Signal Sequence Cleavage of Peptidyl-tRNA Prior to Release from the Ribosome and Translocon

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Many secretory polypeptides undergo cleavage of their signal sequence. In this study, we observed and quantitated the presence of a tRNA-bound, ribosome-associated polypeptide subpopulation present in vitro. This subpopulation was accessible to signal peptidase on ribosome-associated polypeptides longer than 114 amino acids. This demonstrates that it is possible for a peptidyl-tRNA species, in the midst of translation, to be processed by the endoplasmic reticulum signal peptidase implying that the peptidase is closely associated with the mammalian translocon.

“Signal sequence” refers to a stretch of amino acids necessary and sufficient to target a polypeptide to a cellular location (1, 2) such as the peroxisomal (3, 4), chloroplast (5, 6), or mitochondrial (7, 8) membrane or lumen, eukaryotic or prokaryotic plasma membranes (9), the Gram-negative bacterial periplasm (10), or extracellular milieu (11–13). Many secretory proteins possess an amino-terminal signal sequence (13). This sequence is a substrate for a family of conserved proteases called type I signal peptidases (SPase I) (2), which cleave the signal sequence before the protein is secreted from the cell (14). Functional assays show that bacterial proteins can be cleaved by peptidyl-tRNA in vitro. There is evidence that the peptidyl-tRNA that have dissociated from their ribosomes (24). Puromycin increases the electrophoretic mobility of ribosome-associated polypeptide subpopulations present in vitro (23, 27). This resulting peptidyl-tRNA contains a tRNA bound to the ribosome (23, 27). This resulting peptidyl-tRNA migrates more slowly on SDS-PAGE and can be quantified when labeled with [*5S]methionine. Concomitantly, the peptidyltransferase of the ribosome may add puromycin to the carboxyl end of the stalled chain thereby releasing the tRNA (28, 29). Puromycin increases the electrophoretic mobility of ribosome-bound, nascent peptidyl-tRNA but has no effect on peptidyl-tRNA that have dissociated from their ribosomes (24).

Several Higher Molecular Mass Bands Are Present in lac FL-1 Trp, translation product—The transcription/translation for beta-lactamase without stop codons, beta-lactamase, was predicted to encode a 31.6-kDa preprotein of 286 amino acids and a 28.9-kDa mature form of 263 amino acids. For both beta-lactamase and beta-lactamase, translation products at 31 kDa (major) and 25 kDa (minor) were observed in the absence of RM (Fig 1A). In the presence of RM (RM +), an additional, major band at 28 kDa was visible (Fig 1A, lanes 2 and 4). The 31-kDa and the 28-kDa bands have been characterized as the full-length polypeptide with and without signal sequence, respectively. The 25-kDa product has not been characterized but...
treated with 2 mM puromycin, both the 44- and 40-kDa bands gained intensity (Fig. 1, lane 4), the percentage of total tRNA-bound, ribosome-associated peptidyl-tRNA was decreased an average of 91 ± 3% by addition of puromycin (Table I).

Truncated Eukaryotic Constructs Show Results Similar to β-Lactamase—To observe whether the signal sequence can be cleaved from polypeptide intermediates of eukaryotic origin, we synthesized several bovine preprolactin and rat κ light chain constructs. Construct pPl 0.75 Pro was predicted to encode a 19.1-kDa preprotein of 172 amino acids and a 15.9-kDa mature protein of 142 amino acids. When pPl 0.75 Pro was translated in the absence of RM, two major bands of ~35 and 20 kDa were observed (Fig. 2A, lanes 1 and 2). In the presence of RM, two additional bands appeared at 32 and 16 kDa (Fig. 2A, lanes 3 and 4). In the presence of RM, 27 ± 6% of the pPl 0.75 Pro (n = 4) tRNA-bound polypeptide species were without a signal sequence, while in the absence of RM 2 ± 3% of the peptidyl-tRNA lacked a signal sequence (paired, two-tailed Student’s t test, p < 0.01) (Table I). Puromycin released an average of 95 ± 5% of tRNA-bound, processed polypeptide chains. We observed lower but similarly patterned results for construct pPl 0.57 Pro (Table I).

The longer IgG κ light chain construct, κLC 0.89 Ser, coded for a 22.5-kDa preprotein of 206 amino acids and a 19.4-kDa mature protein of 177 amino acids. For translation products of both κLC constructs, the results were similar to β-lactamase and preprolactin truncated constructs (Fig. 2B and Table I).

Signal Sequence Cleavage of β-Lactamase tRNA-bound Constructs Is Length-dependent—To test whether length of the polypeptide intermediate affected processing of the signal sequence, we created truncated mRNA constructs encoding various lengths of β-lactamase. When primed with mRNA coding for a polypeptide greater than 114 amino acids, translations in the presence of RM contained a significantly greater amount of processed peptidyl-tRNA than translations in the absence of RM. Translation mixtures primed with truncated β-lactamase constructs coding for less than 132 amino acids did not show a similar, significant difference (Fig. 3 and Table I, paired, two-tailed Student’s t test).

When mRNA constructs encoding more than 114 amino acids of β-lactamase were translated in the presence of RM, the tRNA-bound translation products were without a signal sequence. These translation products, upon puromycin addition, were reduced in excess of 80–90% of their original intensity. Similarly, all constructs (except βPl 0.27) coding for less than 132 amino acids showed release of tRNA-bound, signal sequence present species in excess of 60% upon puromycin addition (Table I).

**DISCUSSION**

Can ER signal peptidase cleave the signal sequence from polypeptide chains still associated with functional ribosomes? To address this question we built on previous experimental paradigms (24, 26, 31, 32) to resolve and quantitate the existence of tRNA-bound, ribosome-associated polypeptide subpopulations present in vitro.

In this study, typically 25% or greater of the polypeptide was tRNA-bound, and of that generally more than 60% was associated with functional ribosomes (Table I). Our results show that the percentage of tRNA-bound, ribosome-associated polypeptide is a variable quantity, specific to, but not uniformly predicted by, the identity of the parental mRNA. The results indicate that in many in vitro translations, and potentially in many in vivo reactions, as much as 85% of the nascent polypeptide is either not tRNA-bound or ribosome-associated. This tRNA-bound, ribosome-associated subpopulation is the only
ER Signal Peptidase Processes Ribosome-bound Peptidyl-tRNA

Quantitative data from in vitro translations of various mRNA constructs

Table I

<table>
<thead>
<tr>
<th>n</th>
<th>Construct</th>
<th>Length (amino acid)</th>
<th>% total polypeptide as tRNA-bound form (±S.D.)</th>
<th>% total tRNA-bound polypeptide without signal sequence (±S.D.)</th>
<th>% tRNA-bound polypeptide (±S.D.)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>RM−</td>
<td>RM+</td>
<td>RM−</td>
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<td>4</td>
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<td>5</td>
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<td>60 ± 8</td>
<td>62 ± 10</td>
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<td>4</td>
<td>βlac 0.75 Pro</td>
<td>217</td>
<td>44 ± 4</td>
<td>23 ± 2</td>
<td>3 ± 4</td>
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<td>177</td>
<td>46 ± 4</td>
<td>20 ± 2</td>
<td>2 ± 3</td>
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<td>4</td>
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<td>143</td>
<td>47 ± 9</td>
<td>43 ± 22</td>
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<tr>
<td>4</td>
<td>βlac 0.46 Ala</td>
<td>132</td>
<td>25 ± 3</td>
<td>18 ± 7</td>
<td>2 ± 3</td>
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<tr>
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<td>114</td>
<td>19 ± 10</td>
<td>12 ± 7</td>
<td>3 ± 5</td>
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<td>3</td>
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<tr>
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<td>17 ± 11</td>
<td>3 ± 6</td>
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<tr>
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<td>49 ± 6</td>
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<tr>
<td>4</td>
<td>sLC 0.89 Ser</td>
<td>206</td>
<td>36 ± 5</td>
<td>60 ± 5</td>
<td>3 ± 4</td>
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<tr>
<td>3</td>
<td>sLC 0.75 Val</td>
<td>181</td>
<td>84 ± 2</td>
<td>79 ± 6</td>
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<td>4</td>
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<td>76 ± 11</td>
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<tr>
<td>3</td>
<td>pPl 0.57 Pro</td>
<td>139</td>
<td>54 ± 11</td>
<td>64 ± 3</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

Without signal sequence, released by puromycin addition. With signal sequence, released by puromycin addition.

Biologically relevant intermediary in protein translation, a factor that must be taken into consideration in all studies.

To assess the activity of ER signal peptidase, we used this system to observe the differences between polypeptide species in translation mixtures containing or lacking RM. Without RM in the translation mixture, the tRNA-bound population was generally homogenous (e.g. Fig. 1B, lane 5). When the same translation was done in the presence of RM, the tRNA-bound population became heterogeneous, with one band similar to the RM–lane representing unprocessed, tRNA-bound polypeptide and one band of generally 1–3 kDa lower apparent molecular mass representing the tRNA-bound polypeptide without its signal sequence (e.g. Fig. 1B, lanes 5 and 7). Therefore, in the RM + translation mixtures, some tRNA-bound polypeptides were processed and some were unprocessed, and a reproducible percentage of these processed constructs were susceptible to puromycin release (Table I). These results demonstrate that tRNA-bound, ribosome-associated polypeptides can be accessed by ER signal peptidase in vitro.

Once it was established that polypeptides still attached to the ribosome could be processed by the ER signal peptidase, we examined how early during the biosynthesis of a polypeptide a signal sequence could be cleaved. We synthesized increasingly shorter β-lactamase mRNA constructs and translated them. Ribosome-associated polypeptides 114 amino acids and shorter could no longer be resolved from their signal sequence cleaved form (Fig. 3, Table I). However, by our puromycin release assay, tRNA-bound polypeptide chains from these shorter constructs were still attached to functional ribosomes (Table I).

Of the 114 amino acids in the longest unprocessed β-lactamase construct, 70 are buried in the translation and translocation machinery of the cell (24, 33–35) and −25 are the signal sequence itself. This allows −40 amino acids to be exposed to the lumen of the ER. We hypothesize that, in this study, these 20–50 amino acids span the distance from the active site of the signal peptidase to the point where the translocon protects the nascent chain from protease. This would necessitate the subcellular location of the ER signal peptidase.
We have previously demonstrated that nascent polypeptides, still attached to their biosynthetic ribosomes in the cytosol, can be modified by the oligosaccharyltransferase in the lumen of the endoplasmic reticulum (24). The analysis of a nascent polypeptide containing both a cleaved signal sequence and glycosylation site would allow us to assay the relative timing of these events and further our in vitro biochemical understanding of the functional organization of the translocon complex.

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REFERENCES