A cell-free system for target discovery of small membrane proteins

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Abstract
Numerous small proteins have been discovered across all domains of life, among which many are hydrophobic and predicted to localize to the cell membrane. Based on a few that are well-studied, small membrane proteins are regulators involved in various biological processes, such as cell signaling, nutrient transport, drug resistance, and stress response. However, the function of most recently identified small membrane proteins remains elusive. Their small size and hydrophobicity make protein production challenging, hindering function discovery. Here, we combined a cell-free system with lipid sponge droplets and synthesized small membrane proteins in vitro. Lipid sponge droplets contain a dense network of lipid bilayers, which accommodates and extracts newly synthesized small membrane proteins from the aqueous surroundings. Using small bacterial membrane proteins MgrB and SafA as proof of principle, we showed that the in vitro produced membrane proteins reached sufficient concentrations for downstream analysis, and more importantly, they are functionally active. The cell-free system was also suitable for synthesizing other small membrane proteins, including one from human, indicating its high level of versatility. Furthermore, we showed that small proteins produced in this system can be used for in vitro pull-down assays to identify interaction partners. This work presents a robust alternative approach for producing small membrane proteins, which may aid their function discovery in all domains of life.

Keywords
Cell-free synthesis, small membrane proteins, lipid sponge droplets

Introduction

Small proteins also named mini-proteins [1], micro-proteins [2], or small peptides [3], were overlooked in the past due to their small size (less than 50 amino acids in prokaryotes and less than 100 amino acids in eukaryotes) [3, 4]. Small proteins are directly translated from small open reading frames (sORFs) that often start with noncanonical start codons, which increases the difficulty of differentiating valid sORFs from noncoding RNAs [3, 4]. With the advances in bioinformatics and application of ribosomal profiling, hundreds to thousands of sORFs have been identified in recent years [5-7]. While some have low stability and have no apparent contribution to cell fitness in the conditions tested [8], many are conserved, predicted to have a putative transmembrane helix, and may be involved in cell signaling and communication [5].

Small membrane proteins constitute a significant portion of newly discovered small proteins. They are involved in diverse biological processes across domains of life, ranging from bacterial virulence regulation to immunosurveillance Fmiin mammalian cells (reviewed in [9, 10]). Well-characterized bacterial small membrane proteins primarily act as regulators by integrating into large protein complexes, such as KdpF facilitating potassium transport complex formation [11], AcrZ specifying substrates for the AcrBA-TolC drug efflux pump [12], and Prli42 anchoring the stressosome to the bacterial membrane [13]. Small membrane proteins also target individual larger membrane proteins, regulating their stability and function. For example, MgrB and SafA participate in cell signaling by

regulating the activity of the sensor kinase PhoQ [14-17]. MgtS and MntS regulate magnesium and manganese transport by targeting the respective transporter proteins [18, 19]. Lastly, some small membrane proteins directly target the cell membrane, acting as a detector for membrane curvature [20] or as type I toxins regulating membrane permeability [21].
Compared to the number of well-characterized small membrane proteins, the vast majority of them have unknown functions or require functional verification. In contrast to soluble proteins, small membrane proteins are challenging to produce via chemical synthesis due to their hydrophobicity. Overexpression of recombinant small membrane proteins in vivo may induce general stress or even be toxic to cells. These difficulties become major roadblocks for functional investigations of small membrane proteins.

Cell-free protein synthesis (CFPS) has been used for membrane protein production in vitro to avoid potential cytotoxicity and protein aggregation, to improve yield, and to characterize membrane protein functions [22-24] [25]). Commonly used hydrophobic supplements in a cell-free system for membrane protein production include detergents, nanodiscs, and liposomes, which prevent precipitation and support the synthesis and folding of membrane proteins during synthesis. Liposomes closely mimic cellular membranes. Their membrane surface area correlates with the yield of folded and active membrane proteins [26, 27], underscoring the importance of a suitable membrane environment for functional membrane protein production in CFPS. Liposomes also generate separate compartments, which are required for functional assessment of channel and transporter proteins. However, a major drawback of liposomes is their instability in time and response to osmolarity changes. Nanodiscs [28] are a more stable alternative to liposomes, but both require specialized equipment and multiple steps for their preparation. In addition, both systems are limited in the membrane surface area they can provide.

Unlike liposomes and nanodiscs, lipid sponge droplets contain dense non-lamellar lipid bilayer networks, providing an extensive membrane surface for membrane protein insertion. They have a high water content, harbor nanometric aqueous channels, and thus simultaneously accommodate hydrophilic components of a cell-free system. Lipid sponge droplets are simple to prepare and generated via spontaneous assembly of the single-chain galactolipid N-oleoyl β-D-galactopyranosylamine (GOA) and non-ionic detergents such as octylphenoxypolyethoxyethanol (IGEPAL). In aqueous solutions, the two amphiphiles form micrometer-sized coacervate-like droplets that are stable in a wide range of conditions [29, 30]. Despite being a nonbiological lipid, the thickness of GOA membranes (35.25 Å)[31] is comparable to that of a biological membrane (37-40 Å) [32], which may explain why membrane proteins such as diacylglycerol kinase and cytochrome c oxidase were functional when reconstituted in lipid sponge droplets [29].

In this study, we combined lipid sponge droplets with a minimal, defined cell-free system (PURE system) [33] and produced small membrane proteins in *vitro*. We chose the PURE system because of its compatibility with the droplets and to avoid potential interference with downstream functional studies of the synthesized small membrane proteins. Using *E. coli* proteins MgrB and SafA as proof of principle, we showed that the small proteins were successfully synthesized and localized in the droplets. The yield was estimated to be around 500 nM, and the droplets were required to reach this amount. The synthesized MgrB and SafA were functionally active, modifying the kinase activity of their target protein PhoQ in *vitro*. Successful synthesis of two other small membrane proteins AcrZ from *E. coli* and sarcolipin from human demonstrates the versatility of this cell-free system. Furthermore, using the synthesized MgrB, SafA, and AcrZ, we co-immunoprecipitated their interacting proteins from cell lysate, suggesting that this cell-free system is suitable for target discovery of small membrane proteins.

Results and Discussion

Cell-free biosynthesis of small membrane proteins MgrB and SafA

We started with two well-studied *E. coli* proteins MgrB (47aa) and SafA (65aa) to test the cell-free system for small membrane protein production. DNA templates containing T7 promoter and the ORFs of MgrB and SafA were constructed (**Fig. 1**) and used to initiate protein synthesis in the cell-free system with lipid sponge droplets. To visualize CFPS, we added an N-terminal mNeonGreen tag to the small membrane proteins. Additionally, a DNA template for synthesizing only mNeonGreen was used as a control. We observed a gradual increase in green fluorescence during incubation, which reached a plateau after around three hours (**Fig. 1, Movie S1**). For the synthesis of mNeongreen-MgrB and mNeongreen-SafA, the fluorescence primarily localized to lipid sponge droplets (**Movie S1**). Conversely, the mNeongreen control sample showed diffuse fluorescence in the field of view with lipid sponge droplets having a slightly lower fluorescent intensity (**Fig. 1C**). These results suggested that the mNeongreen-tagged small membrane proteins were produced in the cell-free system and that their hydrophobicity led to sequestration of the fusion proteins into the membrane-rich droplet phase. To quantify MgrB and SafA synthesized in the cell-free system, we introduced a FLAG epitope tag to the N-terminus of the proteins (**Fig. 2A**). Using a FLAG-tag standard protein as a reference in Western blot analysis, we quantified that around 608 nM FLAG-SafA was produced in three hours and about 327 nM for FLAG-MgrB (**Fig. 2B, Fig. S1**). This concentration level is sufficient for downstream functional analysis. It is also about one magnitude higher than the reported 10-40 nanomolar concentrations reached for larger membrane proteins in PURE reactions supplemented with liposomes [34].

Small membrane proteins, such as MgrB and SafA, typically contain a single transmembrane helix (**Table S1**). To determine whether lipid sponge droplets were required for such bitopic integral membrane protein synthesis in vitro, we produced and compared FLAG-SafA with the PURE system in the presence and absence of lipid sponge droplets. The Western blot results showed that a higher amount (2.73 times) of SafA was synthesized in the presence of lipid sponge droplets (**Fig. 2C, Fig. S2),** indicating that the hydrophobic environment is beneficial despite the seemingly simple tertiary structure of SafA. The lipid sponge droplets facilitated the reconstitution of the synthesized protein, likely prevented protein aggregation, and thus increased the yield of SafA during CFPS. Along similar lines, previous work on CFPS of membrane channel proteins showed that the production of folded and active protein correlated with the amount of supplemented liposomes [26, 27]. While the membrane surface area liposomes can offer is limited, lipid sponge droplets are filled with a dense membrane environment and can be added to CFPS reactions at high concentrations [29].

Functional analysis of the synthesized MgrB and SafA

The function of MgrB and SafA can be tested by following the activity of their target protein PhoQ. The sensor kinase PhoQ catalyzes an autophosphorylation reaction, where it utilizes ATP to phosphorylate a conserved histidine residue. MgrB and SafA directly interact with PhoQ. MgrB inhibits the autophosphorylation of PhoQ, while SafA is an activator. To test the regulatory function of the synthesized small proteins, we performed PhoQ autophosphorylation assays with mNeonGreen as a negative control (the experimental scheme shown in **Fig. 3A**). To introduce the synthesized FLAG-SafA and FLAG-MgrB to the reaction, we spun down the droplets after CFPS and replaced the supernatant with purified recombinant PhoQ protein in phosphorylation buffer. The reaction mix was divided into two aliquots. One proceeded to autophosphorylation assays by adding radioactively labeled [γ-^{32}P]ATP. The other was used for Western blot analysis to verify the production of the small proteins. The Western blots showed SafA and MgrB bands with anti-FLAG antibodies (**Fig. 3C**), indicating the production of the small membrane proteins in the cell-free system. In the autophosphorylation assay, we observed an

increase in radioactively labeled PhoQ in the presence of the synthesized FLAG-SafA and a decrease in autophosphorylation with the synthesized FLAG-MgrB compared to the negative control (**Fig. 3B**), indicating that the synthesized small membrane proteins are functionally active.

With the same setup, we synthesized and tested the function of MgrB and SafA without epitope tags, and observed similar results in the autophosphorylation assays (**Fig. S3**). Therefore, the cell-free system is also suitable for producing native small membrane proteins, which is advantageous for studying small proteins that are sensitive to epitope or affinity tags. Altogether, the results suggested that our cell-free system can produce functional small membrane proteins sufficient for downstream analysis in vitro.

Cell-free biosynthesis of other small membrane proteins

To test the versatility of the cell-free system, we applied it to synthesize two other small membrane proteins, AcrZ from *E.coli* and sarcolipin from human. AcrZ is an integral membrane protein. It interacts with the drug efflux protein AcrB and forms a 3:3 hexameric membrane protein complex, which binds to AcrA and TolC, forming a complete drug efflux pump [12, 35]. Functionally, AcrZ regulates the substrate specificity of the drug efflux transporter [12, 35]. Sarcolipin is an essential regulator of the sarco-/endoplasmic reticulum Ca2+-ATPase in human myocytes [36]. It stops the pumping of calcium cations out of the cytosol without inhibiting the ATPase activity, thus converting the chemical energy stored in ATP to heat [36]. After codon optimization, we constructed the DNA templates and performed CFPS of AcrZ and sarcolipin in the presence of lipid sponge droplets. A FLAG-tag was introduced for quantification via Western blot analysis. The synthesis of SafA was used as a positive control. We observed that both AcrZ and sarcolipin were produced using the cell-free system. The yield of AcrZ was about 2.4 times more than SafA reaching 1.48 µM (**Fig. 4A, Fig. S4**). Sarcolipin was synthesized less (about 97.3 nM), possibly due to a polar residue (Asn11) in its transmembrane helix (**Table S1, Fig. 4B, Fig. S5**) hindering protein partitioning to lipid sponge droplets. Indeed, an N to L substitution increased the yield (2.44 times) of sarcolipin (**Fig. S6**). Taken together, our results demonstrate the versatility of the cell-free system and indicate that the system could be applied to synthesize small membrane proteins from both prokaryotes and eukaryotes.

Target discovery using the synthesized small membrane proteins

To determine whether the in vitro synthesized small membrane proteins were suitable for target discovery, we used MgrB, SafA, and AcrZ as bait to identify their interaction partners via co-immunoprecipitation (**Fig. 5A**). FLAG-tagged MgrB and SafA were synthesized using the cell-free system as described above. Meanwhile, we isolated the total membrane fraction from *E. coli* cells overexpressing PhoQ-His_6. The membrane was solubilized with buffer containing detergent and incubated with the synthesized MgrB and SafA overnight. Notably, the detergent dissolves most lipid sponge droplets, allowing interactions between the synthesized small protein and components in the membrane. Using anti-FLAG magnetic beads, we immuno-precipitated the FLAG-tagged MgrB and SafA as expected (**Fig. 5B left**). Additionally, we detected PhoQ-His_6 using an anti-His_6 antibody only in samples that contained MgrB or SafA (**Fig. 5B right**), indicating that the synthesized small proteins interacted with their target protein PhoQ and could be used to isolate PhoQ from a crude mix in an overexpression condition.

For the discovery of unknown interaction partners, it will be necessary to fish out interaction partners at their natural concentrations without overexpression. To test this, we synthesized the FLAG-tagged AcrZ and performed co-immunoprecipitation experiments with membrane fractions isolated from *E. coli* MG1655 wild-type cells (**Fig. 5C**). To detect AcrB, the protein that AcrZ interacts with, we obtained anti-AcrB antibody from Dr. Klaas Martinus Pos (Goethe University Frankfurt). Our Western blot results

showed that AcrZ was synthesized and AcrB was co-immunoprecipitated with AcrZ from *E. coli* membrane at its native concentration. The data strongly support the notion that small membrane proteins synthesized with our cell-free system are suitable for target discovery.

Conclusion

In this study, combining lipid sponge droplets with a cell-free system, we have developed an efficient and robust approach to produce functional small membrane proteins up to micromolar concentrations. This allows protein function verification in vitro and assists function discovery of small membrane proteins by identifying their targets. The membrane-rich environment created by lipid sponge droplets provides a large membrane surface area in a three-dimensional space, supporting membrane protein synthesis with high yield and simultaneously sequestering the produced protein into the droplets. This unique feature greatly simplifies downstream experimental procedures (i.e. purification and functional assays), and reduces protein loss. Small membrane proteins that are sensitive to lipid composition could be synthesized in the presence of lipid sponge droplets doped with natural lipids or transferred to liposomes after their synthesis. Encouragingly, however, both MgrB and SafA were active without separation from lipid sponge droplets, greatly simplifying the experimental procedure. The success of identifying AcrZ's target from the wild-type *E. coli* membrane highlights the system's potential in future functional studies of other small membrane proteins. Providing simple protocols for function assays and target identification combined with the small reaction volumes and high protein yields, our cell-free system opens up new possibilities for high throughput screens that will advance our understanding of small membrane protein biology.

Materials and methods

Chemical reagents and linear DNA templates

N-oleoyl β-D-galactopyranosylamine (GOA) with 99.6% purity was synthesized and analyzed by GLYCON Biochemicals. Octylphenoxypolyethoxyethanol (IGEPAL) was purchased from Sigma (I8896). The protein standard with the C-terminal FLAG tag (E-PKSH032870.10) was from Biomol. Linear DNA templates were produced by PCR and were PCR purified. The complete list of linear DNA templates used in this study is summarized in Table S2.

Cell-free synthesis

The experiments were performed as previously described [29, 30]. Briefly, the PURExpress® kit containing Solution A and Solution B (NEB) was used to prepare the transcription-translation reaction. A lipid film was prepared by evaporating 42 µL GOA (10 mM) mixed with 100 µl chlorofom in a glass vial. The lipid film was rehydrated by vortexing with 21 µL rehydration solution (14 µL PURExpress Solution A, 0.875 µL RNase Inhibitor (NEB), 17 µM IGEPAL (90mM), 3.465 µL H_2O) to form lipid sponge droplets. To initiate protein synthesis, 3µl of the droplet solution were combined with 1.5µl PURExpress solution B and 0.5µl DNA template solution to achieve a final concentration of 15 nM. The reactions were carried out at 37 °C at the indicated time. Lipid film and IGEPAL were omitted for cell-free synthesis without lipid sponge droplets.

Fluorescence microscopy

A cell-free reaction mix (3 µL) was pipetted into a lumox dish (Sarstedt) and sealed with a cover glass. The reaction was incubated at 37 °C on stage and observed using a Nikon Eclipse Ti-E inverted fluorescence microscope with a 40× objective. The images and movies were acquired at the indicated time.

Western blot assay

An equal volume of 2X SDS sample buffer was added to a cell-free reaction mix and heated at 90 °C for 10 minutes. Then, the sample was loaded onto the 10-20% SDS tris-tricine polyacrylamide gel (Thermo Fischer) or 7.5% tris-glycine polyacrylamide gel (Bio-Rad). Proteins were separated using electrophoresis and transferred to a polyvinylidene fluoride or nitrocellulose membrane. Anti-FLAG (Sigma), anti-His_6 (Sigma), anti-AcrB antibody (a gift from Dr. Klass Martinus Pos, Goethe University Frankfurt), and an IRDye 800CW-conjugated secondary antibody (LI-COR) were used to detect epitope-tagged proteins and AcrB, respectively. The protein bands were visualized using the Odyssey CLx imaging system (LI-COR) and quantified using ImageJ software.

The overexpression and purification of PhoQ

E.coli phoQ with a C-terminal His_6 tag gene was cloned into pET Duet-1 vector at NcoI/BamHI restriction sites and transformed into *E.coli* BL21(DE3) strain. The strain was grown in the lysogeny broth (LB) medium with ampicillin (100 µg/ml) at 37 °C till OD 0.8 with vigorous shaking. The gene in the pETduet-1 vector was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. Cells were incubated at 16 ^{o}C overnight and harvested by centrifugation. Cells were resuspended in the resuspension buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 10% glycerol, and 0.1 mM PMSF), and lysed using the LM10 microfluidizer at 4 °C. After removing cell debris by centrifugation (11,000 xg) for 10 minutes, the supernatant was centrifuged again at 100,000 xg for 2 hours. The pellet obtained from the ultracentrifuge was then dissolved in the resuspension buffer containing 1% lauryl maltose neopentyl glycol (LMNG) (wt/vol) with gentle shaking at 4 °C for one hour. The insoluble fraction was removed by centrifugation at 21,000 xg for 45 min. The resulting supernatant (resuspended crude membrane) was mixed with TALON Superflow resins at 4 °C for one hour. Subsequently, the TALON Superflow resins were packed into a column and washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.1 mM PMSF). PhoQ was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 200 mM imidazole, 0.1 mM PMSF) and stored in the final buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl).

Autophosphorylation of PhoQ in the presence of synthesized small proteins

Synthesized small proteins were pelleted together with droplets from a 10 µL cell-free reaction mix by centrifugation at 16,200 g for 5 min. The supernatant was removed, followed by the addition of purified recombinant PhoQ-His_6 (14.29 µM) in the phosphorylation buffer (50 mM Tris-HCl pH 7.5, 200 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1 mM MgSO4). The autophosphorylation reaction was initiated by adding 0.1 mM ATP containing 10 µCi of [γ-32P] ATP and incubated at room temperature for 30 min. The reaction was stopped by adding the SDS sample buffer. The samples were then heated at 37 °C for 2 min, and subsequently loaded onto a 12% precast SDS-polyacrylamide gel (Bio-Rad). Phosphorylated PhoQ was separated from free ATPs by electrophoresis. The gel was exposed to an imaging plate overnight and analyzed with a phosphor imager (Azure Biosystems).

In *vitro* pull-down assay

The synthesized FLAG-tagged small proteins from a 30 µL cell-free reaction mixture were used as bait for the pull-down assay. Crude membrane with overexpressed PhoQ-His_6 was prepared from 3L culture and solubilized in the resuspension buffer containing 1% LMNG with the final concentration of 1 mg/ml. *E. coli* MG1655 crude membrane without overexpression was prepared from 1 L culture and solubilized in the resuspension buffer containing 1% LMNG with the final concentration of 6 mg/ml. The synthesized small protein was incubated with 100 µL solubilized crude membrane at 4 °C overnight with gentle rotation. Possible residual lipid droplets were removed by centrifugation. Then, anti-FLAG® M2 magnetic beads (Sigma) were added to the supernatant and the incubation continued at 4 °C for 16 hours. The anti-FLAG® M2 magnetic beads were washed three times with 1 ml wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM

NaCl, 0.1% LMNG). Proteins retained on the beads were eluted with SDS sample buffer at 95 °C for 10 minutes. The eluates were then analyzed by Western blot.

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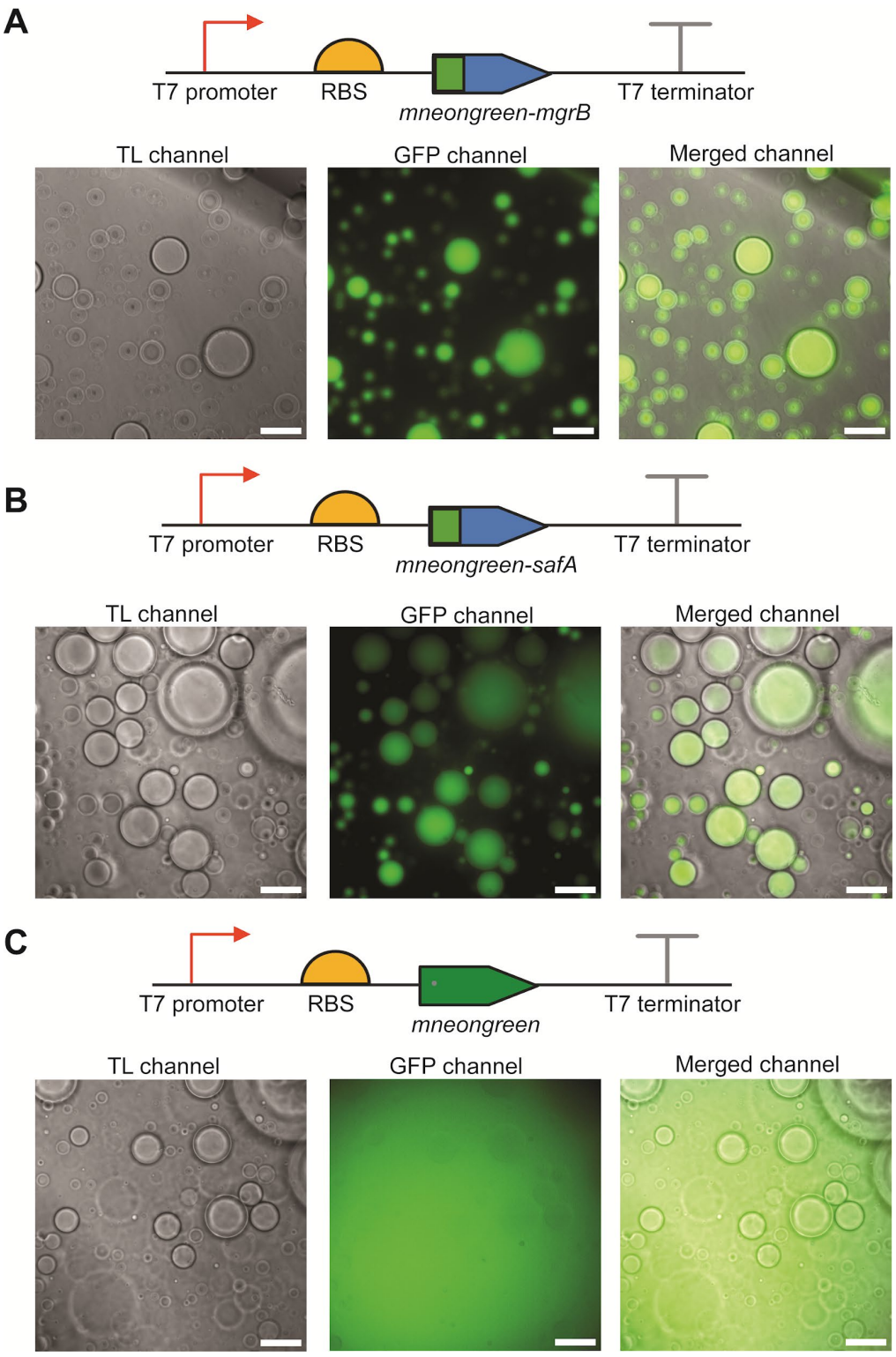

Fig. 1. Cell-free synthesis of small membrane proteins in the presence of lipid sponge droplet. The mNeonGreen labeled MgrB (**A**), SafA (**B**), and mNeonGreen control (**C**) were synthesized in the cell-free system in the presence of lipid droplets. Representative images from the transmitted light (TL), GFP fluorescence and merged channels are shown with the corresponding DNA template scheme above

(Scale bar, 50 µm). Data are representative of at least three independent experiments.

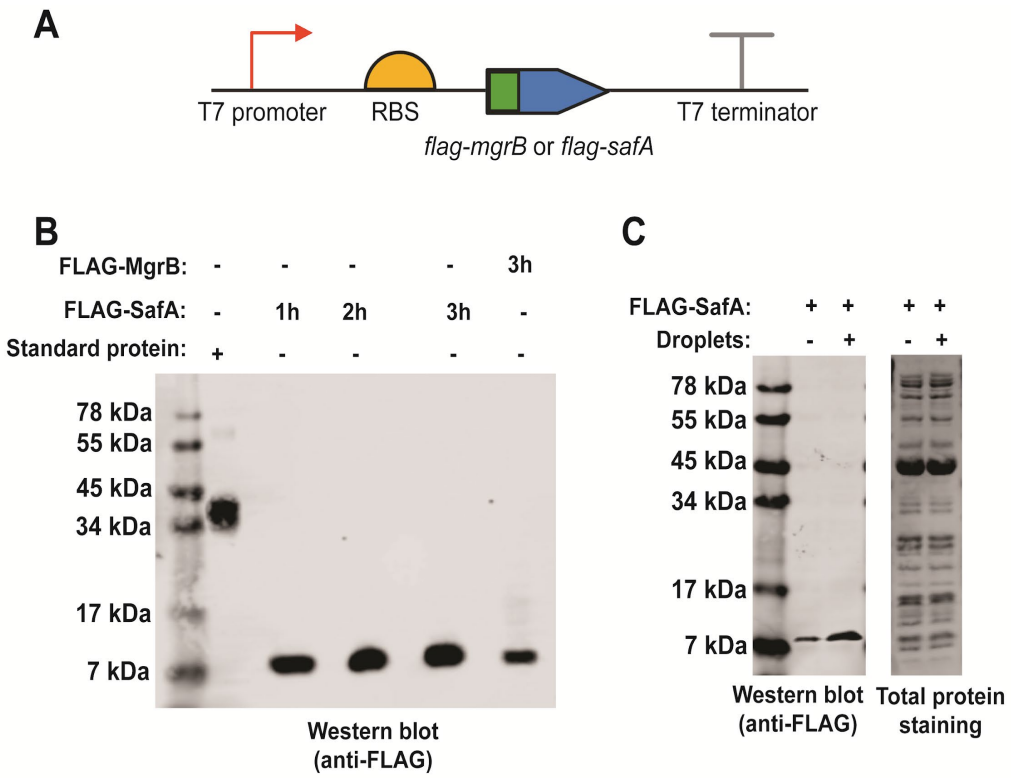

Fig. 2. The yield of synthesized small membrane proteins in cell-free systems. (**A**) Design of the linear DNA templates. (**B**) Western blot analysis of the synthesized MgrB and SafA in a 5 µL reaction. FLAG-tagged standard protein (0.1 µg) was used as a positive control. (**C**) Cell-free synthesis of SafA with or without lipid sponge droplets. Data are representative of three independent experiments**.**

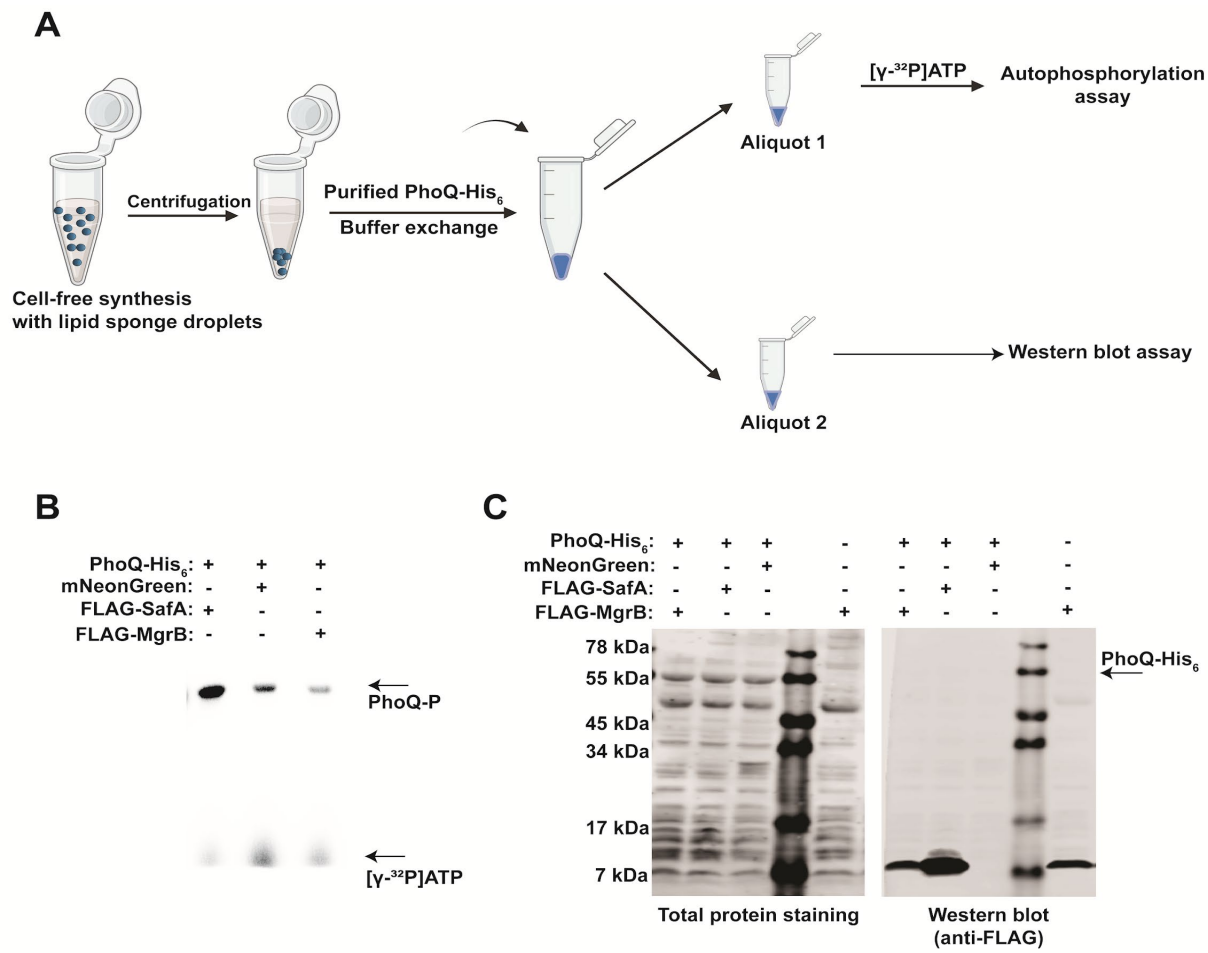

Fig. 3. Functional assay of synthesized small membrane proteins MgrB and SafA. (**A**) The experimental scheme of the functional assay. (**B**) Autophosphorylation of PhoQ in the presence of synthesized proteins. The reactions were carried out at room temperature for 30 minutes. The phosphorylated PhoQ was separated from free [γ-^{32}P]ATPs by SDS-PAGE and detected with a phosphorimager. (**C**) The total protein and western blot analysis of the reaction mix in the functional assay. Data are representative of at least three independent experiments.

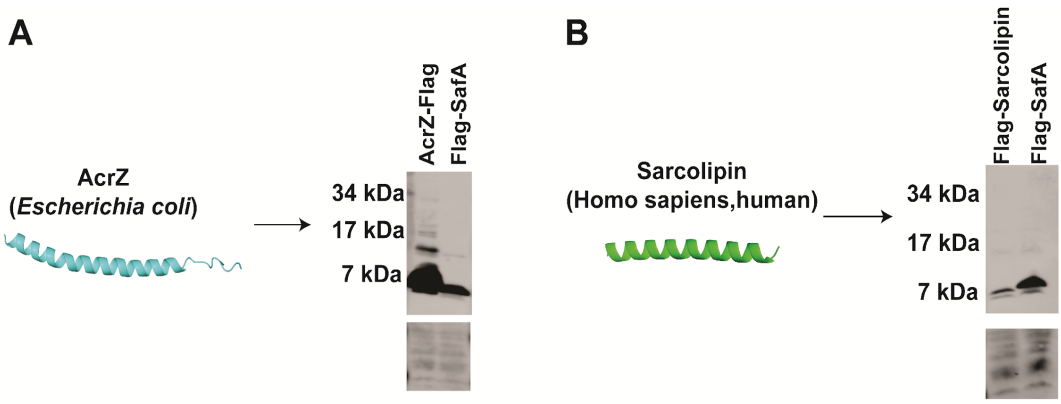

Fig. 4. Cell-free synthesis of AcrZ from *E. coli* and sarcolipin from human. The Alphafold structural prediction of the native proteins and the Western blot analysis of the synthesized FLAG-tagged AcrZ (A) and sarcolipin (B) are shown with the synthesis of SafA as a control. Data are representative of three independent experiments.

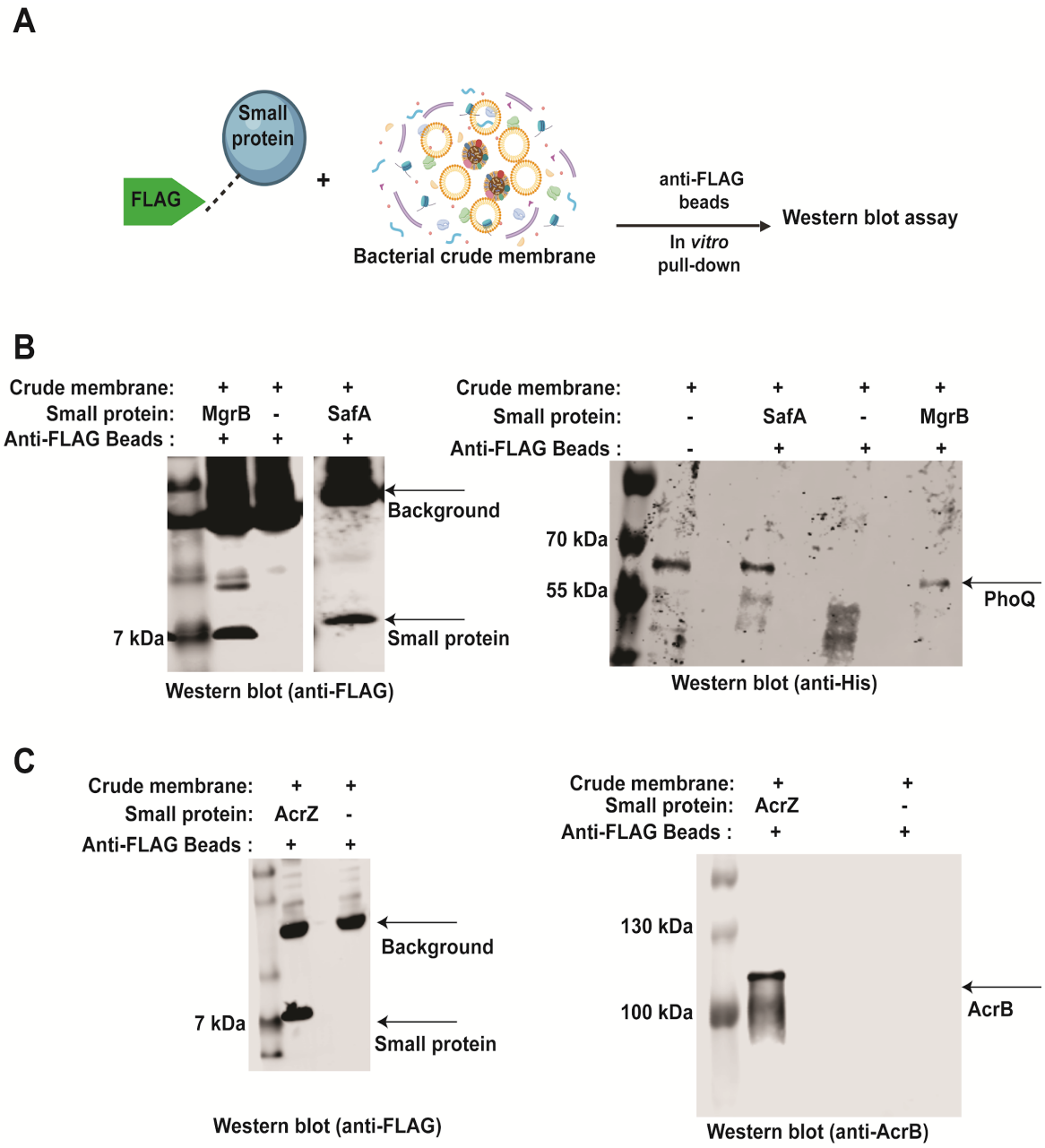

Fig. 5. Co-immunoprecipitation for identifying interaction partners of small membrane proteins. (**A**) The scheme of the in vitro pull-down assay. (**B**) Western blot analysis of the in vitro synthesized FLAG-tagged MgrB and SafA (left) and the co-immunoprecipitated target protein PhoQ-His_6 (right). The pull-down assays were performed using membrane fractions isolated from *E. coli* cells overexpressing recombinant PhoQ-His_6. Data are representative of three independent experiments. (**C**). Co-immunoprecipitation using in vitro synthesized AcrZ-FLAG as bait. The production of AcrZ was verified with Western blot (left) using anti-FLAG antibody. AcrB from the *E.coli* MG1655 crude membranes without overexpression was co-purified with AcrZ and detected with anti-AcrB antibody via Western blot analysis (right). Data are representative of two independent experiments.