Biogenesis of the Magnesium Transporter CorA

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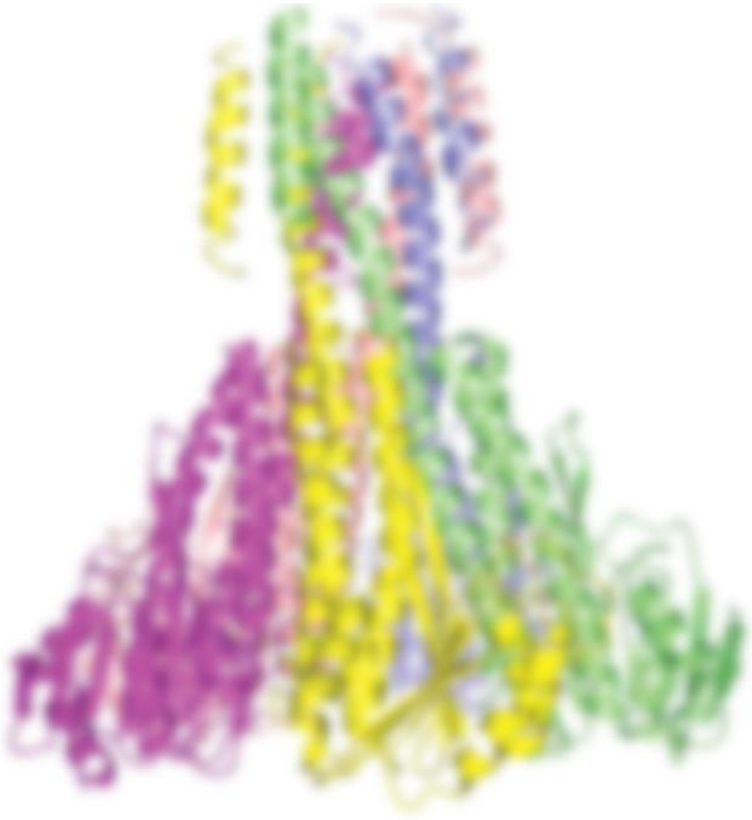

Biogenesis of the Mg^{2+} transporter CorA

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On the cover: artistic side view of the Thermotoga maritime CorA magnesium transporter.
-- Although still blurry, the biogenesis is getting clearer..

Abstract

To maintain proper intracellular Mg^{2+} concentrations prokaryotes utilize three classes of transporters. One of which is the major contributor for Mg^{2+} uptake, CorA. Crystal structures of this transporter showed an unique and unusual pentameric conformation, with a large cytosolic domain with two C-terminal membrane spanning domains per monomer. The targeting of a monomer to the cytosolic membrane probably occurs via these domains, however, a complete picture of the biogenesis remains unclear. A recent study on the membrane proteome of *E. coli* cells depleted of the small insertase YidC, revealed that YidC may be involved in the biogenesis of CorA. In this study the biogenesis of CorA was studied using *in vitro* transcription, translation and insertion experiments. The data suggest a role for both the SecYEG translocon and YidC in the insertion of CorA.

Table of content

1. Introduction

A great portion of the proteins of prokaryotes are destined for insertion into or secretion across the cell's cytoplasmic membrane. These proteins have diverse functions, ranging from energy production to signal transduction and transporting proteins across membranes. The prokaryote's general machinery for inserting and secreting proteins across the cytoplasmic membrane, is a highly conserved heterotrimeric complex consisting out of the SecY, -E and -G proteins that works in concert with a set of cytosolic proteins (1) (2) (3). Because secretory proteins and membrane proteins are synthesised in the cytosol, they have to travel to the insertion or secretion site, a process called protein targeting. This process is highly regulated and starts at the synthesis, where these proteins are recognized by their signal sequences or hydrophobic trans-membrane domain (TMD) (1) (4). Depending on these signal features, one of the two major targeting routes is taken that directs the protein to the SecYEG translocon (5) (figure 1). Most secretory proteins are targeted post-translationally (figure 1.a), whereas for membrane proteins the co-translational (figure 1.b) targeting route is taken. These two pathways diverge at an early stage when the N-terminus emerges from the ribosome exit tunnel (6). At this point the signal recognition particle (SRP) and peptidyl-prolyl cis-trans isomerase trigger factor (TF) compete for binding the ribosome nascent chain (7) (8).

In the post-translational route, the N-terminal signal sequence is recognized by TF. During the nascent chain elongation, SecB takes over the function of TF as a chaperone. The SecB protein stabilizes the synthesised polypeptide, keeps it in an unfolded state and directs the fully synthesised form to SecYEG-bound SecA (9). SecB dissociates from the peptide upon binding of ATP to the motor protein SecA, which drives the unfolded peptide through the

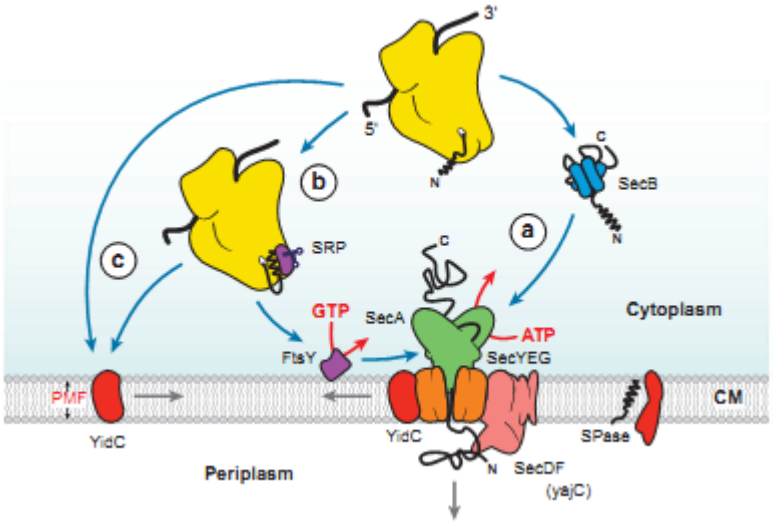


Figure 1 - Schematic representation of the bacterial SecYEG pathway. (a) Secretory proteins are targeted via the post translational pathway. Fully synthesised unfolded pre-proteins are translocated by SecYEG (yellow) following binding of SecB (Blue) to SecYEG-bound SecA (b) Membrane proteins are targeted via the co translational pathway. Proteins destined for insertions are identified by SRP (purple), binding of FtsY (purple) to SRP-bound RNC directs the complex to the SecYEG translocon following insertion into the membrane. (c) YidC-only pathway. YidC can independently insert small hydrophobic proteins, or work in concert with the SecYEG complex. *From A.J.M. Driessen and N. Nouwen, Protein translocation across the bacterial cytoplasmic membrane, 2008*

translocon in an ATP dependent manner (10).

In contrast to secretory proteins, membrane proteins generally do not contain a N-terminal signal sequence, instead their TMD functions as a signal for co-translational targeting (1). During synthesis, the ribosome-bound nascent chain (RNC) is bound by SRP, which in turn is bound by its receptor, FtsY (6). The latter facilitates docking of the complex to the translocon. The polypeptide emerging from the ribosome exit tunnel is inserted into the SecYEG channel following insertion into the lipid bilayer. This last step is made possible by the unique structure of the SecY. This protein contains short helices which is proposed to form a lateral gate through which the TMD can be transported (11). Two other proteins associating with the SecYEG translocon are SecD and –F. Forming a complex together, it is suggested that they are involved in the later stages of translocation as it may pull the proteins through the channel at the periplasmic side of the cytoplasmic membrane (10).

Another protein associated with the SecYEG translocon is YidC insertase. Functional studies of this protein suggests that it may aid in the folding and quality control of membrane proteins (12) (13) and can act together with SecYEG, or insert proteins independently in the YidC-only pathway (figure 1c). During Sec-dependent YidC insertion, the YidC protein interacts with the SecDF components of the Sec translocon, which probably facilitates the association of YidC to SecYEG (14) (15). The site at which YidC interacts with the Sec translocon, however, remains unclear. An electron cryomicroscopy study speculates that these interactions take place at the lateral gate of SecY (16), creating a secluded environment in which the translocated proteins are inserted. Despite extensive research, the mechanism by which proteins are targeted and inserted via the YidC-only pathway remains to be elucidated, upon today only a few small and highly hydrophobic proteins were reported to be inserted using YidC (17) (18). The identification of new substrates may help to clarify the mechanisms involved, and an important contribution in this search was made by Price and colleagues (19). Using YidC depleted *Escherichia coli* cells to identify YidC substrates, it was found that the depletion resulted in protein aggregation, degradation and misfolding in the cytosol as well as in the cytosolic membrane. Furthermore, the results also identified a number of possible YidC substrates, one of which is the small magnesium transporter CorA.

As early as the 1980s the importance of Mg^{2+} as a cofactor in ATP-requiring reactions and membrane stability was described (20) (21). To maintain optimal intracellular Mg^{2+} concentrations prokaryotes utilize three classes of transporters: CorA,

MgtA/MgtB and MgtE (22), of which the CorA is the major contributor for Mg^{2+} uptake. To elucidate mechanisms by which CorA functions, crystal structures were made which revealed an unusual protein (23) (24). The crystallisation of the transporter showed a homopentameric structure (figure 2b) in which each monomer can be divided into three parts (figure 2a); a short C- terminus transmembrane domain constituting two transmembrane helices, a middle part which is largely represented by one long α-helix and finally a large N-terminus cytoplasmic domain. In its pentameric state the middle part is shaped like a funnel, narrow at the C-terminus and wide at the cytoplasmic site. The large N-terminal domain lies exterior to the funnel and consists of seven parallel/antiparallel β-sheets spaced between two sets of α-helices. It is due to the resemblance of a willow tree that these helices are called willow helices (22) (23) (24). This unique structure makes CorA one of a kind with no connection to all other known structures of ion channels or transporters. However, this unique structure gives rise to the question; what are the mechanisms of targeting and insertion? Due to the lack of a signal sequence, targeting to the cytoplasmic membrane and insertion likely occurs via CorA's two C-terminal TMDs.

Nearing the end of CorA's translation, the first TMD emerges from the ribosome exiting tunnel, at this stage the targeting factors may compete for binding the nascent chain and direct the polypeptide to the insertion machinery. In this respect, the CorA transporter

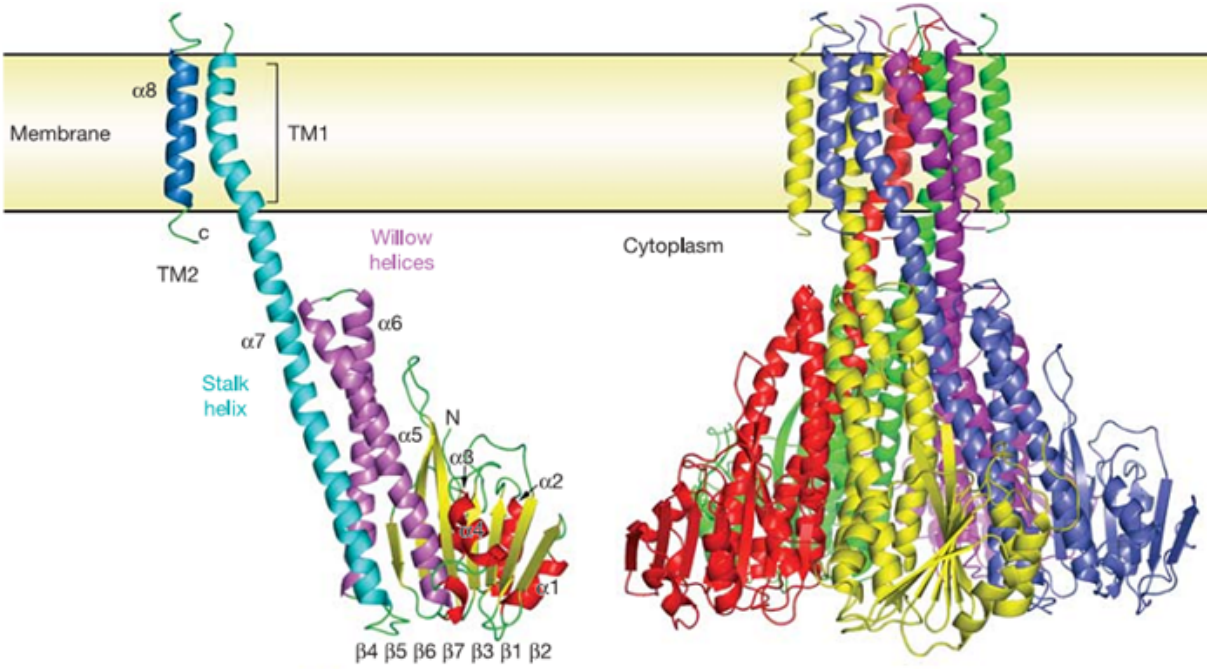


Figure 2 - Mono- and Pentameric structures of the CorA Mg^{2+} transporter.
(a) Ribbon diagram of the monomeric structure. The transmembrane part consists of the transmembrane (TM) segment 1 and 2. The cytosolic part consist of the long stalk helix and willow helices with N-terminus parallel/antiparallel β-sheets spaced between two sets of α-helices (b) Pentameric structure shows the transporter complex in its biological active state.
Modified from V.V. Lunin et al., Crystal structure of the CorA Mg^{2+} transporter, 2006

shows some structure resemblance to tail-anchored (TA) membrane proteins. These proteins are characterized by a functional N-terminus domain located in the cytosol that is anchored to the membrane by a TMD close to the C-terminus (25). Because this TMD only emerges from the ribosome after translation is completed, TA proteins are obligated to be inserted post-translationally into the membrane (26). However, despite the structural resemblance between TA proteins and CorA, the transporter is not characterized as a tail-anchored protein, since the first TMD emerges from the ribosome exiting tunnel before translation is finished. Because of this difference, CorA's targeting might also be differently than that of TA proteins. The study by Price and colleagues revealed CorA as a possible YidC substrate. This implies an role of YidC in the biogenesis of CorA, e.g. insertion into, oligomerization or folding in the cytoplasmic membrane.

This study aimed for elucidating the biogenesis of the CorA Mg^{2+} transporter. Using radiolabeled methionine in combination with *in vitro* transcription and translation in the presence of vesicles the insertion was assayed. The data obtained in this study suggest a role for both the SecYEG translocon and YidC in the insertion of the magnesium transporter CorA.

2. Materials & Methods

2.1 Bacterial strains, plasmids and culturing

The bacterial strains and plasmids used in this study are listed in table 1 and 2 respectively. Overnight cultures were maintained on Lysogeny broth (LB) media supplemented 0.2% glucose or arabinose and 25 µg/ml kanamycin or 100 µg/ml ampicillin at 37 °C.

Table 1 - Strains used in this project

Strain	Description	Reference
SF100	*E. coli* F- lacX74 galE galK thi rpsL (strA) ΔphoA(pvuII), ΔompT	(27)
DH5α	*E. coli* F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–	(28)
FTL10	*E. coli* MC4100-A, ΔyidC, attB::(araC+, PBAD, yidC+); Kan	(29)
HDB51	*E. coli* WAM113, *secB*$^+$ *zic-4901*Tn*10*	(30)

Table 2 – Plasmids used in this project

Plasmid	Description	Reference
pET302 SecYEG$^+$	pET324-derived vector containing a His tag, an enterokinase site and kanamycin resistance	(31)
pET610 SecYEG^{++}	Vector containing a His tag, the Sec Y, E and G genes and kanamycin resistance	(31)
pEH1 YidC$^+$	pEH1 vector containing a His tag and ampicillin resistance	(32)
pEH1 YidC^{++}	pEH1-derived vector containing a His tag, YidC gene and ampicillin resistance	(32)
pTRC99a YidC$^+$	Vector containing trc promoter, YidC gene and ampicillin and kanamycin resistance	(14)
pTRC99a YidC$^-$	Vector containing trc promoter and ampicillin and kanamycin resistance	(14)

2.1.1 SecYEG and YidC IMVs

SecYEG or YidC Inner Membrane Vesicles (IMVs) were prepared from *Escherichia coli* SF100 cells transformed with plasmids pET610-SecYEG or pEH1-YidC respectively. Overnight cultures were shifted to fresh LB with appropriate antibiotics and cultured to an OD_{600} of 0.6. Following the addition of 1 mM IPTG, the cells then were incubated for two hours at 37 °C. Cells were harvested by centrifugation, resuspended in 20% sucrose/50 mM Tris pH 8.0 and stored at -80 °C and used for IMV preparation (see below section 2.2).

2.1.2 YidC depleted IMVs

IMVs depleted of YidC were acquired from the FTL10 strain in which *yidC* is under control of the arabinose promotor. Cells were grown overnight in LB supplemented with 0.2% arabinose and appropriate antibiotics. Overnight cultures were diluted 100-fold in LB supplemented with 0.2% arabinose and growth was continued to an OD_{600} of 0.8. The cells were harvested in sterile tubes by centrifugation (10 min, 6000g, 21 °C) and washed with LB. For YidC+ IMVs, the washed cells were diluted 20-fold in LB supplemented with 0.2% arabinose and growth was continued to an OD_{600} of 0.8 before cells were harvested for IMV preparation. For YidC depleted IMVs (YidC-) the

resuspended cells were diluted 25-fold in LB supplemented with 0.2% glucose and growth was continued to an OD_{600} of 0.8. The cells were then diluted 2-fold in the same medium and the procedure was repeated until the cells ceased to grow. Cells were harvested by centrifugation, resuspended in 20% glycerol/50 mM Tris pH 8.0 and used for IMV preparation (see below section 2.2).

2.2 Creation of Inner Membrane Vesicles

Cells were passed two times through a cell disrupt chamber at 13.000 psi. After the first pass, 0.5 mM PMSF was added to inhibit proteases. Cell debris was removed by centrifugation (10 min, 3000 rpm, 4 °C) using a Beckman Allegra 6R centrifuge. The resulting supernatant was centrifuged again (5 min, 5000g, 4 °C) before pelleting the total membrane fraction by ultra-centrifugation (60 min, 40.000rpm, 4 °C). This fraction was resuspended in 50 mM Tris-HCl pH 8.0 and added to a 36-55% (w/v) sucrose gradient before centrifugation (30 min, 90.000rpm, 4 °C). The orange coloured band containing the IMVs, was taken from the gradient and diluted with 50 mM Tris-HCl pH 8.0. Membranes were collected by centrifugation (60 min, 40.000rpm, 4 °C) and resuspended in 20% glycerol/50 mM Tris-HCl pH 8.0, snap frozen in liquid nitrogen and stored at -80 °C. All further analysis requiring IMVs, were performed using these vesicles.

2.3 Ffh depleted lysate

E. coli HDB51 overnight cultures were grown on LB supplemented with 0.2% arabinose, tetracyclin, 25 µg/ml kanamycin and 100 µg/ml ampicillin. These cultures were 200 times diluted in LB supplemented with 0.2% arabinose and growth was continued to and OD_{600} of 0.6. The cells then were harvested by centrifugation (10 min, 6000g, 21 °C) and resuspended in LB. The cells were collected by centrifugation, resuspended in LB and divided into two fractions, Ffh+ and Ffh-. The Ffh^+ culture was diluted 100-fold in LB supplemented with 0.2% arabinose and growth was continued to an OD_{600} of 0.8 before cells were harvested for lysate preparation. The Ffh- culture was diluted 40-fold in LB supplemented with 0.2% glucose and continued growing to an OD_{600} of 0.6. The cells were then diluted 2-fold in the same medium and the procedure was repeated until the cells ceased to grow. Cells were harvested for lysate preparation. First harvested cells were resuspended in 10 mM Tris-Acetate pH 8.0, 14 mM $Mg(OAc)_2$, 60 mM KOAc and 10 mM (final concentraion) PMSF. Cells were then passed through a cell disruption chamber at 10.000 psi. Cell debris was removed by centrifugation (two times MLA80; 30 min, 25.000 rpm, 4 °C) and the supernatant was incubated for 90 minutes at 37 °C, followed by the last centrifugation step (MLA80; 30 min, 25.000 rpm, 4 °C). The supernatant was transferred to dialysis tubing and dialysed overnight against 10 mM Tris-Acetate pH 8.2, 60 mM KOAc and 14 mM $Mg(OAc)_2$. Dialysis was continued for four hours with two changes of dialysis buffer. The resulting lysate was aliquoted, snap frozen using liquid nitrogen and stored at -80 °C.

2.4 SecYEG and YidC purification

SecYEG and YidC his tagged proteins were purified from their corresponding IMVs. Purification was done using Ni-NTA agarose (qiagen). First a 2 ml solubization mixture consisting of 2 mg IMVs, 100 mM KCl, 2% DDM (w/v), 50 mM Tris-HCl pH 8.0 and 20% glycerol (v/v) was prepared. This mixture was incubated for 30 minutes at 4 °C on a rollerbench to solubilize the proteins. Non-solubilised material was spun down in an Eppendorf centrifuge (10 min, 13.000 rpm, 4 °C). The supernatant was transferred to a clean tube an imidazole was added to a 10 mM final concentration. Thereafter, Ni-NTA agarose resin was added to the supernatant and incubated at 4 °C on a rollerbench. Biospin columns were loaded with the resin and the flowthrough was collected as the 2nd sample. Subsequently, the columns were washed three times with purification buffer, consisting of 50 mM Tris-HCl pH 8.0, 20% glycerol (v/v) 100 mM KCl, 0.1% DDM (w/v) and 10 mM imidazole for SecYEG or 50 mM for YidC. Proteins were eluted by incubating the columns 10 minutes at 4 °C on a rollerbench with purification buffer containing 300 mM imidazole. This procedure was repeated three times. Fractions were analysed on a 12% SDS-PAGE gel and peak fractions snap frozen in aliquots and stored at -80 °C.

2.5 Reconstitution of SecYEG and YidC

The Avanti *E. coli* total lipid extract was used for creating liposomes. *E. coli* lipids (4 mg/ml) in 50 mM Tris-HCl pH 8.0/20 mM DTT were mixed with 0.5% Triton X-100. Purified SecYEG and/or YidC was added and the mixtures were incubated for 30 minutes at 4 °C on a rollerbench. Next, 20 mg Biobeads in buffer K or N for Δϕ proteoliposomes were added, followed by a 2 hour incubation at 4 °C on a rollerbench. Buffer K or N consisted of 50 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 1 mM DTT and 100 mM KCl or Na_2SO_4 respectively. The biobeads were replaced by 20 mg fresh biobeads and incubated for 2 more hours at 4 °C. Finally, the reconstitution mixture was overnight incubated at 4 °C with 40 mg Biobeads. Supernatant was centrifuged (TLA100.4; 30 min, 90.000 rpm, 4 °C), the remaining biobeads washed with buffer K or N and centrifuged again (TLA100.4; 30 min, 90.000 rpm, 4 °C). The resulting pellet was resuspended appropriate buffer.

2.6 *In vitro* transcription, translation and insertion assay

In vitro transcription and translation was carried out as described by Saller et. al. (33). The *in vitro* reactions were carried out for 20 min at 37 °C in the presence of 5 µg IMVs or proteoliposomes. A small sample of the reaction mixture was removed as synthesis control and the remainder was added to 7 M urea/50 mM Tris-HCl pH 8.0 following 30 minutes incubation on ice. Vesicles were collected using centrifugation (A-100/18; 30 min, 30 psi, RT) and analysed by SDS-PAGE and phosphorimaging. Alternatively, the assay mixtures were treated with 5000 U/ml Trypsin for 30 minutes at 21 °C. Vesicles were tricholoroacetic acid-precipitated and

analysed by SDS-PAGE and phosphorimaging. For assaying insertion into proteoliposomes with an Δϕ, *in vitro* transcription translation reactions were performed as described by van der Laan et al. (34). The K^+ concentration in the assay mixture was adjusted to 100 mM, and valinomycin was added at 0.5 μM.

3. Results

3.1 CorA insertion into IMVs is stimulated by SecYEG overexpression

To address the question whether CorA is inserted by SecYEG, the prokaryote major insertion machinery, the translocon was overexpressed in the *E. coli* strain SF100 using plasmid pEK610. IMVs were prepared from wild type and SecYEG overexpressing cells and used in an *in vitro* transcription, translation and insertion experiment. CorA was expressed *in vitro* using the cytomime system (35) in the presence of radiolabeled ^{35}S-methionine and IMVs to allow co-translational insertion. Insertion was assayed using 6 M urea to remove membrane associated proteins. Collecting the IMVs by centrifugation yielded a pellet containing inserted radio-labelled proteins, analysis on SDS-PAGE and phosphorimaging resulted in figure 3. By comparing the SecYEG (figure 3, YEG^{++}) and wild type (figure 3, Wild type) co-precipitates, an increase in band intensity for the SecYEG overexpressing vesicles is observed (figure 3, co-precipitates). This indicates that CorA insertion into IMVs is stimulated by the SecYEG translocon.

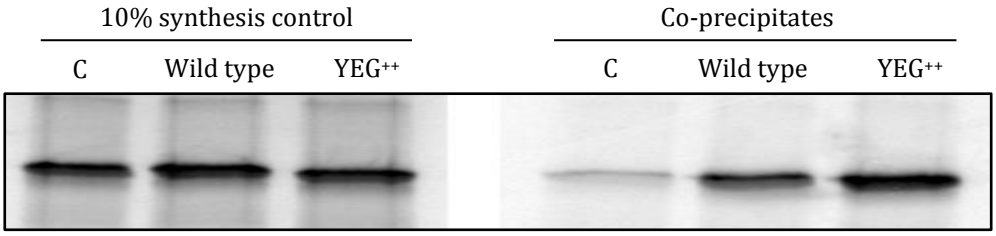


Figure 3 - SecYEG^{++} IMVs show an increase in CorA insertion compared to wild type IMVs
In vitro transcription translation using the cytomime system in the prescence of IMVs. Insertion was assayed using urea extraction.
abbreviations: C, control (20% sucrose/50 mM Tris-HCl pH 8.0); YEG^{++}, SecYEG overexpressing IMVs

3.2 CorA insertion is decreased in YidC depleted IMVs

Examination of the obtained insertion data with SecYEG IMVs gave rise to the question if CorA is only inserted by this translocon, or does insertion by SecYEG depend on involvement of other proteins? Besides SecYEG, the YidC insertase was discovered to insert proteins together or independently of SecYEG (36). To test whether YidC is necessarily for inserting CorA, YidC depleted IMVs were created using the *E. coli* FTL10 strain in which *yidC* is under control of an arabinose promotor. These cells were depleted of YidC by growth in LB supplemented with 0.2% glucose to an OD_{600} of 0.8. The cultures were then diluted 2-fold in the same medium and the procedure was repeated until the cells ceased to grow. To analyse the efficiency of the depletion, the IMVs were analysed by SDS-PAGE western blotting (figure 4a, b respectively). SDS-PAGE analysis of the depleted IMVs showed an up regulation of the PspA stress-response protein, which is an indicator for membrane integrity (37) (figure 4a, arrowhead PspA). This response, however, was not as high as observed by van der Laan et. al. (37), suggesting that the YidC depletion was not complete. Western blot analysis of the depleted IMVs showed a strong decrease of YidC present in the depleted vesicles compared to the wild type (figure 4b).

However, traces of YidC were still present (figure 4b, YidC$^-$), confirming the incomplete depletion. Nevertheless, the insertion of CorA into these IMVs was assayed. F_0c was chosen as a control as it has been shown to inserted via the YidC-only pathway (18). Insertion data from F_0c experiment did, however, not show a clear decrease with the depleted YidC vesicles. This could be due the residual YidC proteins in the depletion IMVs, which might still be enough to insert all the F_0c present in the assay mixture. Optimizing the ratios between IMVS and F_0c may result in a clearer decrease in insertion. Although, the F_0c insertion results did not show a clear decrease, insertion of CorA showed a drastic decrease in YidC depleted vesicles compared to the wild type (figure 4c-d). This decrease suggests that YidC plays a role in the insertion processes of CorA, which is in line with the findings of Price and colleagues (19).

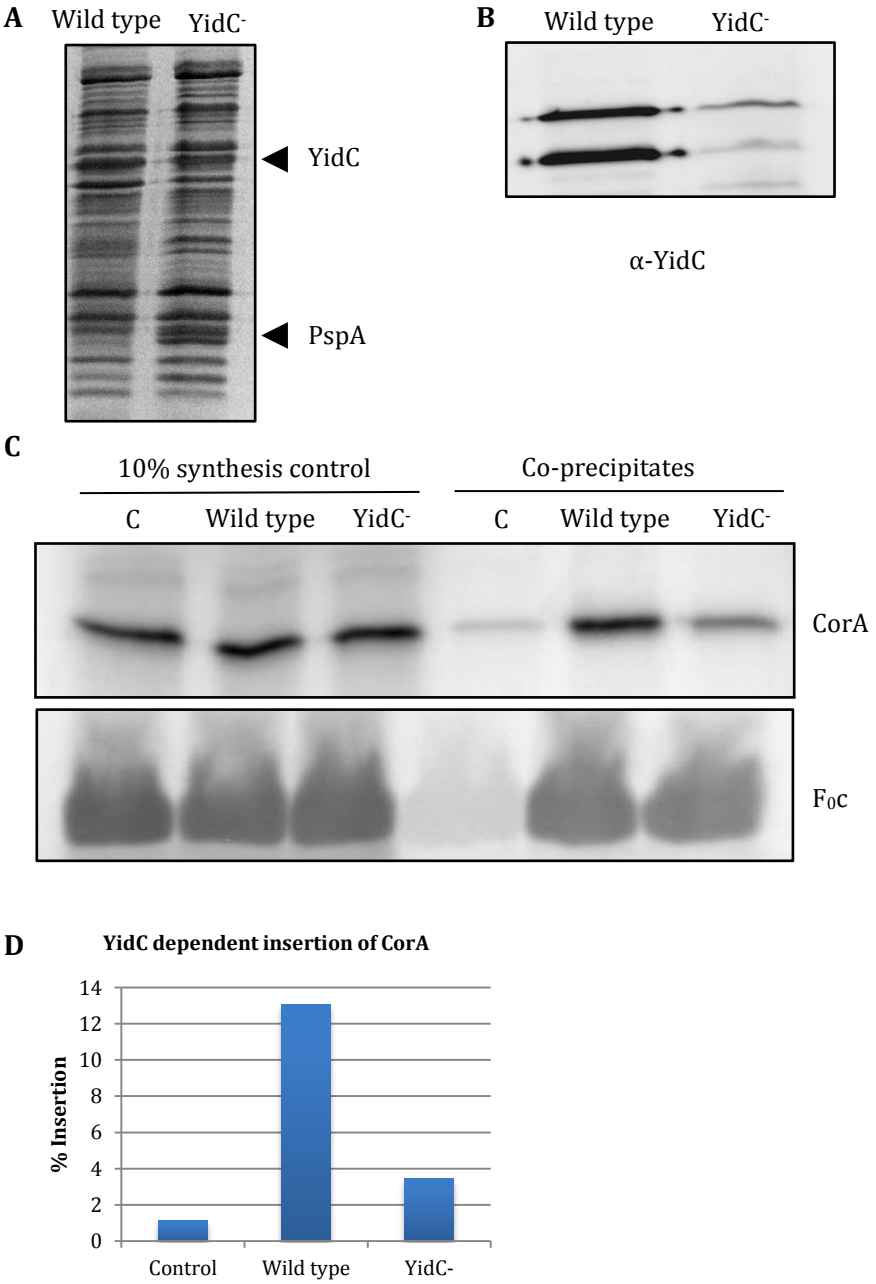


Figure 4 - YidC depleted IMVs show and decrease in CorA insertion
(a) SDS-PAGE of wild type and YidC depleted IMVs stained with Coomassie brilliant blue (b) Western blot of wild type and YidC depleted IMVs blotted with an antibody against YidC. (c) *in vitro* transcription, translation and insertion assay of CorA and F_0c. Insertion was assayed using urea extraction. (d) Quantification graph of the CorA phosphor image.
abbreviations: C, control (20% sucrose/50 mM Tris-HCl pH 8.0); YidC$^-$, YidC depleted IMVs

3.3 CorA needs SecYEG and YidC for insertion into proteoliposomes

Data from the previous experiments suggest that both SecYEG and YidC are involved in the insertion of CorA. To study the insertion dependencies in a more defined system, proteoliposomes were used instead of inner membrane vesicles. Purified SecYEG and YidC were reconstituted in proteoliposomes (figure 5a) and an *in vitro* transcription, translation and insertion assay was performed using these proteoliposomes (figure 5b). ElaB and F_0c were used as controls, as these proteins have been shown to be inserted by SecYEG and YidC respectively (Oldebesten, A. unpublished) (18). ElaB and F_0c inserted into the SecYEG and YidC proteoliposomes respectively, indicating that both the reconstituted SecYEG and YidC were active. The increased F_0c insertion observed with the SecYEG vesicles could be due to an excessive SecYEG reconstruction, which might drive this protein into the SecYEG translocation pathway. The slight increase of CorA insertion observed for SecYEG/YidC proteoliposomes agrees with data obtained from the previous experiments and suggest that CorA needs both SecYEG and YidC for correct insertion. However, the overall activity of the proteoliposomes for CorA insertion is low and barely exceeds the background levels.

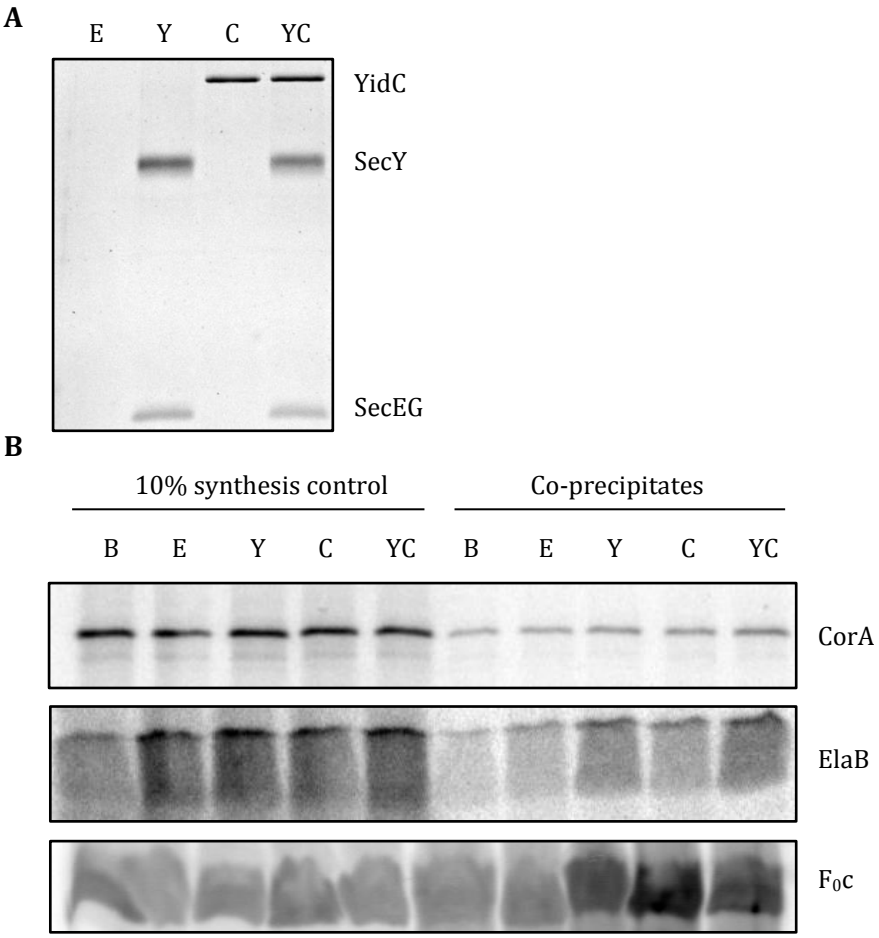


Figure 5 - CorA needs SecYEG and YidC for insertion into proteoliposomes.
(a) SDS-PAGE of proteoliposome reconstitution. (b) *in vitro* transcription, translation and insertion assay of CorA, ElaB and F_0C using proteoliposomes and the cytomime system. abbreviations: B, control (Buffer K); E, Empty liposomes; Y, SecYEG liposomes; C, YidC liposomes; YC, SecYEG/YidC liposomes.

3.4 Role of an ΔΦ in CorA insertion

The *in vitro* transcription and translation assays in combination with IMVs showed that CorA insertion is stimulated by SecYEG and YidC. Subsequently, using the same assay in combination with proteoliposomes showed a slight increase in CorA insertion for the SecYEG/YidC vesicles. Because the insertion efficiency in the proteoliposomes was low, the question arose whether CorA needs a electrochemical gradient for insertion. To investigate this, proteoliposomes were reconstituted in sodium sulphate buffer and *in vitro* transcription translation was performed as described in van der Laan et al (34). The sodium-loaded proteoliposomes were diluted into the reaction mix containing 100 mM potassium chloride, the highly selective potassium ionophore valinomycin was added to generate a potassium gradient (38). FtsQ was used as a control for the ΔΦ dependent insertion (data not shown), as it has been shown to inserted dependent of a gradient (34). Unfortunately, no FtsQ protein synthesis was observed after phosphorimaging, Insertion data of CorA showed no significant increase in insertion into SecYEG and/or YidC proteoliposomes compared to empty liposomes (figure 6a,b). Only a ~1% CorA insertion increase compared to empty liposomes was observed with YidC reconstituted vesicles (figure 6b). In contrast to the insertion results with proteoliposomes without a gradient (figure 5), co-reconstitution of SecYEG and YidC showed a decrease in insertion (figure 6a,b). The lack of a significant CorA insertion in this experiment might be due to a non-existent or too weak gradient, which could not be confirmed by the FtsQ control rendering the obtained results questionable.

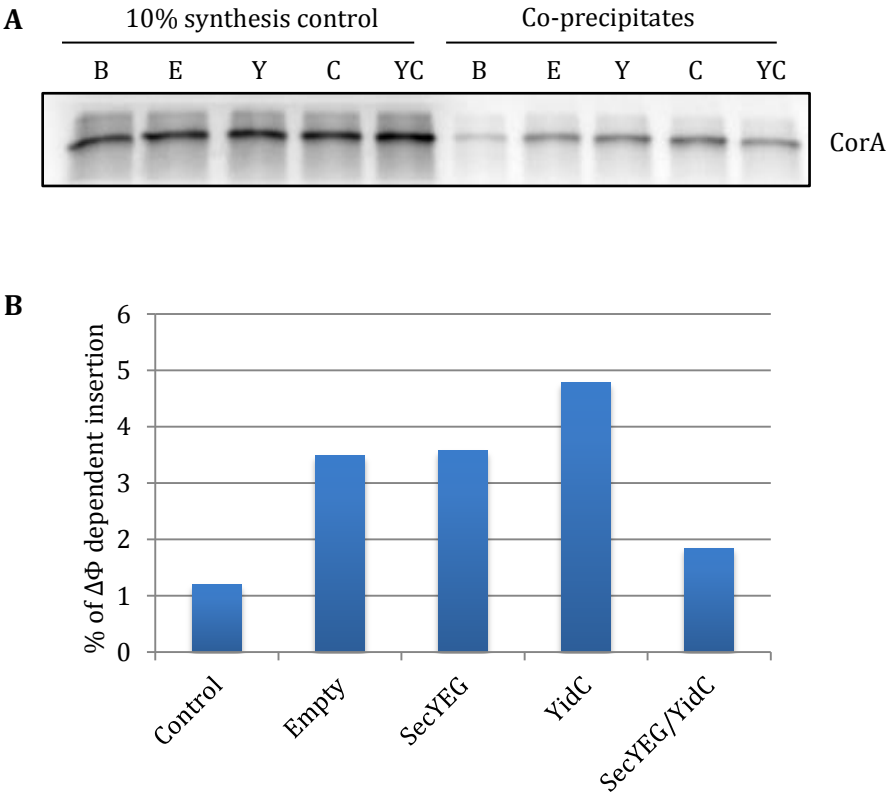
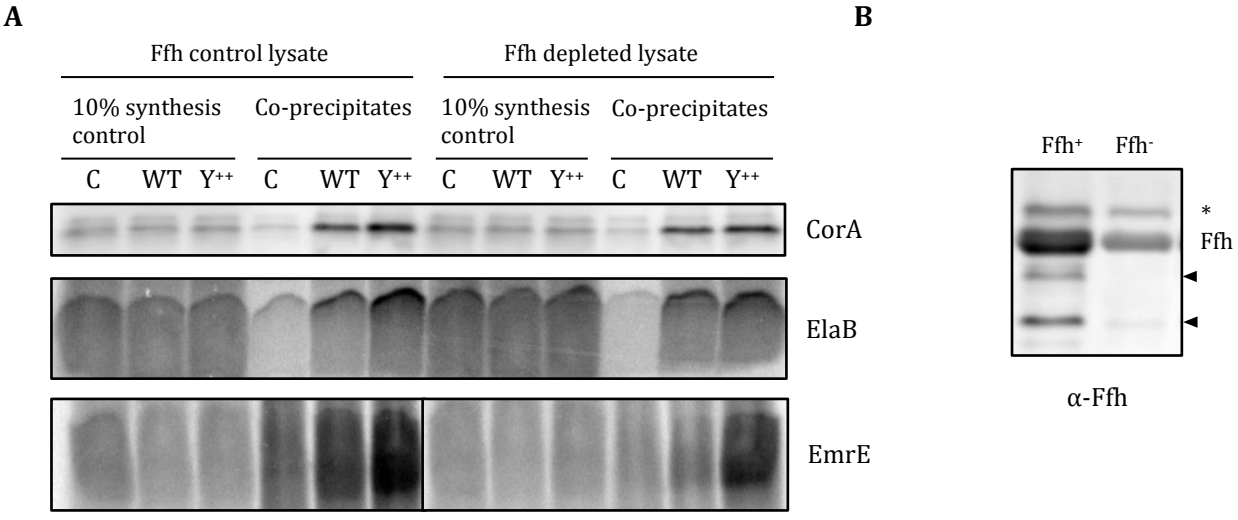


Figure 6 – Inconclusive ΔΦ dependence for CorA insertion. (a) *in vitro* transcription, translation and insertion assay of CorA using proteoliposomes with an electrochemical gradient. (b) quantification graph of the CorA phosphor image
abbreviations: B, control (Buffer N); E, Empty liposomes; Y, SecYEG liposomes; C, YidC liposomes; YC, SecYEG/YidC liposomes.

3.5 Role of SRP in the biogenesis of CorA

To investigate whether the targeting of CorA is mediated by the prokaryote's SRP homolog, Ffh, a Ffh depleted lysate was made. For this purpose, the *E. coli* strain HBD51 was used in which the *ffh* gene is under control of the arabinose promotor. Cells were grown to an OD_{600} of 0.6 in the presence of 0.2% arabinose (control cells, Ffh^+) or glucose (Ffh depleted lysate, Ffh^-) and diluted 2-fold in the same medium, this procedure was repeated for 4 hours to the point where the cells ceased to grow. Lysate was prepared from the harvested cells and the amount of Ffh present was determined by western blotting using an antibody against Ffh (figure 7b). The data showed that there was still Ffh present in the lysate (figure 7b, Ffh^-). However, the concentration was much reduced compared to the non-depleted lysate (figure 7b, Ffh^+). Next, an *in vitro* transcription, translation and insertion assay was performed using the Ffh lysates in combination with IMVs and the cytomime system to assay co-translational insertion (figure 7a). The TA protein ElaB and multidrug resistance efflux transporter EmrE functioned as controls in this experiment. Recent data on ElaB suggest that it inserts via a post-translational SRP-independent pathway (Oldebesten, A. unpublished. Shofiyah, S. unpublished). EmrE however, is expected to be targeted co-translationally by Ffh. Indeed, insertion of EmrE was less efficient in the Ffh depleted lysate (figure 7a, e), suggesting a role for Ffh in EmrE targeting. Surprisingly, ElaB also showed a slightly decreased insertion with the Ffh depleted lysate (figure 7a, d). This is in contrast to previous data, which suggested that ElaB is targeted to the membrane Ffh independently. Insertion of CorA was also slightly affected upon depletion of Ffh (figure 7a, c). However, CorA seems less affected by the depletion than the controls (figure 7 c-e). As the decrease in EmrE insertion is much more pronounced than that of CorA upon Ffh depletion, the significance of the obtained CorA results remains unclear.



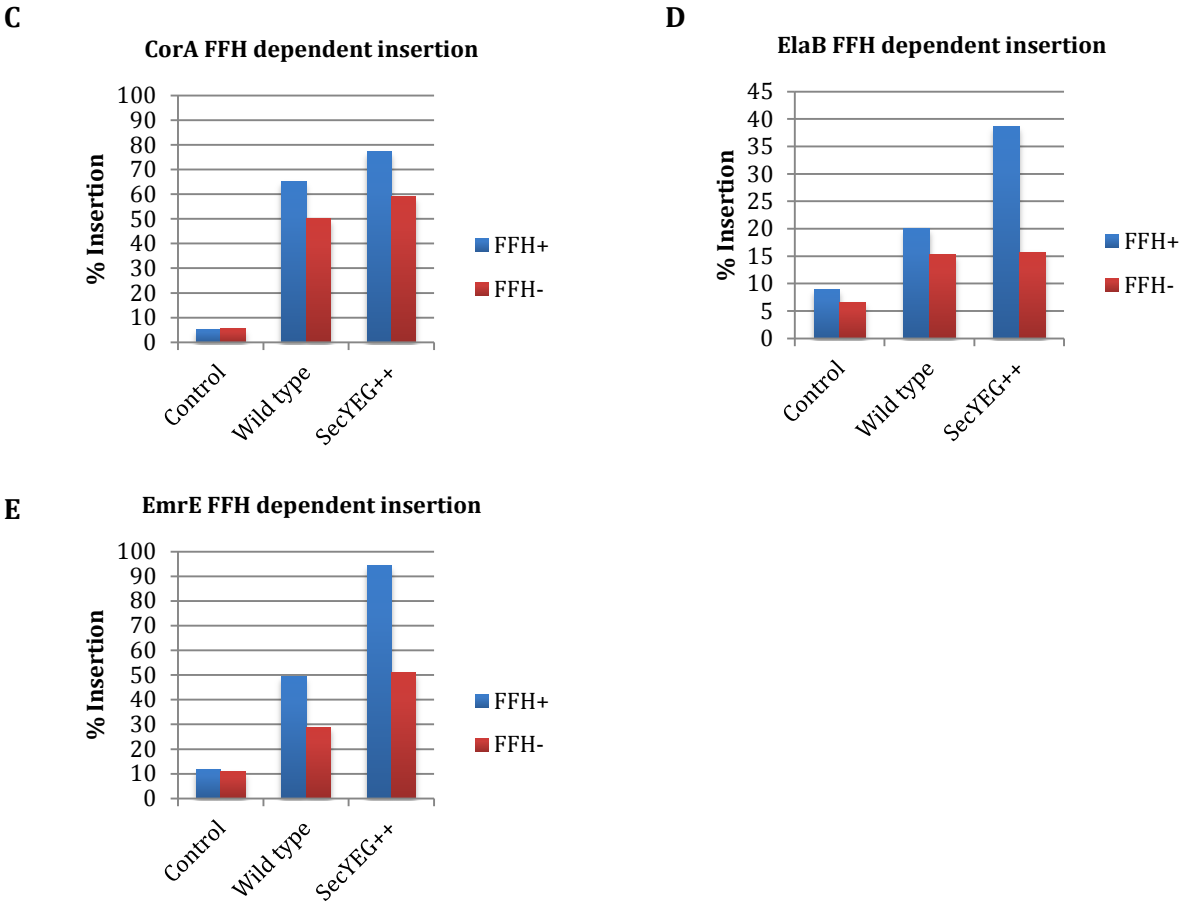


Figure 7 - CorA seems to depend on Ffh for insertion into IMVs. (a) *in vitro* transcription, translation and insertion assay of CorA, ElaB and EmrE using Ffh control and depleted lysate. (b) Western blot of Ffh control and Ffh depleted lysate blotted with primary α-Ffh. Degradation products are indicated by the arrowhead. Asterisk indicates a loading dye contamination. (c-e) quantification graphs of the phosphor images. Quantification was done using ImageJs build-in gel analyser option. Co-precipitate band intensities were calculated from its own synthesis sample and plotted against an arbitrary percentage scale.
abbreviations: C, control (20% sucrose/50 mM Tris-HCl pH 8.0); CL, Control lysate (ABS lysate); WT, wild type IMVS; SecYEG++ and Y^{++}, SecYEG overepxressing IMVs; Ffh+, Ffh control lysate; Ffh-, Ffh depleted lysate.

3.6 Proteinase protection assays was inconclusive and needs optimisation

In the previous experiments the urea extraction assay was used to test for protein insertion into vesicles. However, this assay may not distinguish between proteins that are inserted or strongly associated with the membrane. For this purpose it was attempted to develop a proteinase protection assay. CorA was expressed *in vitro* using the cytomime system in the presence of wild-type or SecYEG overexpressing IMVs. Reaction mixtures were treated with 5000 U/ml trypsin. After incubation, vesicles were tricholoroacetic acid-precipitated and analysed by tricine gel electrophoresis and phosphorimaging. These samples resulted in fragment pattern with no significant differences in the band intensities between the wild type and SecYEG overexpressing vesicles (figure 8a). To clarify this pattern, the *in vitro* insertion reaction mix was first treated with urea or spun through a sucrose cushion. The resulting pellets were dissolved in buffer and treated with trypsine. Treatment with the protease resulted in multiple protease protected fragments in both wild type as well as the SecYEG overexpressing IMVs, possibly indicating that CorA is inserted into the vesicles (figure 8b, c). The height of some of the smaller protection bands might correspond to the two trans-membrane domains of CorA of about 7 kDa (figure 8a, b ◂). Unfortunately the exact size could not be determined as 7 kDa is beyond the range of the ^{14}C labelled marker available and needs to be confirmed to give a decisive answer. The results of this experiment are questionable, perhaps due to the quality of IMVs used, and need to be repeated to give a decisive answer.

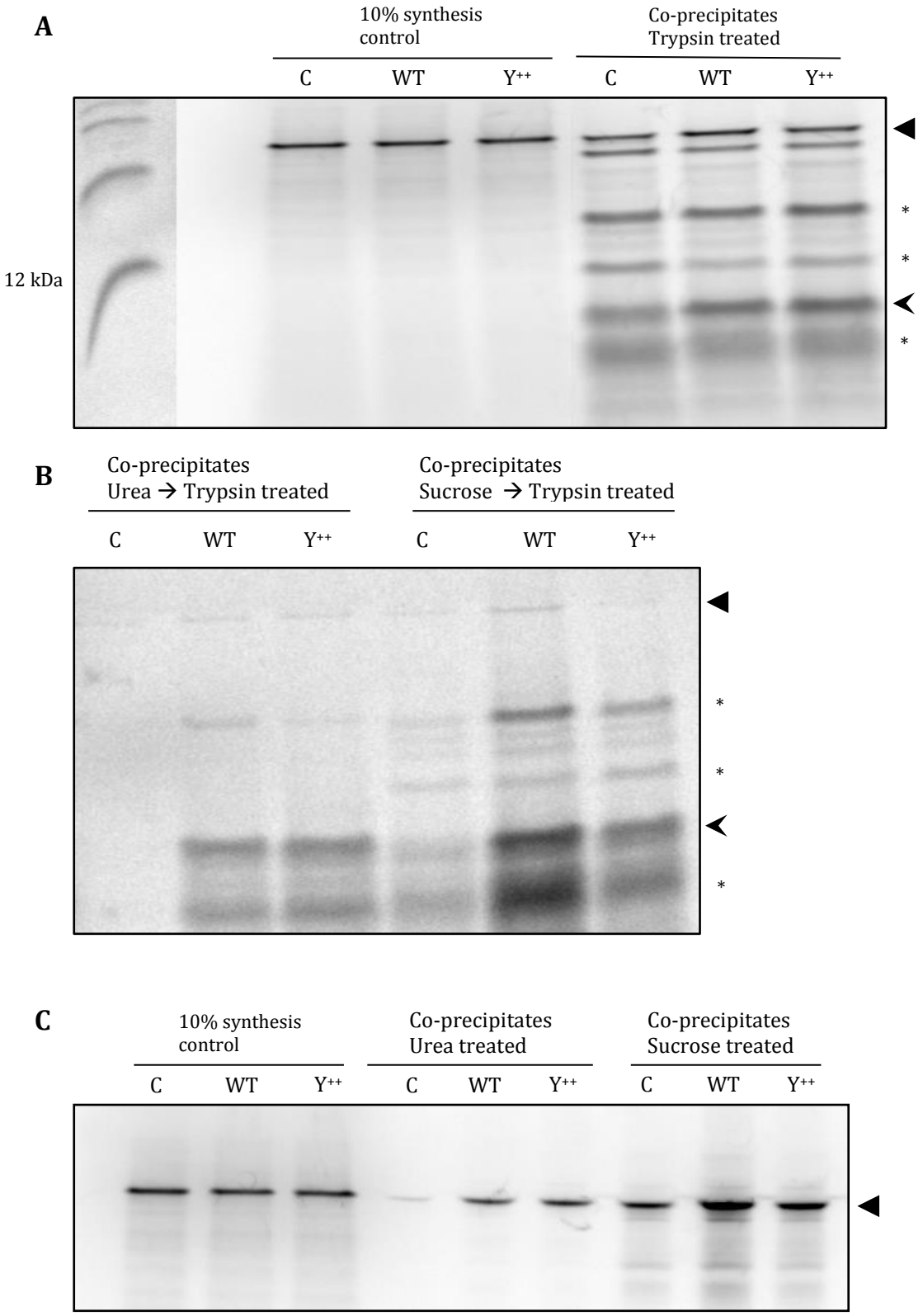


Figure 8 – Test for CorA insertion using a proteinase protection assay. (a-c) *in vitro* transcription, translation and insertion assay of CorA using IMVS. (a) Treatment of wild type and SecYEG overexpression IMVs with Trypsin. (b) Wild type and SecYEG overexpressing IMVs first treated with 6 M urea or sucrose then Trypsin. (c) Assay controls, insertion was assayed using urea extraction and influence of sucrose was visualized. Full length CorA (◀) and its possibly two TMDs (➤) are indicated by the arrowheads. Asterisks indicate alternative protease protected fragments. Abbreviations: C, control (20% sucrose/50 mM Tris-HCl pH 8.0); WT, wild type IMVS; Y++, SecYEG overepxressing IMVs.

Discussion

The magnesium transporter CorA is anchored to the cytosolic membrane via two C-terminal TMD domains. A recent study using *E. coli* cells depleted of YidC suggested that YidC may be involved in the biogenesis of CorA (19). In this study, *in vitro* transcription, translation and insertion experiments were used to study CorA's biogenesis. At the basis was the urea assay, in which membrane integration of *in vitro* synthesized CorA was tested by its resistance to urea.

Insertion data using IMVs overexpressing SecYEG suggested an increase in the CorA protein insertion by the SecYEG translocon. The drastically decreased level of CorA insertion in IMVs depleted from YidC suggested also a role for YidC for CorA insertion. This corresponds to the YidC depletion study of Price and colleagues (19), were a decrease in CorA levels was observed which suggests an YidC involvement in the biogenesis of the CorA transporter. Unfortunately, a complete depletion of YidC was not achieved in this study as western blotting showed small traces of the protein still present in the vesicles. Additionally, the weak PspA response also supports the observed western blot data. In a similar study, this response was shown to be stronger upon complete YidC depletion from *E. coli* cells (37). The actual effect of YidC mediated insertion of CorA, could possibly show an even greater effect using stronger YidC depleted vesicles. Furthermore, it has been suggested that YidC is involved in the generation of an electrochemical gradient by inserting proteins involved in energy production which generate a proton motive force (18) (34). If CorA is indeed dependent on a gradient to be inserted, the lack of such in the YidC depleted vesicles might also explain the observed decrease in insertion. Regrettably, the role of a gradient in the insertion of CorA could not be elucidated in this study. Attempts to insert CorA into proteoliposomes in which an electrochemical gradient was generated did not result in significant insertion. Possibly, the gradient was too weak, which could not be verified with the FtsQ control due to the lack of synthesis. A possible optimization step would be to purify the SecYEG and YidC proteins in a buffer containing sodium sulphate. The insertion experiments in this study using inner membrane vesicles as well as proteoliposomes, however, showed a trend that suggests a co-dependence of SecYEG and YidC for the insertion of the CorA monomers.

The urea extraction assay was used in this study to test for protein insertion into vesicles. However, a different approach using a protease could give a more direct indication of whether a protein is inserted or not. For this purpose the protease trypsin was used in a protease protection assay. The results of this experiment were rather contradictory to the data of the urea extraction assay. Although treatment with trypsin

resulted in multiple protease protected fragments in both wild type as well as the SecYEG overexpressing IMVs, suggesting that CorA is inserted into the vesicles, no effect of the SecYEG overexpression could be observed. One explanation could be that the *in vitro* synthesized CorA has only a strongly association with the IMVs, although it may be difficult to envision how SecYEG would stimulate this association. Unfortunately, attempts to confirm this hypothesis and optimize the assay failed due to a lack of time.

The targeting of CorA might occur via the prokaryote's SRP homolog, Ffh. The obtained results showed insertion levels of CorA similar to those of EmrE, which showed a decrease in SecYEG stimulated insertion with the Ffh depleted lysate. CorA however, seems less affected by the depletion and the overall low synthesis levels and the incomplete depletion of Ffh, make it difficult to interpret the significance of the data. Previous preliminary results suggested that ElaB is targeted post-translational and independent of SRP (A. Oldebesten and S. Shofiyah unpublished). The data observed in this study suggests otherwise, as an slight decrease of ElaB insertion is seen with de Ffh depleted lysate, suggesting a SRP dependent insertion. Further investigation is needed to determine whether these results are significant or not. A possible explanation for the obtained data might be the incomplete depletion of the Ffh proteins in the lysate. The remaining Ffh might still target the assayed proteins to the insertion site. Optimizing the ratios between lysate and the proteins of interest could result in a greater difference between CorA and ElaB insertion.

Attempts to determine via which translational pathway CorA is targeted to the insertion site in the cytosolic membrane, a co-/post-translational insertion assay was carried out. Unfortunately, this experiment failed, as there was no protein synthesis observed. Given the structure of CorA's subunits and the manner of translation, the co-translational pathway might be chosen for its targeting to the insertion machinery. As suggested by the targeting experiment, this might occur via Ffh to YidC and/or SecYEG translocon. Alternatively, one monomer of the pentameric CorA transporter has two C-terminus TMDs, and resembles the structure of a tail anchored protein. It may be because of this resemblance that CorA might also be post-translationally targeted to the membrane, perhaps with the aid of Ffh. This type of targeting is not yet observed in prokaryotes, but eukaryotes utilize the Ffh homolog SRP to post-translational target TA proteins to the endoplasmic reticulum (39) (40). Further investigation is needed to elucidate the targeting mechanism of CorA, however, both SecYEG and YidC seem necessarily for correct insertion. YidC might possibly also fulfil the role of chaperone in the cytoplasmic membrane (12) (13). This feature of YidC might help correct folding of CorA into the lipid bilayer and perhaps even aids in the assembly of the pentameric state of the

transporter. Furthermore, in the absence of YidC, CorA might be degraded as seen for other membrane proteins in the YidC depletion study of Price and colleagues.

Taken together, the data obtained in this study suggests a dual role of SecYEG and YidC in CorA biogenesis. The involvement of YidC implies that CorA is a substrate for the small insertase, which agrees with the findings of the study by Price and colleagues. A collaborative between SecYEG and YidC is observed for the lipoprotein CyoA (41), and could also apply for the correct biogenesis of CorA.

In conclusion, these results point to a dual role of SecYEG and YidC and form a step towards elucidating the complete biogenesis of the magnesium transporter CorA.

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