Artificial Cells

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In Vitro Synthesis of the *E. coli* Sec Translocon from DNA**

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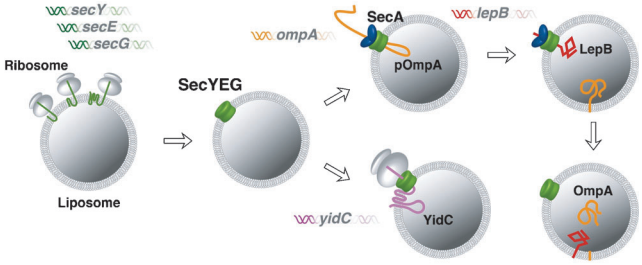
Abstract: *Difficulties in constructing complex lipid/protein membranes have severely limited the development of functional artificial cells endowed with vital membrane-related functions. The Sec translocon membrane channel, which mediates the insertion of membrane proteins into the plasma membrane, was constructed in the membrane of lipid vesicles through in vitro expression of its component proteins. The components of the Sec translocon were synthesized from their respective genes in the presence of liposomes, thereby bringing about a functional complex. The synthesized E. coli Sec translocon mediated the membrane translocation of single- and multi-span membrane proteins. The successful translocation of a functional peptidase into the liposome lumen further confirmed the proper insertion of the translocon complex. Our results demonstrate the feasible construction of artificial cells, the membranes of which can be functionalized by directly decoding genetic information into membrane functions.*

An artificial living cell is a sustainable and reproducible cell-like entity composed of biological components, such as proteins, DNA, RNA, and phospholipids.[1] The practical strategy for producing such an artificial cell is assembling biomolecules that imitate the architectures and functions of biosystems in living organisms.[2] Previously, we reconstructed the translation machinery with the minimal number of purified translation factors required for an artificial gene expression system. The *p*rotein *s*ynthesis *u*sing *r*ecombinant *e*lements (PURE) system[3] enables the information encoded in the DNA sequence to be converted into functional proteins and enzymes.

Based on the PURE system, we have synthesized several membrane machines that are important and indispensable for vital cell functions.[4] In particular, the membrane enzymes that catalyze phospholipid biosynthesis were synthesized within vesicles. Although the synthesized enzymes produced new phospholipid, morphological changes to the vesicle were not observed because of low efficiency of the enzymes. Therefore, a more efficient membrane protein insertion is needed to express membrane proteins and achieve real membrane function, which is a serious challenge for the production artificial cells. The previous approach for constructing membrane protein machinery depended on non-regulated spontaneous membrane insertion. This limits the secretion of exocellular proteins, which must pass a lipid membrane. Our novel approach to this problem is the initial synthesis, through spontaneous insertion, of a specialized translocation complex (Sec translocon[5]) that conducts the membrane integration and exportation of proteins. Any other prokaryotic membrane proteins, including inner or outer membrane proteins, can be synthesized as downstream reactions (Scheme 1).



Scheme 1. A schematic representation of the translocation system, which is based on the in vitro synthesis of the SecYEG translocon from DNA. Genes encoding SecYEG are expressed with the PURE system. The synthesized SecYEG proteins are spontaneously integrated into the lipid membrane, thereby resulting in Sec translocon equipped vesicles. Subsequently, other membrane proteins (pOmpA or YidC) are also synthesized and translocated or integrated in a process mediated by the synthesized SecYEG. Further synthesis of LepB following pOmpA synthesis leads to digestion of the signal sequence of pOmpA within the vesicle lumen. SecA is a motor protein required for the translocation of hydrophilic regions of the substrate proteins.

In bacterial cells, the Sec translocon is synthesized within the cytosol and transported to the cell membrane (plasma membrane). The function of the Sec translocon is to transport outer membrane proteins or periplasmic proteins from the inside to the outside of the cell. In this study, however, we synthesized the *E. coli* Sec translocon on the outside of liposomes. Therefore, the orientation of the synthesized Sec translocon was inverted; that is, the inside and outside of the liposome correspond to the exterior and interior of cell, respectively (Scheme S1 in the Supporting Information). This study is an important intermediate step toward the synthesis

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of the Sec translocon within a large liposome (giant vesicle) as in living cells. Such an artificial cell model would enable the coupling of the protein synthesis and secretion events that are essential for keeping a cell alive.

To realize this idea, we synthesized three major component proteins of the *E. coli* Sec translocon; SecY (48.5 kDa), SecE (13.6 kDa), and SecG (11.3 kDa) by using the PURE system (Figure 1a). The stoichiometry of SecY, SecE, and SecG in the SecYEG complex is 1:1:1. Balanced production was obtained after titrating the amount of input DNA (Figure S1 in the Supporting Information). SecYEG synthesized in the presence of liposomes was obtained in the liposome fraction after centrifugation (Figure S2). This result indicates that the SecYEG proteins were spontaneously inserted into the liposome membrane. Spontaneous insertion occurs as a result of hydrophobic interactions between a membrane protein and the lipid bilayer, and the orientation of the inserted protein is not regulated.[6] We investigated the proportion of SecYEG proteins with the native-like orientation in the liposome membrane. The results of a partial proteolysis assay suggested that about 50–60% of the integrated SecY or SecE maintained native-like membrane topology (Figure S3). Furthermore, complex formation between the SecYEG proteins on the membrane was visualized by blue native PAGE (BN-PAGE; Figure 1b). A bold clear band was observed when all three SecYEG proteins were synthesized together. A slightly smaller band was also observed when the SecG gene was omitted, thus suggesting the formation of a SecYE complex. Since no band appeared when SecY and SecG were synthesized together, it is likely that a SecYE complex is first assembled before the incorporation of SecG to complete the SecYEG complex. The presence of each component in the obtained bands was confirmed by using antibodies against SecY, SecE, and SecG (Figure S4).

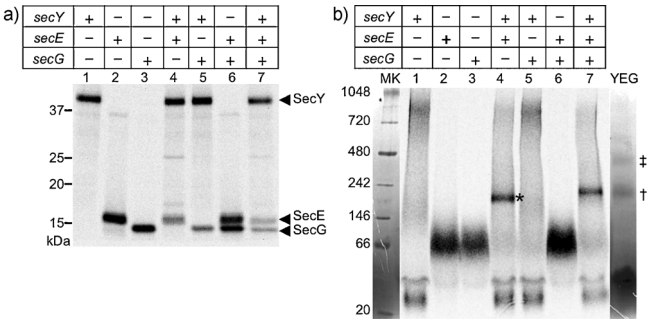

Figure 1. In vitro syntheses of SecYEG. a) SecY, SecE, and SecG were synthesized in various combinations with the PURE system supplemented with liposomes and [^{35}S]methionine. The synthesized proteins were analyzed by SDS-PAGE after washing with acetone. b) BN-PAGE analysis of the synthesized SecYEG. SecY, SecE, and SecG were synthesized in various combinations in the presence of liposomes and subjected to BN-PAGE in the presence of 0.4% DDM. The presumed monomeric and dimeric SecYEG complexes are indicated with crosses and double crosses, respectively. The SecYE subcomplex is indicated with an asterisk. Only the protein bands labeled by [^{35}S]methionine are visible on the gels, except in the MK and YEG lanes. MK = molecular marker, YEG = SecYEG isolated from cell membrane as a positive control, DDM = *n*-dodecyl β-D-maltopyranoside.

Importantly, complex formation was achieved through self-assembly of the component proteins, without any assistance from a membrane chaperone. In the PURE system, the synthesized SecYEG proteins were inserted into the liposome membranes without disruption of either the native-like topology or formation of the heterotrimeric complex. We next tested the functions of the SecYEG complex with several types of membrane protein substrates.

The precursor of the secreted protein outer membrane protein A (pOmpA) was synthesized after the synthesis of SecYEG (Figure 2a). pOmpA can bind the lipid membrane because of its N-terminal hydrophobic segment, but the following domain that corresponds to the mature protein

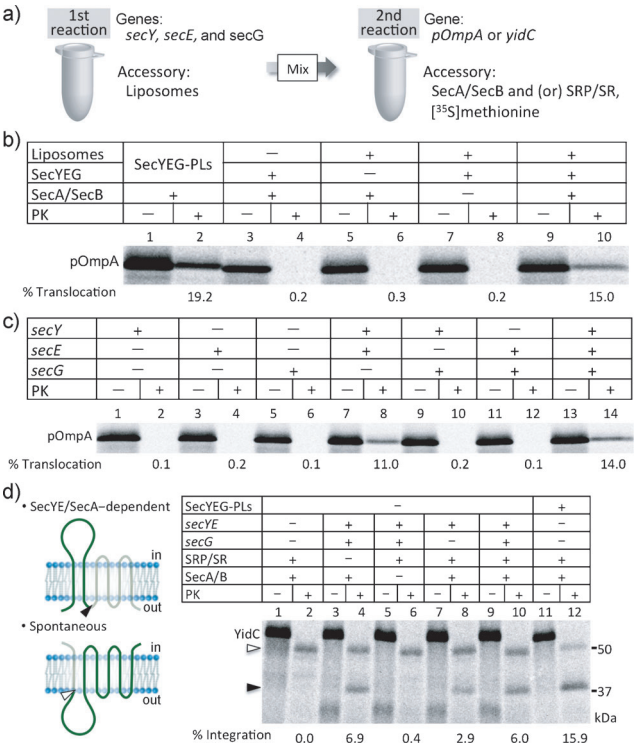

Figure 2. Translocation activity of in vitro synthesized SecYEG. a) A schematic representation of the translocation assay. SecYEG was synthesized in the first reaction in the presence of liposomes. The reacted mixture was mixed with the second reaction containing the accessory proteins SecA/SecB or (/and) SRP/SR and DNA for the substrate proteins. The product was digested with proteinase K (PK) and analyzed by SDS-PAGE. b) Translocation activity was evaluated in the presence or absence of liposomes, SecYEG synthesis, and SecA/SecB, as indicated. Proteoliposomes reconstructed from purified SecYEG and lipids (PLs-SecYEG) were used as a positive control. c) Translocation activity was tested with various combinations of the SecY, SecE, and SecG syntheses. d) Integration of YidC was performed with various combinations of SecYE and SecG in vitro syntheses, SecA/SecB, and SRP/SR. Proteoliposomes reconstructed from purified SecYEG and lipids (PLs-SecYEG) were used as a positive control. The membrane topologies of YidC are described in the left panel and the positions of the membrane-protected fragments resulting from SecYE/SecA dependently insertion and spontaneously insertion of YidC are indicated by filled and open triangles, respectively. 25 % of the PK samples were loaded.

cannot translocate across the membrane without SecA activity. The results show that the synthesized pOmpA was successfully translocated depending on the presence of SecYEG in the liposome membrane and free SecA/SecB (Figure 2b, lanes 9–10). The bands for the proteinase K positive samples (PK^+; even lanes) represent pOmpA that was not digested by PK, thus indicating translocated pOmpA. SecA is the molecular motor that translocates proteins across the membrane by using energy from ATP.[7] SecB is a cytosolic chaperone that keeps proteins loosely folded before translocation.[7] No translocation was observed when any of these components (SecYEG or SecA/B) was omitted (Figure 2b, lanes 3–8). In this experiment, the protein synthesis and membrane translocation of pOmpA were performed simultaneously. Since the SecA-dependent translocation of pOmpA is known to occur independent from protein synthesis, the pOmpA translocation was also performed under uncoupling conditions by adding antibiotics or RNase instead of performing simultaneous pOmpA synthesis. The result showed that similar translocation was observed in such posttranslational translocation assays (Figure S5). In order to characterize the function of each subunit, the same experiments were performed with various combinations of SecY, SecE, and SecG. The results show that pOmpA translocation occurred in the presence of SecYE and SecYEG (Figure 2c, lanes 7–8 and 13–14). These data correlate with the BN-PAGE results (Figure 1b) and with a previous report,[8] which indicated that SecY and SecE are essential but SecG is not. About 10–20% of the synthesized pOmpA was translocated to the inside of the liposome. This efficiency was somewhat lower than in previous studies on the Sec translocon in proteoliposomes[9] or inverted membrane vesicles,[10] but translocation of the synthesized pOmpA was clearly achieved. On the other hand, enhanced translocation activity for the SecY C385Y mutant was discovered in this study through mutational analysis of SecY (Figure S6c), and with this more active SecY variant, about 70% of the pOmpA was translocated.

In addition to pOmpA, we tested the multi-span membrane protein YidC. YidC is known to act as a membrane protein insertase for simple membrane proteins such as the *c*-subunit of ATP synthase or virus coat proteins,[11] and as a chaperone for multi-span membrane proteins in cooperation with the Sec translocon.[12] YidC has six membrane-spanning helices and a large hydrophilic loop in the periplasmic domain.[13] Based on this feature, the successful membrane insertion of YidC regulated by SecYEG can be evaluated by observing the appearance of a 37 kDa peptide band on SDS-PAGE after PK digestion. As shown in Figure 2d, the specific PK-resistant band at around 37 kDa was observed in the presence of SecYE(G) and SecA/B (Figure 2d, lanes 4, 8, 10, and 12). A nonspecific band at 50 kDa, resulting from spontaneous insertion with the reverse topology, was also observed. Successful specific translocation in the presence of SecY was emphasized when the C385Y mutant was used (Figure S6e). The resultant band pattern is consistent with previous reports.[10b,14] In bacterial cells, YidC is thought to be first led to the Sec translocon by the signal recognition particle (SRP) and SRP receptor (SR)[15] in a process coupled with translation. However, enhancement of the membrane integration of YidC by SRP and SR was not observed in this in vitro reaction system. By contrast, a dependence on SecA for translocating the hydrophilic loop into the liposome lumen was clearly observed. In summary, synthesized SecYEG mediates the membrane translocation of both single-span and multi-span membrane proteins. This is the first evidence that the SecYEG translocon is functional in the liposome membrane when synthesized in vitro from DNA.

To demonstrate that the translocated membrane protein maintains its functional activity inside the liposome lumen, we next synthesized another membrane protein, LepB, which is a type I signal peptidase capable of digesting the N-terminal signal sequence of pOmpA. LepB was synthesized after the synthesis of pOmpA. The successful translocation of pOmpA (37 kDa), accompanied by the removal of the signal sequence that results in OmpA (35 kDa), was observed in the $LepB^+$/PK^+ sample (Figure 3, lane 12). Although the band intensity

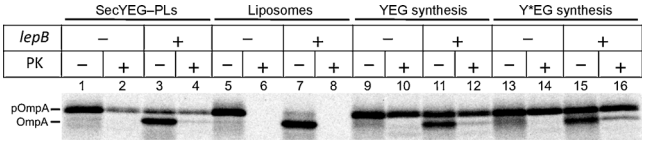

Figure 3. A cascade of membrane protein insertions. LepB was synthesized after the stepwise syntheses of SecYEG and pOmpA, with plain liposomes and proteoliposomes reconstructed with SecYEG (PLs-SecYEG) as negative and positive controls, respectively. The SecY C385Y mutant (Y*) was also tested. After all syntheses, the samples were processed by PK. The positions of pOmpA (37 kDa) and the product of signal digestion OmpA (35 kDa) are indicated.

of OmpA is not significant, the appearance of OpmA in the $LepB^+$/PK^+ sample was clearly observed when the SecY C385Y mutant was used (Figure 3, lane 16). These data confirm that the cascading reactions (i.e., SecYEG synthesis, pOmpA synthesis, LepB synthesis, and signal digestion) successfully occurred. Note that pOmpA without its signal sequence cannot be translocated by SecYEG and SecA (Figure S7). The membrane integration of LepB itself was verified in the same manner as that of pOmpA or YidC (Figure S8). Although the ratio of correctly integrated LepB was low, a dependence on SecYE and SecA was clearly shown, thus indicating that a fraction of the synthesized LepB was properly integrated into the liposome membrane and able to digest the signal sequence of the pOmpA. These results suggest that the translocated membrane protein is functional inside the liposome lumen.

We calculated that one liposome carries about 3.3 SecYEG complexes on the membrane (see the Supporting Information). The initial speed of pOmpA translocation by in vitro synthesized SecYEG was comparable to that for the reconstructed proteoliposomes reconstructed from purified SecYEG and lipids (SecYEG-PLs) that are generally used for in vitro analyses of the Sec translocon (Figure S9).

In summary, we have successfully reconstructed the Sec translocon by synthesizing its component proteins in vitro. The synthesized SecYEG components were spontaneously

inserted into the liposome membrane and formed a functional complex. All of the protein syntheses in this study were performed with the PURE system. The characteristic high purity achieved the reconstructed system makes this approach suitable for the construction of artificial cells. Lack of purity is often a limitation for cell-free systems based on cell extracts, which often contains unfavorable molecules for protein synthesis, such as DNase, RNase, Protease, or cell membrane fraction.

The next goal of this research is to synthesize the SecYEG translocon within giant unilameller vesicles (GUVs). GUVs are micrometer-size vesicles that are capable of efficient encapsulation of a cell-free system and DNA.[16] Although several cellular reactions have been performed within GUVs to date, the construction of a functional phospholipid bilayer that includes membrane proteins remains a major challenge for the development of artificial cells. Our approach enables the correct installation of integral membrane proteins into lipidic bilayers. This approach should thus accelerate the construction of artificial cells in the laboratory. SecA and SecB were purified from *E. coli* for use in this study owing to the capacity limitation of the PURE system but future development should involve the cell-free synthesis of these factors from DNA as well.

Our in vitro system can also be useful for modeling cellular processes that are difficult to explain in vivo. Since our system is based on a reconstructed cell-free system, interference by other cellular components in the reaction of interest is eliminated. For instance, in mutation analyses, the direct effects of interesting mutations on the function of the protein of interest can be clearly obtained. Indeed, we have discovered a highly active SecY mutant that was not identified by an in vivo approach.[17] This advantage would be applicable to rapid and easy mutational analysis not only of the Sec translocon but also of other membrane protein complexes.

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