Components required for membrane assembly of newly synthesized K^+ channel KcsA

Annemieke van Dalen[a,*], Martin van der Laan[b], Arnold J.M. Driessen[b], J. Antoinette Killian[a], Ben de Kruijff[a]

[a] *Department of Biochemistry of Membranes, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*
[b] *Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

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Abstract An *Escherichia coli* in vitro transcription–translation system was used to study the components involved in the biogenesis of the homotetrameric potassium channel KcsA. We show that a functional signal recognition particle pathway is essential for tetramer formation, probably to direct correct monomer insertion in the membrane. In the absence of YidC or at reduced SecYEG levels, KcsA assembly occurs with lower efficiency. Strikingly, the highest efficiency of tetramerization was observed when transcription–translation was carried out in the presence of pure lipid vesicles, demonstrating that a phospholipid bilayer is the minimal membrane requirement to form the KcsA tetramer. It is concluded that SecYEG and YidC are not required for the formation of tetrameric KcsA in vitro.

Key words: Assembly; Lipid bilayer; Membrane insertion; Oligomerization; Potassium channel; Targeting

1. Introduction

Many membrane proteins fulfil their function in the membrane as part of oligomeric complexes. For example, ATP synthases, drug transporters, protein secretion systems and ion channels are composed of several identical and non-identical subunits. K^+ channels are widespread in nature and play important roles in for example osmoregulation and signal transduction. Many different types of K^+ channels are known, in the human genome over 50 genes are assigned to encode various K^+ channels, and in most classes the functional channel is composed of four identical subunits. The first identified prokaryotic K^+ channel, KcsA, is smaller than eukaryotic channels, but is still a homotetrameric protein with a pore region that is homologous to that of eukaryotic channels [1]. Very recently it was established that the ion conduction pore is indeed functionally conserved from KcsA to different classes of eukaryotic K^+ channels [2]. KcsA can easily be expressed in *Escherichia coli*, purified and functionally reconstituted. The electrophysiological properties of this channel protein are well described and its structure has been elucidated at an atomic level [3]. Each KcsA subunit contains two transmembrane spanning segments and an extracellular loop which folds back into the pore and contains the conserved K^+ signature sequence. Four subunits are symmetrically aligned around the central pore and form a highly stable tetramer [4]. The recent breakthrough in knowledge about this protein [5,6] makes it a paradigm for ion channel proteins. However, very little is known about the biogenesis of this important class of membrane proteins. Here we study which components are involved in the biogenesis of KcsA using an *E. coli* transcription–translation system.

Insertion into and translocation across the inner membrane of *E. coli* of monomeric proteins is the subject of many studies. Genetic and biochemical experiments have revealed many components that are involved in these processes (for reviews see for example [7,8]). The actual channel through which most proteins are inserted or translocated is formed by the protein complex of SecY, SecE and SecG, also called the translocon. Translocation of many periplasmic and outer membrane proteins in *E. coli* can occur post-translationally, in which the cytosolic chaperone SecB keeps the preproteins in a translocation-competent state. The SecB–preprotein complex is targeted to SecA, which can bind with high affinity to the translocon. SecA mediates translocation through SecYEG by ATP-driven cycles of insertion and de-insertion.

Many inner membrane proteins are not dependent on SecB for targeting, instead they use the signal recognition particle (SRP) pathway that supports co-translational targeting and membrane insertion [9]. The SRP consists of a 4.5S RNA and a 48 kDa protein, called fifty-four homologue (Ffh), since it is homologous to the 54 kDa main component of the more complex eukaryotic SRP. Ffh binds to ribosome-nascent chains that expose particularly hydrophobic stretches of amino acids, such as the signal anchor sequences of membrane proteins. Targeting of the nascent chain to the membrane and the translocon requires the bacterial homologue of the SRP receptor FtsY that is found both in the cytosol and at the membrane. Release of the nascent chain from the SRP–FtsY complex is a GTP-controlled process, followed by insertion into the membrane usually via SecYEG, although transloca-

*Corresponding author. Fax: (31)-30-2522478.
E-mail address: a.vandalen@chem.uu.nl (A. van Dalen).

Abbreviations: IMV, inner membrane vesicle; SRP, signal recognition particle; TLE, total *Escherichia coli* lipid extract

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tion and membrane insertion might be mechanistically distinct events for the translocon [10]. Moreover, some membrane proteins have been shown to be Sec-independent [11,12]. In studies on membrane protein biogenesis, recently a new membrane component, YidC, was identified. Cross-linking experiments demonstrated that YidC is in the vicinity of the transmembrane segment of nascent membrane proteins, while depletion of YidC in vivo was found to interfere with the insertion of several membrane proteins [13–15]. Besides ATP and GTP as energy sources, also the proton motive force (pmf) can promote efficient insertion and translocation and it can direct correct topology [16,17]. In addition to proteins, lipids play an important role both in the SRP pathway [18] and in protein translocation [19].

All of the components discussed above have been shown to be important for protein translocation or insertion of monomeric membrane proteins. However, it is never studied whether these components are involved in the biogenesis of oligomeric proteins. Because of the importance of ion channels in membranes, the large amount of knowledge and the simplicity of the protein, we use KcsA to study membrane insertion and oligomerization. We have already shown that oligomerization takes place in the membrane and is stimulated by the pmf [20]. In this study, we investigate the effect of selectively removing SRP and Sec components on KcsA assembly. It is shown that for efficient oligomerization of KcsA in the membrane a functional SRP pathway is required and that the role of the translocon seems to be less important than for protein translocation. Also no absolute requirement for YidC was found, as the highest amount of assembled tetramers was found to be formed in a pure lipid bilayer.

2. Materials and methods

2.1. Plasmids, strains and growth conditions

Plasmids pT7-KcsA [20], pT7phoE (from J. Tommassen) and pTacompA [21] were used to direct the transcription of the *kcsA*, *prePhoE* and *proOmpA* genes by T7 or *E. coli* RNA polymerase. *E. coli* strains MRE600 [22] and MC4100 [23] were used to isolate wild type lysate and inverted inner membrane vesicles (IMVs), respectively. Strain MM52 (*secA51*(*ts*)) [24] was grown for several generations at the non-permissive temperature (42°C) to isolate IMVs containing a SecA mutant that is defective in protein translocation. Strain PC2977 (*secY24*(*ts*)) [25] was grown for 3 h at the non-permissive temperature (42°C) to obtain IMVs with reduced SecY levels. The Ffh conditional strain WAM121 [26] was used to obtain lysate and IMVs depleted for Ffh. FtsY-depleted lysate and IMV were obtained from the FtsY conditional strain FJP10 (from J. Luirink). YidC- or SecE-depleted IMVs were isolated from the YidC or SecE conditional strains JS7131 or CM124, respectively [14,27]. Cultures of the Ffh, FtsY, YidC or SecE conditional strains were grown as described in the studies mentioned above and depletion was obtained after the cells were washed twice with LB and back-diluted in the same medium but with fructose replacing arabinose. Where appropriate, antibiotics were added to the medium.

2.2. S-135 lysate, IMV and lipid vesicle preparations

S-135 lysate and IMVs were isolated as described [28]. Depletion of S-135 lysate for SecA or SecB was accomplished by immunoprecipitation of wild type lysate. Antibodies against SecA or SecB (from H. de Cock) were coupled to protein-A Sepharose by incubation for 1 h at room temperature of 40 μl antiserum with 10 mg Sepharose suspended in 30 mM Tris, pH 8.6, 150 mM NaCl. After washing the Sepharose twice with lysate buffer (10 mM triethanolamine, 10 mM $Mg(OAc)_2$, 22 mM NH_4OAc, 1 mM dithiothreitol (DTT), pH 7.5), 100 μl wild type S-135 lysate was incubated with the antibody-coupled Sepharose for 2 h at room temperature. The Sepharose was pelleted by centrifugation (5 min, 14 000 rpm) and the resulting supernatant contained S-135 lysate depleted for SecA or SecB. Possible non-specific effects resulting from this long pre-treatment were checked by PhoE antibody treatment of the lysate. Depletion of all different lysates and IMVs was checked on immunoblot using the antibody against the protein of interest. For comparison, an equal amount of wild type lysate or IMV, based on protein content, was included in the immunoblot analysis. Phospholipid content of IMVs was determined by phosphorus assay [29] after extraction of the lipids according to the method of Bligh and Dyer [30].

A total wild type *E. coli* lipid extract (TLE) was prepared as described [31]. Large unilamellar vesicles were prepared by drying an appropriate amount of the *E. coli* lipids from its chloroform stock solution and subsequently hydrating the lipid film in 20 mM HEPES, pH 8, 100 mM K_2SO_4, 250 mM sucrose. Unilamellar vesicles were obtained by the method of Hope et al. [32] using 200 nm membrane filters (Anotop 10, Whatman, UK). Proteoliposomes were prepared by reconstitution of 20 μg SecYEG, 60 μg YidC or both in *E. coli* phospholipids using 25 mM HEPES, pH 8, 100 mM Na_2SO_4 as reconstitution buffer. The method of reconstitution was performed as described [33]. The final lipid concentration was determined by phosphorus assay [29].

2.3. In vitro transcription, translation and membrane assembly

In vitro transcription, translation and membrane assembly of KcsA were performed as described [20]. In the co-translational membrane assembly, IMVs (20 μg protein) were added within 3 min after the start of the translation reaction and further incubated for 30 min. The reaction was stopped by the addition of chloramphenicol (30 μg/ml) and chilling on ice. For post-translational membrane assembly, KcsA translation was continued during 25 min, radioactive methionine incorporation was chased by the addition of an excess of non-radioactive methionine and a continued incubation for 5 min after which synthesis was stopped by addition of chloramphenicol and incubation on ice (at least 5 min). Subsequently, IMVs were added and the mixture was incubated for 20 min at 37°C.

Membrane association of KcsA was analyzed by sucrose flotation analysis (based on the method described in [34]). The in vitro translation sample was mixed with a sucrose solution (in 50 mM triethanolamine, 70 mM KOAc, 8 mM $Mg(OAc)_2$, 1 mM DTT, pH 7.5) to obtain 100 μl with a final sucrose concentration of 1.6 M. 200 μl 1.4 M and 50 μl 0.2 M sucrose solution were subsequently layered on top. The gradient was centrifuged (90 min, TLA 120.2 rotor, 100 000 rpm, 4°C) and analyzed for soluble, membrane and aggregate fractions. The top 40 μl represents the soluble fraction; the next 200 μl was taken as membrane fraction, while the bottom 50 μl represented the aggregated material. Since from the samples containing IMVs less than 2% of radioactive protein was recovered from the soluble fraction, this fraction is not depicted in the results. Membrane and pellet fractions were analyzed by SDS–PAGE [35], except that the samples were never boiled before electrophoresis to maintain a stable tetramer. Gels were scanned in a Phosphor Imager (Molecular Dynamics) and quantified with the program Image Quant. The amount of tetramers is expressed as the relative amount of monomers detected in the tetramer form. Immunodepletion of the lysate with an irrelevant antibody resulted in 55% reduction of tetramer formation efficiency. Results obtained with the $SecA^-$ and $SecB^-$ immunodepleted lysates were corrected for this non-specific effect. KcsA assembly in pure lipid vesicles and proteoliposomes was performed by co-translationally adding 50 nmol vesicles (based on lipid phosphate). The sucrose flotation analysis was adjusted to mixing the sample with sucrose to yield 100 μl of 1.3 M final sucrose concentration and 125 μl 1 M sucrose solution and 50 μl buffer layered on top. After centrifugation for 60 min (TLA 120.2 rotor, 100 000 rpm, 4°C), the lipid vesicles were floated to the buffer–sucrose interface on top. To maintain these floated vesicles separated from the lower part of the gradient, first the bottom fraction of the gradient was removed. The remaining of the gradient contained only the floated vesicles. This sucrose flotation analysis was checked by introducing 0.5 mol% *N*-rhodamine-PE in the lipid vesicles and measuring the fluorescence at 560 nm excitation and 580 nm emission wavelength in the pellet and membrane fractions. The total recovery of lipids from the gradient was $>70\%$ and usually less than 1.5% of the recovered lipids was detected in the pellet fraction.

PrePhoE and proOmpA transcription, translation and translocation were performed as described [28]. Translocation was monitored by protease protection.

3. Results

3.1. In vitro assembly of KcsA

In order to investigate which components of the SRP and Sec machinery are involved in membrane insertion and oligomerization of KcsA, an in vitro transcription–translation system was used. In such a system the presence of different components can easily be controlled and the effect of depletion on assembly can be readily analyzed. We have shown before [20] that formation of the KcsA tetramer in vitro cannot occur in aqueous medium only, but requires the presence of IMVs. Since KcsA has a high tendency to aggregate, the extent of tetramer formation should be determined from the membrane-associated proteins. The association of KcsA with the vesicles can in principle be monitored by carbonate extraction or pelleting of the vesicles, but this is hampered by the fact that also in the absence of IMVs, with or without carbonate, most of the monomeric KcsA can be pelleted, probably due to aggregation. Therefore, a two-step sucrose flotation gradient was used to distinguish membrane-associated proteins from aggregates. In this procedure, the aggregated protein will be pelleted, while the membrane-associated protein will float with the vesicles to the interface of two sucrose steps (see Section 2). In Fig. 1A the result of the in vitro synthesis of KcsA in the absence and presence of IMVs is shown after sucrose flotation analysis. It is clear that in the absence of IMVs, most synthesized KcsA ends up as monomer in the pellet fraction. Also layering the membrane-free sample on top of a high concentration sucrose cushion resulted in pelleting of most of the synthesized KcsA (not shown). Addition of IMVs during translation (co-) resulted in a large amount (80%, Fig. 1B) of KcsA floating with the membranes, a fraction of which (12%, Fig. 1C) is present as tetramer. The faint tetrameric band observed in the pellet fraction is most likely caused by a slight contamination of the pellet fraction with membranes. Fig. 1A,B shows that when IMVs were added after protein synthesis was completed and stopped (post-), the amount of KcsA recovered from the membrane fraction was significantly decreased (50%). Some tetrameric KcsA could be detected in the membrane fraction, but this is three-fold reduced compared to the co-translational assay (Fig. 1C). Membrane association of monomeric and tetrameric KcsA was not affected by flotation through a high salt (500 mM KOAc) containing sucrose gradient (not shown), excluding the possibility of membrane association only via electrostatic interactions. Dimers or trimers were never detected in these and in the forthcoming experiments nor were they observed in vivo [20]. It can be concluded that both membrane association and tetramerization in vitro occur with the highest efficiency when the inner membranes are present during translation. All subsequent experiments were therefore performed co-translationally.

Table 1
Translocation (%)[a] of secretion proteins under the various depletion conditions

	wt	SecA$^-$	SecB$^-$	SecY$^-$	SecE$^-$	YidC$^-$	Ffh$^-$	FtsY$^-$
prePhoE	20.4	0.8	0.9	9.2	9.3	18.4	15.7	14.8
proOmpA	16.5	1.8	7.0	4.1	4.9	14.1	9.1	11.2

[a]The % translocation was calculated as the amount of protease-protected precursor and mature protein relative to the amount of synthesized protein.

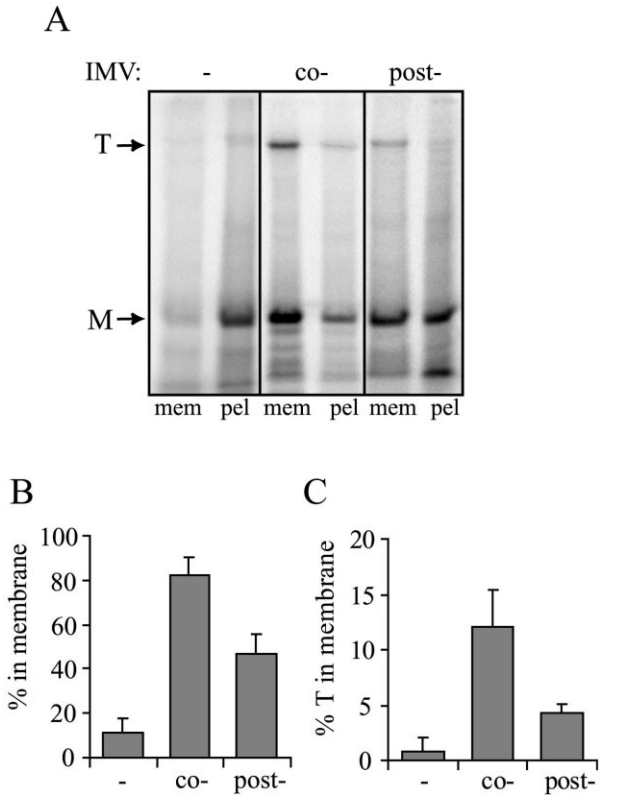

Fig. 1. In vitro transcription–translation of KcsA in the absence or presence of IMVs. IMVs (20 μg protein content) were omitted (−) or added within 3 min after the start of translation (co-translationally) or after translation was completed and stopped (post-translationally). Samples were separated by a flotation gradient centrifugation and the membrane (mem) and pellet (pel) fractions were analyzed on SDS–PAGE (A). Monomeric (M) and tetrameric (T) KcsA are indicated. The total amount of KcsA recovered from the membrane fraction relative to the total amount of synthesized KcsA (B) and the relative amount of tetrameric KcsA formed in the membrane fraction (C) was determined. Without IMVs, the total amount of KcsA recovered from the soluble and membrane-representing part of the gradient is included in the quantification. The average and standard deviations are calculated from at least three different experiments.

3.2. Depletion conditions

Before studying the effect of the removal of any of the SRP and Sec components on KcsA biogenesis, the degree of depletion was checked on immunoblot (not shown). All components were $>98\%$ removed from the lysate or IMV, except the translocon level, which was 60% and 35% reduced by SecY and SecE depletion, respectively, as tested by immunodetection of SecY.

The various depletion conditions were next tested for translocation of the well studied precursor proteins prePhoE and proOmpA (Table 1). Translocation in the absence of SecA or SecB, or into the vesicles with lower levels of SecY and SecE, was largely reduced, confirming the role of these proteins in protein translocation and demonstrating that the used depletion conditions can display functional defects. Translocation of prePhoE and proOmpA into IMVs depleted for YidC, Ffh or FtsY was much less affected, in accordance with the known functions of these proteins.

3.3. Requirement of components involved in targeting of KcsA

To investigate which route is used by KcsA to be targeted

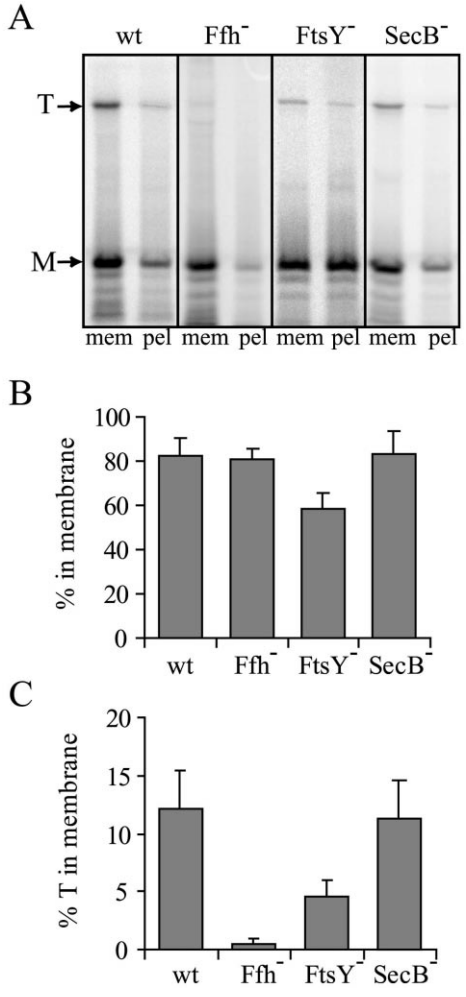

Fig. 2. The influence of targeting and docking components on KcsA assembly. KcsA translation, membrane association and oligomerization were performed with wild type (wt) lysate and IMVs, Ffh- or FtsY-depleted lysate and IMVs (Ffh^- or $FtsY^-$) and SecB-depleted lysate in combination with wild type IMVs ($SecB^-$). After a sucrose gradient flotation, the samples were analyzed on SDS–PAGE (A) and quantified (B and C) as described in the legend of Fig. 1. Tetramer formation in the absence of SecB was corrected for the decreased tetramerization efficiency resulting from the lysate preparation.

to the membrane, the roles of Ffh, FtsY, representing the SRP pathway, and SecB, representing the Sec targeting pathway, were studied. The most prominent effect was observed under Ffh depletion conditions. From the gel in Fig. 2A it is clear that tetramer formation is severely hampered. Quantification reveals a 30-fold reduction of the amount of tetramers in the membrane. This demonstrates that Ffh is essential for membrane assembly of the KcsA tetramer. The synthesized monomers that accumulate under Ffh depletion are still associated with the membrane, but they are probably not correctly inserted for tetramerization. Most likely, they are associated via non-specific hydrophobic interactions, as flotation through a high salt containing sucrose gradient did not reduce the amount of membrane-associated KcsA monomers (data not shown). The importance of the SRP pathway for KcsA membrane assembly is further demonstrated by the reduced tetramerization efficiency upon FtsY depletion (Fig. 2A). Quantification shows that the amount of formed tetramers is three-fold reduced and now also a slight reduction in membrane association is observed (Fig. 2B,C). The less stringent effect of FtsY depletion on KcsA assembly compared to Ffh depletion is possibly caused by the large excess of FtsY over Ffh in the cell [36]. Under depletion conditions, remaining FtsY molecules are more likely to exist, which can fulfil their Ffh binding and targeting functions, although the overall efficiency is reduced which results in a lower KcsA assembly efficiency in the membrane. Finally, in Fig. 2 it is shown that depletion of SecB has no effect on membrane association and tetramerization.

3.4. Requirement of membrane components

The previous results showed that the SRP pathway is involved during the biogenesis of KcsA. Next, we studied which proteins are necessary for assembly of KcsA into the membrane as a tetramer. In vivo studies already suggested that the SecA ATPase activity is not required for this process [20]. In agreement with this, it was found that the lysate completely

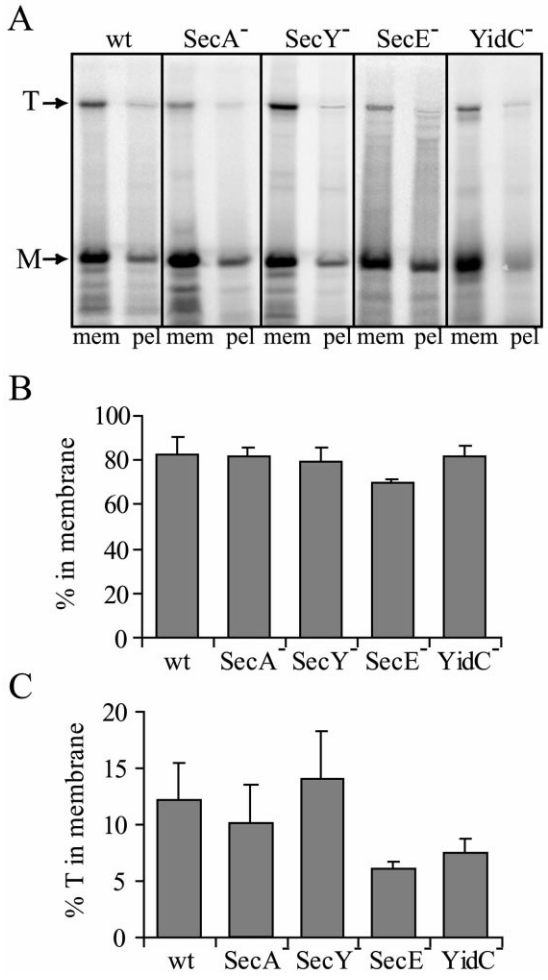

Fig. 3. The influence of membrane components on KcsA assembly. KcsA translation, membrane association and oligomerization were performed with SecA-depleted lysate and IMVs with an inactive mutant of SecA ($SecA^-$) and with IMVs depleted for SecY, SecE or YidC. After a sucrose gradient flotation, the samples were analyzed on SDS–PAGE (A) and quantified (B and C) as described in the legend of Fig. 1. Tetramer formation in the absence of SecA was corrected for the decreased tetramerization efficiency resulting from the lysate preparation. For comparison the results under wild type conditions are shown.

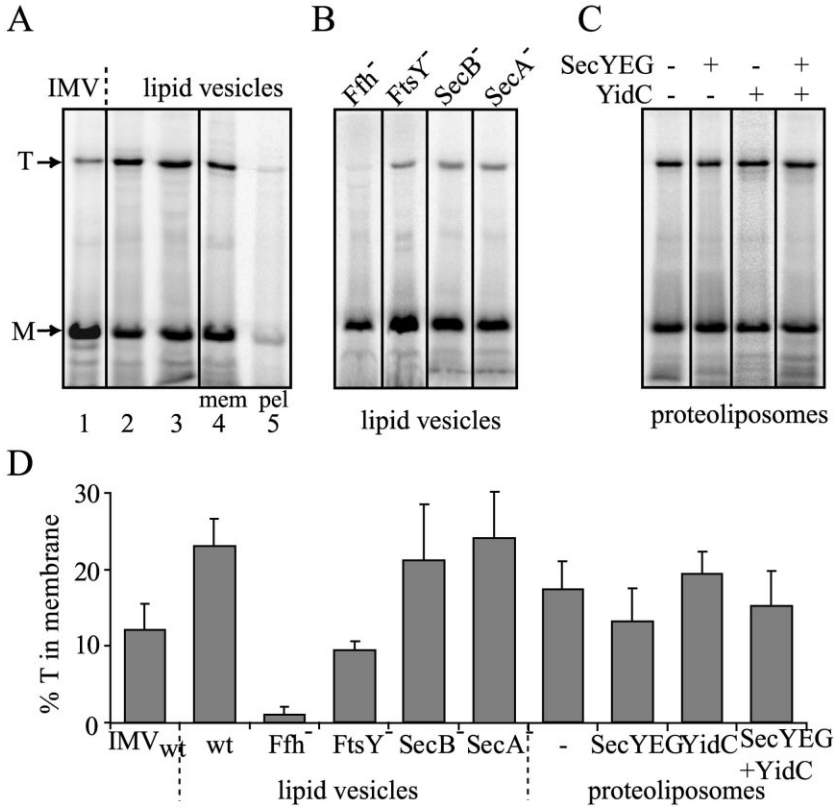

Fig. 4. KcsA assembly in pure lipid vesicles and proteoliposomes. A: KcsA translation was performed in the co-translational presence of wild type IMVs (lane 1; 20 μg protein content = 6 nmol P_i) or pure lipid vesicles (lane 2: 6 nmol P_i; lane 3: 50 nmol P_i) prepared from a TLE. Samples were directly or after flotation through a sucrose gradient (with 50 nmol P_i; lanes 4 and 5) analyzed on SDS–PAGE. B: SDS–PAGE analysis of the membrane fraction after KcsA assembly in lipid vesicles using Ffh-, FtsY-, SecB- or SecA-depleted lysate. C: SDS–PAGE analysis of the membrane fraction after KcsA assembly in the proteoliposomes. D: Quantification of the relative amount of tetrameric KcsA formed in the membrane fraction of samples containing lipid vesicles with wild type or various depleted lysates and in the proteoliposomes. Tetramer formation in the absence of SecA and SecB was corrected for the decreased tetramerization efficiency resulting from the lysate preparation. For comparison the value obtained in wild type IMVs is included. The data are expressed as the average of at least three different experiments.

depleted for SecA in combination with IMVs containing an inactive SecA mutant still supports KcsA assembly (Fig. 3A). The membrane association and tetramer formation are comparable to the wild type conditions (Fig. 3B,C). Also pre-incubation of the IMVs with 10 mM azide at the non-permissive temperature, or with an antibody against SecA had no effect on KcsA membrane association and tetramerization (not shown).

To study the importance of the integral components of the translocon, IMVs with 60% reduced levels of SecY were used ($SecY^-$ in Fig. 3). It is clear from the gel (Fig. 3A) and as quantified in Fig. 3B,C that in the inner membranes prepared from the SecY temperature sensitive strain the association and tetramerization of KcsA are not changed compared to the wild type inner membranes, whereas prePhoE and proOmpA translocation into these vesicles was significantly reduced (see Table 1). This indicates that KcsA assembly is not sensitive to 60% reduction of SecY. When vesicles from the SecE conditional strain were tested (Fig. 3A, $SecE^-$), a two-fold reduction in tetramerization was observed and membrane association was not largely affected (Fig. 3B,C). This implies that depletion of SecE reduces tetramerization, suggesting that SecE is involved in KcsA assembly. To further test the influence of the translocon on KcsA assembly, membrane association and tetramerization in IMVs with largely increased SecYEG levels were analyzed. Whereas proOmpA translocation was two-fold increased in these vesicles, KcsA assembly was unaffected (data not shown). Thus it seems that KcsA assembly is less sensitive to SecYEG changes compared to translocation of secretory proteins.

For insertion of several membrane proteins it has been shown that YidC is involved, even when no Sec proteins were required [13,14]. From Fig. 3A,B it is clear that association of KcsA to YidC-depleted membranes is not disturbed. Tetrameric KcsA is also visible (Fig. 3A), but compared to the wild type condition the intensity of the band is lower. Quantification confirms that the efficiency of tetramer formation in the absence of YidC is reduced (Fig. 3C). These data imply that YidC makes oligomerization of KcsA more efficient.

3.5. Assembly in the lipid bilayer

To establish the minimal requirements for KcsA assembly, pure lipid vesicles were used in which putative assembly supporting components were incorporated. Therefore, first protein-free large unilamellar TLE vesicles were added in the co-translational in vitro assay. Unexpectedly, a much more intense band of the KcsA tetramer was seen on the gel in the presence of an equal amount of TLE vesicles (on phospholipid basis) than observed in the presence of IMVs (Fig. 4A, compare lanes 1 and 2). In contrast to the situation in IMVs [20],

this tetramerization in the liposomes was not dependent on the presence of a membrane potential (not shown). Yet, the KcsA tetramers in both systems display a similar electrophoretic behavior. In addition, it is found that the thermo-stability of the tetramer formed in the presence of TLE vesicles or IMVs is similar (manuscript in preparation). These observations strongly suggest that the tetramer formed in both membrane systems is the same. Quantification of the intensity of the bands shows that the ratio between tetrameric and monomeric KcsA using TLE vesicles is four-fold increased over that of the IMVs (0.53 compared to 0.13), indicating that a lipid bilayer only is sufficient to support KcsA assembly. To be able to measure association of KcsA to the lipid vesicles, the sucrose flotation gradient had to be adapted in the sense that a larger amount of vesicles had to be added to the sample to obtain sufficient lipid recovery from the gradient. Addition of an almost 10-fold increased amount of TLE vesicles did not affect the efficiency of tetramerization (Fig. 4A, compare lanes 2 and 3) and the association of KcsA to the TLE vesicles is shown to be comparable to IMV association (lanes 4 and 5; 85% of the total amount of KcsA is found in the membrane fraction), which is not affected by flotation through a high salt containing sucrose gradient. Membrane association to the TLE vesicles, as analyzed by sucrose flotation, was not changed upon removal of any of the cytosolic components and was insensitive to high salt treatment (not shown). Similar to the situation in IMVs, analysis of the membrane fraction demonstrates that the absence of Ffh results in a largely diminished tetramerization. Depletion of the lysate for FtsY also showed a largely reduced efficiency in tetramer formation (Fig. 4B and the quantification in Fig. 4D). KcsA assembly in the absence of SecB or SecA is similar to wild type conditions. These results are comparable to the described effects in the experiments with IMVs, suggesting a similarity in targeting to the different membrane systems and implying that the presence of membrane proteins is not essential for KcsA assembly. This similarity in targeting requirements again supports the notion that the KcsA tetramer formed in both membrane systems is the same. A possible explanation for the surprising differences in tetramerization efficiency between the IMVs and TLE vesicles will be given in Section 4.

Finally, proteoliposomes, containing no protein, SecYEG, YidC or both, were tested in the in vitro assay. Secretory protein translocation and membrane protein insertion have been shown previously to be functional in these vesicles [30]. From Fig. 4C and as quantified in Fig. 4D it is clear that tetramer formation was not increased in the proteoliposomes containing either SecYEG only, YidC only, or both. This demonstrates that these membrane protein components do not largely improve the KcsA tetramerization efficiency compared to a pure lipid bilayer.

4. Discussion

Membrane protein insertion in *E. coli* has been studied for a limited amount of monomeric membrane proteins. Using an in vitro approach, we present here for the first time studies on the requirement of proteinaceous components for the biogenesis of an oligomeric membrane protein, the K^+ channel KcsA. It has been shown before that KcsA can be expressed in and purified from the *E. coli* inner membrane as tetramer and reconstituted in lipid bilayers as a functional protein [1]. The formation of the tetramer has been demonstrated to occur in the membrane [20] and in the present study we investigated which cytoplasmic and membrane bound components that are generally involved in protein translocation are important for KcsA assembly.

Our results clearly show that the SRP pathway is important for efficient assembly of the KcsA tetramer, most likely for correct insertion of the monomer allowing tetramer formation. For several other monomeric membrane proteins the importance of the SRP pathway was also shown and suggested to be required for functional membrane assembly [9,37]. Interaction of SRP with nascent polypeptides was demonstrated to be dependent on the presence of a particular hydrophobic targeting signal [38,39]. For endoplasmic reticulum targeting signals in yeast it was determined that SRP-dependent signals have a hydrophobicity value approaching 3.0 (according to the Kyte and Doolittle scale, [40,41]). Calculating the hydrophobicity of the transmembrane segments of KcsA using the same method reveals a value of 3.2 for the first and 3.0 for the second transmembrane segment. Considering the homology between the *E. coli* and yeast SRP systems, one can suggest that these transmembrane segments mediate the interaction with SRP. Several studies, using purified proteins, have shown that Ffh binds to FtsY in aqueous medium in a GTP-dependent manner [42,43]. Depletion of either of these proteins largely affected KcsA tetramerization. The non-productive association of the KcsA monomers to the membrane observed under these conditions is most likely driven by hydrophobic interactions. This might involve the hydrophobic transmembrane segments of KcsA, but also the N-terminal 24 residues that precede the first transmembrane segment, since the N-terminus was shown by site-directed spin-labelling to be located as α-helix at the membrane–water interface [44]. These mis-targeted monomers under SRP depletion conditions most likely are incorrectly inserted or misfolded, thereby blocking tetramerization. The importance of the SRP pathway is in line with the observation that KcsA assembly occurred most efficiently co-translationally. Considering the high hydrophobicity of membrane proteins, SRP most likely targets these proteins already during synthesis to the membrane to prevent aggregation in the aqueous environment of the cytosol. Here we have shown that in the post-translational presence of inner membranes indeed more aggregated protein was detected and the efficiency of tetramerization of the membrane-associated monomers was much lower as compared to the co-translational assay. The degree of post-translational KcsA assembly was similar to the effect observed after disruption of the SRP pathway by FtsY depletion. This indicates that the absence of FtsY results in such delayed docking of the nascent protein to the membrane that its membrane assembly is turned into a post-translational event.

Many membrane proteins that are dependent on the SRP route have been shown to be inserted into the membrane via the Sec translocase [8] and also YidC was found in the vicinity of transmembrane segments of several proteins during insertion [13–15,45]. However, here we show that KcsA assembly by itself does not require the presence of SecA, SecY, SecE or YidC. The SecA independence is in agreement with our previous in vivo results [20] and with the SecA-independent membrane insertion of many other membrane proteins containing short periplasmic loops [46–49]. The absence of an absolute requirement for SecY or SecE during KcsA assembly resem-

bles the insertion requirements of the polytopic membrane protein MelB that contains short periplasmic loops, like KcsA. This protein was found to insert correctly under similar SecY depletion conditions used in the present study and also under SecE depletion conditions in vivo, that blocked OmpA translocation [48]. In contrast, insertion of Lep and MalF, both polytopic membrane proteins with a long periplasmic loop, was largely affected upon SecE depletion in vivo [27,38]. In addition, Lep translocation was found to be blocked in SecY depletion vesicles [50]. Under the depletion conditions used here it is possible that KcsA makes use of residual SecYEG, because particularly after SecE depletion KcsA membrane assembly is slightly less efficient. However, from the observation of a highly efficient membrane association and tetramerization in the pure lipid membrane, it can be concluded that the presence of SecYEG is not absolutely required.

KcsA assembly was slightly less efficient in YidC-depleted vesicles, while insertion of Lep that also contains two transmembrane helices was two-fold reduced (data not shown), consistent with the reduced insertion efficiency in vivo [14]. Such differential dependence on YidC was also reported for single spanning membrane proteins, as YidC depletion caused only a minor effect on FtsQ insertion, whereas insertion of M13 procoat was severely inhibited [14,45]. Still, nascent chain constructs of both FtsQ and Lep could be clearly cross-linked to YidC. These results suggest a subtle and protein-dependent role of YidC in membrane insertion.

The possibility to form a tetramer in the presence of only a lipid bilayer demonstrates that KcsA has the intrinsic ability for tetramerization once it is correctly targeted to the bilayer via the SRP pathway. A possible explanation for the higher efficiency of tetramerization in the pure lipid membrane is that in the *E. coli* inner membrane the oligomerization process is slowed down due to molecular crowding. In the lipid bilayer, newly synthesized monomers can only interact with other monomers, leading to a rapid and pmf-independent tetramerization. One could speculate that in the *E. coli* inner membrane, where there are many possibilities for non-specific interactions, an unknown component interacts specifically with the KcsA monomer, thereby sustaining its potential for tetramerization. Subsequent efficient tetramerization may then require the presence of a pmf.

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