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Cleavage of a tail-anchored protein by signal peptidase

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Abstract Tail-anchored proteins are post-translationally targeted and inserted into the endoplasmic reticulum membrane. They do not use the co-translational signal-recognition particle (SRP)-dependent pathway, but rather utilize an ill-defined, ATP-dependent mechanism. Here, we show that a tail-anchored protein can be cleaved by signal peptidase and that the sequence requirements for efficient cleavage seem to be the same as for cleavage of co-translationally targeted SRP-dependent proteins.

Key words: Membrane protein; Signal peptide; Signal peptidase; Tail-anchor

1. Introduction

The 'tail-anchored' proteins constitute an interesting subclass of the integral membrane proteins. These proteins do not have an N-terminal signal sequence and are targeted post-translationally to the endoplasmic reticulum (ER) membrane by virtue of a C-terminal hydrophobic segment [1]. The C-terminal tail downstream of the hydrophobic segment is translocated across the ER membrane by an ill-defined, ATP-dependent process [2–6], leaving the bulk of the protein facing the cytosol.

Previous work has shown that membrane integration of the tail-anchored protein synaptobrevin (Syb) can be effected by poly-Leu segments of 12–18 residues in place of the wild-type transmembrane domain [7]. Here we show that signal peptidase, an enzyme complex that associates with and can be crosslinked to the Sec61 translocon [8], can efficiently cleave Syb constructs with transmembrane segments composed of a poly-Leu stretch followed by a canonical signal peptidase cleavage cassette, but only if the poly-Leu stretch is shorter than ~16 residues. This maximal length is very similar to what we have previously found for signal peptidase cleavage of a poly-Leu signal sequence in a signal-recognition particle (SRP)-dependent protein [9], strongly suggesting that proteins utilizing the tail-anchored assembly pathway and those targeted via the SRP pathway have access to the signal peptidase in the same fashion.

2. Materials and methods

2.1. Enzymes and chemicals

Unless otherwise stated, all enzymes, plasmid pGEM3βG1, and rabbit reticulocyte lysate were from Promega (Madison, WI, USA) or New England Biolabs (Boston, MA, USA). T7 DNA polymerase, [^{35}S]Met, ^{14}C-methylated marker proteins, ribonucleotides, deoxyribonucleotides, dideoxyribonucleotides, and the cap analogs m7G(5′)ppp(5′)G were from Amersham-Pharmacia Biotech (Uppsala, Sweden, and Piscataway, NJ, USA). Dog pancreas rough microsomes (RMs) were prepared as described in [10]. The competitive glycosylation inhibitor peptide benzoyl-Asn-Leu-Thr-methylamide was from Quality Controlled Biochemicals (Hopkinton, MA, USA). The signal peptidase inhibitor *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was from Sigma Chemical (St. Louis, MO, USA).

2.2. DNA manipulations

Site-specific mutagenesis was performed using the Quick Change Site Directed Mutagenesis kit from Stratagene. All mutants were confirmed by sequencing of plasmid DNA. All cloning steps were done according to standard procedures.

2.3. Expression plasmids

Syb2-L_n-CC constructs were made from the previously described Syb2-17L-G13 construct and were cloned into the pGEM3βG1 plasmid behind the T7 promoter as a *Pst*I–*Bam*HI fragment as described in [7]. The signal peptidase cleavage cassette was introduced by ligating double-stranded oligonucleotides between the *Spe*I and *Nde*I sites in the Syb2-17L-G13 construct. The amino acid sequence ...LVQQQYV... at the C-terminal end of the 17L stretch was changed to ...LVPSAQA↓AYV... (the intended signal peptidase cleavage site is indicated by the arrow) to make the Syb-L_{17}-CC construct. The length of the poly-Leu segment in Syb-L_{17}-CC was changed by PCR mutagenesis to make the full series of Syb-L_n-CC constructs. To make Syb-L_{15}-CC(Q_{-1}), the signal peptidase cleavage cassette ...LVPSAQA↓AYV... was changed to ...LVPSAQQ↓AYV... in Syb-L_{15}-CC. A version of Syb-L_{15}-CC truncated after the intended signal peptidase cleavage site was made by introducing two stop codons (TGA TAG) immediately downstream of the ...LVPSAQA sequence.

2.4. Expression in vitro

DNA template for in vitro transcription of full-length Syb mRNA was prepared by transcription of the Syb-pGEM3βG1 plasmid with T7 RNA polymerase for 1 h at 37°C. The transcription mixture was as follows: 1–5 μg DNA template, 5 μl 10× T7 buffer (400 mM Tris–HCl, pH 7.9, 60 mM $MgCl_2$), 5 μl bovine serum albumin (1 μg/μl), 5 μl m7G(5′)ppp(5′)G (10 mM), 5 μl dithiothreitol (50 mM), 5 μl rNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP), 18.5 μl H_2O, 1.5 μl RNase inhibitor (40 U/μl), and 2 μl T7 RNA polymerase (20 U/μl) in a total volume of 50 μl. Translation of 1 μl Syb mRNA in 9 μl nuclease-treated reticulocyte lysate, 1 μl RNase inhibitor (40 U/μl), 1 μl [^{35}S]Met (10 μCi/μl), 1 μl amino acids mix (1 mM of each amino acid except Met), 1 μl RM (four equivalents) was performed as described in [11] at 30°C for 1 h. After translation, membranes were sedimented through a sucrose cushion in a Beckman airfuge at 4°C for 5 min at 20 psi, and the supernatant and pellet fractions were separated.

For post-translational translocation of Syb-L_n-CC constructs, the incubation was first performed without microsomes at 30°C for 30 min.

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Microsomes were then added and the incubation was continued for another 30 min. When relevant, apyrase was added to a final concentration of 50 mU/μl [12] and the translation mix was incubated at 30°C for 10 min before the addition of microsomes.

Proteins were analyzed by SDS–PAGE and gels were visualized on a Bio-Rad Molecular Imager FX using the Quantity One Quantitation software.

3. Results

We have previously shown that the tail-anchored protein Syb can be integrated into microsomal membranes when the wild-type C-terminal hydrophobic segment is replaced by a poly-Leu segment that is at least 12 residues long [7]. In an attempt to study processing by signal peptidase of such Syb-L_n constructs (where *n* denotes the number of consecutive Leu residues), we engineered a canonical signal peptidase cleavage cassette ...L_nVPSAQA↓AYV... at the C-terminal end of the poly-Leu stretch, Fig. 1. These Syb-L_n-CC constructs were expressed in vitro in the presence of dog pancreas RM and were analyzed by SDS–PAGE. An acceptor site for N-linked glycosylation (underlined in Fig. 1) was included 21 residues downstream of the hydrophobic segment to serve as a convenient marker for translocation of the C-terminal tail into the lumen of the microsomes.

To demonstrate that the integration of the Syb-L_n-CC constructs into the membrane is post-translational and ATP-dependent, the Syb-L_{18}-CC construct was expressed with microsomes added post-translationally, Fig. 2A. Translocation of the C-terminal tail was readily apparent from the glycosylation of the C-terminal acceptor site (lanes 1, 3); addition of the competitive glycosylation peptide inhibitor benzoyl-Asn-Leu-Thr-methylamide prevented modification of the C-tail (lanes 5, 6). When apyrase was added together with the microsomes in order to deplete ATP from the reaction mixture, no translocation was observed (lane 4). C-tail translocation was somewhat more efficient when RMs were present from the start of the translation reaction (lane 2), presumably because this minimizes aggregation of the Syb product. Similar results were obtained with the two other constructs tested in this way, Syb-L_{13}-CC and Syb-L_{15}-CC (data not shown).

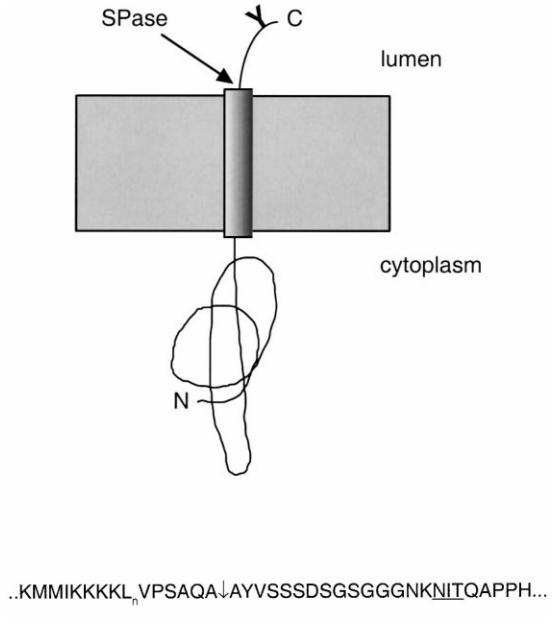

Fig. 1. The Syb-L_n-CC series of constructs. The wild-type transmembrane segment has been replaced by a poly-Leu sequence ending with a signal peptidase cleavage cassette (the intended cleavage site is indicated by the arrow), and the C-terminal tail has been replaced by a stretch of polar amino acids including an acceptor site for N-linked glycosylation (indicated by **Y** in the cartoon and underlined in the amino acid sequence).

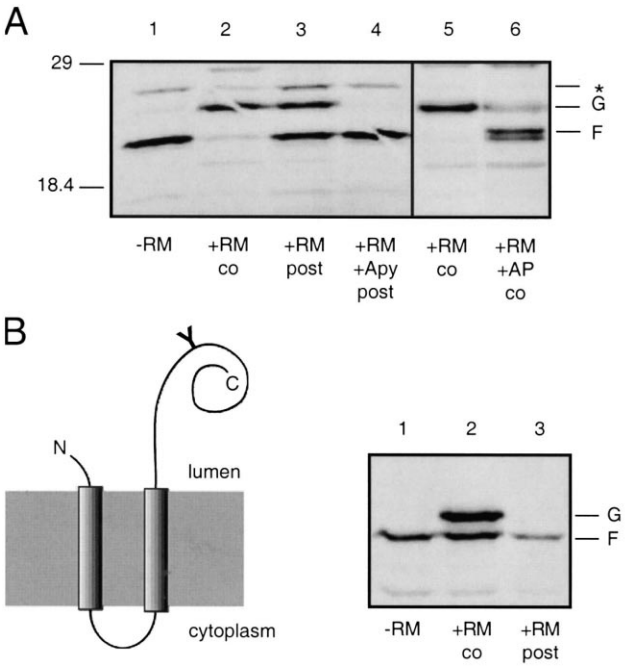

Fig. 2. Post-translational translocation of the Syb-L_{18}-CC C-tail across the microsomal membrane. A: Syb-L_{18}-CC was translated in the absence (lanes 1, 3–4) or presence (lanes 2, 5, 6) of RM. In lanes 3 and 4, RMs were added post-translationally (after 30 min) and incubation was continued for another 30 min; in lane 4 apyrase was included to remove ATP from the reaction mixture. In lane 6, the competitive glycosylation inhibitor benzoyl-Asn-Leu-Thr-methylamide was included during translation. F: Full-length, unglycosylated protein, G: full-length, glycosylated form. A background band present both in the absence and presence of RMs is indicated by an asterisk. B: The SRP-dependent protein leader peptidase (left) was translated in the absence of RMs (lane 1), in the presence of RMs (lane 2), and with RMs added post-translationally (lane 3). F: Full-length, unglycosylated protein, G: full-length protein that has been glycosylated on Asn^{214} (indicated by **Y** in the cartoon) in the large C-terminal domain, cf. [14].

As a comparison, the SRP-dependent protein leader peptidase was translated under the same conditions, Fig. 2B. A glycosylation site in the lumenal C-terminal domain served as a convenient marker for proper translocation. As expected, co- but not post-translational translocation was seen in this case (compare lanes 2 and 3).

Having established that Syb-L_n-CC constructs were inserted into the microsomes by the post-translational tail-anchored pathway, we next expressed a series of constructs where the length of the hydrophobic transmembrane segment was varied from 18 down to 13 residues, Fig. 3A. The full-length, glycosylated protein (G) was only seen for constructs with $n \geq 15$, whereas constructs with shorter hydrophobic segments gave rise to products of lower molecular mass (C1, C2) than the unglycosylated full-length protein (F). Only the G and C1 products were efficiently pelleted in the membrane fraction (lanes 7–12). As demonstrated in Fig. 3B, the main C1-prod-

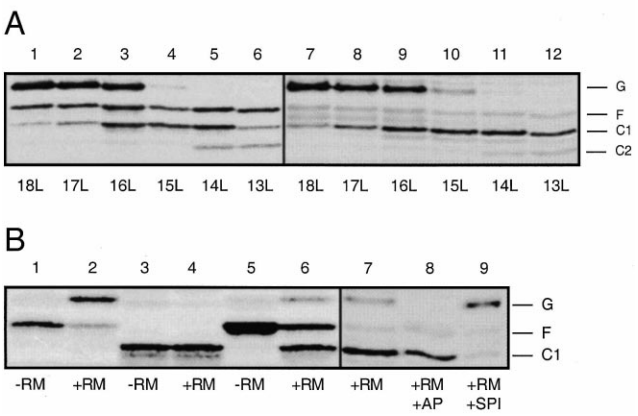

Fig. 3. The C-terminal tail in the Syb-L_n-CC constructs is removed by signal peptidase when $n \leq 16$. A: The indicated constructs were translated in the presence of RM. Total samples are shown in lanes 1–6 and the membrane pellets are shown in lanes 7–12. F: Full-length, unglycosylated proteins, G: full-length, glycosylated proteins, C1: proteins cleaved by signal peptidase (these are unglycosylated since the glycosylation site is in the C-terminal tail, cf. Fig. 1), C2: a second cleavage product of unknown origin. B: Syb-L_{15}-CC(Q_{-1}) (lanes 1, 2), Syb-L_{15}-CC truncated at the intended signal peptidase cleavage site (lanes 3, 4), and Syb-L_{15}-CC (lanes 5–9) were translated in the absence (–) or presence (+) of RM either with no additions (lanes 1–7), with addition of the competitive glycosylation inhibitor peptide benzoyl-Asn-Leu-Thr-methylamide (lane 8), or with the addition of the signal peptidase inhibitor *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (lane 9). Total samples are shown in lanes 1–6 and membrane pellet fractions are shown in lanes 7–9.

uct in Syb-L_{15}-CC co-migrated with a version of Syb-L_{15}-CC that was truncated at the intended signal peptidase cleavage site (lanes 3, 4), and the formation of C1 was inhibited both by a $Ala^{-1} \rightarrow Gln$ mutation at the signal peptidase cleavage site (lanes 1, 2) and by the signal peptidase inhibitor *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (lanes 7–9; this inhibitor has been shown to inhibit microsomal signal peptidase in vitro – M. Lively, personal communication). C1 therefore represents a Syb derivative that has been shortened by signal peptidase-mediated cleavage of its lumenally exposed C-terminal tail. The F product presumably represents full-length proteins that have not been properly targeted to the microsomes, cf. Fig. 2A. The C2 product may represent molecules that have initially been inserted into the membrane and then released into the supernatant after cleavage in or just before the hydrophobic C-terminal segment. We have not yet established the identity of the C2 fragment; it may result from processing of the poly-Leu segment by the recently identified signal peptide peptidase in the ER [13].

4. Discussion

We have analyzed a series of constructs derived from the tail-anchored protein Syb. All constructs have a C-terminal hydrophobic segment composed of one valine and between 13 and 18 leucines, followed by a canonical signal peptidase cleavage cassette (Fig. 1). Just as observed for wild-type Syb [2], these constructs can be inserted post-translationally into microsomal membranes in vitro and their C-terminal tail can be translocated into the microsomal lumen in an ATP-dependent reaction (Fig. 2). Their mode of targeting and insertion into the ER membrane is thus distinct from the co-translational SRP-dependent insertion pathway [1], yet, surprisingly, we observe efficient cleavage by signal peptidase of the shorter constructs (Fig. 3).

Interestingly, the maximal length of the hydrophobic poly-Leu stretch that allows efficient cleavage is 16 residues (15 L plus one V), which is close to the maximal length (16 L plus one V) determined previously for signal peptidase cleavage of poly-Leu signal-anchor sequences in a co-translationally targeted protein [9]. Thus, although integrated into the microsomal membrane by distinctly different mechanisms, proteins assembled through the co-translational SRP pathway and proteins utilizing the post-translational tail-anchored pathway both end up being accessible for cleavage by the signal peptidase enzyme. Each polypeptide therefore must gain access to and be recognized by the signal peptidase active site. Given that the signal peptidase complex can be crosslinked to and is thought to be associated with the Sec61 complex [8], this means either that the active site in a translocon-bound signal peptidase can be approached by a tail-anchored protein diffusing in the membrane, that some signal peptidase complexes are found free in the bilayer, or that tail-anchored proteins also utilize the Sec61p translocon for membrane insertion even though they are not targeted by SRP. The recent finding that a newly synthesized tail-anchored protein can be crosslinked to components of the Sec61p translocon (S. High, personal communication) suggests that the last possibility may be the most likely. More work will be required to provide definitive evidence for this however.

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