Nascent SecM chain interacts with outer ribosomal surface to stabilize translation arrest

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SecM, a bacterial secretion monitor protein, posttranscriptionally regulates downstream gene expression via translation elongation arrest. SecM contains a characteristic amino acid sequence called the arrest sequence at its C-terminus, and this sequence acts within the ribosomal exit tunnel to stop translation. It has been widely assumed that the arrest sequence within the ribosome tunnel is sufficient for translation arrest. We have previously shown that the nascent SecM chain outside the ribosomal exit tunnel stabilizes translation arrest, but the molecular mechanism is unknown. In this study, we found that residues 57–98 of the nascent SecM chain are responsible for stabilizing translation arrest. We performed alanine/serine-scanning mutagenesis of residues 57–98 to identify D79, Y80, W81, H84, R87, I90, R91, and F95 as the key residues responsible for stabilization. The residues were predicted to be located on and near an α-helix-forming segment. A striking feature of the α-helix is the presence of an arginine patch, which interacts with the negatively charged ribosomal surface. A photocross-linking experiment showed that Y80 is adjacent to the ribosomal protein L23, which is located next to the ribosomal exit tunnel when translation is arrested. Thus, the folded nascent SecM chain that emerges from the ribosome exit tunnel interacts with the outer surface of the ribosome to stabilize translation arrest.

Introduction

Translation is a fundamental biological process via which ribosomes decode genetic information in mRNA and convert it into amino acid sequences that form proteins. The process is strategically regulated to ensure that proteins are synthesized with precise timing and at specific subcellular locations in response to changes in the cellular environment [1,2]. Under some circumstances, translation arrest occurs at the elongation or termination step to control the expression of specific genes. This phenomenon, termed translation arrest, is genetically programmed and mediated by nascent (poly)peptides. Several such (poly)peptides have been identified in both prokaryotes and eukaryotes. They interact with the ribosome interior to block specific translation steps. Interestingly, they have little sequence similarity and induce translation arrest via distinct mechanisms; some require cofactors such as metabolites and antibiotics, whereas others do not [3,4]. The best characterized of these (poly)peptides is SecM from Escherichia coli.

E. coli SecM, a 170-amino-acid secretion protein, has a specific sequence at its C-terminus (150-FSTPVWISQAQGIRAGP-166) called the arrest sequence, which is required to induce translation elongation arrest [5,6]. SecM regulates the translation of the downstream gene secA, which encodes a secretion-driving ATPase, in response to protein secretion activity in the cell [7]. When a cell is secretion-proficient, nascent SecM is pulled by SecA in association with the SecYEG translocon immediately after translation arrest [8]. At this point, a secondary structure encompassing the secM–secA Shine–Dalgarno sequence in the secM–secA mRNA inhibits secA translation [5,9]. However, under
secretion-defective conditions, SecM translation is subjected to prolonged arrest, inducing a conformational rearrangement in the mRNA that exposes the Shine–Dalgarno sequence. This in turn enables ribosome binding and subsequent secA translation [5–7,10].

The arrest sequence of SecM can cause translation arrest of unrelated proteins, which can be utilized to generate nascent chain–ribosome complexes (e.g. Ref. [11]). Hence, it is widely accepted that the SecM arrest sequence is necessary and sufficient for sustained translation arrest. However, recent investigations suggest that the arrest sequence alone is not always adequate to provide stable translation arrest when fused to the C-terminus of unrelated proteins [11–15]. For instance, we have demonstrated that the efficiency and stability of translation arrest correlates with the length of the spacer sequence between the HaloTag protein and the arrest sequence [14]. In addition, Goldman et al. [15] reported that nascent chain folding near the ribosome tunnel exit can result in translation arrest release via peptide stretching in the tunnel. The most likely explanation is that the SecM arrest sequence is susceptible to a pulling force exerted by the nascent chain outside the ribosome. Interestingly, we have found that the nascent SecM chain outside the ribosome exit tunnel helps stabilize the arrest [14]. However, the molecular mechanism underlying the stabilization is unknown. To clarify this issue, we performed mutational analysis of SecM and found that the folded nascent SecM chain interacts with the ribosomal surface to stabilize translation arrest.

**Experimental**

**Prediction of the secondary structure**

The secondary structure of *E. coli* SecM was predicted using the PSIPRED program (v3.3; http://bioinf.cs.ucl.ac.uk/psipred/) [16].

**Preparation of templates for *in vitro* transcription and translation**

To introduce the TC tag (CCPGCC) [17] into the N-terminus of SecM and SecM(Δ1–37), the gene encoding SecM was amplified from pTA-SecM [14] using PCR with the primers shown in Supplementary Table S1. The PCR products were digested with *Nde* I and *Bam* H I and then ligated to the same sites in the pET23b vector (Merck Millipore) to yield the plasmids pTCSecM and pTCSecM(Δ1–37). Deletion mutants of TC-SecM were generated from pTCSecM using the KOD -Plus- Mutagenesis Kit (Toyobo). Point mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) or KOD -Plus- Mutagenesis Kit. An expression plasmid for HaloTag-SecM with a 23-amino acid glycine–serine linker [Halo-SecM(23GS)] was generated from pHaloSecM(8GS) [14] using the KOD -Plus- Mutagenesis Kit. The primer sets are presented in Supplementary Table S2–S4. The resultant plasmids were used as templates for PCR amplification to prepare template DNA for *in vitro* transcription and translation, as described previously [14,18].

**Analyzing the translation arrest duration**

Each construct was transcribed and translated using the PUREfex 1.0 system (Gene Frontier Co., Ltd.) in a 20-μl reaction containing 10 μl of solution I, 1 μl of solution II, 1 μl of solution III, 2 μl of template DNA, 1 μl of SUPERase In RNase Inhibitor (Thermo Fisher Scientific), 1 μl of 20 μM FAMsH-EDT2 (Thermo Fisher Scientific), and 4 μl of nuclease-free water (Qiagen). The mixtures were incubated for 20 min at 37°C, and then 2 mM puromycin (Sigma–Aldrich) was added to release nascent polypeptides from elongation-competent ribosomes and block elongation of newly initiated translation. After further incubation for the indicated time at 37°C, aliquots (4 μl) were sampled and mixed with the same volume of the 2× sample buffer [125 mM Tris–HCl pH 6.8, 4% (w/v) SDS and 20% (v/v) glycerol] treated with RNaseure (Thermo Fisher Scientific) [19]. The sample buffer was employed to reduce background staining. SDS–PAGE was performed using NuPAGE 10% Bis-Tris Gel with MES SDS Running Buffer (Thermo Fisher Scientific) to detect polypeptidyl-tRNA [20]. Fluorescently-labeled polypeptides were visualized using the ChemiDoc Touch MP imaging system or Molecular Imager FX (Bio-Rad Laboratories). Band intensities were measured using ImageJ (https://image.nih.gov/ij/). Before image analysis, the background of each image was subtracted using the ImageJ rolling ball background subtraction algorithm. The lifetime of translation arrest for each construct was determined as described previously [14]. Data fitting was performed using the KaleidaGraph program (Synergy Software). Statistical analyses were performed using the R software (https://www.r-project.org/).
Photocross-linking assay

p-Benzoyl-L-phenylalanine (Bpa) [21] was introduced into TC-SecM and Halo-SecM(23GS) using the amber suppression method with the PUREfrex 1.0 system lacking release factor 1 (RF1) to avoid competition between the release factor and suppressor tRNA [22]. TC-SecM and TC-SecM(P166A) with an amber mutation at Y80, W81, or F95 were translated in the presence of an amber suppressor tRNA with Bpa (Bpa-tRNA; ProteinExpress Co., Ltd.). Each reaction contained 10 μl of solution I, 1 μl of solution II without RF1, 1 μl of solution III, 2 μl of template DNA, 2 μl of Bpa-tRNA solution, and 4 μl of nuclease-free water. The mixtures were incubated for 40 min at 37°C in the dark. After the addition of puromycin (2 mM), the mixtures were irradiated with a 355-nm laser (Genesis CX355-40 STM, Coherent) for 10 min at room temperature. The resultant products were stained with 2 μM FlAsH-EDT₂ for 30 min at 37°C and then treated with 400 μg/ml RNase A (Thermo Fisher Scientific) for 10 min at 37°C. Polypeptides were separated on 12% Laemmli SDS–PAGE gels. Fluorescently-labeled polypeptides were visualized using Molecular Imager FX. To identify a photocross-linking partner for SecM, Halo-SecM(23GS) with an amber mutation at Y80 was translated in a PAGE gels. Gels were stained with Coomassie Brilliant Blue (EzStain Aqua, ATTO corporation). Each reaction contained 10 μl of template DNA, 2 μl of solution I, 1 μl of solution II without RF1, 1 μl of solution III, 2 μl of Bpa-tRNA solution, and 4 μl of nuclease-free water. The mixtures were incubated for 40 min at 37°C in the dark. After the addition of puromycin (2 mM), the mixtures were treated either with or without UV irradiation. The products were biotinylated using 1 μM HaloTag PEG Biotin Ligand (Promega) and treated with RNase A. Unbound biotin ligands were removed using NAP5 columns (GE Healthcare). The biotinylated products were purified using streptavidin-modified magnetic beads (Dynabeads MyOne Streptavidin C1, Thermo Fisher Scientific), according to the manufacturer’s instructions. Bound proteins were eluted by boiling in Laemmli sample buffer and then resolved on 7% Laemmli SDS–PAGE gels. Gels were stained with Coomassie Brilliant Blue (EzStain Aqua, ATTO corporation). Gel pieces of ∼65 kDa were excised and digested in-gel with trypsin gold (Promega). The digested peptides were extracted with 50% acetonitrile. After evaporation using a centrifugal evaporator, the desalted gel pieces were dissolved in 2% acetonitrile and 0.1% TFA solution and desalted with a C18 Stage Tip (Nikkyo Technos). The resulting peptides were subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) as described elsewhere [23]. MS/MS spectra were searched against the GenoBase database [24] using the Sequest algorithm within Proteome Discoverer 2.1 (Thermo Fisher Scientific).

Results

Identification of the segment responsible for stable translation arrest

The nascent SecM chain outside the ribosome exit tunnel cannot be seen in the currently available cryo-electron microscopy structure [12,25,26]. We predicted the secondary structure of SecM using the PSIPRED program [16], which is one of the most popular and reliable methods available on the web. The prediction indicated that SecM forms several α-helices (Supplementary Figure S1). In considering the predicted structure and length of the ribosome exit tunnel (the tunnel can accommodate ∼30 amino acids of a stretched peptide chain [27]), we constructed a series of deletion mutants of SecM [TC-SecM(Δ1–37), TC-SecM(Δ38–56), TC-SecM(Δ57–73), TC-SecM(Δ74–98), and TC-SecM(Δ99–132)] to identify the segment responsible for stabilizing translation arrest. These mutants were fused with a tetracysteine (TC) tag at the N-terminus, enabling fluorescent labeling and translation product detection (Figure 1). The TC tag is composed of six amino acids CCGPGCC, which covalently bind to biarsenical dyes such as FlAsH-EDT₂ with high affinity [17].

TC-SecM and the deletion mutants were translated in the presence of FlAsH-EDT₂ using a commercially available version of a reconstituted E. coli cell-free translation system (PURE system) [22] to examine the durations of translation arrest [14]. The products were treated with puromycin to release nascent polypeptides from elongation-competent ribosomes and block elongation of newly initiated translation. SecM-arrested ribosomes, which contain a prolyl-tRNAPro in the A site, prevent the entry of puromycin, but allow it when the arrest is released [20]. Then, aliquots of the mixture obtained at the indicated time points were separated using SDS–PAGE at a neutral pH to analyze the fraction of the translation arrest product (polypeptidyl-tRNA) remaining [14,20] (Supplementary Figure S2). The lifetime was determined by fitting a single exponential curve to the plot of the fraction against the incubation time with puromycin to evaluate the duration of translation arrest [14] (Supplementary Figure S2). The deletion of residues 1–37, corresponding to a secretion signal sequence [28], significantly prolonged the duration of translation arrest (Figure 1). Consistent with our result, the signal sequence has been shown to facilitate the release of translation arrest [10,29]. However, the absence of residues 57–73 and 74–98 significantly shortened the duration of translation arrest, whereas the absence of residues 38–56 and 99–132 did not (Figure 1). These results implied that residues within the region 57–98 are necessary for stable translation arrest.

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Identification of the residues responsible for stable translation arrest

Next, we performed alanine/serine-scanning mutagenesis of residues 57–98 to identify the amino acid residues important for stable translation arrest. A total of 42 TC-tagged mutants were translated in the PURE system, and the durations of translation arrest were examined (Supplementary Figure S3 and Figure 2A). Most of the mutants displayed shorter durations of translation arrest than the wild type. In particular, the durations of D79A, Y80A, W81A, H84A, R87A, I90A, R91A, and F95A mutants were <50% of that of the wild type (Figure 2A). The key residues responsible for stable translation arrest are expected to be present on and near helix 4, which is the predicted fourth α-helix composed of residues 81–95 (Supplementary Figure S1 and Figure 2B). Strikingly, H84, R87, and R91 will line one face of the helix, presuming the residues form a positively charged patch (Figure 2B). Substitutions of H84 for negatively and positively charged residues significantly reduced the duration of translation arrest (Supplementary Figure S4). However, substitutions of R87 and R91 for glutamate greatly diminished the durations of translation arrest, whereas substitutions for lysine did not (Supplementary Figure S5 and Figure 2C). These results indicate that positively charged residues at positions 87 and 91 are required for stable translation arrest. Given that the ribosome has a highly negatively charged surface [30], it is likely that the arginine residues associates directly with the ribosomal surface via electrostatic interactions, resulting in sustained translation arrest. On the other hand, mutation of Q83 to alanine significantly increased the duration of translation arrest (Figure 2A). Because Q83 is predicted to be located on helix 4 (Figure 2B) and alanine has the highest helix-forming propensity [31], the stability of translation arrest is assumed to be positively correlated with the structural stability of helix 4. Consistent with this idea, substitutions of Q83 for glycine and proline, which are normally unfavorable in α-helices [31], decreased the durations of translation arrest (Supplementary Figure S6). These findings support the hypothesis that the helical structure of a nascent SecM chain emerging from the ribosome exit tunnel is important in its potential interaction with the ribosomal surface.

Photocross-linking between SecM and the ribosome

To demonstrate spatial proximity between helix 4 of SecM and the ribosomal surface, we employed a site-specific photocross-linking approach using the photoreactive amino acid Bpa. Bpa generates biradical species upon irradiation with UV light (350–365 nm), and the species react covalently with carbon-hydrogen bonds within 3 Å [21]. Aromatic residues (Y80, W81, and F95) were selected to be substituted with Bpa, because the aromatic character of Bpa substantially affected the stability of translation arrest such as in the case of R87 (Supplementary Figure S7). The translation products of Y80Bpa, W81Bpa, and F95Bpa mutants were subjected to UV irradiation and separated using SDS–PAGE with RNase A pretreatment. TC-SecM, not containing Bpa, predominantly migrated as a band of ~20 kDa (Figure 3, lane 1), whereas the Y80Bpa, W81Bpa, and F95Bpa mutants rendered multiple bands (Figure 3, lanes 2, 5, and 8), indicating that the bands with apparently higher...
**Figure 2. Lifetimes of translation arrest of alanine/serine-scanning mutants.**

**Part 1 of 2**

(A) Lifetimes of translation arrest for TC-SecM (WT) and alanine/serine-scanning mutants. Values represent the mean ± standard deviation of three or four independent experiments. Statistical significance was determined by Dunnett’s multiple comparison of means testing (*P < 0.001). Representative gel images and quantitative data are shown in Supplementary Figure S3. (B) Predicted structure of residues 57–98. The structure was predicted by PSIPRED v3.3 (Supplementary Figure S1). The highlighted amino acid residues are critical to stable translation arrest: positively and negatively charged, and aromatic.

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molecular masses correspond to photocross-linked products. RNase A treatment did not alter the mobility of the products (Supplementary Figure S8), suggesting that SecM was linked to proteins. Intriguingly, the introduction of the arrest-weakened mutation (R87A) (Figure 2A) into the mutants reduced the apparent 32-kDa-photocross-linked products (Figure 3, lanes 3, 6, and 9). Also, the introduction of the arrest-deficient mutation (P166A) [6] completely eliminated the products (Figure 3, lanes 4, 7, and 10). These results indicate that the cross-linking degree is compatible with the stability of translation arrest.

The notable cross-linking product of SecM(Y80Bpa) was analyzed using mass spectrometry to identify the cross-linking partner, because the translation arrest stability of TC-SecM(Y80Bpa) was similar to that of TC-SecM (Supplementary Figure S7). SecM(Y80Bpa) was translated in fusion with the C-terminus of HaloTag via a 23-amino acid GS linker, which enabled purification of the cross-linking product through a biotin–strep-tavidin interaction [32]. We have previously shown that the fusion of HaloTag to SecM does not affect the stability of translation arrest [14]. The translation products of Halo-SecM(Y80Bpa) with and without UV irradiation were purified and resolved using SDS–PAGE. UV irradiation resulted in a cross-linking product with a molecular mass of 65 kDa, whereas no cross-linking product was observed without UV irradiation (Supplementary Figure S9). In the absence and presence of UV irradiation, gel pieces containing 65 kDa proteins were excised, treated with trypsin, and subjected to analysis by LC–MS/MS. Two independently prepared samples were analyzed. To reduce false positive identifications, a cutoff was applied where proteins had at least two peptide annotations per protein (number of peptides ≥ 2) under UV irradiation. All of those proteins are listed in Supplementary Table S5. A total of 27 proteins were found in two samples, of which 9 met the
requirement (<20 kDa) for the cross-linking partner of SecM (Table 1). Among them, the ribosomal protein L23, which is located next to the ribosomal exit tunnel on the 50S ribosomal subunit [33,34], showed a significant increase in the number of peptide spectrum matches after being exposed to UV light (Table 1). The result suggests that helix 4 of SecM is in close proximity to L23, due to a specific interaction between SecM and the outer surface of the ribosome.

### Discussion

In this study, we examined the molecular mechanism via which the nascent SecM chain outside the ribosomal exit tunnel stabilizes translation arrest. First, we showed that the residues 57–98 are important for stable translation arrest (Figure 1), which is consistent with the findings of an earlier study [35]. Second, we found that the stability of SecM translation arrest can be modulated by single amino acid changes in the residues 57–98 (Figure 2 and Supplementary Figures S4, S5). Alanine/serine-scanning mutagenesis identified eight key residues (D79, Y80, W81, H84, R87, I90, R91, and F95) responsible for stabilization (Figure 2A). These residues were predicted to be located near and on helix 4 of SecM (Figures 1, 2B). Positively charged residues at position 87 and 91 were shown to be essential for stable translation arrest, suggesting that the residues may interact with the negatively charged ribosome surface (Figure 2C). Finally, we demonstrated the spatial proximity of the helix and ribosome surface. A photocross-linking experiment showed that when translation is arrested, Y80 is located adjacent to ribosomal protein L23, which is near the nascent chain exit site of the ribosome (Figure 3 and Table 1). L23 is a universal ribosomal protein, which serves as the docking site for nascent chain-associated factors that facilitate protein folding, maturation, and localization, including trigger factor [36,37], methionine

### Table 1 LC–MS/MS identification of proteins contained in the photocross-linking product

| Protein                      | Accession Number | Mw (kDa) | Replicate 1 | Replicate 2 |
|------------------------------|------------------|----------|-------------|-------------|
|                              |                  |          | Number of peptides | Number of peptide spectrum matches | Number of peptides | Number of peptide spectrum matches |
|                              |                  |          | +UV | −UV | +UV | −UV | ΔPSMs | +UV | −UV | +UV | −UV | ΔPSMs |
| Halo-SecM (Y80Bpa)           |                  | 53.913   | 10  | 9   | 111 | 53  | 58   | 10  | 11  | 116 | 65  | 51   |
| 50S ribosomal protein L23    | JW3280           | 11.192   | 5   | 45  | 45  | 35  | 35   | 4   | 35  | 35  | 35  | 35   |
| 50S ribosomal protein L17    | JW3256           | 14.356   | 2   | 1   | 6   | 3   | 3    | 3   | 3   | 11  | 8   | 3    |
| 30S ribosomal protein S11    | JW3259           | 13.836   | 3   | 2   | 9   | 6   | 3    | 3   | 3   | 9   | 9   | 0    |
| 30S ribosomal protein S13    | JW3260           | 13.091   | 3   | 3   | 8   | 7   | 1    | 4   | 4   | 11  | 12  | −1   |
| 50S ribosomal protein L14    | JW3272           | 13.532   | 3   | 2   | 7   | 6   | 1    | 2   | 2   | 6   | 6   | 0    |
| 50S ribosomal protein L20    | JW1706           | 13.489   | 4   | 4   | 11  | 12  | −1   | 4   | 4   | 9   | 10  | −1   |
| 50S ribosomal protein L28    | JW3612           | 9.001    | 2   | 2   | 5   | 6   | −1   | 3   | 1   | 8   | 2   | 6    |
| 50S ribosomal protein L6     | JW3267           | 18.892   | 2   | 4   | 6   | 10  | −4   | 2   | 4   | 4   | 4   | 4    |
| 30S ribosomal protein S21    | JW3037           | 8.495    | 2   | 4   | 5   | 14  | −9   | 4   | 3   | 13  | 12  | 1    |

Proteins with a molecular mass of <20 kDa are adapted from Supplementary Table S5. The number of peptides and peptide spectrum matches (PSMs) represent the total number of distinct peptide sequences identified in the protein and the total number of identified peptide sequences (peptide spectrum matches), respectively. +UV, with UV irradiation; −UV, without UV irradiation; ΔPSMs, (the number of PSMs)_{+UV}−(the number of PSMs)_{−UV}. 

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a stable translation arrest. Although SecM exhibits limited phylogenetic distribution [61], it can be argued that SecM is a highly sophisticated regulatory protein.

Abbreviations
Bpa, p-benzoyl-L-phenylalanine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PSMs, number of peptides and peptide spectrum matches; RF1, release factor 1; TC, tetracysteine.

Author Contribution
R.I. and T.F. conceived the study and designed the experiments; M.M., R.I. and Y.G. performed the experiments and analyzed the data; T.N. and H.T. performed the LC–MS/MS analysis; and M.M., R.I. and T.F. wrote the manuscript.

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Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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