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The plasticity of a translation arrest motif yields insights into nascent polypeptide recognition inside the ribosome tunnel

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Abstract

The recognition of a C-terminal motif in *E. coli* SecM (^{150}FXXXXWIXXXXGIRAGP^{166}) inside the ribosome tunnel causes translation arrest, but the mechanism of recognition is unknown. While single mutations in this motif impair recognition, we demonstrate that new arrest-inducing peptides can be created through remodeling of the SecM C-terminus. We found that R163 is indispensable, but that flanking residues that vary in number and position play an important secondary role in translation arrest. The observation that individual SecM variants showed a distinct pattern of crosslinking to ribosomal proteins suggests that each peptide adopts a unique conformation inside the tunnel. Based on the results, we propose that translation arrest occurs when the peptide conformation specified by flanking residues moves R163 into a precise intra-tunnel location. Our data indicate that translation arrest results from extensive communication between SecM and the tunnel and help explain the striking diversity of arrest-inducing peptides found throughout nature.

INTRODUCTION

During translation nascent polypeptides traverse a long aqueous tunnel in the large ribosomal subunit before emerging into the cytoplasm (Milligan and Unwin, 1986; Yonath et al., 1987). The ribosome tunnel has a length of ~100 Å from the peptidyl transferase center (PTC) to the exit site and an average diameter of ~15 Å (Nissen et al., 2000). The tunnel is highly irregular in shape and contains numerous grooves and cavities. While the surface of the tunnel is comprised mainly of 23S rRNA, it also contains non-globular segments of several ribosomal proteins. Two of the proteins, called L4 and L22 in bacteria, reside near the tunnel entrance, and a third protein, L23, is located near the exit. A narrowing of the tunnel ("constriction point") is seen ~30 Å from the PTC where a conserved β hairpin loop of L22 is in close proximity to L4.

Although the functional significance of the ribosome tunnel is poorly understood, there is growing evidence that it is not simply a passive conduit. Early studies showed that ribosomes protect ~30–40 amino acids of a typical nascent chain (Malkin and Rich, 1967) and thereby suggested that polypeptides transit the tunnel in an extended conformation (~3.5 Å /residue). Recent work, however, has indicated that at least some polypeptides adopt a more compact conformation inside the tunnel (Hardesty and Kramer, 2001; Lu and Deutsch, 2005; Woolhead et al., 2004 and 2006). Moreover, specific polypeptides that are too short to emerge from the

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ribosome tunnel have been shown to arrest translation in both prokaryotes and eukaryotes (Tenson and Ehrenberg, 2002). This *cis*-acting translational attenuation serves to regulate the expression of a downstream gene and often requires a threshold concentration of a small molecule such as an amino acid or antibiotic. For example, a 24 amino acid leader peptide encoded by the *E. coli tnaC* gene inhibits its own termination in the presence of tryptophan and thereby activates the transcription of a downstream operon (Gong and Yanofsky, 2002). Likewise, peptides encoded in small open reading frames located at the 5' end of fungal carbamoyl phosphate synthetase (the arginine attenuator peptide, or AAP), plant cystathionine γ-synthase, and mammalian S-adenosylmethionine decarboxylase mRNAs stall their own translation in response to high concentrations of arginine, methionine and polyamines, respectively (Tenson and Ehrenberg, 2002). Curiously, the ribosome-stalling polypeptides identified to date vary considerably in length and share no sequence homology.

Physiological signals that do not involve small molecules can also trigger translation arrest. In *E. coli*, a secreted 170 amino acid protein called SecM regulates the translation of the co-transcribed *secA* gene in response to the secretion status of the cell (Oliver et al., 1998). Under normal conditions, the SecA Shine-Dalgarno sequence is masked by secondary structure in the *secM-secA* mRNA. Transient stalling of the ribosome at P166 destabilizes the structure and facilitates the synthesis of a basal level of SecA. The stalling is released by a "pulling" force exerted by the Sec machinery on SecM (Nakatogawa and Ito, 2001; Butkus et al., 2003). In contrast, under secretion-impaired conditions, a prolonged translation arrest increases the exposure of the SecA Shine-Dalgarno sequence and leads to overproduction of the protein (Nakatogawa and Ito, 2001). Consistent with the notion that stalling occurs in the ribosome tunnel, a short segment derived from the C-terminus of SecM can function autonomously to inhibit the translation of unrelated downstream sequences (Nakatogawa and Ito, 2002).

There is considerable evidence that translation arrest is initiated by the interaction of specific nascent polypeptide sequences with tunnel components. Mutations in L4, the L22 β hairpin loop and segments of 23S rRNA that line the top third of the ribosome tunnel impair SecM- and TnaC-mediated translation arrest (Nakatogawa and Ito, 2002; Cruz-Vera et al., 2005; Lawrence et al., 2008). Furthermore, despite their vast sequence diversity, all arrest-inducing peptides examined to date have been shown to contain one or more specific residues that play a critical role in regulating translation (Alderete et al., 1999; Fang et al., 2000; Vazquez-Laslop et al., 2008). Mutation of any of the nine key residues in the *E. coli* SecM motif ^{150}FXXXXWIXXXXGIRAGP^{166} (which are located at the top of the tunnel at the time of ribosome stalling) reduces translation arrest activity (Nakatogawa and Ito, 2002). Likewise, W12, D16, and the terminal P24 residue are essential for TnaC-mediated arrest (Cruz-Vera, et al., 2005 and 2008). The mutational data suggest that the recognition of specific motifs inside the ribosome tunnel transmits a signal outward that ultimately inhibits further translation. Consistent with this hypothesis, an analysis of SecM-stalled ribosomes by cryo-EM revealed a dramatic change in ribosome structure (Mitra et al., 2006). Presumably due to conformational changes near the PTC that inhibit the transpeptidation reaction, P166-tRNA remains bound to the A site and is not incorporated efficiently into the SecM nascent chain during translation arrest (Muto et al., 2006).

Beyond this basic phenomenology, the mechanism by which specific nascent polypeptide sequences are recognized in the ribosome tunnel remains obscure. The observation that the spacing between critical residues in arrest-inducing peptides cannot be altered (Gong and Yanofsky, 2002; Nakatogawa and Ito, 2002) suggests that translation arrest might entail the interaction of the nascent chain with multiple sites along the tunnel. In this regard it is intriguing that TnaC, SecM and AAP all have an essential residue 11–12 amino acids from the arrest point. Because many mutations that impair translation arrest affect components of the ribosome around the constriction point (where this key residue would be located if the nascent chain

were in an extended conformation), it has been proposed that the narrow part of the tunnel might be a "discriminating gate" (Nakatogawa and Ito, 2002). Consistent with this hypothesis, a recent study showed that antibiotic-dependent ribosome stalling is sensitive to mutations in the same region (Vazquez-Laslop et al., 2008). It is unclear, however, how unrelated sequences can all be deciphered at the constriction point. Even in the simplest scenario, the observation that mutations in L4, L22 and neighboring rRNA segments differentially affect SecM-, TnaC-, and antibiotic-dependent ribosome stalling suggests that sequence discrimination occurs in an elaborate binding site. To complicate matters, a recent study showed that ribosomes induce the SecM C-terminus to adopt a compact conformation during translation arrest and thereby suggest that the recognition of specific sequence motifs involves a complex series of events (Woolhead et al., 2006).

To gain insight into the mechanism of peptide recognition inside the ribosome tunnel, we explored the degree to which the SecM translation arrest motif tolerates sequence variation. We hypothesized that if mutations that impair arrest perturb critical interactions between the ribosome and the nascent chain, then it might be possible to isolate second-site suppressor mutations that either restore these interactions or strengthen other interactions. In this way we hoped to clarify the nature and number of interaction sites. Surprisingly, we found that extensively remodeled motifs containing as few as three of the nine key residues in the *E. coli* SecM motif are completely functional. Further analysis suggested that a single invariant residue (R163) is "recognized" by the ribosome, while other more variable residues primarily influence the conformation of the nascent chain in the tunnel. Based on the data, we propose that translation arrest occurs when the conformation of nascent SecM facilitates the positioning of R163 at a specific intra-tunnel site and that mutations near the constriction point only indirectly affect peptide recognition. In addition, we propose that, like SecM, all arrest peptides consist of both essential and context-dependent sequence elements, and that this feature helps to explain their diversity.

RESULTS

Second-site mutations that suppress translation arrest defects reveal an unexpected plasticity of the *E. coli* SecM translation arrest motif

Although alanine-scanning mutagenesis indicated that only residues 150, 155–156 and 161–166 of *E. coli* SecM [SecM(Eco)] are required for complete translation arrest (Nakatogawa and Ito, 2002), we found that the mutation of residues 157–160 to proline also causes severe translation arrest defects (Woolhead et al., 2006). This observation suggested that constraining the polypeptide backbone between residues 157–160 might perturb the positioning of one or more key residues inside the ribosome tunnel and thereby prevent recognition of the translation arrest motif. We reasoned that second-site suppressor mutations might be isolated that would either restore the proper positioning of these key residues or introduce conservative substitutions at key positions that would restore recognition. To test this hypothesis, we used an assay based on the finding that the fusion of the SecM(Eco) arrest motif to LacZα inhibits synthesis of the reporter, but that mutations that impair translation arrest restore LacZα production and turn colonies dark blue on indicator plates (Nakatogawa and Ito, 2002). We randomly mutagenized the SecM moiety of a SecM-LacZα fusion carrying the Q160P mutation, which impairs arrest, and screened for pale blue colonies (white colonies contained nonsense or frameshift mutations). Ultimately we obtained six plasmids designated Sup1-Sup6 that produced colonies of the desired color and that encode one or more second-site mutations (Fig. 1A).

We confirmed that the suppressor mutations isolated in the genetic screen restore SecM function using an in vivo translation arrest assay. In these experiments a secretion-deficient *E. coli* strain (CU164) was transformed with a plasmid harboring full-length SecM(Eco) (pSecM),

pSecM containing an arrest-compromising mutation (Q160P or P166A), or pSecM containing one of the suppressor sequences. Cells were subjected to pulse-chase labeling, and SecM was immunoprecipitated. Consistent with previous results (Nakatogawa and Ito, 2002), only the translation-arrested fragment of wild-type SecM (166 residues) was observed; no full-length protein (170 residues) was detected even after a 2 min chase (Fig. 1B, lane 1–3). As also previously observed, the P166A mutation abolished translation arrest and led to the production of only full-length SecM (Fig. 1B, lanes 25–27). The Q160P mutation was less severe in that about half of the pulse-labeled SecM was isolated as the translation-arrested fragment. After a 2 min chase, however, only full-length protein was observed, suggesting that the mutation destabilizes the translation-arrested state (Fig. 1B, lanes 4–6). Interestingly, Sup1–3 yielded a single polypeptide that co-migrated with the stalled fragment of wild-type SecM(Eco) (Fig. 1B, lanes 7–15). The identity of this band as a translation-arrested product was verified using a coupled transcription-translation assay (Fig. S1A). These results indicate that the second-site mutations in Sup1–3 completely restore translation arrest activity. Sup4–6 only partially restored activity in vivo in that only ~50–60% of the SecM was isolated as a translation-arrested fragment after a 2 min chase (Fig. 1B, lanes 16–24 and Fig. S1A).

Surprisingly, only some of the suppressor mutations appeared to support our original hypothesis. Sup5 and Sup6 harbor mutations in the “non-essential” residues 157–159 that might restore the proper positioning of key amino acid side chains. In addition, Sup4 contains a modest mutation of a key residue (I156T) that might promote translation arrest in the context of a nascent polypeptide constrained by the Q160P mutation. Sup4–Sup6, however, are only relatively weak suppressors. All of the strong suppressors (Sup 1–Sup3) contain at least one highly non-conservative alteration of a key residue (G161P and, in the case of Sup3, W155E). The observation that a severe alteration of residue W155 can be compatible with translation arrest is especially striking because W155 has been predicted to be located near the tunnel constriction point (Berisio et al., 2003). The isolation of suppressors lacking elements of the canonical SecM(Eco) arrest motif provided the first indication that at least some of the key residues might not be involved in direct interactions with the ribosome and that the constriction point might not be a “discriminating gate”.

Distantly related SecM homologs effectively stall translation in *E. coli*

Interestingly, the C-terminal sequences of Sup1–Sup3 closely resemble those of uncharacterized SecM homologs found in the genomes of Pasteurellales (van der Sluis and Driessen, 2006). Like the SecM homologs found in Enterobacteriales, these homologs are completely divergent in overall sequence and length, but they are directly upstream of *secA* and their C-terminal sequences can be aligned (Fig. 2A). Like Sup1–3, *Haemophilus ducreyi* and *Pasteurella multocida* SecM homologs both contain prolines at positions that are equivalent to Q160 and G161 of SecM(Eco). These homologs also contain the same sequences as Sup2 and Sup3 at positions that are equivalent to W155 and I156 of SecM(Eco).

The fact that the outcome of our genetic screen seemed to mimic the natural evolution of SecM raised the possibility that C-terminal sequences of divergent SecM homologs might stall translation in *E. coli*. To test this hypothesis, we replaced the last 22 residues of SecM(Eco) with moderately divergent (5/9 key residues conserved) and highly divergent (3/9 key residues conserved) C-terminal SecM sequences derived from from *Haemophilus influenzae* or *Mannheimia succiniciproducens* (Fig. 2B). CU164 transformed with plasmids encoding the chimeras [SecM(C-Hi) and SecM(C-Ms)] or the chimeras containing the P166A mutation were subjected to pulse-chase analysis, and the chimeric proteins were immunoprecipitated. Remarkably, SecM(C-Hi) and SecM(C-Ms) produced a single polypeptide that co-migrated with the translation-arrested fragment of SecM(Eco) (Fig. 2C, lanes 7–9 and 13–15). In contrast, SecM (C-Hi) and SecM (C-Ms) containing the P166A mutation yielded products that

co-migrated with full-length SecM (Fig. 2C, lanes 10–12 and 16–18). We confirmed that these polypeptides correspond to translation-arrested and full-length versions of SecM, respectively, using coupled in vitro transcription-translation assays (Fig. S1B). We also found that the native *H. influenzae* SecM protein undergoes elongation arrest in *E. coli* cell-free extracts (Fig. S1C). These data show that distantly related SecM homologs are completely functional in *E. coli* despite only weak sequence conservation.

Diverse SecM variants share a common mechanism of translation arrest

Based in part on the observation that P166 plays an essential role in SecM-mediated translation arrest even when the arrest motif is substantially altered, we conjectured that Sup1–6, SecM (C-Hi) and SecM(C-Ms) stall translation by the same mechanism as SecM(Eco). To test this idea, we first compared the position at which stalling occurs in *E. coli* SecM and each of the SecM variants. Coupled transcription-translation reactions were programmed with full-length SecM DNA templates and peptidyl-tRNAs were resolved on low pH Tris-acetate gels (Kirchdoerfer et al., 2007). Northern blot analysis was conducted using oligonucleotide probes complementary to $tRNA^{Gly}$ and $tRNA^{Pro}$, the tRNAs that would be linked to the nascent chain if stalling occurs at residues 165 and 166, respectively. Because residue 165 in SecM(C-Ms) is a serine instead of a glycine, we also used a probe that can detect $tRNA^{Ser}$. Consistent with previous results showing that G165 is the terminal residue of stalled SecM(Eco) (Garza-Sanchez et al., 2006; Muto et al., 2006), we found that a significant fraction of the SecM(Eco) nascent chain was attached to $tRNA^{Gly}$ (Fig. 3, top panel, lane 1). Interestingly, we found that all of the variant SecM nascent chains were also attached to $tRNA^{Gly}$ except for SecM(C-Ms), which was attached to $tRNA^{Ser}$ (Fig. 3, top and middle panels). While we found that a fraction of each stalled product contained $tRNA^{Pro}$ (Fig. 3, bottom panel), our results are consistent with the observation that a subset of stalled SecM(Eco) products contain P166 in vivo (Collier et al., 2004; Sunohara et al., 2004). Furthermore, we found that like SecM(Eco) RNCs, SecM (C-Hi), SecM(C-Ms) and Sup 1–6 RNCs were resistant to attack by puromycin, presumably because access to the A site is blocked by the presence of $tRNA^{Pro}$ (Woolhead et al., 2006; Muto et al., 2006) (Fig. S2). Taken together, these data provide evidence for a universal SecM stalling mechanism that is independent of variation in the arrest peptide sequence.

SecM arrest motifs consist of functionally distinct classes of residues

The results described above show that the SecM translation arrest motif tolerates considerable sequence variation. While the data indicate that P166 cannot be altered, this amino acid has a unique role in the arrest process as an aminoacyl tRNA, and it never enters the ribosome tunnel. The plasticity of the SecM sequence N-terminal to P166, however, challenges the idea that translation arrest results from the precise positioning of most or all of the key residues in the SecM(Eco) motif at fixed recognition sites located along the ribosome tunnel and instead suggests a more complex interaction between the nascent chain and the tunnel wall. To gain further insight into the relationship between sequence and function, we performed alanine/serine scanning mutagenesis on residues 149–165 of SecM(C-Ms) to identify residues that are required for translation arrest.

Interestingly, we found that the mutation of only one residue, R163, completely abolished translation arrest mediated by SecM(C-Ms). R163 is one of only three amino acids that is invariant in all SecM homologs sequenced to date, and mutation of this residue also abolishes translation arrest mediated by SecM(Eco) (Nakatogawa and Ito, 2002; Fig. S3A). Mutation of R163 to other amino acids (including lysine) abolished translation arrest activity in vivo, but changing the CGC codon to synonymous arginine codons had no effect on function (Fig. 4A, lanes 46–48 and Fig. S3B, lanes 4–24). Shifting R163 -1 or +1 in the SecM(C-Ms) sequence also resulted in the production of only full-length protein (Fig. S3B, lanes 31–36). These results

demonstrate that both the identity of arginine at residue 163 and its exact position in the amino acid sequence play a decisive role in SecM-mediated translation arrest.

Beyond the common requirement for R163, we found a dramatic disparity in the role played by other amino acids in the arrest mediated by SecM(Eco) and SecM(C-Ms). Most notably, while the alteration of residues 159–166 in SecM(C-Ms) impaired elongation arrest activity in vivo, mutation of residues 149–158 had no effect (Fig. 4A). This is a striking result because F150, W155 and I156 all play a very significant role in the stalling mediated by SecM(Eco) (Nakatogowa and Ito 2002; Fig. 5, lanes 10–18). The data show that while the SecM(Eco) arrest motif spans 17 amino acids, the SecM(C-Ms) arrest motif spans only 8 residues. Moreover, we found that the relative contribution of each residue between 159 and 166 to translational stalling is different in SecM(Eco) and SecM(C-Ms) (Fig. 4B). For instance, H159 and A160 play a modest role in SecM(C-Ms)-mediated translation arrest, but the equivalent residues in SecM(Eco) do not appear to play any role. In addition, mutation of P161 in SecM (C-Ms) strongly impairs translation arrest activity, but mutation of G161 in SecM(Eco) has only a minor effect. These differences strongly suggest that all SecM arrest motifs contain a class of residues that play a secondary role in translational stalling, and that the number, position and identity of these residues is context-dependent (i.e., dependent on the overall sequence of the C-terminal peptide). Consistent with this interpretation, A164 in SecM(Eco) is required for complete arrest but, conversely, the presence of an alanine at this position in SecM(C-Ms) causes an arrest defect. Because the secondary residues are so variable, it seems unlikely that they contribute directly to the transmission of a signal through the ribosome tunnel.

An examination of the Sup1–3 sequences as well as of the natural evolution of SecM suggested a plausible explanation for the existence of relatively short translation arrest motifs. Although *E. coli* and its close relatives require a glycine at position 161, Sup1–3 and the SecM homologs of Pasteurellales all contain a proline at this position (Figs. 1A and 2A). In addition, the sequence N-terminal to P161 is not conserved in the Pasteurellales. Finally, while the Q160P mutation severely impairs arrest mediated by SecM(Eco), a proline is found at position 160 in Sup1–3 and in some of the Pasteurellales homologs. The sequence information suggests that prolines at positions 160 and 161 might reduce the role of F150, W155 and I156 in translation arrest. To test this hypothesis, we combined the F150A, W155A and I156A mutations with either the G161P mutation or the double Q160P/G161P mutation and examined the effect of the mutations on SecM(Eco)-mediated translation arrest in vivo. Consistent with our hypothesis, we found that the defect produced by the W155A/G161P and I156A/G161P double mutations was similar to that produced by the G161P mutation alone but was markedly less severe than that produced by either W155A or I156A alone (Fig. 5A, lanes 13–30). Furthermore, when residues 160 and 161 were both converted to proline, F150, W155 and I156 could all be mutated to alanine (either singly or in combination) without producing any discernable arrest defect (Fig. 5A, lanes 34–42). R163, however, was still strictly required for arrest activity (Fig. S3A lanes 19–21). The observation that a +1 shift of Q160P/G161P completely abolished translation arrest indicates that the position of the prolines is important (Fig. 5A, lanes 31–33). Taken together, the data suggest that prolines at positions 160 and 161 reduce the arrest–inducing peptide to the simple core motif ^{162}IRXXP^{166} (where X is a small amino acid).

Differential crosslinking of divergent SecM arrest peptides to ribosomal proteins

Based on the finding that SecM arrest peptides contain residues that appear to provide contextual information and that prolines profoundly influence the size and functionality of arrest motifs, we conjectured that divergent arrest peptides might adopt unique conformations inside the ribosome tunnel (or might be situated in distinct positions relative to the tunnel walls). Presumably such unique configurations would result from inherent constraints (e.g., the

presence of prolines), interactions between the nascent polypeptide and tunnel components, or constraints imposed on the polypeptide by the tunnel environment. Consistent with the idea that the conformation of the nascent chain is relevant to arrest induction, proline mutations at positions 158 and 160 of SecM(Eco) each cause strong arrest defects, but the combination of the two prolines (in Sup4) almost completely restores translational stalling (Fig. 5B). The simplest explanation of this result is that the two prolines cancel out the contribution that they each make individually to polypeptide conformation.

We used a site-specific photocrosslinking approach to obtain more direct information about the disposition of SecM arrest peptides inside the ribosome tunnel. We modified an amber suppression system involving the co-expression of an amber suppressor tRNA and an amino acyl-tRNA synthetase from *M. jannaschii* that are orthogonal to the *E. coli* translation machinery (Xie and Schultz, 2006). While this method has previously only been used to incorporate “unnatural” amino acids into proteins in a site-specific fashion in vivo, we adapted the technology for use in cell-free transcription-translation reactions. Reactions were programmed with full-length SecM(Eco), Sup1 and SecM(C-Ms) DNA templates containing single amber mutations. Sup1 was chosen for these experiments because it differs from SecM (Eco) at only two positions, and SecM(C-Ms) was chosen because it is the most divergent SecM variant that we analyzed. Amber suppression led to the incorporation of photoreactive *p*-benzoyl-L-phenylalanine (Bpa) or *p*-azido-L-phenylalanine (Azp) into the protein. To reduce the frequency of termination at the amber codon and thereby enhance the efficiency of amber suppression, we made extracts from a strain that contains the release factor 1 (RF1) mutant allele *prfA1* (Zhang et al., 1996). Based on the ratio of arrested SecM to SecM that was terminated at the amber codon, the incorporation efficiency was ~10–20% (Fig. 6, left panel, compare “arrested” to “no suppression”). Although the incorporation of amino acid analogs at some “non-essential” positions abolished translation arrest (Fig. S4), the incorporation of Bpa or Azp at residues 141, 144, 149, 151, 152, and 158 did not impair the function of the arrest peptide (except as noted below). After the cell-free reactions were completed they were exposed to UV light and immunoprecipitations were conducted with antisera raised against L4, L22 and L23 to assess the proximity of each photoprobe to tunnel components.

Interestingly, we found that each SecM variant exhibited a distinct pattern of crosslinking to ribosomal proteins. When Bpa was incorporated into Sup1 and SecM(C-Ms) at residue 141, photoadducts of ~29–30 kD were clearly visible in the UV-irradiated samples (Fig. 6A, lanes 10–12). These bands corresponded to SecM (~18 kD) crosslinked to roughly equal amounts of L22 and L23 (~11–12 kD) (Fig. 6A, right panel). Smaller amounts of these crosslinked products were observed in reactions programmed with SecM(Eco), but the disparity corresponds to a reduction in the efficiency of translation arrest (Fig. 6A, lane 1). The incorporation of Bpa at residue 144 did not affect translation arrest, and SecM variants containing the photoprobe at this position were all crosslinked to L22 with similar efficiencies (Fig. 6B). The crosslinking of sites in the nascent chain that are ~22–25 residues from the PTC to L22 and L23 is consistent with previous results (Houben et al., 2005) and suggests that the proximity of each SecM variant to the tunnel wall is similar near the distal end of the tunnel. Striking differences between the variants were observed when the probes were placed closer to the PTC, however. Incorporation of Bpa or Azp at residue 149, 151 or 152 resulted in significant crosslinking between SecM(C-Ms) and L22 and/or L4 (Fig. 6C–E). Crosslinking between Sup1 and the same ribosomal proteins was observed at all of these positions, but at positions 151 and 152 the signal was much weaker. In contrast, no crosslinking was observed when the photoprobe was incorporated at residue 149 or 152 of SecM(Eco) (position 151 cannot be evaluated because Bpa perturbs translation arrest). Finally, crosslinks were observed between Sup1 and SecM(C-Ms) containing Azp at residue 158 and L4, but not between the corresponding SecM(Eco) derivative and L4 (Fig. 6F). Taken together, these results strongly suggest that the segment of each of the three SecM isoforms adjacent to the arrest motif core

has a distinct conformation (or spatial relationship to the tunnel walls) that might extend into the core itself. Because the Sup1 crosslinking pattern is "intermediate" between those of SecM (Eco) and SecM(C-Ms), it appears that conformational differences between two polypeptides correlate with the degree to which their sequences diverge.

The notion that SecM variants adopt unique conformations that result at least in part from interactions with tunnel components predicts that changes in tunnel structure would produce a variable effect on the activity of each variant. Consistent with our prediction, we found that mutations in both L22 and 23S rRNA impaired the arrest mediated by the SecM variants to different degrees (Fig S5).

DISCUSSION

In this report we show that SecM mutants isolated in a genetic screen as well as divergent SecM homologs mediate translation arrest in secretion-deficient *E. coli* as effectively as SecM(Eco). The identification of fully functional arrest peptides that contain as many as six mutations in the arrest motif ^{150}FXXXXWIXXXXGIRAGP^{166} was counterintuitive because single mutations reduce the efficiency of translation arrest. Despite the striking sequence diversity of the SecM variants, they all appear to mediate translational stalling by the same mechanism. Interestingly, we found that while mutation of R163 and P166 abolished the translation arrest mediated by both SecM(Eco) and the most divergent SecM variant, SecM(C-Ms), partial arrest defects resulted from the mutation of other C-terminal residues that vary in position, number and side chain chemistry. Furthermore, photocrosslinking data provided evidence that the sequence variation that we observed correlates with a distinct positioning of the C-terminus of individual SecM variants relative to L4 and L22. Because the presence of proline residues (which often impose conformational constraints) strongly affects arrest activity, it seems likely that the photocrosslinking data reflect conformational differences among the nascent polypeptides. Additional studies will be required, however, to determine the degree to which the structure of each nascent chain (as opposed to the structure of the tunnel) varies. In any case, the observation that individual nascent polypeptide chains are situated differently inside the ribosome tunnel strongly suggests that it provides an interactive, rather than a passive, environment.

The remarkable plasticity of the SecM arrest motif most likely arises from the presence of a class of residues that play a context-dependent role in the recognition process. While R163 appears to perform a singular function in translational stalling, the other residues between 150 and 165 that play a "secondary" role probably fall into at least one other functional class. The variable contribution of these residues to translation arrest and their variable nature suggests that they work in different combinations to achieve the same end. Indeed a subset of unique residues [e.g., P160 and P161, which are the only two residues found in Sup1 but not SecM (Eco)] must specify the differences in the crosslinking patterns that we observed. Presumably the requirement for these context-dependent residues reflects the unusual circumstances of polypeptide recognition in the ribosome tunnel, an environment in which the composition and location of potential ligands is in constant flux. An alternative explanation of the plasticity of the SecM arrest motif is that both R163 and the secondary residues are coordinately recognized, but that the latter simply form less significant interactions with the ribosome than R163. This explanation is very unlikely, however, because it would imply the existence of an exceptionally complex recognition process in which components of the ribosome tunnel integrate information obtained through interactions with a potentially very large number of different sequences.

Based on the notion that the C-terminal residues of SecM perform distinct functions, we propose a new model for the recognition of the arrest peptide in the ribosome tunnel (Fig. 7). In our model, SecM residues near the top of the tunnel specify the conformation of the nascent

chain and thereby influence the positioning of R163. Translation arrest occurs when the conformation of the nascent chain moves R163 into a precise intra-tunnel site where it either transmits a signal by interacting with a critical ribosomal component or simply prevents further movement of the polypeptide chain. In this model, nascent chain "discrimination" occurs relatively close to the PTC. Nascent chain conformation might be influenced at least in part by microenvironments in the tunnel. In principle, nascent chain conformation might change dynamically during polypeptide elongation and be subject to constant monitoring by the ribosome. Alternatively, a specific conformation might become locked in only when P166-tRNA occupies the A site and initiates a series of events that alter the tunnel environment or, as previously proposed, transiently stalls translation (Muto et al., 2006). Consistent with this possibility, part of the SecM(Eco) nascent chain that resides inside the tunnel undergoes a dramatic conformational change when P166-tRNA binds to the ribosome (Woolhead et al., 2006). In any case, we propose that the presence of a proline at residue 161 enhances the positioning of R163 by constraining nascent chain conformation. In contrast, when residue 161 is a flexible glycine, residues further down the tunnel such as F150 and W155 are required to stabilize the arrest peptide possibly by interacting with tunnel components. Regardless of their mode of action, F150 and W155 promote translation arrest only when compaction of the nascent chain between residues 135 and 159 moves them into an appropriate location (Woolhead et al., 2006).

An implicit postulate of this model is that the conformation of the nascent chain near the top of the tunnel is determined by multiple residues, including residues that can be located relatively far away. The prevalence of partial arrest defects that we observed is consistent with this idea because if multiple residues contribute to the positioning of R163, then a single mutation might create only a small structural perturbation. In this regard it is noteworthy that I162 is invariant in all SecM homologs, but an I162A mutation causes only partial defects in the arrest mediated by both SecM(Eco) and SecM(C-Ms). It seems likely that this amino acid plays a particularly important, yet non-essential role in the positioning of the adjacent R163 residue. The observation that the incorporation of Bpa into SecM(Eco) at positions N-terminal to the arrest motif (e.g., 140) abolishes translation arrest (Fig. S4), however, clearly suggests that distant residues can also influence events close to the PTC. Likewise, the finding that L22 mutations impair SecM(C-Ms)-mediated arrest even though the arrest motif does not reach the constriction point in the tunnel (Fig. S5) raises the possibility that the mutations act indirectly by perturbing the disposition of distant residues that normally do not participate in translational stalling. Indeed the constriction point may appear to be a universal "discriminating gate" largely because mutations in this region often exert indirect effects on the arrest process.

Finally, our results may help to explain the striking heterogeneity of arrest peptides that have been identified in both prokaryotic and eukaryotic systems. In essence, we found that the SecM arrest motif is comprised of only two essential elements plus an equal or greater number of variable elements. Consequently the SecM sequence diverges to such a degree that identification of SecM homologs would be difficult if they were divorced from their genomic context. If one postulates that all arrest peptides likewise contain only a few essential elements and that arrest peptide discrimination involves the positioning of a key residue (which is clearly not always arginine) at one of multiple sites throughout the tunnel (rather than at a fixed "gate"), then the sequence and size diversity of arrest peptides can be explained. Consistent with this hypothesis, TnaC contains only two invariant residues, an aspartate and a tryptophan located 8 and 12 residues, respectively, from the PTC. The mutation of either residue abolishes TnaC-mediated arrest (Cruz-Vera and Yanofsky, 2008). Interestingly, the residues located between the invariant residues and those C-terminal to the aspartate are loosely conserved (suggesting that they play a significant, though less critical role in arrest) whereas the N-terminal residues show no conservation. It is also noteworthy that most characterized translation arrest phenomena require the presence of a small molecule, such as an antibiotic that targets the

ribosome. The binding of specific macrolides in the tunnel, which is required for translation arrest mediated by the ErmC peptide, might alter the tunnel so as to create new sites of peptide discrimination. It is also possible, however, that the binding of other small molecules such as tryptophan, which appears to bind near the A site to promote TnaC-mediated stalling (Gong and Yanofsky, 2002), directly or indirectly affects the environment in the tunnel. Indeed by binding to different locations on the ribosome small molecules might induce the formation of a multitude of discrimination sites that initiate arrest in response to only one or two critical amino acids.

EXPERIMENTAL PROCEDURES

Bacterial strains

CU164 (MC4100 *zhd::tet secY39*^{cs}) (Baba et al., 1990), C41 (Miroux and Walker, 1996) or SQ351 (MG1655 Δ7*rrn* Δ*lacZYA* pKK3535 pTRNA67) (Cruz-Vera et al., 2005) or their derivatives were used in all experiments.

Pulse-chase experiments

To examine the kinetics of SecM translation arrest, *E. coli* harboring pSecM derivatives were grown at 37°C in M9 medium containing 0.2% glycerol, 100 μg/ml ampicillin, and all the L-amino acids (40 μg/ml) except methionine and cysteine. Overnight cultures were washed and diluted into fresh medium at OD_{550}=0.02. When the cultures reached OD_{550}=0.2, synthesis of plasmid-borne genes was induced for 30 min by the addition of 200 μM IPTG. Pulse-chase labeling was conducted as described (Ulbrandt et al., 1997). SecM-containing polypeptides were then immunoprecipitated with rabbit polyclonal antibodies against synthetic peptides corresponding to residues 30–54 and 127–142 of SecM (Nakatogawa and Ito, 2001). Except where noted, immunoprecipitated proteins were resolved by electrophoresis on 12% NuPAGE minigels (Invitrogen) with MOPS running buffer. In these and all other experiments that involved the detection of a radioactive signal, gels were scanned using a Fuji BAS-2500 phosphorimager.

Coupled transcription-translation reactions and photocrosslinking

E. coli C41 S-30 extract preparation, coupled transcription-translation reactions and cetyl trimethylammonium bromide (CTABr) fractionations were performed essentially as described (Woolhead et al., 2006). In a typical experiment in which radiolabeling was required, 10 μCi Tran^{35}S-Label (MP Biomedical) was added to a 25 μl reaction. Samples were resolved by PAGE as described (Woolhead et al., 2006) except where noted. For photocrosslining experiments S-30 extracts were prepared from strains MNY9 (C41 *prfA1 zcg*174::Tn10 pBpaCm) and MNY10 (C41 *prfA1 zcg*174::Tn10 pAzpCm) grown at 37°C in medium supplemented with chloramphenicol (10 μg/ml). RF1 was inactivated by heating the extracts at 42°C for 16 min. Reactions (200 μl) were programmed with full-length SecM variants harboring the desired amber mutation and contained 10 μCi Tran^{35}S-Label and either 1 mM Bpa or 2 mM Azp (Bachem). After a 30 min incubation at 37°C, reactions were placed on ice for 5 min. A portion (5%) of the reaction was withdrawn for CTABr fractionation followed by immunoprecipitation with anti-SecM. The RNC complexes in the remaining portion were isolated by centrifugation through a 0.5 M sucrose cushion and resuspended in 200 μl RNC buffer (Woolhead et al., 2006). A portion (50 μl) representing untreated RNC complexes was TCA precipitated and subjected to immunoprecipitation with anti-SecM. The remainder was transferred to a 96-well flat bottom plate and UV-irradiated (365 nm, Spectroline SB-100P) on ice for 15 min at a distance of 3–4 cm. A portion (50 μl) of the irradiated RNC complexes was treated as described above and represented the UV-treated samples. The remaining ~90 μl were TCA precipitated and subjected to immunoprecipiation using rabbit antisera raised against synthetic peptides corresponding to the N-terminus of L4, L22 and L23

(MELVLKDAQSALTVSETTFGC, METIAKHRHARSSAQKVRLVC, MIREERLLKVLRAPHVSEKAC). Finally, all samples were resolved on 12% NuPAGE minigels with MOPS running buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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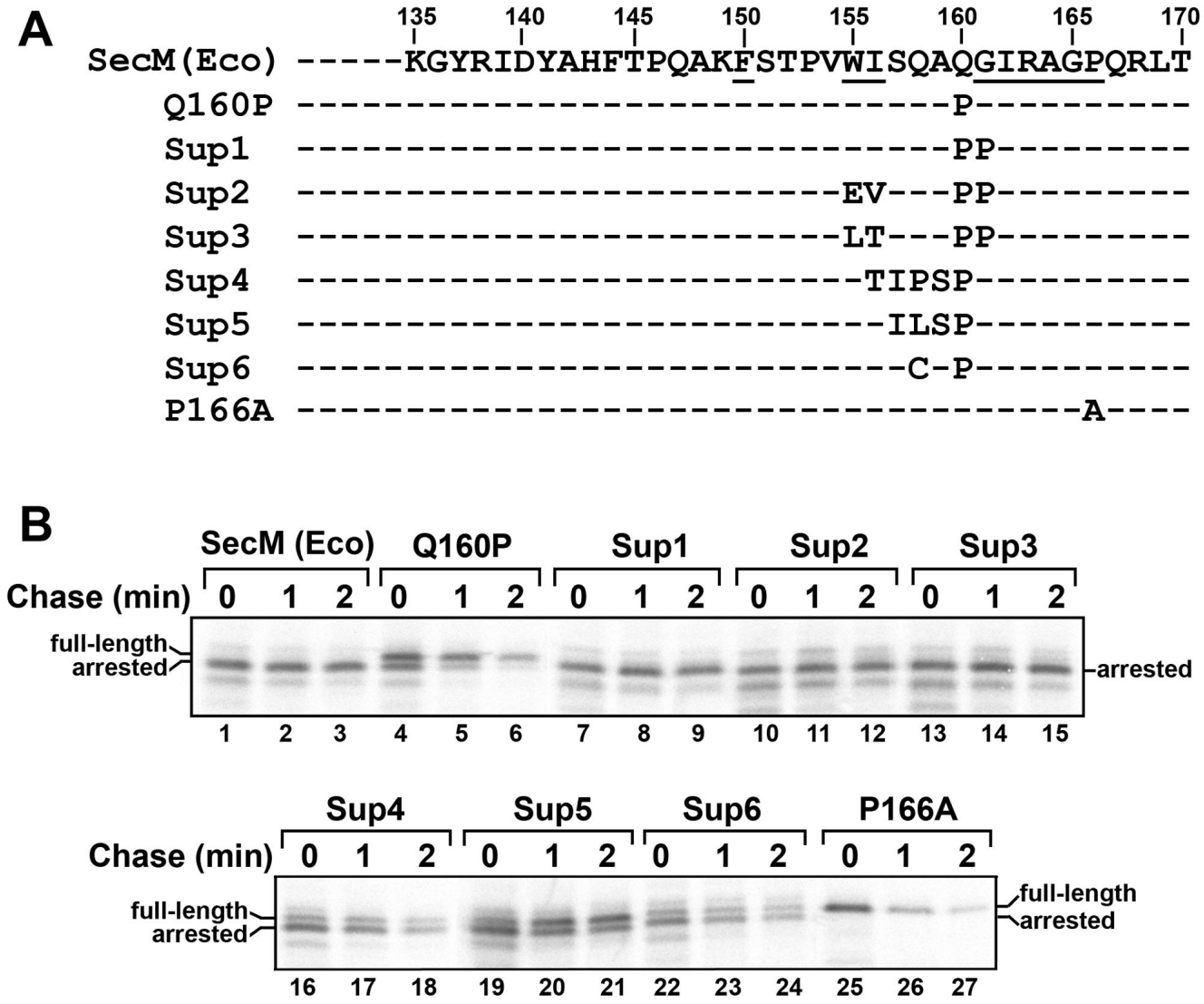


Fig. 1. Isolation of second-site mutations that suppress a defect in SecM-mediated translation arrest

(A) A plasmid encoding the translation arrest-deficient Q160P allele of SecM(Eco) was randomly mutagenized, and intragenic mutations that suppress the defect were isolated in a LacZα reporter screen. Positions where the sequence of the suppressor mutants (Sup1–6) and wild-type SecM differ are indicated. SecM(Eco) residues that were previously shown to be required for complete translation arrest are underlined. **(B)** CU164 (*secY39*) transformed with a plasmid encoding SecM(Eco), a SecM(Eco) mutant, or Sup1–6 were subjected to pulse-chase labeling after the addition of IPTG to induce expression of the plasmid-borne gene. Full-length and translation-arrested forms of SecM were immunoprecipitated and resolved by SDS-PAGE.

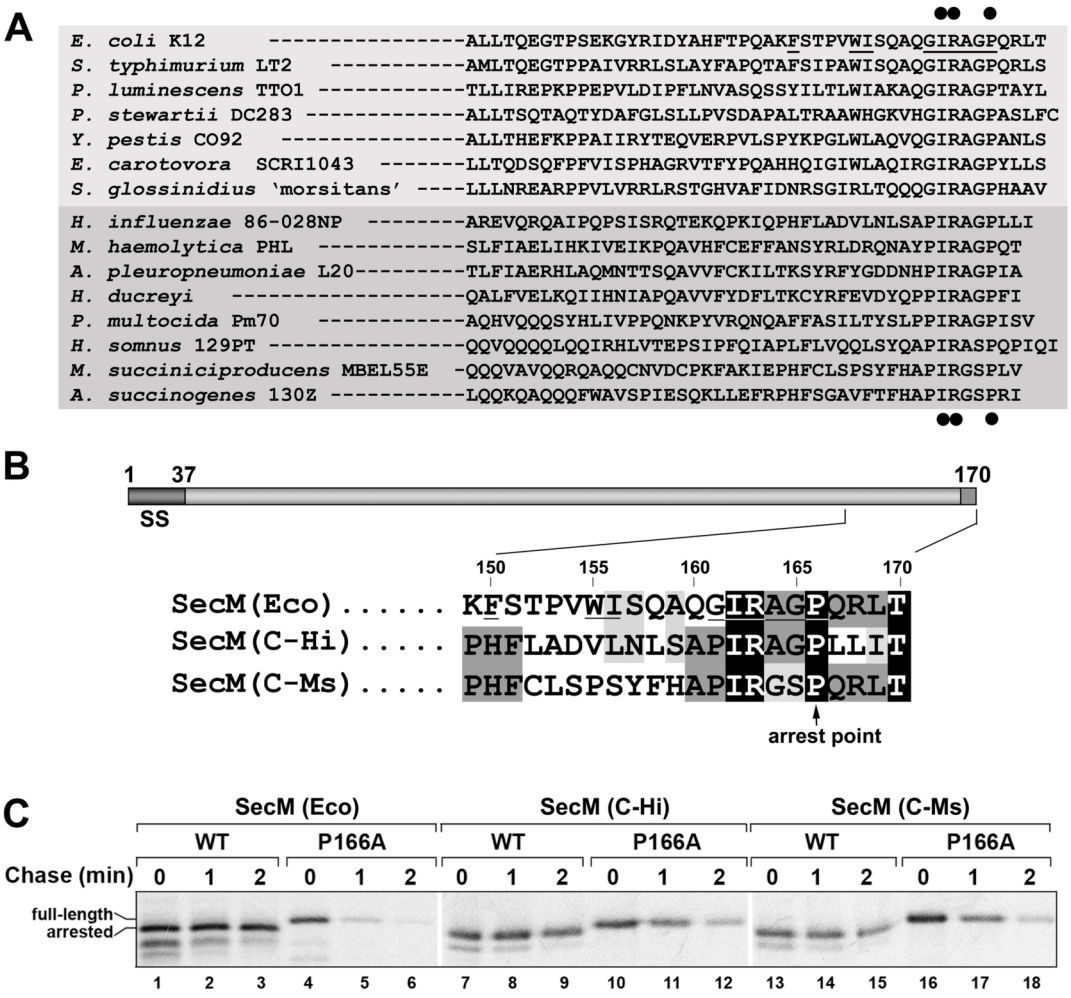


Fig. 2. C-terminal segments of distantly related SecM homologs induce translation arrest in *E. coli*

(A) Sequence alignment of the C-terminus of representative SecM homologs from Enterobacteriales (light gray) and Pastuerellales (dark gray). Invariant residues are indicated (●). Residues of SecM(Eco) that were previously shown to be required for full translation arrest are underlined. **(B)** Illustration of SecM(Eco) and SecM chimeras containing a C-terminal segment from *H. influenzae* 86-028NP SecM [SecM (C-Hi)] or *M. succiniciproducens* MBEL55E [SecM(C-Ms)]. Four residues were introduced after the arrest point (P166) to maintain a sequence length of 170 residues. Invariant, conserved and similar residues are depicted in black, dark grey and light grey, respectively. SS: signal sequence. **(C)** CU164 transformed with a plasmid encoding the indicated SecM derivative were subjected to pulse-chase labeling after the addition of IPTG, and SecM-containing polypeptides were immunoprecipitated.

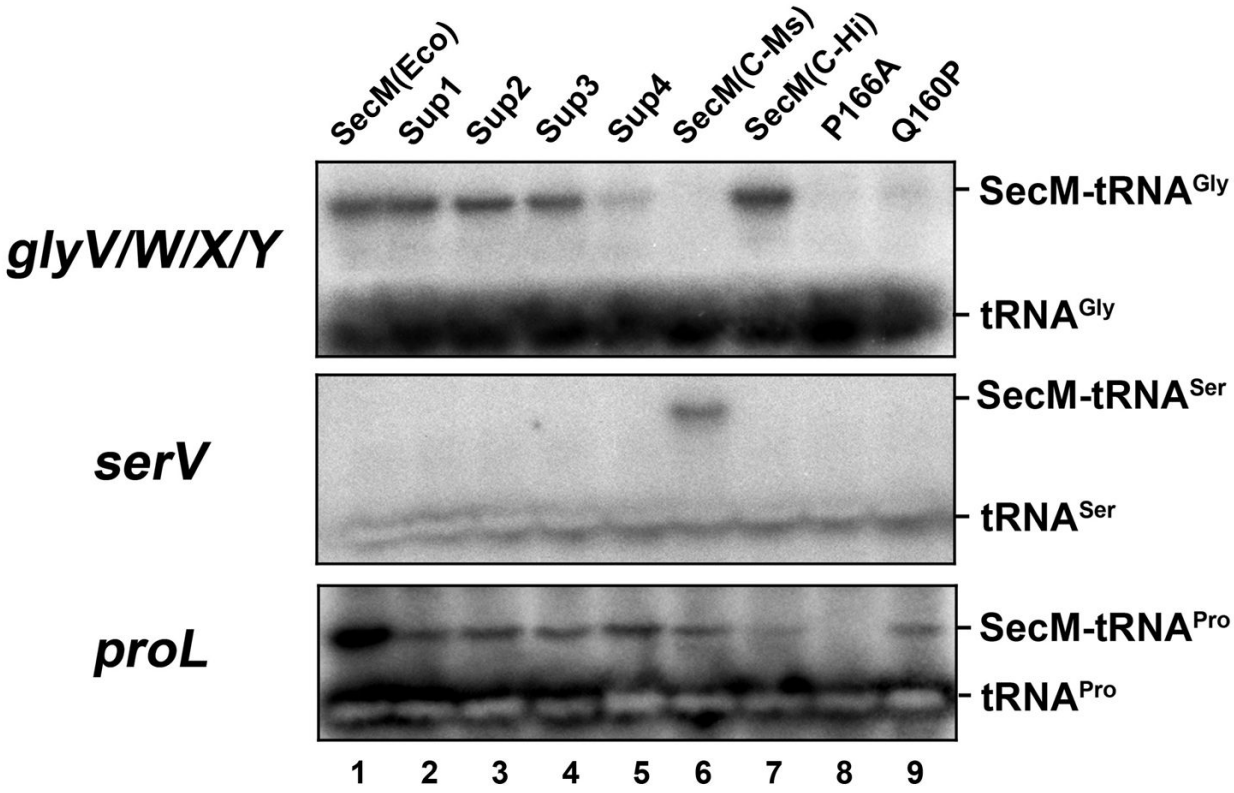
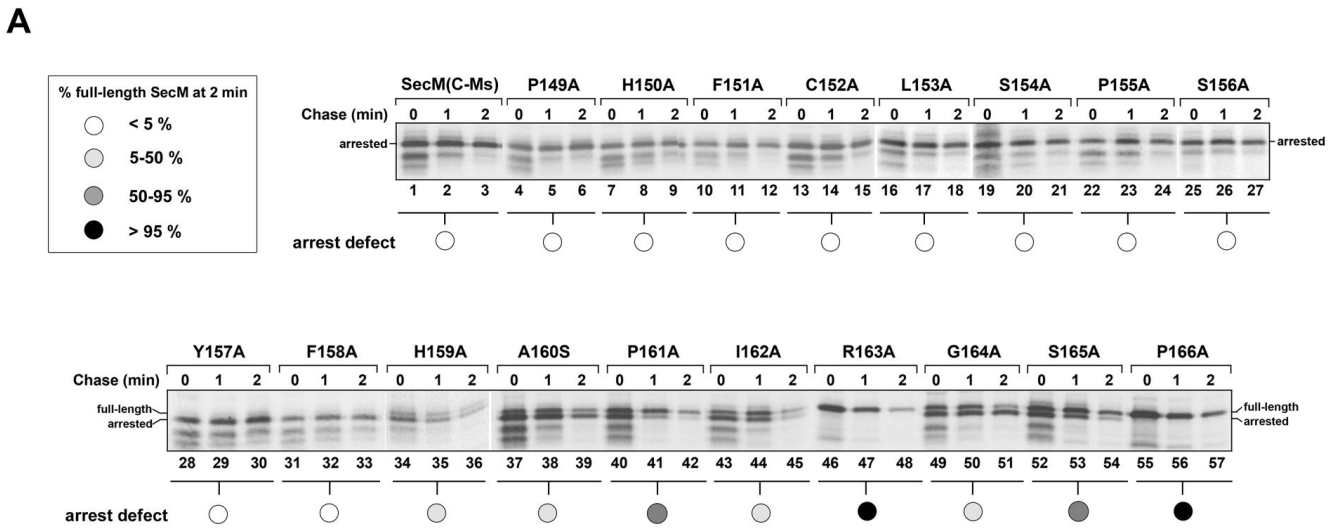


Fig. 3. The translation of distantly related SecM variants stalls at the same position

The indicated SecM variants were synthesized in a coupled transcription-translation reaction and stalled peptidyl-tRNAs were analyzed by Northern blot using [γ-^{32}P]-ATP labeled oligonucleotides complementary to *glyV/W/X/Y*, *proL*, and *serV* tRNAs.



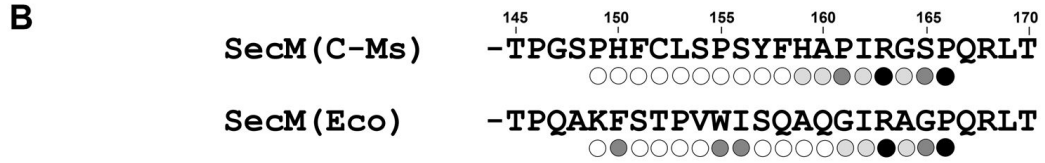


Fig. 4. The translation arrest motif in SecM (C-Ms) spans only eight residues

(A) CU164 transformed with a plasmid encoding SecM (C-Ms) or the indicated SecM (C-Ms) mutant were subjected to pulse-chase labeling after the addition of IPTG, and SecM-containing polypeptides were immunoprecipitated. **(B)** Summary of the data in part A and previous results (Nakatagowa and Ito, 2002).

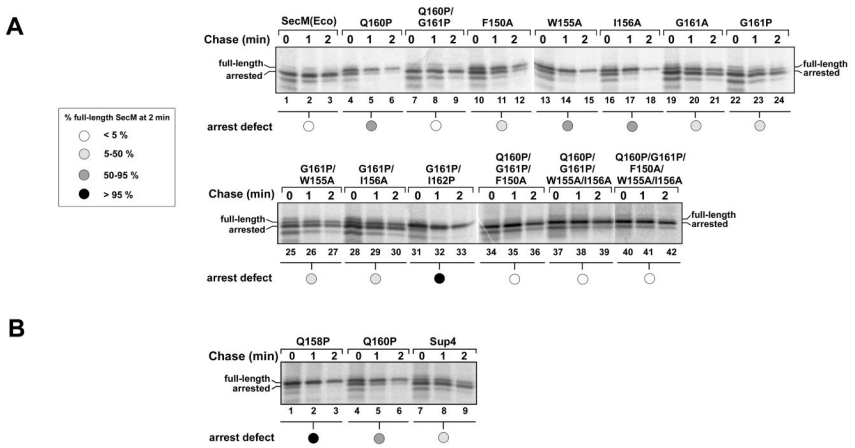


Fig. 5. Prolines at positions 160 and 161 of SecM (Eco) reduce the role of F150, W155 and I156 in translation arrest

(A) and **(B)** CU164 transformed with a plasmid encoding SecM (Eco) or the indicated SecM (Eco) mutant were subjected to pulse-chase labeling after the addition of IPTG, and SecM-containing polypeptides were immunoprecipitated. The effect of combining prolines at positions 158 and 160 is illustrated in part B.

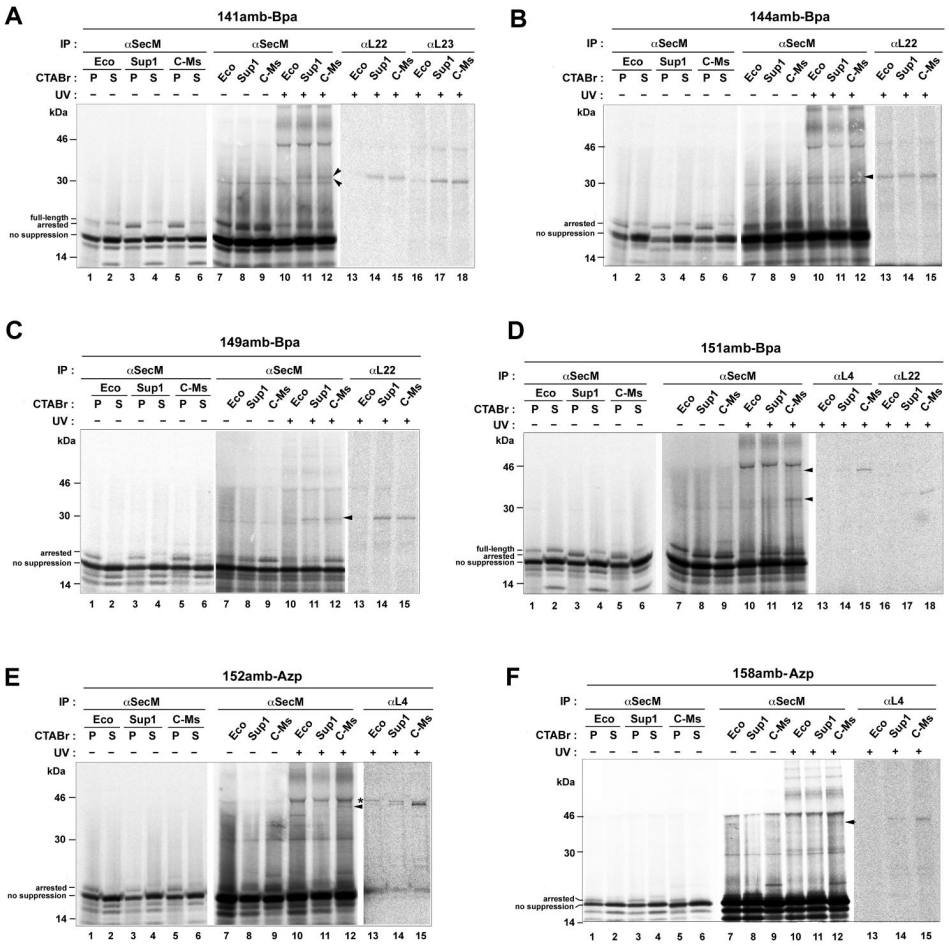


Fig. 6. SecM(Eco), Sup1 and SecM(C-Ms) nascent chains are located in distinct positions relative to ribosomal tunnel proteins

Photoreactive Bpa **(A–D)** or Azp **(E–F)** residues were incorporated into SecM(Eco), Sup1, and SecM(C-Ms) by amber suppression at the indicated position in coupled transcription-translation reactions. A portion of each sample was withdrawn for CTABr fractionation followed by immunoprecipitation with anti-SecM (lanes 1–6). P, CTABr pellet; S, CTABr supernatant. RNCs isolated from the remainder of each sample were subjected to immunoprecipitation with anti-SecM (lanes 7–9) or UV-irradiated and divided into portions that were subjected to immunoprecipitation with anti-SecM (lanes 10–12) or with anti-L4, L22 or L23 antisera (lanes 13–18). Prominent photoadducts are denoted with an arrowhead. In panel E, the asterisk denotes a nonspecific background band.

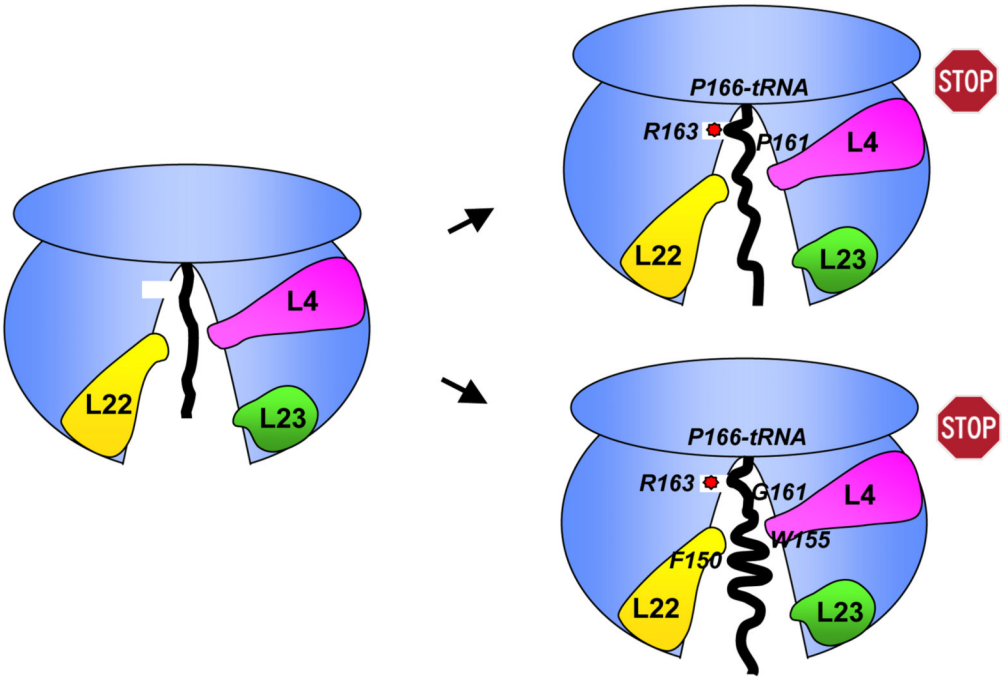


Fig. 7. Model for the recognition of SecM arrest peptides inside the ribosome tunnel

SecM-mediated translation arrest occurs when P166-tRNA is bound to the A site and the conformation adopted by the nascent chain inside the tunnel moves R163 into a precise intra-tunnel location. The presence of a proline at position 161 stabilizes the nascent chain conformation and effectively reduces the arrest peptide to the segment between residues 159–165. The presence of a flexible glycine at position 161, however, requires the stabilization of the nascent chain by residues farther down the tunnel (e.g., W155 and F150).