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Profiling of signal sequence characteristics and requirement of different translocation components

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ABSTRACT

The N-terminal signal sequence (SS) on proteins targeted to the endoplasmic reticulum (ER) is surprisingly diverse in hydrophobicity, in the number of preceding N-terminal residues (N-length), and in charged residues flanking the sequence. However, it remains unclear how these sequences despite their heterogeneity bind to the same site and open the Sec61 translocon. We assessed varying features of SSs and their efficiencies in initiating translocation across the ER by using 5-min radiolabeling in yeast. We found that while hydrophobic SSs with a short N-length efficiently initiated translocation in Sec62 mutant, Sec63 mutant and Sec72 deletion strains, most SSs showed varying degrees of translocation defect in these strains. In particular, Sec71 was required for internal hydrophobic SSs to efficiently initiate translocation. These results suggest that different combinations of Sec62, Sec63, Sec71 and Sec72 dynamically associate with the Sec61 translocon for the optimal binding of incoming SSs of broad characteristics and the initiation of protein translocation *in vivo*.

1. Introduction

In eukaryotes, approximately 30% of proteins enter the endoplasmic reticulum (ER), and their entry into the ER begins the process of protein trafficking along the secretory pathway. A prerequisite to enter this pathway is the presence of a signal sequence (SS) [1]. Generally it is said that N-terminally positioned SSs or so-called signal peptides (SPs) are cleaved by signal peptidase, whereas more internally located SSs, conventionally referred to as signal-anchored (SA) sequences, are not cleaved by signal peptidase. However, SPs and SAs harbor similar sequence contexts and the presence of a signal peptidase cleavage site is not the sole determinant for whether an SS is cleaved or not [2], which makes it difficult to distinguish SPs and SAs. When a hydrophobic SS of a nascent chain emerges from the ribosome exit tunnel, it is recognized by the signal recognition particle (SRP) and guided to the ER membrane [3–5]. Less hydrophobic SSs are not recognized by SRP but are targeted to the ER by cytosolic chaperones that bind the mature part of the nascent chain [6–8]. After reaching the ER, SSs initiate protein translocation through the Sec61/α/Y translocon, a main pore-forming channel in the ER membrane [9–17].

Recently, the SRP-independent targeting (SND) pathway has been suggested to target and translocate proteins containing a transmembrane domain (TMD) located in the middle of the protein or toward the C-terminus of the protein, whereas the guided entry of tail-anchored proteins (GET) pathway serves to translocate proteins harboring a C-terminal tail-anchored sequence [18–20].

SSs consist of three parts: the hydrophobic core (H) and the flanking N- and C-terminal regions. While the basic structure of SSs is relatively simple, great diversity lies in the length and hydrophobicity of the H-region and the length and the charge distribution of the N- and C-terminal flanking regions [21–23]. Numerous studies have revealed that these features influence the orientation of SSs at the Sec translocon [24–35]. However, how various types of SSs bind to the Sec translocon and open the channel for protein translocation is poorly defined.

The Sec62/63 complex is composed of Sec62, Sec63, Sec71 and Sec72 and associates with the Sec61 channel for post-translational translocation [36–38]. However, Sec62 and Sec63 are also shown to mediate SRP-dependent and co-translational translocation of SA and membrane proteins [39–43]. Further, mammalian homologs of Sec62 and Sec63 are found to be associated with ribosomes [44]. Beyond the role in post-translational translocation, these studies implicate an expanded function of the Sec62/63 complex in co-translational translocation. A proximity-specific ribosome profiling study suggests that neither SRP dependence nor independence confer a strict preference of

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proteins to co- or post-translational translocation [45]. Thus, increasing evidence points to a more intricate interplay between SSs, SRP, and the Sec62/63 complex than is previously thought, and we undertook to investigate their relationships in the context of varying features of SSs.

First, we assessed the heterogeneity of SSs in terms of their hydrophobicity and the length of the N-terminus preceding the SS (N-length) and determined how these characteristics affect SS efficiency. Our data show that increasing N-length impaired the function of moderately hydrophobic SSs but not that of highly hydrophobic SSs, indicating that short N-length is an important feature for moderately hydrophobic SSs. Our analysis of natural proteins shows that moderately hydrophobic SSs indeed have a shorter N-length than hydrophobic SSs. Next, we assessed how the diversity in SSs function in SRP, Sec62, Sec63, Sec71 and Sec72 defective yeast strains by metabolic labeling. We found that moderately hydrophobic SSs with a short N-length required all four components of the Sec62/63 complex for efficient translocation initiation whereas more hydrophobic SSs with the same N-length were independent of Sec62, Sec63 and Sec72, indicating that the latter are more translocation efficient SSs. Surprisingly, Sec71 was critical for efficient translocation initiation of hydrophobic SSs, especially internal ones. Overexpression of Sec62, Sec63 or Sec72 in *sec71*Δ did not complement translocation defects of Sec71-dependent substrates, implying a distinctive role of Sec71 in sorting internal hydrophobic SSs. These results suggest that different targeting and translocation components decode and sort signal sequence characteristics for proper initiation of protein translocation *in vivo*.

2. Materials and methods

2.1. Yeast strains

The *Saccharomyces cerevisiae* haploid W303-1α (*MATα*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*) was used as WT strain [46]. Construction of the *sec62 35DDD* mutant strain (*MATα*, *sec62Δ::HIS3*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3, p415 1 kb upstream + sec62 35DDD*) was described in [39]. *sec63 A179T* strain was generated as follows. W303-1α was transformed with pRS416 1 kb upstream + *SEC63*. Genomic *SEC63* copy was deleted by homologous recombination of transformed *HIS3* cassette amplified from pCgH [47]. The resultant cells were transformed with pRS415 1 kb upstream + *sec63 A179T*. pRS416 1 kb upstream + *SEC63* was removed by FOA selection. The genomic ORF of *SEC71* or *SEC72* in W303-1α were substituted by *HIS3* marker amplified from pCgH vector by homologous recombination, and *sec71*Δ (*MATα*, *sec71Δ::HIS3*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*) and *sec72*Δ (*MATα*, *sec72Δ::HIS3*, *ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*) strains were generated. For overexpression of Sec components, pRS425GPD overexpression vector carrying *SEC62-FLAG*, *SEC63-FLAG*, *SEC72-FLAG*, or *SEC71-3XHA* was transformed into either the *sec71Δ* strain or the genomic Sec71 HA-tagged strain (*MATα*, *sec63Δ::HIS3*, *sec71::HA-G418 ade2*, *can1*, *his3*, *leu2*, *trp1*, *ura3*, pURA-SEC63). For endogenous expression in *sec71Δ* strain, pRS415 1 kb upstream + *SEC62-FLAG*, *SEC63-FLAG* or *SEC72-FLAG* was transformed into the *sec71*Δ strain.

2.2. Construction of plasmids

To generate a vector encoding CPY, a pair of oligonucleotide primers containing 30 bases complementing the upstream and downstream sequences of the *SmaI* site in pRS424GPDHA vector [40] and annealing sequences for 5′ and 3′ end of the *PRC1* (*CPY*) gene were synthesized. The *PRC1* (*CPY*) ORF was amplified from genomic DNA of W303-1α by using these primers, and the resulting PCR products were transformed into W303-1α with a *SmaI* digested pRS424GPDHA vector for homologous recombination as described in [48]. Using the resultant plasmid (pRS424GPD*CPYHA*) as template, a *SmaI* site was introduced between the 2nd and 3rd residues of *CPY* by site-directed mutagenesis following manufacture's protocols (Toyobo, Japan). The N-terminal sequences of Dap2 and Sec71 were amplified from genomic DNA and inserted into a *SmaI* digested pRS424GPD*CPYHA* vector to generate pRS424GPD*D27CPY(1.9)HA* and pRS424GPD*S30CPY(1.5)HA* vectors, respectively. All other CPY variant plasmids were created by site-directed mutagenesis from these two vectors. To construct overexpression vectors containing *SEC62*, *SEC63* or *SEC72*, the corresponding ORFs were cloned under a GPD promoter in pRS425 plasmid by homologous recombination or using the Gibson assembly kit following the manufacture's protocol. *FLAG* tag was introduced by site-directed mutagenesis. Sec71 was cloned into pRS425GPDHA vector by homologous recombination.

2.3. Western blot analysis

To check overexpression of Sec components, 1.6 OD_{600} unit cells overexpressing each Sec component and a model protein were harvested after an overnight incubation at 30 °C. Cells were mixed with 60 μl of SDS sample buffer (50 mM Tris-HCl, pH 7.5, 5% SDS, 5% glycerol, 50 mM EDTA, pH 8, 50 mM DTT, 1 × protease inhibitor cocktail (Quartett, Germany, PPI1015), 1 mM PMSF) and boiled at 95 °C for 5 min. Samples were subjected to SDS-PAGE and Western blotting.

2.4. Pulse-labeling and immunoprecipitation

For W303-1α, *sec62 35DDD, sec71*Δ and *sec72*Δ strains, protein radiolabeling with [^{35}S]Met and immunoprecipitation were carried out as described in [40]. For *sec63 A179T*, cells were starved for 15 min at a non-permissive temperature (37 °C). For *sec65-1*, cells were shifted to 37 °C for an additional 30 min incubation prior to the 15min starvation at 37 °C. Immunoprecipitated proteins were prepared with 60 μl of sample buffer, subjected to SDS-PAGE and autoradiography, visualized with Typhoon™ FLA 7000, and then quantified with the Multi-GaugeV3.0 software.

2.5. Tunicamycin treatment

Prior to radiolabeling with [^{35}S]Met, 1.5 OD_{600} unit cells per reaction were harvested and pre-incubated in 1 ml of –Met synthetic defined medium with tunicamycin (Sigma, 100 μg·ml) for 15 min at 30 °C. After pre-incubation, cells were pelleted down and resuspended in 150 μl of –Met synthetic defined medium containing tunicamycin to maintain the same concentration throughout radiolabeling time.

2.6. Carbonate extraction

5–10 OD_{600} units of cells were grown overnight and harvested. Cells were resuspended in 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 100 mM NaCl, 300 mM sorbitol, 1 mM PMSF, 1 × protease inhibitor) and vortexed with glass beads for 10 min at 4 °C. The lysate was transferred to a new pre-chilled tube after a quick spin down. The remaining glass beads were washed with 200 μl of lysis buffer, the lysate of which was added to the final lysate. Unbroken cells were removed by centrifugation for 30 s at 14,000 RPM on a desk-top centrifuge and the lysate was transferred to two pre-chilled tubes, one for the 'total' fraction and the other for carbonate extraction. For carbonate extraction, 300 μl of 0.1 M Na_2CO_3 (pH 11.5) was added to the lysate, and the reaction was incubated for 30 min on ice prior to centrifugation for 20 min at 14,000 RPM. The resulting supernatant was saved as the 'supernatant' fraction in another E-tube, and the pellet was resuspended and washed with 200 μl of Na_2CO_3 and centrifuged for 20 min at 14,000 RPM. After removal of Na_2CO_3 solution from the pellet, 'total', 'supernatant' and 'pellet' fractions were incubated with TCA (final concentration 12.5%) for 30 min on ice. Precipitated proteins were washed with 100% acetone, saved for sample preparation with 60 μl of sample buffer and analyzed by SDS-PAGE and Western blotting.