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Tail-extension following the termination codon is critical for release of the nascent chain from membrane-bound ribosomes in a reticulocyte lysate cell-free system

Michiyo Takahara, Haruka Sakaue, Yukiko Onishi, Marifu Yamagishi, Yuichiro Kida, Masao Sakaguchi *

Graduate School of Life Science, University of Hyogo, Kouto, Ako-gun, Hyogo 678-1297, Japan

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Nascent chain release from membrane-bound ribosomes by the termination codon was investigated using a cell-free translation system from rabbit supplemented with rough microsomal membrane vesicles. Chain release was extremely slow when mRNA ended with only the termination codon. Tail extension after the termination codon enhanced the release of the nascent chain. Release reached plateau levels with tail extension of 10 bases. This requirement was observed with all termination codons: TAA, TGA and TAG. Rapid release was also achieved by puromycin even in the absence of the extension. Efficient translation termination cannot be achieved in the presence of only a termination codon on the mRNA. Tail extension might be required for correct positioning of the termination codon in the ribosome and/or efficient recognition by release factors.

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1. Introduction

Cell-free translation systems are utilized for various objectives, such as the production of cytotoxic proteins [1]; incorporation of non-natural amino acids into polypeptide chains [2]; and as experimental systems for protein folding [3], including cotranslational folding [4], post-translational protein localization to mitochondria and peroxisomes [5], and studies of protein conformations [6]. Cell-free systems supplemented with vesicles derived from rough endoplasmic reticulum membrane (RM) provide an ideal tool for the biochemical analysis of cotranslational translocation across the membrane. These systems can reproduce complex processes for membrane insertion of proteins by membrane-bound ribosomes. The RM vesicles are prepared from dog pancreas through differential centrifugation and density fractionation. In this system, newly synthesized polypeptide chains with signal peptides or signal-anchor sequences are cotranslationally targeted to RM vesicles and transported to the lumenal side of the membrane or inserted into the membrane. N-linked glycosylation is also reproduced. Among various cell-free systems, the reticulocyte system provides an efficient system for these processes because it contains various cytosolic factors for protein transport, signal recognition particles, and hsp70 chaperons. Synthesized polypeptide chains are almost completely targeted to and inserted into the RM. The cell-free experiments have revealed various dynamic aspects of nascent polypeptide chains on the RM, e.g., charges of nascent chains moving through the translocation channel tentatively arrest movement [7,8], and hydrophobic segments and positive charges cooperate on the membrane to induce the transmembrane orientation of the protein [9]. In some experiment, we utilized RNA lacking termination codon, by which the synthesized nascent chain is retained in the ribosome peptidyl transferase center (PTC) as peptidyl-tRNA. Using this system, we demonstrated various intermediates of membrane insertion and nascent chain folding [10,11]. Cell-free experiments have also revealed the dynamic interactions between ribosome and protein translocation channels [12,13] and novel modes of membrane protein insertion into the endoplasmic reticulum membrane [14,15].

Membrane-bound ribosomes provide a convenient system for easily monitoring nascent chain release from the ribosome; when a potential glycosylation site is incorporated near the termination codon, the site is accessible to the glycosylation enzyme in the lumen only after release of the polypeptide chain from the ribosome. In our ongoing studies of the function of membrane-bound ribosomes, we found that nascent chain release is regulated not only by the presence of the termination codon but that it also depends on the context of the termination codon. Here we examined the effects of the context of the termination codons on the efficiency of their function in membrane-bound ribosomes.

Abbreviations: PTC, peptidyl transferase center; RM, rough microsomal membranes; RNC, ribosome nascent chain complex.

* Corresponding author. Fax: +81 791 58 0207.
E-mail address: sakag@sci.u-hyogo.ac.jp (M. Sakaguchi).

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2. Materials and methods

2.1. Materials

RM were prepared from dog pancreas and treated with EDTA and then with *Staphylococcus aureus* nuclease (Roche) to remove endogenous mRNAs, as described previously [16]. Puromycin (Sigma) and DNA-manipulating enzymes (Takara and Toyobo) were obtained from the indicated sources.

2.2. Construction of model proteins

A model polypeptide was derived from rat serum albumin as described previously [9]. The cDNA fragment of the model protein was subcloned between the XbaI and ApaI sites of pRcCMV. The cloned DNA fragment contained the Kozak sequence at the 5′-region. The glycosylation sites, Asn^{67}-Ser-Thr and Asn^{180}-Ser-Thr, were included. Several residues around the 2nd site were optimized for efficient glycosylation by the oligosaccharyl transferase in the lumen.

2.3. Cell-free transcription

Template DNA for cell-free transcription was generated by polymerase chain reaction using the following primers. The forward primer had a sequence at the 50-base upstream of the T7-RNA polymerase promoter of pRcCMV: GCAGAGCTCTCTGGCTAACT.

Reverse primers, including the termination codon and various tail-extensions, were used as follows. To generate the ribosome nascent chain complex (RNC), the termination codon and the tail-extensions were omitted. Three nucleotides corresponding to the termination codon, TAA, are underlined, and were changed for other termination codons where indicated.

GGTGTAAGCAGCAGTGCTATT for RNC
GGATATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 13
ATATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 11
ATTTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 9
TTGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 7
TGTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 6
GTATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 5
TATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 4
ATATTAGGTGTAAGCAGCAGTGCTATT for TAA + 3
TATTAGGTGTAAGCAGCAGTGCTATT for TAA + 2
ATTAGGTGTAAGCAGCAGTGCTATT for TAA + 1
TTAGGTGTAAGCAGCAGTGCTATT for TAA + 0

Polymerase chain reaction was performed using KOD-Plus DNA polymerase (Toyobo) according to the supplier's manual. The reaction cycle was as follows: initial denaturing at 95 °C for 2 min; cycle reaction, including denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. The amplified DNA fragment was purified by agarose gel electrophoresis, band excision, and column purification using the DNA Gel band purification system (Qiagen). The transcription mixture of 50 µl included 50 units of T7 RNA Polymerase (Takara), 40 mM Tris–HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, 0.5 mM 4NTPs, RNase Inhibitor (Promega), and 1 µg DNA fragment, and was incubated at 37 °C for 2 h. The RNA products were subjected to phenol/chloroform extraction, then precipitated with ethanol precipitation, dissolved in 20 µl of water, and stored until use at −20 °C.

2.4. Preparation of reticulocyte lysate

The reticulocyte lysate was prepared from male New Zealand White rabbits as previously described [17]. All procedures were performed in accordance with the guidelines established by the University of Hyogo for the care and use of experimental animals. Blood from the phenyl hydrazine-treated rabbits was immediately mixed with heparin solution (Mochida) and transferred to an ice-cold 50-ml centrifuge tube, then centrifuged using a swing rotor centrifuge at 2000*g* at 4 °C for 5 min (3500 rpm, HITACHI, T4SS31 rotor). The supernatant, including the white fluffy layer on the precipitate, was removed and the pellet was resuspended up to a 50-ml total volume with buffer A (0.14 M NaCl, 5 mM KCl, 7.5 mM $Mg[OAc]_2$). The cells were subjected to the same washing step for two more times, then vigorously mixed with an equal volume of ice-cold water for 2 min. The lysed cell-suspension was centrifuged at 20,000*g* (14,000 rpm, HITACHI, R18A rotor) at 4 °C for 10 min. The supernatant was treated with 100 U/ml *S aureus* nuclease in the presence of 1 mM $CaCl_2$ and the reaction was terminated with 2 mM EGTA. The nuclease-treated lysate was divided into 50 µl aliquots, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

2.5. Cell-free translation in reticulocyte lysate

Cell-free translation of in vitro-synthesized mRNAs was performed essentially as described previously [18], except that translation reactions with RM contained castanospermine to prevent heterogeneity of the products due to trimming of the core sugar chain. The translation reaction included 100 mM potassium acetate (KOAc), 1.2 mM magnesium acetate ($Mg[OAc]_2$), 32% reticulocyte lysate, castanospermine (20 µg/ml), and 15.5 kBq/µl [^{35}S]-EXPRESS protein-labeling mix (Perkin Elmer). Translation was performed at 30 °C for the indicated time period in either the absence or presence of RM. The translation reaction mixtures were treated by RNase in sample buffer and subjected to SDS–polyacrylamide gel electrophoresis. Radio-labeled polypeptide chains were visualized with an imaging analyzer (BAS1800, Fuji Film), and quantified using Image Gauge software (Fuji Film). In the presence of RM, the synthesized nascent chains were almost completely targeted to membrane vesicles and glycosylated. The translocation percentage was estimated as the percentage of the diglycosylated product among monoglycosylated and diglycosylated products.

3. Results and discussion

3.1. Function of the termination codon depends on tail-extension

To examine the effect of tail-extension on the termination codon function, we generated two classes of mRNA. One class possessed no tail-extension and the open reading frame ended just at the termination codons (Fig. 1A, +0). Others possessed a 13-base tail-extension (Fig. 1A, +13) downstream of the termination codon. As a control, one mRNA ended at the open reading frame and possessed no termination codon. In this situation, the nascent chain is retained in the ribosome as peptidyl-tRNA and forms the static ribosome-nascent chain complex (RNC) (Fig. 1B, RNC). The model protein consisted of a rat serum albumin backbone of 186 residues, an N-terminal signal peptide, and artificial potential glycosylation sites (Figs. 1A and 2). The glycosylation sites were optimized for the glycosylation reaction. When the model protein is synthesized in the presence of RM, the N-terminal signal peptide initiates translocation of the nascent chain through the translocation channel in the microsomal membranes. The signal peptide is cleaved off during elongation. The upstream glycosylation site (Asn^{67}) is translocated in the earlier phase of synthesis and glycosylated irrespective of the release of the nascent chain from ribosome (Fig. 1B, center). This glycosylation is a decisive indication of translocation initiation. On the other hand, the downstream second glycosylation

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