Video Article

Using SecM Arrest Sequence as a Tool to Isolate Ribosome Bound Polypeptides

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Abstract

Extensive research has provided ample evidences suggesting that protein folding in the cell is a co-translational process[1-5]. However, the exact pathway that polypeptide chain follows during co-translational folding to achieve its functional form is still an enigma. In order to understand this process and to determine the exact conformation of the co-translational folding intermediates, it is essential to develop techniques that allow the isolation of RNCs carrying nascent chains of predetermined sizes to allow their further structural analysis.

SecM (secretion monitor) is a 170 amino acid *E. coli* protein that regulates expression of the downstream SecA (secretion driving) ATPase in the *secM-secA* operon[6]. Nakatogawa and Ito originally found that a 17 amino acid long sequence (150-FSTPVWISQAQGIRA**G**P-166) in the C-terminal region of the SecM protein is sufficient and necessary to cause stalling of SecM elongation at Gly165, thereby producing peptidyl-glycyl-tRNA stably bound to the ribosomal P-site[7-9]. More importantly, it was found that this 17 amino acid long sequence can be fused to the C-terminus of virtually any full-length and/or truncated protein thus allowing the production of RNCs carrying nascent chains of predetermined sizes[7]. Thus, when fused or inserted into the target protein, SecM stalling sequence produces arrest of the polypeptide chain elongation and generates stable RNCs both *in vivo* in *E. coli* cells and *in vitro* in a cell-free system. Sucrose gradient centrifugation is further utilized to isolate RNCs.

The isolated RNCs can be used to analyze structural and functional features of the co-translational folding intermediates. Recently, this technique has been successfully used to gain insights into the structure of several ribosome bound nascent chains[10,11]. Here we describe the isolation of bovine Gamma-B Crystallin RNCs fused to SecM and generated in an *in vitro* translation system.

Video Link

The video component of this article can be found at http://www.jove.com/video/4027/

Protocol

1. DNA Template Preparation and *in vitro* Transcription

1. The gene of interest is cloned in any T7 and/or e.g. SP6 promoter based plasmid. To obtain RNCs of interest, C-terminus of the target polypeptide is extended by adding arrest inducing sequence of SecM FXXXXWIXXXXGIRAGP[7]. In order to ensure that the polypeptide fragment of interest will extrude out of the ribosomal tunnel, the C-terminal part of the target protein has to be extended by at least 30 amino acids[12-14]. A flexible Glycine-Serine rich linker can be introduced between the protein and the SecM arrest sequence to avoid any possible conformational constraints.
2. For *in vitro* transcription, template DNA should be linearized with restriction enzyme cutting downstream of the ORF. One needs to verify complete linearization of the plasmid DNA by running the restriction digestion product on agarose gel electrophoresis.
3. The linearized plasmid is further used for *in vitro* transcription reaction. Different concentration of template DNA can be tested to identify optimum DNA concentration required for *in vitro* transcription. Generally with Ambion's MEGAscript High yield Transcription Kit (Ambion/Life Technologies, Grand Island, NY), 1 µg linearized DNA yields 40-60 µg mRNA. *In vitro* transcription is done following manufacturer's instruction (Ambion/Life Technologies, Grand Island, NY).
4. Following *in vitro* transcription, mRNA is purified by lithium chloride precipitation according to manufacturer's instruction (Ambion's MEGAscript High yield Transcription Kit, Ambion/Life Technologies, Grand Island, NY).
5. mRNA integrity is further verified by electrophoresis using acrylamide or agarose gels.

2. *In vitro* Translation

For *in vitro* translation using the RTS 100 E. coli HY Kit (5 Prime, Gaithersburg, MD) follow the steps below:

1. Prepare 100 µl of *in vitro* translation reaction following manufacturer's instruction. Briefly, in nuclease free water add 24 µl amino acid mixture minus methionine (1 mM), 10 units of Ribonuclease Inhibitor (Invitrogen/Life Technologies, Grand Island, NY), 20 µCi of radioactive [^{35}S]-Methionine (MP Biomedicals, Solon, OH), 20 µl Reaction mix, 24 µl Reconstitution buffer and 24 µl of *E. coli* Lysate.
2. Incubate the reaction at 30 °C for 5 min to pre warm the translation reaction. Add 1-2 µg of mRNA to the translation reaction and incubate at 30 °C for 10-15 min.
3. Stop the reaction by placing the translation reaction on ice.

3. Isolation of Nascent Polypeptide from *in vitro* Translation Reaction

1. To isolate RNCs, the translation reaction is layered on top of 4.5 ml of 5-30% sucrose gradient in 20 mM HEPES-KOH pH 7.5, 15 mM $MgCl_2$, 100 mM Potassium acetate, 1 mM DTT and centrifuged using Beckman Coulter SW55-Ti rotor at 41,000 rpm, 4 °C for 2 hrs.
2. Following centrifugation, sucrose gradient is fractionated to isolate different ribosomal populations and associated nascent chains. Separation is monitored using the ISCO Programmable Density Gradient System with continuous recording at 254 nm using an ISCO UA-6 absorbance detector.
3. The fraction(s) containing 70S ribosomes are collected and analyzed further depending on the aim of the experiment.

4. Observation of Protein Bound to Ribosome with Tris-tricine SDS PAGE

To check, whether the SecM extended protein remains attached to the 70S ribosome, gradient fractions were collected and treated as follows:

1. Protein in each fraction was precipitated by adding Trichloroacetic acid (TCA) to a final concentration of 10% and incubated at 4 °C overnight.
2. After overnight incubation, samples were centrifuged at 14,000 X g for 15 min to pellet the protein. Following centrifugation supernatant was removed and the pellet washed twice with solution containing Acetone: 1 mM Tris-HCl pH 7.6 (4:1). Pellet was further air dried and dissolved in SDS-PAGE loading buffer.
3. The treated sample was resolved and analyzed by Tris-tricine SDS-PAGE.
4. After electrophoresis, gels were fixed, dried using vacuum gel dyer and subjected to autoradiography. The distribution of nascent polypeptides were observed using phosphoimaging.

5. Representative Results

Here we present an experiment describing the isolation of the full-length bovine Gamma-B Crystallin stably bound to the 70S ribosome. **Figure 1** depicts steps involved in the isolation of bovine Gamma-B Crystallin RNCs. The C-terminus of the Gamma- B Crystallin was extended overall by 32 amino acids to ensure that full-length protein extrudes out of the ribosomal tunnel; this also includes the SecM stalling sequence placed at the very C-terminus of the fusion polypeptide. Following *in vitro* translation, the 70S ribosomes were isolated by sucrose gradient centrifugation **(Figure 2.1)**. In order to ensure that the protein remains stably bound to the ribosome, the 70S containing fractions were pooled, desalted, buffer exchanged and subjected to an additional round of sucrose gradient centrifugation **(Figure 2.2)**. The result presented in **Figure 2** clearly suggests that SecM can efficiently induce translational arrest of the Gamma-B Crystallin RNCs.

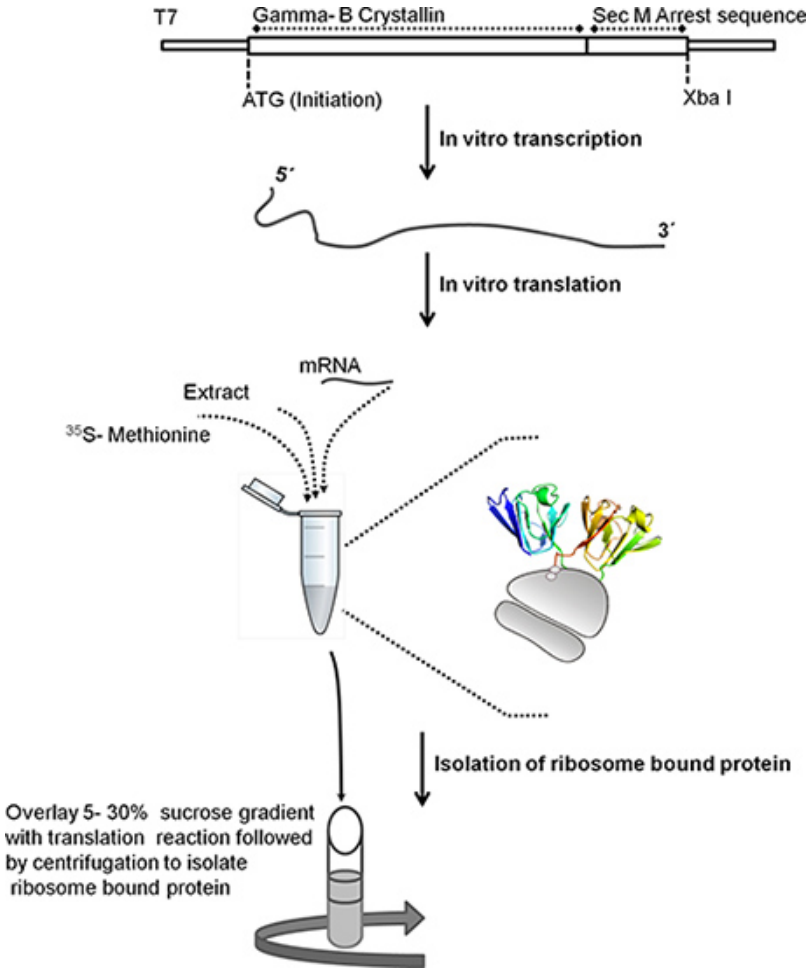

Figure 1. Schematic explanation of the steps involved in isolation of RNCs. C-terminus of bovine Gamma-B Crystallin was extended by 32 amino acid sequence. This extended C-terminal regions also includes SecM arrest sequence. Gamma-B Crystallin with SecM sequence was cloned in pIVEX 2.3 (T7 based) plasmid. For *in vitro* transcription the template DNA was linearized by XbaI. Linearized template was further used for *in vitro* transcription. The T7 in the DNA template is recognized by T7 RNA polymerase that transcribes the Gamma-B Crystallin gene located downstream of the T7 promoter. mRNA was purified using lithium chloride precipitation method. The purified mRNA was then used for in vitro translation. Following incubation, the translation reaction was layered on top of 5-30% sucrose gradient and centrifuged in Beckman Coulter SW55-Ti rotor at 41,000 rpm, 4 °C for 2 hrs.

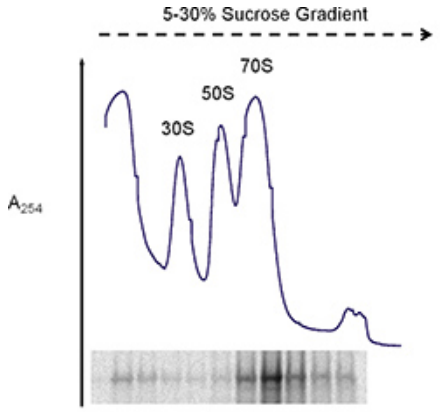

Figure. 2.1

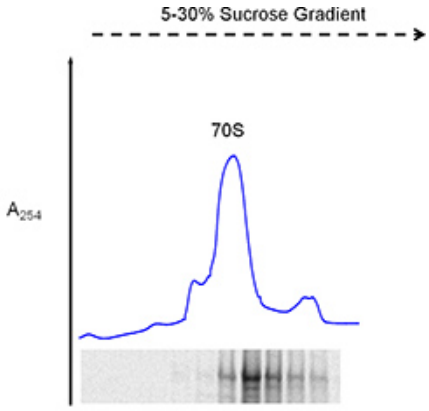

Figure. 2.2

Figure 2. Isolation of bovine Gamma-B Crystallin RNCs stably bound to the 70S ribosome. 1 µg mRNA was mixed in 100 µl reaction *E. coli* S30 Extract System as mentioned in the protocol section with 20 µCi [^{35}S]-Methionine and incubated at 30 °C for 15 min. Following incubation, the translation reaction was layered on top of 5-30% sucrose gradient in 20 mM HEPES-KOH pH 7.5, 15 mM $MgCl_2$, 100 mM Potassium acetate, 1 mM DTT and centrifuged in Beckman Coulter SW55-Ti rotor at 41,000 rpm, 4 °C for 2 hrs. Following velocity sedimentation, the gradient was unloaded and the ribosome pro le was obtained. The data were recorded by the PeakTrak program (ISCO gradient density gradient fractionation system). Fractions were collected, the protein was TCA precipitated and resolved on 16.5% T, 6% C Tris-Tricine PAGE gel[15]. The gel was dried and exposed for autoradiography. Following exposure the gel was scanned using Typhoon 9410 imaging scanner. **Figure 2.1** shows that full-length Gamma-B Crystallin is present in 70S ribosome fractions. Thus, SecM stalling sequence allows the isolation of the stable bovine Gamma-B Crystallin RNCs. Data in **Figure 2.2** clearly indicate that the isolated RNCs are indeed stable. In the current experiment the 70S fractions after first round of sucrose gradient centrifugation were pooled and the sucrose was removed using Amicon Ultra-4 Centrifugal Filter Unit (Millipore), followed by buffer exchange in solution containing 20 mM HEPES-KOH pH 7.5, 15 mM $MgCl_2$, 100 mM Potassium acetate, 1 mM DTT. This sample was further subjected to an additional round of centrifugation through 5-30% sucrose gradient and fractionated. Each fraction was treated and analyzed like in **Figure 2.1.**

Discussion

For reproducible results, quality and concentration of the components used for *in vitro* transcription and translation are critical. We have used commercially available *in vitro* transcription and translation extracts and they give efficient and reproducible results, if handled carefully. Quality of mRNA can affect the translation, so it is of utmost importance to test the integrity of mRNA before using it for *in vitro* translation. Incubation time for *in vitro* translation varies with the length of the protein. Also, the time/speed of centrifugation can be adjusted accordingly, in case, the 70S ribosomal fraction is not well resolved and separated from the 50S. Further, ribosome bound protein complexes should be handled carefully to avoid RNase contamination. Moreover, buffer conditions should be monitored carefully and chelating agents that might chelate Mg^{2+} should be avoided.

SecM arrest sequence, upon its translation interacts with the ribosomal proteins L4 and L22 as well as 23S rRNA in the ribosomal tunnel[7,8]. A number of critical residues, constituting the SecM arrest motif, **F**XXXX**WI**XXXX**GIRAGP**[7](in bold type) are of immense importance, ensuring the efficiency of the translational arrest. Mutations, or deletions of these critical residues may lead to the relief of the translation elongation arrest[7,8]. Thus, maintaining the sequence of the SecM arrest motif **F**XXXX**WI**XXXX**GIRAGP**[7] intact is absolutely critical to ensure that the protein under study would remain stably bound to the ribosome.

This technique can be used for analysis of the structure of co-translational intermediates and co-translational folding of various proteins. It has been recently successfully used to determine the exact structure of several ribosome bound nascent chains with the help of NMR[10-11]. Additionally, this technique can also be used to generate nascent peptides of predetermined sizes for analysis of their tertiary interactions with accessory proteins, cofactors or ligands.

An alternative approach to produce RNCs complexes would involve the use of truncated mRNAs lacking stop codon. This approach has been widely used by many researchers to study the co-translational protein folding[see e.g. 12,16]. However, it has a number of drawbacks. This approach can not be employed *in vivo* and also problematic for the use *in vitro* with *E. coli* extract due to the presence in *E. coli* of SsrA system that mediates addition of the C-terminal peptide tag (AANDENYALAA) to proteins translated from mRNAs without in-frame stop codons, leading to their degradation[17]. Therefore, in the later case, one has to use a completely reconstituted system and/or a system, lacking/suppressing SsrA-tagging machinery. SecM-directed stalling is efficient and unique as it has been proven to produce stalled ribosome complexes *in vivo* and *in vitro* with almost similar efficiency[18].

Disclosures

No conflicts of interest declared.

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