel in the inner membrane of bacteria, is a homopentameric complex [22] that is required for the survival after hypo-osmotic shock [23,24]. MscL has recently been shown to require YidC for insertion in the membrane or for oligomerization in the membrane [25,26]. We previously showed that MscL can be produced *in vitro* in the presence of various detergents or non-detergent surfactants [2,7]. In the present work, we determine whether the protein MscL can integrate directly into preformed pure lipid vesicles added to an *in vitro* system devoid of detergent or surfactant.

## 2. Materials and methods

### 2.1. In vitro synthesis

Plasmid DNA (0.5 μg) carrying the coding region of *mscL* [2] was incubated in a commercial bacterial lysate (Roche RTS 100 HY or RTS 100 Disulfide Kits containing 50 μl) for 6 h at 30 °C in accordance with the instructions. For large scale preparation, we used the RTS 500 lysate, working under exchange conditions: a 1 ml reaction chamber containing the *E. coli* lysate and the plasmid DNA is separated from a 10 ml feeding chamber by a semi-permeable membrane that allows continuous supply of substrates and removal of inhibitory by-products, thus extending the duration of expression and the protein yield. Plasmid DNA (15 μg) was incubated for 20 h in the RTS 500 HY lysate. In some cases, the lysate was centrifuged at 109,000×*g* for 10 min before use. Where indicated, the lysate was supplemented with Asolectin liposomes (up to 4 mg/ml).

### 2.2. Preparation of liposomes

Liposomes were prepared from Asolectin IV-S (Sigma), an extract from soybean lipids enriched with 1,2-diacyl-*sn*-glycero-3 phosphocholine. Lipids were dissolved in chloroform; after evaporation, the lipid film was sonicated in water to a final concentration of 10 to 40 mg/ml.

### 2.3. Proteoliposome recovery and purification

Proteoliposomes were easily recovered by slow centrifugation (23,000×*g* for 10 min) of the reaction mixture diluted five-fold in 25 mM Tris–HCl (pH 7.0) or in 10 mM HEPES–KOH (pH 7.4). For urea treatment, the pellet was resuspended in 200 μl of 20 mM Tris (pH 7.0) containing 4 M urea. After incubation at room temperature for 15 min, the suspension was diluted twice with 20 mM Tris containing 250 mM NaCl before centrifugation for 30 min at 285,000×*g* at 18 °C in a Beckman TLA-100.2 rotor. For electrophysiology experiments, the pellet recovered after the slow centrifugation was resuspended in 200 μl of 10 mM HEPES–KOH (pH 7.4), 100 mM KCl. Aliquots of this suspension were then used to form giant liposomes as described below.

Proteoliposomes were also purified by floatation sucrose gradient centrifugation essentially as for *E. coli* membrane proteins [27]. For each gradient, two RTS 100 lysates containing the synthesized protein were mixed with sucrose and diluted with 25 mM HEPES–KOH (pH 7.4) to a final volume of 300 μl and a final w/w sucrose concentration of 60%. Steps (350 μl) were created using 56.2, 53.2, 50.2, 47.1, 44.2, 41.2, 38.1, and 35.9% sucrose solutions, and the tubes were centrifuged in a Beckman SW55Ti centrifuge for 40 h at 285,000×*g* at 10 °C. Fractions were collected by the top and proteins in a sample of each fraction were precipitated in 10% trichloroacetic acid and analyzed by SDS-PAGE in gels containing 15% acrylamide followed by immunoblotting to detect MscL.

### 2.4. SDS-PAGE and immunoblotting

For SDS-PAGE analysis, samples from *in vitro* synthesis reactions were precipitated in 80% of acetone at −20 °C. Pellets were resuspended in an SDS buffer containing 10 M urea and incubated at 60 °C during 15 min. Proteins were separated by SDS-PAGE in ready gel precast gels (Bio-Rad) containing a 4–20% gradient acrylamide using Tris–HCl glycine buffers. Proteins in the gels were either stained with Coomassie brilliant blue or transferred onto nitrocellulose membranes and detected using a specific antibody against the histidine tag, secondary antibodies coupled with horseradish peroxidase, and chemiluminescence using Pierce ECL reagents. Cross-linking experiments of MscL channels were performed as described previously [2], using 2% formaldehyde.

### 2.5. Freeze fracture electron microscopy

Samples were centrifuged and the pellets were resuspended in 30% glycerol-containing buffer. An aliquot of the sample was placed on the copper holder and quenched in liquid propane cooled with nitrogen. The frozen sample was fractured at −125 °C *in vacuo* of about $10^{-5}$ Pa with a liquid nitrogen-cooled knife in a Balzers 300 freeze-etching unit as described [28]. The fractured sample was replicated with a 1–1.5 nm deposit of platinum-carbon, and coated with 20 nm carbon film. The Pt/C replica was cleaned with 2% SDS, washed with pure water, transferred onto copper EM grid and observed with a Philips CM100 electron microscope.

### 2.6. Formation of giant proteoliposomes

Proteoliposomes obtained by centrifugation or by sucrose gradient fractionation were supplemented with pure liposomes in order to achieve a given lipid to protein ratio (from 40 to 140). After sonication, the mixture was subjected to 3 freeze–thaw cycles and ultra-centrifuged (300,000×*g* for 25 min). To obtain giant proteoliposomes amenable to patch-clamp recordings, the pellet was resuspended in 20 μl of 10 mM HEPES–KOH, pH 7.4, and the resulting suspension was subjected to a dehydration/rehydration cycle as previously described [29]. Rehydration was performed in 10 mM HEPES–KOH, 100 mM KCl, pH 7.4. A 2 μl sample of the giant proteoliposome suspension was deposited in a patch-clamp chamber and diluted with 2 ml of bath solution (10 mM HEPES–KOH, 100 mM KCl, pH 7.4).

### 2.7. Patch-clamp recording

Single-channel activity was recorded using standard patch-clamp methods. Patch electrodes were pulled from Pyrex capillaries (Corning

code 7740) using a P-2000 laser pipet-puller (Sutter Instruments Co.) and were not fire polished before use. Micropipettes were filled with a buffer similar to that of the patch-clamp chamber consisting of 5 mM $MgCl_2$ and 2 mM $CaCl_2$. A negative pressure (suction) was applied to the patch pipette with a syringe and monitored with a piezoelectric pressure transducer (Bioblock Scientific). Records were filtered at 1 kHz (−3 dB point) through a four-pole Bessel low-pass filter, digitized at a rate of 2 kHz, and analyzed on a personal computer, with a pClamp (Axon). The membrane potential refers to the potential in the bath minus the potential in the pipette.

## 3. Results

### 3.1. In vitro synthesis of MscL protein in the presence of pure lipid vesicles

*In vitro* synthesis of MscL, without any addition to the lysate, yielded about 1 ml of protein per ml of lysate, under exchange conditions. We reported previously that a similar amount of protein can be obtained in the presence of some detergents and surfactants at concentrations above the critical micellar concentration (CMC) [2,7]. We observed here that MscL can be produced with the same efficiency when liposomes, obtained by sonication of azolectin lipids, were added to the lysate up to a concentration of 4 mg lipids per ml. Routinely, we used a concentration of 2 mg lipids per ml of lysate (Fig. 1A).

Floatation gradient centrifugation of total synthesis mixtures was used to distinguish between liposome association and aggregation of MscL in liposome-supplemented fraction. All the MscL protein produced floated to the top of the gradient (35% sucrose), whereas MscL aggregates, obtained in the liposome-free control mixture, remained at the bottom of the tube (Fig. 1.B). When MscL, solubilized from *E. coli*, or synthesized *in vitro* in the presence of detergent, is incubated with a cross-linking agent and later subjected to SDS-PAGE, five bands corresponding to the different levels of oligomerization can be observed [2]. When cross-linking was performed with the MscL protein synthesized *in vitro* in the presence of liposomes, the same pattern was observed (Fig. 1.C). As noted before [2], MscL protein synthesized *in vitro* in the absence of detergent and lipids is also able to oligomerize.

Although sedimentation of pure lipid vesicles normally needs high speed ultracentrifugation, we recently made the surprising observation that liposomes added to the RTS lysate mixture are readily and quantitatively sedimented by centrifugation at speeds as low as 23,000 ×*g*, for 10 min [16]. The same observation was made here. After centrifugation at 23,000 ×*g* for 10 min, the *in vitro* synthesized MscL was mainly detected in the pellet fraction of the lysate, irrespective of the absence or presence of 2 mg/ml of lipids (Fig. 2). Even though these pellet fractions were enriched with MscL proteins, they also contained proteins from the lysate. A comparison of different conditions (centrifugation before or after synthesis, absence or presence of liposomes) presented in the Fig. 2 revealed that pellet fraction contamination was independent of the presence of lipids and existed only when protein synthesis had occurred, possibly due to association of some proteins in the lysate, such as poly-ribosomes that have been formed during synthesis.

To distinguish between simple association of MscL proteins to liposomes and true insertion, the material from liposome-supplemented reactions was treated with 4 M urea. Upon centrifugation, the MscL proteins were recovered with the liposome pellet (data not shown). These data which show that MscL was not readily released by urea, strongly suggest that it was inserted in the liposomes.

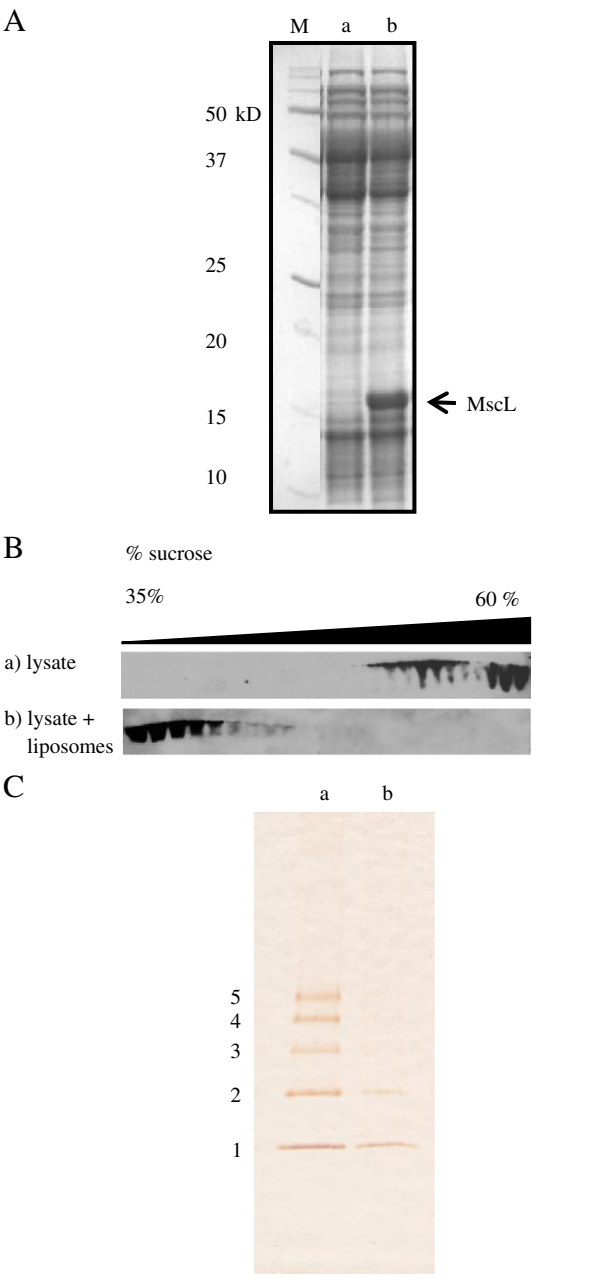


**Fig. 1.** *In vitro* synthesis and localization of MscL in the presence of liposomes. A. The protein MscL was produced *in vitro* in the presence of liposomes. Extracts of lysate of RTS 100 Disulfide before (a) and after (b) protein production with 2 mg/ml of azolectin liposomes. Samples were analyzed by SDS-PAGE after Coomassie Blue staining. Each lane was loaded with 2.5 µl (out of 50 µl) of the RTS100 lysate. M: molecular weight standards. B. Floatation sucrose gradient analysis of *in vitro* synthesized MscL in the presence of liposomes. RTS 100 HY reaction mixtures with (b) or without (a) liposome supplementation were loaded on the bottom of a sucrose gradient, as indicated in Materials and methods. After ultracentrifugation, fractions were collected from the gradients and dissolved in SDS. MscL proteins were detected by SDS-PAGE and immunoblotting. C. Cross-linking of MscL channels synthesized *in vitro* in the presence (a) or in the absence (b) of liposomes. Samples were treated with 2% formaldehyde for 1 h at room temperature. After a treatment for 15 min with 8 M urea at 60 °C, MscL proteins were detected by SDS-PAGE and immunoblotting.

### 3.2. Visualization of proteoliposomes by freeze-fracture electron microscopy

After MscL synthesis, the lysate was centrifuged and freeze-fracture electron microscopy of the pellet was used to visualize directly the insertion of MscL protein into liposomes. When synthesis was performed in the absence of added liposomes, large aggregates, presumably corresponding to precipitated MscL, were detected. When synthesis was performed in the presence of liposomes which

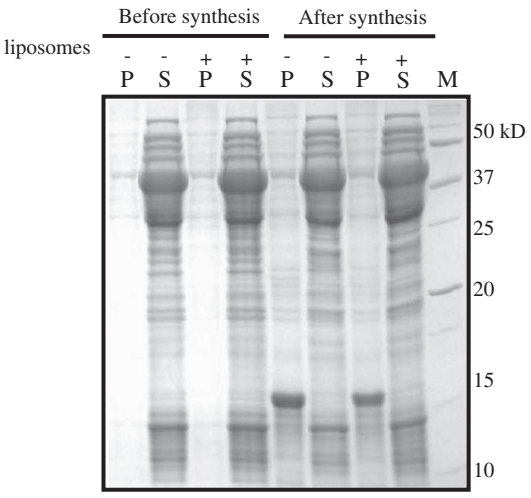


**Fig. 2.** MscL is detected in the pellet fraction. The synthesis is carried out in the absence (−) or in the presence (+) of 2 mg/ml of azolectin liposomes. In each case, lysates before or after synthesis, as indicated, were diluted 5 times and centrifuged at 23,000 ×g for 10 min. Pellet (P) and supernatant (S) extracts were analyzed as in Fig. 1A. Each lane was loaded with the equivalent of 2.5 μl (out of 50 μl) of the RTS100 lysate. M: molecular weight standards.

were initially smooth, they displayed characteristic particulate surfaces, indicating that membrane proteins had inserted in the lipid bilayers. Aggregates were not observed under these conditions (Fig. 3).

### 3.3. Patch-clamp recordings of functional MscL channels inserted into liposomes

The functionality of the MscL protein produced in the presence of liposomes was assayed by the patch-clamp technique. Proteoliposomes obtained by centrifugation or by sucrose gradient fractionation were supplemented with pure liposomes in order to achieve a given lipid to protein ratio and the mixture was subjected to a dehydration–rehydration cycle to yield giant proteoliposomes suitable for patch-clamp recording. Since in these experiments MscL was not purified, its exact concentration could not be determined. On the basis of previous experiments, the yield was assumed to be around 1 mg/ml lysate. When MscL was purified, the variation around this mean, which depends on the batch, does not exceed 50%. In initial experiments, a lipid to MscL protein ratio (weight/weight) of 40 was used. In previous experiments with MscL produced either *in vitro* in the presence of detergents or in bacteria and then solubilized in detergent purified and reconstituted, the average number of activated channels per patch was around 8–9 [2]. This number is obtained by averaging the maximum number of channels that were observed to open under pressure until the rupture of the patch. Surprisingly, in experiments performed with MscL synthesized in the presence of liposomes, a much larger number of channels per patch was observed. Fig. 4 A shows a typical recording where the application of pressure elicits a robust mechanosensitive current. Individual openings are not resolved but the current corresponds to the sum of 20–25 channel unitary currents, even at a rather moderate applied pressure (40 mm Hg). Due to these large currents, the patches were often so unstable that a lower lipid to protein ratio (140) had to be used. Under these conditions the mean number of channels per patch was $6.6 \pm 2.3$ ($n = 8$). As shown in Fig. 4B, at membrane voltage of −10 mV, application of negative pressure in symmetrical 100 mM KCl media resulted in the opening of typical 1500 pS MscL channels that closed upon release of the pressure.

A high resistance seal could not be obtained in most patches. We speculated that the leak current was flowing through open porin

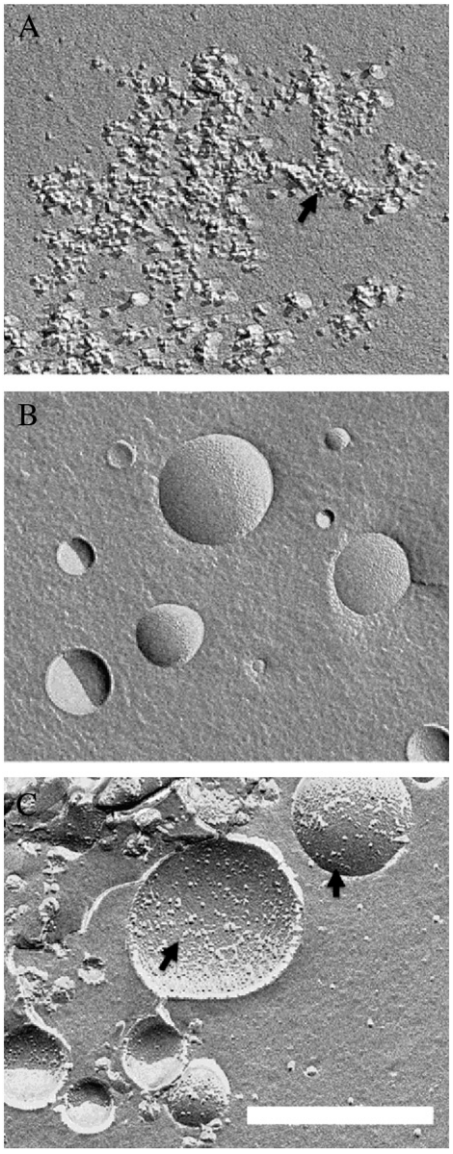


**Fig. 3.** Electron micrograph of MscL synthesis carried out in the absence (A) or in the presence (C) of liposomes. Arrows indicate aggregated protein in A and reconstituted MscL particles into liposomes in C. B is an electron micrograph of freeze-fractured liposomes used in C. Scale bar is 500 nm.

channels. Porin channels are permanently open at low potential and close at high voltage. Indeed, when, in the absence of applied pressure, the voltage was stepped to high voltage (120–140 mV), the closure of channels with all the characteristics of porin channels [30] was frequently observed: these channels exhibited high conductance and fast kinetics superimposed on slow kinetics of closure. We showed previously that contaminating endogenous vesicles could be detected in the commercial lysate from Roche by immunoblotting against proteins of the inner or the outer membrane [2,16]. Residual endogenous vesicles could also be observed by electron microscopy [16]. Clearly, contaminating vesicles that sediment with the proteoliposomes had fused into the giant liposomes during the dehydration–rehydration cycle. Despite the low amount of endogenous vesicles, the fact that porins are abundant proteins in Gram negative bacteria easily explains this contamination, which is particularly troublesome in electrophysiology experiments.

In the next step, the crude RTS lysates were centrifuged at 109000 ×*g* for 10 min before use. As reported previously [16], this protocol eliminates more than 80% of the remaining protein OmpA

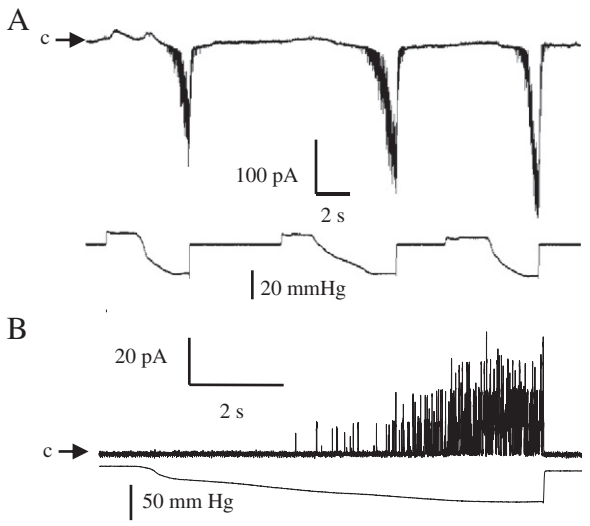


**Fig. 4.** Electrophysiological activity of MscL produced *in vitro* in the presence of liposomes. Patch-clamp experiments were performed in giant proteoliposomes in the inside-out excised configuration. The upper trace shows the current and the lower trace shows the pressure applied to the membrane patch. Application of suction resulted in the opening of channels that closed upon the release of the pressure. Time, current and pressure scales are indicated by small bars, and the arrow indicates the closed level of the mechanosensitive channels. The bath and pipette media were as indicated in the Materials and methods. The applied membrane potential was −10 mV. A: The estimated lipid to protein ratio was 40. B: The estimated lipid to protein ratio was 140.

from the lysate, and residual vesicles were no longer visible. MscL proteins synthesized in this pre-treated lysate supplemented with liposomes floated to the top of the gradient in floatation gradient centrifugation (not shown). Furthermore, liposomes, recovered after MscL synthesis in the pre-centrifuged lysate again displayed particulate surfaces indicative of protein insertion (data not shown). Electrophysiological experiments performed with MscL made *in vitro* with pre-centrifuged lysates and liposomes revealed the absence of contaminating channels and greatly enhanced the ease of seal formation. Here again, the number of active mechanosensitive channels per patch was higher than when proteins were solubilized in detergent. At a lipid to protein ratio of 140, on average, $3.1 \pm 0.85$ channels ($n = 24$) were observed per recording before rupture of the patch.

To summarize, MscL proteins synthesized *in vitro* in the presence of liposomes are not only functional, but the number of active channels is higher than when the protein is produced *in vitro* in detergent or in bacteria and extracted with detergent for further purification and reconstitution.

## 4. Discussion

It is now generally accepted that membrane proteins do not naturally insert directly and unaided into lipid membrane during biogenesis. In *E. coli*, the major route used for integration and export of membrane proteins is the Sec pathway. The multimeric membrane protein complex that constitutes the translocon of the Sec system can associate with the ribosome to catalyze the co-translational insertion of membrane proteins [31]. Small membrane proteins, such as the phage M13 coat protein, were originally thought to be Sec-independent and able insert spontaneously into the membrane. More recently it was found that the M13 coat protein, which spans the membrane twice, as well as the single membrane spanning phage Pf3 coat protein, need the YidC protein for insertion in the membrane [32–34]. The YidC protein is homologous to Oxa1, which is involved in the insertion of proteins into the inner membrane of mitochondria. Further studies led to the discovery of endogenous *E. coli* membrane proteins that are dependent on YidC for insertion. Thus, YidC is involved in the assembly of the $F_1F_0$ ATPase and cytochrome *bo*3 quinol oxidase [35].

YidC (but not Sec) is also involved in the membrane assembly of the mechanosensitive channel MscL. However there are conflicting reports on its exact role. In one report YidC was described to be essential for the insertion of the protein in the membrane [25], while, in a second report it was implicated in the oligomerization of the protein in the membrane [26]. In contrast with the results discussed above, a number of recent studies, in particular those aimed at exploring the potentialities of cell-free synthesis for membrane proteins, have resulted in evidence for direct insertion of membrane proteins in liposomes.

In this study, we showed unambiguously that the MscL protein, synthesized *in vitro*, can be directly inserted in liposomes added to the bacterial lysate prior to synthesis. We first showed that protein synthesized in the presence of liposomes migrated at the top of a sucrose gradient, as expected for proteins inserted into membranes. MscL proteins inserted in endogenous contaminating vesicles were certainly negligible. In a previous study, we estimated the amount of contaminating vesicles in the commercial Roche lysate. Using an antibody against the membrane protein DjlA, we found that the amount of this protein in 1 ml of RTS lysate (9.6 mg total proteins) is 4% of that found in a crude lysate of BL21 *E. coli* cells containing the same amount of total proteins. A 4% contamination of plasma membrane should correspond to 50 μg membrane lipids [2]. Moreover, upon pre-centrifugation of the commercial lysate, 80% of the contaminating vesicles are removed [16], leaving some 10 μg plasma membrane lipids, an amount to be compared to 1 mg of Mscl protein produced in the lysate. Indeed, when MscL was produced in the absence of liposomes, it was found to aggregate and sediment at the bottom of the tube. Second, freeze-etching experiments clearly demonstrated that the proteins were inserted into the preformed liposomes. These preformed liposomes could be easily distinguished from endogenous vesicles which were much smaller, densely packed with proteins, and which had disappeared after pre-centrifugation of the lysate.

Patch-clamp experiments showed that the MscL proteins directly targeted to liposomes had all the characteristics, at the single channel level, of the native protein. Moreover, the proteins were observed to be more active than when they were produced in bacteria, solubilized, purified and reconstituted, or when they were produced by cell-free synthesis in the presence of detergent. A plausible explanation is that MscL proteins targeted directly to liposomes were never subjected to detergent treatment, which might affect MscL protein stability.

It seems reasonable to conclude that MscL, which needs YidC for insertion *in vivo* in biological membranes, inserts spontaneously in liposomes *in vitro* to form properly folded functional channels. The consequences of this conclusion are several-fold. First, requirement for insertase *in vivo* does not mean that a membrane protein cannot be directly targeted to liposomes by cell-free synthesis. Further experiments should test whether other membrane proteins can be produced in this way. It would be particularly interesting to study whether membrane proteins that have both a large cytoplasmic domain and a large external domain can be inserted directly. Second, it is probably incorrect to infer that a membrane protein does not need an insertion machinery, solely from a cell-free synthesis study, as proposed by Hovijtra et al. in the case of the bacterial aquaporin [17]. Third, the real function of an insertase such as YidC needs re-investigation.

Since several different membrane proteins have been shown to insert directly into membranes, it is clear that the surface of a membrane bilayer in itself is not an insurmountable barrier to the penetration of a hydrophobic part of a membrane protein. In their study, Engelhard et al. showed that the only requirement for targeting of bacteriorhodopsin to liposomes was a fluid membrane, a factor

that we did not investigate here [12]. It is possible, therefore, that fluctuations in the lipids result in transient defects through which an unfolded membrane protein can insert directly. Nishiyama and colleagues have recently argued that the mannitol permease, the M13 procoat and Pf3 procoat, can all insert spontaneously into liposomes but that this integration is blocked by diacylglycerol at concentrations found in *E. coli* membranes [10,11]. The authors speculated that the bulky structure of diacylglycerol might allow it to fill open spaces between phospholipids, thus sealing the membrane. An insertase might be necessary to overcome this barrier. However, in preliminary experiments, we found that MscL was still able to insert in azolectin liposomes containing 5% diacylglycerol, as judged from floatation gradient experiments. A biological membrane also differs from a liposome membrane in that it is heavily packed with membrane proteins. At a lipid to protein ratio of 1:1, which is currently found in most biological membranes, the membrane proteins, as visualized in freeze-etching experiments, appear to cover most of the membrane surface, leaving little place for insertion of *de novo* synthesized proteins. The discrepancy between insertion of MscL in liposomes and in biological membranes could point to a more specific function for YidC: that of channeling the inserted protein in a membrane over-crowded with membrane proteins.

The interest of producing membrane proteins directly targeted to liposomes for functional studies cannot be over-emphasized, especially for ion channels. In this area of research, the current approach involves expression of the corresponding gene in cultured cells that can be studied by patch-clamp. However, this method does not work if the channel does not migrate to the plasma membrane or for the study of channels from organelles. Moreover, it may be necessary to study a given channel independently of its cellular context. In all these cases, and also for drug screening, another, more laborious, approach is used. After production in cells, the protein channels are solubilized, purified and reconstituted in a pure bilayer amenable to electrophysiological study (planar lipid bilayer, giant liposome). Cell-free targeting to liposomes would constitute a new expression system which allows the direct production of proteoliposomes. We have shown here with a commercial bacterial lysate that contaminating channels can be eliminated so that the only electrophysiological activity is that of the protein under study.

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