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Sec61α and TRAM are sequentially adjacent to a nascent viral membrane protein during its ER integration

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KEYWORDS

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SUMMARY

Cotranslational integration of a nascent viral membrane protein into the endoplasmic reticulum membrane takes place via the translocon. We have been studying the early stages of the integration of a double-spanning plant viral movement protein to gain insights into how viral membrane proteins are transferred from the hydrophilic interior of the translocon into the hydrophobic environment of the bilayer, where the transmembrane (TM) segments of the viral proteins can diffuse freely. Photocrosslinking experiments reveal that this integration involves the sequential passage of the TM segments past Sec61α and TRAM. Each TM segment is first adjacent to Sec61α and subsequently is adjacent to TRAM. TRAM crosslinking extends for a long period during nascent chain biogenesis. In addition, the replacement of the first viral TM segment with a non-viral TM sequence still yields nascent chain photoadducts with TRAM. TRAM therefore appears to be involved in viral membrane protein integration, and nascent chain recognition by TRAM does not appear to rely solely on the TM domains.

ABBREVIATIONS

ER: endoplasmic reticulum; IP: immunoprecipitation; Lep: leader peptidase; RNC: ribosome-nascent chain complex; SDS-PAGE: sodium dodecylsulfate polyacryamide-gel electrophoresis; TM: transmembrane; TRAM: translocating chain-associating membrane protein.

The vast majority of membrane proteins emerge from the ribosome and are inserted into the membrane through a protein-conducting channel in a process termed 'cotranslational' integration [1]. This channel, the so-called translocon, is evolutionarily conserved across kingdoms and is the same translocation machinery that is used by the secretory proteins. The translocon is therefore a two-way gate that receives elongating nascent polypeptide sequences from the ribosome and directs hydrophilic protein regions across or through the membrane and hydrophobic transmembrane (TM) segments into the lipid bilayer laterally.

The translocon is composed of a Sec61 complex in eukaryotes and a SecYEG complex in bacteria and archaea. The heterotrimeric mammalian Sec61 complex contains Sec61α, Sec61β and Sec61γ subunits, and was first shown to be involved in the insertion of nascent membrane proteins by using a photocrosslinking approach [2; 3] that identified proteins adjacent to the membrane-inserting nascent chains. One additional component of the mammalian translocon, the translocation-associated membrane protein (TRAM), was also found to be required for the integration of some TM segments [4; 5]. Because integration activity can be successfully reproduced by proteoliposomes reconstituted with pure lipids and only these four proteins [6], the heterotrimeric Sec61 and TRAM are considered to be the core components of the mammalian translocon for the integration of membrane proteins.

Many viruses express membrane proteins, and very frequently these proteins mediate essential tasks like membrane fusion or genome transport.

The functional activities of these proteins require that they are targeted, inserted and oriented correctly in infected cell membranes. As a result, viral proteins typically mimic cellular protein signals in order to interact with the cellular machineries that accomplish each of these complex aspects of integration. Importantly, loss or modification of these signals can influence virus infectivity and pathogenesis. In the case of plant viruses, local spreading throughout their hosts can be accomplished by a number of pathways, the most common being cell-to-cell movement through plasmodesmata, the ER connections between adjacent cells. This movement requires the function of virus-encoded movement proteins. Functions assigned to these proteins include nucleic acid binding, targeting to the ER, and modification of the size exclusion limit of the plasmodesmata (recently reviewed in [7]). It is now clear that several of these movement proteins integrate into the ER membranes [8; 9; 10; 11].

While it is generally assumed that most viral membrane proteins exploit the host ER components, the mechanism of viral membrane protein integration has not been explored in detail. Interestingly, however, the TRAM requirement is not universal, since some polypeptides can be integrated in a reconstituted system that lacks TRAM [4; 6]. Yet two different types of viral-encoded proteins, envelope proteins of the baculovirus occlusion-derived virus and a movement protein from plant virus, are targeted to the ER membrane by the signal recognition particle and inserted in proximity to Sec61α and TRAM [12; 13].

To dissect the viral membrane protein integration process, we have used a photocrosslinking approach. The normally transient interactions that occur during membrane protein insertion were stabilized by generating integration intermediates of the nascent chains. By using artificially truncated mRNAs lacking a stop codon, a ribosome translates to the end of an mRNA and then halts, forming a stable ribosome/nascent chain complex (RNC) [14]. The environment of a nascent viral protein at different stages during its integration into the ER was then examined by using different lengths of truncated mRNAs and hence nascent chains.

Plant viral membrane protein p9 is sequentially adjacent to Sec61α and TRAM

We have previously shown that p9, a movement protein from the carnation mottled virus, is a double-spanning membrane protein with both the N- and C-terminus facing the cytosol [8]. More recently, we have demonstrated that the protein integrates into the ER membrane cotranslationally through the Sec61 translocon [13]. But in contrast to what has been observed for other double-spanning proteins both in mammalian and prokaryotic systems [15; 16], p9 remained adjacent to Sec61α until translation was terminated. In addition, p9-derived nascent chains were also found adjacent to TRAM. In these studies, the proximity of the nascent chain to translocon proteins was assessed directly by positioning a photoreactive probe in the TM region where the probe would be expected to photocrosslink to nearby integration machinery upon illumination with UV light [17; 18].

Radiolabeled, fully-assembled integration intermediates for such experiments can be prepared *in vitro* by translating truncated mRNAs in the presence of [^{35}S]Met. A photoreactive probe can then be selectively incorporated into the nascent polypeptide by including in the translation reaction a modified amber suppressor aminoacyl-tRNA (εANB-Lys-tRNA^{amb}) that recognizes and translates an amber stop codon positioned at the desired location in the truncated mRNA sequence [5; 12; 13; 19]. In the experiments reported here, an amber stop codon was substituted roughly in the middle of each TM sequence, at codons 15 and 49 of p9 (p9St15 and p9St49, respectively).

To examine p9 nascent chain proximity to TRAM, integration intermediates containing nascent p9 chains of increasing length (Fig. 1) were prepared in the presence of microsomes to generate integration intermediates. But since native p9 is a small protein with only 87 amino acids, it was necessary to elongate its C-terminal domain to examine RNCs with long nascent chains and thereby trap the C-terminal end of native p9 in the RNC-translocon complex by preventing termination of protein synthesis and the release of p9 from the translocon into the lipid phase. To accomplish this, the extramembraneous P2 domain of the *Escherichia coli* leader peptidase (Lep) was fused to the C-terminal end of p9 (see reference [13] for cloning details), and the resulting plasmid was used to generate DNA fragments of different lengths by PCR using 3' primers that anneal at selected positions (Fig. 1). These fragments were then transcribed to prepare the truncated mRNA of different lengths.

Photoactivatable probes were incorporated by the translation of truncated mRNAs with amber codons at position 15 (p9St15, Fig. 1b) or 49 (p9St49, Fig. 1d) in the presence of εANB-Lys-tRNA^{amb}. After photolysis, the membranes were sedimented to enrich for membrane-integrated material, and the extent of photocrosslinking of each p9 derivative to TRAM was determined by immunoprecipitation (IP) using affinity-purified antibodies to TRAM. As shown in Fig. 1b, the probe in TM1 (p9St15) reacts covalently with TRAM at all nascent chain lengths tested. These data also reveal that some TM1 segments remain adjacent to TRAM long after TM2 reaches the pore.

In contrast, when the probe is located at position 49 (p9St49) in TM2, a nascent chain of at least 100 residues is required to observe photoadducts with TRAM (Fig. 1d). This delayed crosslinking nicely matches the delayed incorporation of the probe into p9 and thus the translocon. As with the TM1 probe, once TM2 photoadducts are detected, the TM segment remains adjacent to TRAM for longer nascent chains (Fig. 1d, lanes 5-9).

A quantitative comparison of the relative amounts of photoadducts containing Sec61α and TRAM was made by splitting each sample and then immunoprecipitating in parallel one half with an affinity-purified antiserum specific for Sec61α [13] and the other half with an affinity-purified antiserum for TRAM. When the probe was positioned in TM1, fewer crosslinks were formed to TRAM than to Sec61α, but the number of TRAM photoadducts increased with increasing RNC lengths (Fig. 1c). This effect was even clearer when the probe was positioned in TM2. As shown

previously for a probe located in TM2, RNCs longer than 80 residues strongly crosslinked to Sec61α ([13], Fig 3C), whilst at least 100 residues were needed to detect photoadducts with TRAM (Fig 1d). These results suggest an orderly transition in the integration process of viral membrane protein TM segments from Sec61α to TRAM as they move laterally through the translocon and into the bilayer, with the two TM segments remaining adjacent to both Sec61α and TRAM even when there are 135 (p9St15) and 91 (p9St49) residues between the probe and the tRNA (for 140 RNC length), which are clearly sufficient to allow the TM sequences to diffuse away from the translocon and into the bilayer. Interestingly, a similar scenario was depicted for a polytopic membrane protein in *E. coli* where the first pair of helices of a RNC remained close to YidC (a putative prokaryotic functional analogue to TRAM) even in a situation where a long cytoplasmic region after the second TM segment would create ample freedom for the first pair of helices to insert into the membrane lipids [20].

Influence of the loop in the integration mechanism

We have previously shown that the loop interconnecting the TM segments of p9 greatly influences TM1 crosslinking to Sec61α [13]. In fact, swapping the short loop that connects TM1 and TM2 of p9 (14 residues) by the P1 domain from Lep (42 residues) results in a transitory contact of both TM1 and TM2 segments to Sec61α (Fig. 2b and 2e). When the probe was positioned in TM1 (p9P1St15), RNCs containing nascent chains 78 and 88 amino acids in length crosslinked to Sec61α (Fig. 2b). For all chain lengths

longer than 98 residues, little or no photocrosslinking to Sec61α was observed (Fig. 2b, lanes 4-10), indicating that TM1 had moved away from Sec61α. With the probe located in TM2 (p9P1St77), adducts with Sec61α were observed for RNC between 108 and 143 residues (Fig. 2e, lanes 13-17), but then TM2 also lost contact with Sec61α as the nascent chain lengthened before translation terminated (Fig. 2e, lanes 18 and 19).

In order to discern whether the protein partitioned to the lipid phase or remained in a proteinaceus environment before ribosome release, the photolysed RNCs were also immunoprecipitated with anti-TRAM antisera. As shown in Fig. 2c, nascent chains longer than 98 residues crosslinked to TRAM when the probe was located in TM1, and photoadducts were detected even for the longest nascent chains. In the case of TM2, photoadducts appeared for nascent chains of 128 residues (Fig. 2f, lane 15), and crosslinking was detected for all the longer nascent chains. These results point towards a sequential movement of both TM segments through the translocon. Initially, TM1 was positioned at the core of the translocon where it strongly crosslinked to Sec61α. But for nascent chains longer than 98 residues, TM1 diffused laterally away from Sec61α. However, instead of leaving the translocon, TM1 was now adjacent to TRAM until ribosome release. Consistent with this scenario, the intensity of Sec61α adducts decreased concomitantly with the appearance of crosslinking to TRAM (see quantification in Fig. 2d). Similarly, TM2 was adjacent to Sec61α for nascent chains between 108 to 128 residues long, but the TM2 crosslinking

to Sec61α decreased as the nascent chain lengthened and crosslinking to TRAM increased (Fig. 2g).

Probing the environment of a non-viral TM sequence in a viral protein context.

To determine if TM sequence influences its proximity to TRAM, we performed a TM sequence swap experiment. The TM1 from p9 was replaced with the first TM segment from Lep (H1) (p9H1, Fig. 3a). Previous experiments with a single-spanning (H1) construct derived from Lep have shown that the protein inserts in a cotranslational and SRP-dependent manner into rough canine pancreatic microsomes [21]. In addition, H1 has been shown previously to interact with Sec61α [19] both in the presence and in the absence of the second TM segment, H2 [15]. In agreement with those results, when replacing TM1 by H1, crosslinks to Sec61α could be observed for all RNCs tested (Fig. 3c). Quantification showed that p9H1 RNC crosslinking efficiency reached a maximum between 70 and 110 residues. Photoadduct formation clearly decreased when the chains reached a length of 115 amino acids (Fig. 3c), but did not disappear.

Lep has been widely used as a model membrane protein for biogenesis studies. In fact, it has been used in photocrosslinking approaches in different laboratories to study membrane protein integration into the ER [19; 21; 22]. To date, crosslinks to TRAM were never observed unless charged residues were introduced into the middle of H1 [21]. In sharp contrast with the established integration models, p9H1 (containing H1 wild type

sequence) showed strong crosslinking to TRAM (Fig. 3d). Crosslinks to TRAM were observed at all nascent chain lengths tested, similar to what was observed with wild type p9 (compare Fig. 3d and Fig. 1b). Notably, despite efficient H1 photocrosslinking to TRAM in the p9 context, TRAM photocrosslinking was never detected when H1 sequence, with a probe located at the same position, was assayed out of the viral protein context (Fig. 4). Hence, when H1 was located in a non-viral context, preceded by a preprolactin-derived signal sequence (SS), that is cleaved by signal peptidase, to ensure proper membrane integration (111p, described in reference [19]), RNCs photocrosslinked to Sec61α for nascent chains longer than 150 residues (Fig. 4, lanes 2-4); no photoadduct formation was observed when the same samples were immunoprecipitated with an affinity-purified antiserum for TRAM (Fig. 4, lanes 7-9). Interestingly, crosslinks to Sec61α decreases as the length of the nascent chain increases (compare Fig. 4, lanes 2 and 3 with lane 4), suggesting that H1 has diffused away from the channel as observed for similar leader peptidase constructs used previously [15; 19; 22]. Together, these results suggest that TM segment recognition by TRAM is not entirely determined by the sequence of the TM segment. Instead, a combination of parameters, which apparently includes other sequences within the nascent chain, seems to determine TM sequence progression through the translocon during the integration process.

TRAM acts as a TM collecting site in viral membrane protein integration.

By probing the environment of the p9 TM segments and some derived proteins, we examined the spatial relationship between the nascent viral

protein and the core components of the ER translocon at different stages of bilayer insertion. As a result, a model for the integration of p9-derived proteins has been proposed (Fig. 5). In the case of p9 (as well as p9H1, Fig. 5a), we found that both TM segments remained in close proximity not only to Sec61α, but also to TRAM, until translation terminated. Furthermore, each TM segment moved sequentially from Sec61α to TRAM during integration, as was observed earlier for the VSV-G TM sequence [5]. It was shown previously that wild type H1 TM segment was adjacent to Sec61α at an early stage of protein integration, but subsequently moved away from the translocon [19; 21; 22]. In contrast, the presence of a charged residue in H1 caused it to move from Sec61α to TRAM, perhaps because the presence of a charged residue forced the protein to remain at the interface of the channel and the lipid phase where H1 contacted TRAM at several different nascent chain lengths [21]. Interestingly, H1 in the p9 context (p9H1) displayed a similar sequence of crosslinking to Sec61α and TRAM, which suggests that TRAM interacts with the viral protein in some way to ensure its proper integration into the lipid phase.

One possible role for TRAM might involve collecting the TM segments of a polytopic viral membrane protein. As was previously shown with wild type p9, both TM segments remained close to Sec61α until the nascent chain was released from the ribosome. However, when TM1 and TM2 were separated by a longer loop, TM1 moved away from Sec61α when the nascent chain was longer than 98 residues [13]. Similarly, TM2 was adjacent to Sec61α for only a limited time when nascent chain lengths were

between 108 and 143 residues (Fig. 2e). However, the present results showed that while proximity to Sec61α decreased, the TM segments remained in close proximity to TRAM until translation terminated (Fig. 2c and 2f). Thus, as depicted in Fig 5b, in p9P1 construct the TM segments do not move directly into the lipid phase upon moving away from Sec61α, but instead are retained adjacent to TRAM. Whether this apparent interaction of p9 TM segments with TRAM is a pre-requisite for proper folding and assembly of viral membrane proteins has yet to be determined. Interestingly, TRAM photoadducts were not detected for a non-viral polytopic membrane protein in similar biogenesis studies [23], while other proteins like PAT-10 that are closely associated with functional Sec61 complexes have been proposed to act by collecting different TM segments of another non-viral polytopic membrane protein [24].

CONCLUSIONS

Using a double-spanning plant viral membrane protein as a model, we have investigated the mechanism of viral TM segment integration, and found several interesting and unexpected results. Among the most relevant are the following: (i) viral TM segments integrate into the lipid bilayer through a sequentially ordered contact with Sec61α and TRAM; (ii) TRAM retains viral TM domains at the translocon until protein partition into the bilayer; and (iii) a non-viral TM sequence never before shown to be in proximity to TRAM is found adjacent to TRAM when inserted in a viral protein. These findings show that the nature of the nascent membrane protein dictates

how it interacts with the protein components of the translocon, and suggest that the translocon proteins play an active role in the proper integration and assembly of nascent membrane proteins.

FIGURE LEGENDS

Figure 1. Photocrosslinking of p9 nascent chains to TRAM. Preparation of truncated mRNA, *in vitro* translation in the presence of a modified amber suppressor aminoacyl-tRNA (εANB-Lys-tRNA^{amb}) and canine microsomal membranes, photocrosslinking and membrane sedimentation were carried out as described previously [12; 13; 19]. After photocrosslinking, equal aliquots from each RNC length were removed and directly analyzed by SDS/PAGE to detect and quantify the total radioactive translation products used for the quantifications. Finally, the samples were immunoprecipitated with TRAM antisera. Immunoprecipitations were performed as before [12; 13; 19]. **(a)** Structural organization of p9. p9 RNCs with radioactive nascent chains of different lengths (60-140 residues) and carrying a single photoreactive probe either at position 15 in TM1 **(b)** or at position 49 in TM2 (**d)** were photolyzed in the presence of membranes as described [13]. The samples were subjected to IP with antibodies to TRAM. The bracket signals the RNC-TRAM photoadducts. Protein generated by read-through of the TAG codon is indicated by asterisk. (<) indicates translation termination at the amber stop codon at position 49. **(c)** and **(e)** Quantification of the photocrosslinking experiments. Relative photoadduct yield (mean values from at least two independent experiments like those in panels (b) and (d)) was plotted as a function of polypeptide length. The radioactivity in the Sec61α (filled symbols, [13]) and TRAM (empty symbols, this paper) crosslinked products (bracket) in each sample was normalized to the same number of total translation products, and is expressed relative to the

maximum photoadduct yield for each photocrosslinking experiment, which means that photoadduct yields are not comparable between different constructs.

Figure 2. Photocrosslinking of p9P1 protein to Sec61α and TRAM. The experiments were performed as in Figure 1. After photocrosslinking the samples were split for IPs with Sec61α or TRAM antisera. **(a)** Structural organization of the p9P1 protein, in which the 14 residues of the loop that connects TM1 to TM2 in p9 are replaced by the P1 domain from Lep. **(b)**, **(c)** and **(d)** A single photoreactive probe was incorporated by positioning the amber stop codon at position 15 (p9P1St15). **(e)**, **(f)** and **(g)** A single photoreactive probe was incorporated by positioning the amber stop codon at position 77 (p9P1St77). After photolysis in the presence of membranes, an aliquot from each RNC length was removed and directly analyzed by SDS/PAGE to detect and normalize the total radioactive translation products (not shown). The remainig sample was split in two and immunoprecipitated with antibodies to Sec61α (b and e), or to TRAM (c and f). Quantifications shown in (d) and (g) were performed as in Fig.1.

Figure 3. Photocrosslinking of p9H1 nascent chains to Sec61α and TRAM. The construct p9H1 was obtained by introducing a Bgl II site after the TM1 segment in the pGEM-p9/P2 vector [13]. Subsequently, the N-terminus of Lep (22 residues that includes H1) was subcloned from PCR amplification of this region using as a template the pGEM-Lep vector (G. von Heijne) and using as primers oligonucleotides carrying flanking Nco I/Bgl II restriction sites. **(a)** Structural organization of the p9H1 protein. RNCs with

radioactive nascent chains of different lengths (60-140 residues) and carrying a single photoreactive probe at position 13 in H1 (replacing Thr13 in the chimera) were photolyzed in the presence of membranes. **(b)** Total samples were analyzed by SDS-PAGE. Photoadducts containing either Sec61α **(c)**, or TRAM **(d)**, were purified by IP. **(e)** Quantifications were performed as in Fig. 1.

Figure 4. H1 Sec61α *versus* TRAM photocrosslinking. The construct with the Lep H1 in a non-viral protein context was obtained by introducing H1 sequence in the construct 111p described previously [5; 19]. **(a)** Structural organization of the chimera. H1 sequence is preceded by a preprolactin-derived signal sequence (SS), which is cleaved by signal peptidase, to ensure proper membrane integration. A single amber stop codon positioned the probe (star) to the same position as in Fig. 3 (Thr13). **(b)** After photolysis of integration intermediates containing RNCs of different lengths, the samples were split in thirds, one third was loaded directly on the gel (lanes 11-14, Totals), one third was immunoprecipitated with antibodies to Sec61α (lanes 1-4) and the other third was immunoprecipitated with antibodies to TRAM (lanes 6-9). Photoadducts are indicated by downward arrowhead. H1 preferentially crosslinked to Sec61α, whereas crosslinking to TRAM were never observed.

Figure 5. Models for the integration of viral-derived membrane proteins. The hypothetical ER membrane integration site is viewed from above. **(a)** p9 TM1 is adjacent to Sec61α in the translocon at all nascent chains tested [13], but for nascent chains longer than 70 residues crosslinking of TM1 to

TRAM suggests a transition of the nascent chain toward the interface between both ER translocon components. For TM2, a nascent chain at least 80 residues long is required to crosslink to Sec61α and at least 100 residues are needed for crosslink to TRAM. Hence, TM segments interact with translocon components until the protein is released from the ribosome. **(b)** Nascent p9 chimeric protein harboring the P1 domain from Lep (p9P1) has a strikingly different integration mechanism. TM1 interacts with Sec61α at an early stage, but loses contact with Sec61α and moves adjacent to TRAM when the nascent chain is longer than 98 residues. TM2 contacts Sec61α for nascent chains between 108 and 128 residues, and then moves away from Sec61α. Both TM1 and TM2 are then adjacent to TRAM until the newly synthesized protein is released from the translocon.

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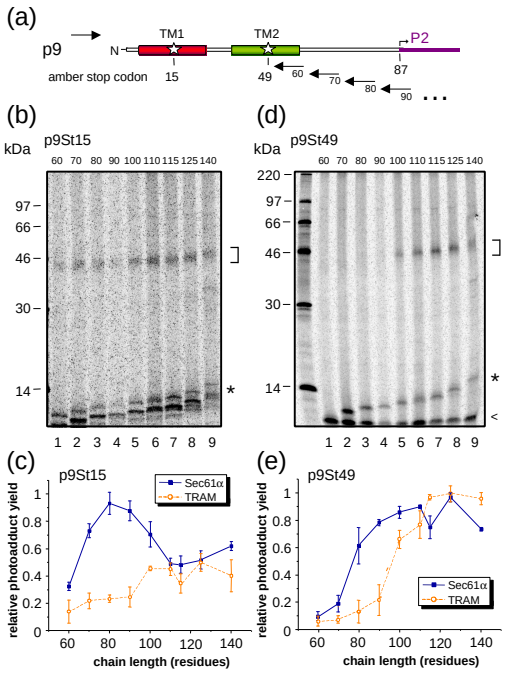
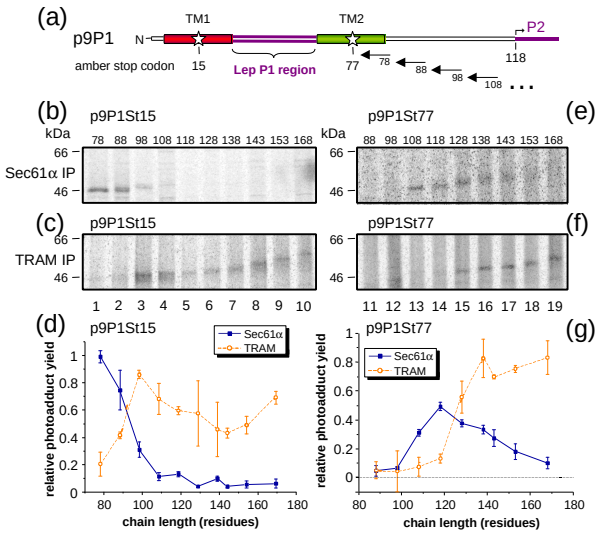
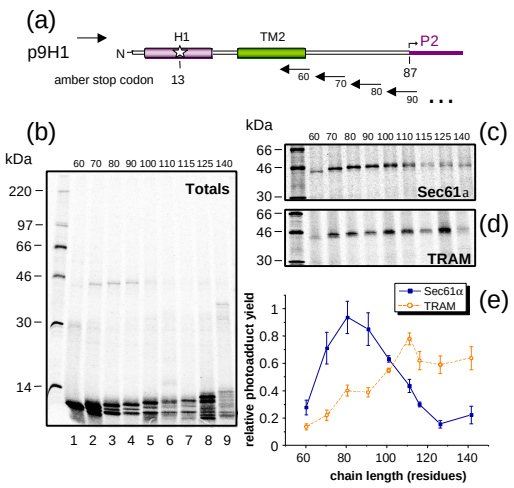
Saurí *et al.* Figure 1



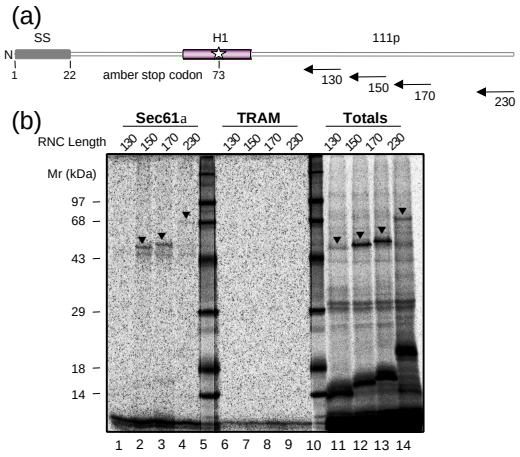
Saurí *et al.* Figure 2



Saurí *et al.* Figure 3



Saurí *et al.* Figure 4



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