

Membrane Insertion and Biogenesis of the *Turnip Crinkle Virus* p9 Movement Protein[∇]

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Plant viral infection and spread depends on the successful introduction of a virus into a cell of a compatible host, followed by replication and cell-to-cell transport. The movement proteins (MPs) p8 and p9 of *Turnip crinkle virus* are required for cell-to-cell movement of the virus. We have examined the membrane association of p9 and found that it is an integral membrane protein with a defined topology in the endoplasmic reticulum (ER) membrane. Furthermore, we have used a site-specific photo-cross-linking strategy to study the membrane integration of the protein at the initial stages of its biosynthetic process. This process is cotranslational and proceeds through the signal recognition particle and the translocon complex.

Cell-to-cell transport of plant virus requires the virally encoded movement proteins (MPs). These proteins specialize in the translocation of the viral genome or, in some cases, the virions from the replication/encapsidation site to adjacent cells. This process takes place through the plasmodesmata (PD), the small pores formed by prolongations of the endoplasmic reticulum (ER) membranes trapped within the center of the plasma membrane-lined cytoplasmic cylinder that connect plant cells. MPs belong to different protein families with unique functional and structural characteristics. The most studied MP is p30 from the *Tobacco mosaic virus*, a 30-kDa RNA-binding protein (4) with two putative transmembrane (TM) segments (2) that has so far been considered an integral membrane protein (13, 42). At an early stage of infection, p30 associates with the ER network (18, 59). Given that the ER is continuous through PD, it was suggested that the movement complex transports cell to cell via the PD. On the other hand, passage through the connecting structure largely remains a mystery, although it seems reasonable that the process again occurs in close juxtaposition to the ER-derived membrane (desmotubule) that runs through the PD (12, 35). Many other plant viruses have a cell-to-cell transport system based not on one but on two (double-gene block [DGB]) or even three (triple-gene block [TGB]) MPs. In some of these cases it has been shown that at least one MP is closely associated with the ER membrane (28, 34, 41, 50, 55). Thus, it has been assumed that other MPs associate similarly with membranes.

The targeting and insertion of an integral membrane protein can occur either posttranslationally, in which the protein is completely synthesized on cytosolic ribosomes before being inserted, or cotranslationally, in which protein synthesis and integration into the ER membrane are coupled. In the latter

case, the targeting of the ribosome-mRNA-nascent chain complex to the membrane depends on the signal recognition particle (SRP) and its interaction with the membrane-bound SRP receptor (11), which is located in close proximity to the translocon. The translocon, a multiprotein complex composed of the Sec61 α , - β , and - γ subunits (16) and the translocating chain-associated membrane protein (TRAM) (15) in eukaryotic cells, facilitates the translocation of soluble proteins into the ER lumen and the insertion of integral membrane proteins into the lipid bilayer (24).

Plant virus infection depends on the proper targeting and association or insertion of the movement proteins with or into the ER membrane. In this report, we investigate the insertion into, topology of, and targeting to the membrane of the p9 MP from *Turnip crinkle virus* (TCV). This is a positive-sense single-stranded RNA virus that belongs to the *Carmovirus* genus and thus to the DGB. Its 4-kb genome encodes five open reading frames (ORFs) (3, 17). Translation of the first two yields p28 and p88, both implicated in viral RNA synthesis. In the central region, two overlapping ORFs encode the small proteins p8 and p9, which have been shown to be involved in cell-to-cell movement (6, 17, 31). The RNA-binding protein p8 (17, 58) overlaps the distal 3' region of the replicase p88. The 3' region of the genome encodes the viral coat protein p38, and its 5' end overlaps p9 (3).

A strong interaction with the membrane is expected for p9 due to the close similarities in the genomic arrangement of TCV (57) with other carmoviruses, like *Carnation mottle virus* (CarMV) and *Melon necrotic spot virus* (MNSV). Both CarMV and MNSV have two small MPs, one an RNA-binding protein (39, 53, 54) and the other a cotranslationally inserted integral membrane protein (34, 47, 55). In this study, we present evidence of the integration of TCV p9 into ER-derived microsomal membranes. Using an *in vitro* translation system based on a model integral membrane protein, we have been able to identify two membrane-spanning domains. Additionally, the membrane topology of the p9 MP was analyzed *in vitro* and found to have an N terminus (N-t)/C terminus (C-t) luminal orientation. Finally, using a site-directed photo-cross-linking

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approach, we demonstrated that the mechanism of p9 insertion into the ER membrane involves SRP and the translocon.

MATERIALS AND METHODS

Enzymes and chemicals. All enzymes, as well as plasmid pGEM1, the RiboMAX SP6 RNA polymerase system, rabbit reticulocyte lysate, and dog pancreas microsomes, were from Promega (Madison, WI). [³⁵S]Met and ¹⁴C-labeled methylated markers were from GE Healthcare. Restriction enzymes and endoglycosidase H (Endo H) were from Roche Molecular Biochemicals. Proteinase K (PK) was from Sigma-Aldrich (St. Louis, MO). The DNA plasmid, RNA clean-up, and PCR purification kits were from Qiagen (Hilden, Germany). The oligonucleotides were from Thermo (Ulm, Germany).

Computer-assisted analysis of the p9 sequence. Prediction of transmembrane (TM) helices was done using up to 10 of the most common methods available on the Internet: DAS (8) (<http://www.sbc.su.se/~miklos/DAS>), ΔG Prediction Server (21, 22) (<http://www.cbr.su.se/DGpred/>), HMMTOP (51) (<http://www.enzim.hu/hmmtop/>), MEMSAT (25) (<http://saier-144-37.ucsd.edu/memsat.html>), SOSUI (23) (<http://bp.nuap.nagoya-u.ac.jp/sosui/>), SPLIT (26) (<http://split.pmfst.hr/split/4/>), TMHMM (29) (<http://www.cbs.dtu.dk/services/TMHMM>), TMPred (http://www.ch.embnet.org/software/TMPRED_form.html), and TopPred (5) (<http://www.sbc.su.se/~erikw/toppred2/>).

DNA manipulation. The full-length TCV p9 sequence (a kind gift from Anne E. Simon, University of Maryland) (40) was fused to the P2 domain of the *Escherichia coli* leader peptidase (Lep) using NcoI/NdeI restriction sites in the pGEM1 plasmid (55). Mutations of Leu47 to Asn, Leu49 to Asp, His77 to Asn, and the sequence encoding Gly12 to an amber codon (TAG) were performed using the QuikChange PCR mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's protocol. The hydrophobic regions (HRs) from p9 were amplified and introduced into the modified Lep sequence from the pGEM1 plasmid (32) using SpeI/KpnI sites. All DNA manipulations were confirmed by sequencing of plasmid DNAs.

In vitro transcription and translation. Lep constructs with HR segment inserts were transcribed and translated as previously reported (34). Full-length p9 DNA was amplified from plasmid pGEM1 using a reverse primer with a stop codon at the end of the p9 sequence. Alternatively, p9 fused to the first 50 amino acids from P2 was amplified using a reverse primer with a stop codon at the 3' end.

In vitro transcription was performed with Ribomax SP-6 (Promega) by following the manufacturer's instructions. The mRNAs were purified using a Qiagen RNeasy clean-up kit and verified on 1% agarose gel. *In vitro* translation of *in vitro*-transcribed mRNA was performed in the presence of reticulocyte lysate, [³⁵S]Met, and dog pancreas microsomes.

The translation reaction mixture was diluted in either 8 volumes of buffer A (35 mM Tris-HCl [pH 7.4] and 140 mM NaCl) for the untreated samples, 4 volumes of buffer A containing 8 M urea for the urea-treated samples, or 4 volumes of buffer A containing 100 mM Na₂CO₃ (pH 11.5) for the alkaline extraction, as previously described (14). The samples were incubated on ice for 30 min and then clarified by centrifugation at 10,000 × *g* and 4°C for 10 min. Subsequently, membranes were collected by layering the supernatant onto a 50-μl sucrose cushion and centrifuged at 100,000 × *g* for 20 min at 4°C in a Beckman tabletop ultracentrifuge with a TLA-45 rotor. Finally, pellets and supernatants were analyzed by SDS-PAGE, and gels were visualized on a Fuji FLA3000 phosphorimager using the ImageGauge software. Endoglycosidase H (Endo H) treatment was done as previously described (34). Briefly, the translation mixture was diluted in 4 volumes of 70 mM sodium citrate (pH 5.6) and centrifuged (100,000 × *g*, 20 min, 4°C). The pellet was then suspended in 50 μl of sodium citrate buffer with 0.5% (wt/vol) SDS and 1% (vol/vol) β-mercaptoethanol, boiled 5 min, and incubated for 1 h at 37°C with 0.1 milliunits of Endo H before SDS-PAGE analysis. For the proteinase K protection assay, the translation mixture was supplemented with 1 μl of 50 mM CaCl₂ and 1 μl of proteinase K (2 mg/ml) and then digested for 40 min on ice. The reaction was stopped by adding 2 mM phenylmethylsulfonyl fluoride (PMSF) before SDS-PAGE analysis.

Phase separation in Triton X-114 solution. Phase separation of integral membrane proteins using the detergent Triton X-114 was performed as previously described (1); Triton X-114 (1%, vol/vol) was added to a translation mixture that had previously been diluted with 180 μl of phosphate-buffered saline (PBS). After being mixed, samples were incubated at 0°C for 1 h and laid over 300 μl of PBS supplemented with 6% (wt/vol) sucrose and 1% (vol/vol) Triton X-114. After 10 min at 30°C, an organic droplet was obtained by centrifugation for 3 min at 1,500 × *g*. The resulting aqueous upper phase (AP; 200 μl) was collected, and the organic droplet at the bottom of the tube was diluted with PBS (organic

TABLE 1. Computer analysis of the TCV p9 amino acid sequence

Algorithm	Integral membrane protein present	No. of TM segments (starting aa/ending aa) ^a
DAS	Yes	2 (5/18, 40/58)
HMMTOP	Yes	1 (33/52)
MEMSAT	Yes	1 (38/57)
SOSUI	Yes	2 (1/18, 39/61)
SPLIT	Yes	2 (2/18, 37/59)
TMHMM	No	0
TMPred	Yes	2 (1/19, 38/57)
ΔG Prediction Server	Yes	2 (1/20, 38/57)
TopPred	Yes	2 (1/21, 37/57)

^a aa, amino acid(s).

phase [OP]). Both the OP and AP were then supplemented with sample buffer and boiled for 10 min prior to SDS-PAGE analysis.

Cotranslational and posttranslational insertion assay. Full-length p9 mRNAs were translated (30°C 1 h) either in the presence (see Fig. 5, + and Co samples) or in the absence (– and Post samples) of microsomal membranes. Translation was then inhibited with cycloheximide (10 min, 26°C, 2-mg/ml final concentration), after which microsomes were added to those samples labeled as posttranslational and incubated for an additional hour at 30°C. Membranes were collected by ultracentrifugation and analyzed by SDS-PAGE.

Photo-cross-linking experiments. Truncated mRNAs were generated by PCR using different reverse primers that lacked a stop codon to obtain nascent chains of a specific length. PCR products were *in vitro* transcribed using purified SP6 RNA polymerase as described above. For SRP photo-cross-linking experiments, *in vitro* translation (typically 50 μl, 26°C, 40 min) of 70- or 110-residue nascent chains was performed as described before (27) in a wheat germ cell extract containing 40 nM SRP, 100 μCi of [³⁵S]Met, and 32 pmol of ε-s-azido-2-nitrobenzoyl-Lys-tRNA^{amb}. After translation, samples were irradiated for 20 min on ice using a 500-W mercury arc lamp. Photolyzed samples were sedimented through a 130-μl sucrose cushion {0.5 M sucrose, 20 mM HEPES (pH 7.5), 4 mM magnesium diacetate [Mg(OAc)₂], 100 mM potassium acetate (KOAc)} using a TLA100 rotor (Beckman Instruments; 100,000 rpm, 4 min, 4°C) to recover the SRP-ribosome-nascent chain complex (RNC). Pellets were resuspended in sample buffer before analysis by SDS-PAGE and detection by phosphorimaging as described before (47).

To assess Sec61α and TRAM photo-cross-linking, truncated mRNAs were translated as described above but in the presence of 8 eq of column-washed, rough ER microsomes (CRM). Samples were photolyzed and sedimented as described above prior to sample immunoprecipitation (IP).

IP. Pelleted membranes were resuspended in 50 μl of 3% (wt/vol) SDS-50 mM Tris-HCl (pH 7.5) and incubated at 55°C for 30 min. Samples were diluted with 500 μl of buffer A (140 mM NaCl, 10 mM Tris-HCl [pH 7.5], 2% [vol/vol] Triton X-100) for Sec61α IP and with buffer B (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.5], 1% [vol/vol] Triton X-100) for TRAM IP. Samples were precleared by rocking them with 30 μl of buffer A/B-washed protein A-Sepharose (Sigma) at room temperature for 1 h. After removal of the beads by centrifugation, supernatants were incubated with affinity-purified rabbit antisera specific for Sec61α or TRAM (Research Genetics, Huntsville, AL) overnight at 4°C. Then, 30 μl of protein A-Sepharose, previously equilibrated with buffer A or B, was added and incubated for 2 h at 4°C. After sedimentation, the beads were washed twice with 750 μl of buffer A or B, followed by a final washing in the same buffer without detergent. Samples were prepared for SDS-PAGE analysis by the addition of sample buffer and incubation at 95°C for 5 min. Results were visualized and processed using the Bio-Rad FX phosphorimaging device.

RESULTS AND DISCUSSION

TCV p9 is an integral membrane protein. Computer-assisted analysis of the TCV p9 amino acid sequence showed that all membrane protein prediction algorithms except TMHMM predicted that p9 is an integral membrane protein (Table 1). To test these predictions *in vitro*, p9 transcription/translation experiments in the presence of ER-derived microsomal membranes and [³⁵S]Met were carried out. After high-speed cen-

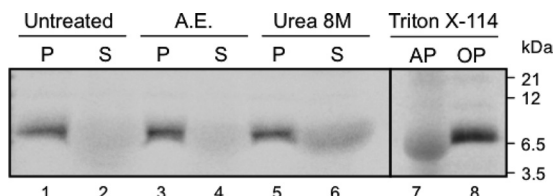


FIG. 1. TCV p9 MP is an integral membrane protein. Segregation of [³⁵S]Met-labeled p9 into membranous and soluble fractions (untreated, lanes 1 and 2) and after alkaline wash (sodium carbonate buffer, lanes 3 and 4) or urea treatment (lanes 5 and 6). P and S denote pellet and supernatant, respectively. For Triton X-114 partitioning experiments, OP and AP refer to organic and aqueous phases, respectively (lanes 7 and 8). A.E., alkaline extraction.

trifugation ($100,000 \times g$), p9 was recovered from the pellet fraction with the sedimented microsomal membranes (Fig. 1, lanes 1 and 2), indicating that it was either a membrane-associated (peripheral or integral) or translocated luminal protein (39). To differentiate between these possibilities, translation mixtures were either treated with 8 M urea or washed with sodium carbonate (pH 11.5). Urea treatment removes proteins weakly or peripherally associated from the membrane (49), whereas alkaline extraction disrupts the membrane and releases any soluble luminal proteins (41). Despite treatments with both agents, p9 remained associated with the membranous pellet fraction (Fig. 1, lanes 1 to 6), suggesting an intimate association with microsomal membranes. To confirm this conclusion, the translation mixture was treated with Triton X-114, a detergent that forms an organic phase (OP), into which membrane lipids and integral membrane proteins are segregated (1), and an aqueous phase (AP), containing soluble and peripheral membrane proteins. After Triton X-114 treatment, p9 was detected entirely in the OP (Fig. 1, lanes 7 and 8). These combined results corroborate the predictions and prove that p9 behaves as an integral membrane protein.

To identify p9 membrane-spanning domains, prediction algorithms were used as a preliminary analysis. Most algorithms suggested the presence of two transmembrane (TM) domains located roughly around amino acids 1 to 21 and 37 to 57, respectively (Table 1). The membrane insertion capacity of these two hydrophobic regions (HRs) was examined using the experimental setup based on N-linked glycosylation and summarized in Fig. 2 (top).

N-linked glycosylation has been extensively used as topological reporter for more than a decade (37). Proteins can be glycosylated only in the lumen of the ER because the active site of oligosaccharyl transferase (OST), a translocon-associated protein responsible for N glycosylation, is located there; no N-linked glycosylation occurs in the membrane or the cytosol. Thus, the HR to be tested is inserted into the P2 luminal domain of the model protein Lep (leader peptidase from *E. coli*), where it is flanked by two N glycosylation acceptor sites (G1 and G2, tripeptide sequences Asn-X-Ser/Thr, where X can be any amino acid except Pro) (21, 22). The chimeric protein is then expressed *in vitro* in the presence of ER-derived microsomal membranes. When the P2 region is translocated across the membrane, both the G1 and G2 sites can be glycosylated (filled circles), since both acceptor sites are located within the lumen (Fig. 2) and are accessible to the OST. However, inser-

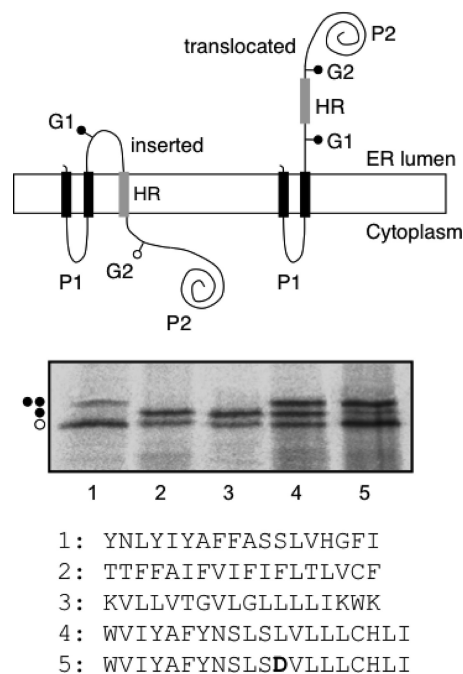


FIG. 2. Insertion of TCV p9 hydrophobic region (HR) 1 and 2 into microsomal membranes. (Top) Schematic representation of the leader peptidase (Lep) construct used to report insertion into the ER membrane of p9 HR1 and HR2. The HR under study is inserted into the P2 domain of Lep, flanked by two artificial glycosylation acceptor sites (G1 and G2). Recognition of the HR by the translocon machinery as a TM domain locates only G1 on the luminal side of the ER membrane, preventing G2 glycosylation. The Lep chimera will be doubly glycosylated when the HR being tested is translocated into the lumens of the microsomes. (Middle) *In vitro* translation in the presence of membranes of the different Lep constructs. Control HRs were used to verify sequence translocation (lane 1) and membrane integration (lane 2) (clones 67 and 68 in reference [44], a kind gift from G. von Heijne's lab). Constructs containing HR1 (residues 3 to 20), HR2 (residues 38 to 57), or HR2 with Leu49 replaced by Asp (lanes 3, 4 and 5, respectively) were transcribed and translated in the presence of membranes. Bands of nonglycosylated protein are indicated by a white dot; singly and doubly glycosylated proteins are indicated by one and two black dots, respectively. The HR sequence in each construct is shown at the bottom.

tion of the tested region into the membrane renders glycosylation only on the G1 site, leaving the G2 site unmodified (Fig. 2, empty circle). Single glycosylation increases the molecular mass of the protein by about 2.5 kDa relative to the observed molecular mass in the absence of microsomal membranes, and double glycosylation increases the apparent mass by 5 kDa.

Translation of a construct containing amino acids 3 to 20 (HR1) from TCV p9 resulted in a single glycosylation of the protein (Fig. 2, lane 3), indicating that this region of p9 was recognized by the translocon machinery as a TM domain. When residues 38 to 57 (HR2) were assayed, ~50% of the membrane-inserted Lep molecules were singly glycosylated (Fig. 2, lane 4), thereby indicating that HR2 can be recognized for membrane insertion, although with lower efficiency. On the other hand, substitution of the central Leu49 of HR2 by Asp (L49D) reduced the singly glycosylated population to <5% (Fig. 2, lane 5); the L49D mutation therefore precludes proper

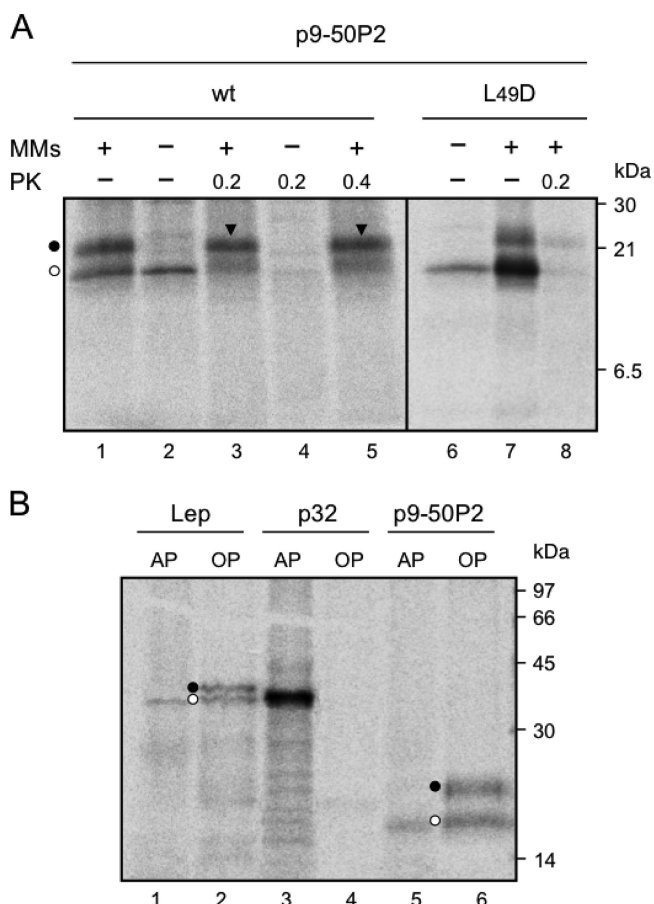


FIG. 3. Insertion and topology of p9-50P2. (A) *In vitro* translation of wild-type (wt) p9 (lanes 1 to 5) and the L49D mutant (lanes 6 to 8) when fused to the first 50 amino acids of the P2 domain from Lep, in the presence (+) and absence (-) of microsomal membranes (MMs) and proteinase K (PK). Bands of nonglycosylated protein are indicated by a white dot; glycosylated proteins are indicated by a black dot. The arrowhead identifies undigested protein after PK treatment (with either 0.2 or 0.4 mg/ml). (B) Triton X-114 partitioning of Lep, PNRSV p32 MP (a previously reported nonintegral membrane protein [33]), and p9-50P2. OP and AP refer to organic and aqueous phases, respectively.

insertion of the HR2 domain into the lipid bilayer by the Sec61 translocon.

p9 integrates into the membrane with an N-t/C-t luminal orientation. Once p9 was established as an integral membrane protein and the TM segments were identified, we sought to study the topology of the protein when it inserted into the ER-derived membranes. Since N glycosylation acceptor sites are absent in the TCV p9 sequence, the first 50 residues of the Lep P2 domain, which contains an N glycosylation acceptor site, were attached to p9 as a C-t reporter. When the resulting fusion protein, p9-50P2, was translated *in vitro* in the presence of microsomal membranes and [³⁵S]Met, the chimeric protein was efficiently glycosylated, as shown by the increase in its molecular mass when translated in the presence of microsomal membranes (Fig. 3A, lanes 1 and 2). These data suggest that the C-t domain is in the lumen. Since replacement of Leu49 by Asp significantly reduced the membrane insertion ability of

HR2 in the Lep context (Fig. 2), this change was introduced into the p9-50P2 fusion, and the glycosylation of the P2 acceptor site was severely reduced (Fig. 3A, lanes 6 and 7). p9 HR2 is therefore normally inserted into the membrane during biogenesis.

The nature of the cytosolic/luminal domains was further examined by proteinase K (PK) digestions. Treatment with PK degrades domains of membrane proteins that protrude into the cytosol, but membrane-embedded or lumenally exposed domains are protected. The addition of PK to a wild-type p9-50P2 translation mixture did not degrade the glycosylated form (Fig. 3, lanes 3 to 5), even when high protease concentrations were used. The soluble C-t domain of the protein is therefore protected from PK action by the microsomal membranes, whereas the nonglycosylated band was PK sensitive, indicating that this protein species was not properly integrated into the membrane and its HR2 region was not inserted. These data also show that the loop connecting HR1 and HR2 is not accessible for PK digestion when HR2 is properly inserted, probably because it is too short and located too close to the membrane, as observed previously for a homologous viral protein (48). In contrast, the L49D p9-50P2 construct was completely digested after PK treatment (Fig. 3A, lane 8). The HR2 and the soluble C-t domain of this mutant were exposed to the cytoplasm by preventing HR2 insertion, thereby allowing PK digestion. These results indicate an N-t/C-t luminal orientation for the chimeric protein p9-50P2.

To verify the insertion of the p9-50P2 chimera into the lipid bilayer, Triton X-114 partition assays were performed. The accumulation of the protein in the OP shows that the presence of the P2 region did not block p9 insertion into the microsomal membranes (Fig. 3B). Parallel experiments were performed using Lep and a viral movement protein (p32 from *Prunus necrotic ring spot virus* [PNRSV]) as controls for membrane-inserted and peripherally associated proteins, respectively (33).

As noted earlier, the TCV p9 sequence lacks natural glycosylation acceptor sites that can be used as topological reporters. To investigate the topology of the TCV MP in the absence of foreign fusion domains, two different glycosylation acceptor sites were introduced into p9 by site-direct mutagenesis. The first one, at position 77 (N77, replacing His77 with Asn), was introduced in the C-t domain of p9, and the second at position 45 (N45, by inserting a Thr after Ser46) within HR2. As shown in Fig. 4, the N77 mutant is significantly glycosylated, as p9-50P2 was, when the protein was translated *in vitro* in the presence of microsomal membranes (lanes 1 to 3). Hence, the p9 C-t domain was translocated into the ER lumen. In contrast, the N45 mutant was not glycosylated, indicating that in those cases where HR2 is inserted, the glycosylation acceptor site is embedded in the membrane, and in those cases where it is not inserted (since the extent of the HR2 insertion in the Lep context was ~50% [Fig. 2]), the acceptor site remained in the cytosol (Fig. 4, lanes 4 to 6). This result also demonstrates an extracellular N-t orientation for the first TM segment (see schemes in Fig. 4, top). Fusion of the N77 and N45 mutants to the first 50 residues of the Lep P2 domain produced, as expected, doubly and singly glycosylated proteins, respectively (Fig. 4, lanes 7 to 9), due to the extra acceptor site provided by P2. Thus, the observed glycosylation of the two new acceptor sites (N45 and N77) corroborate p9 topology.

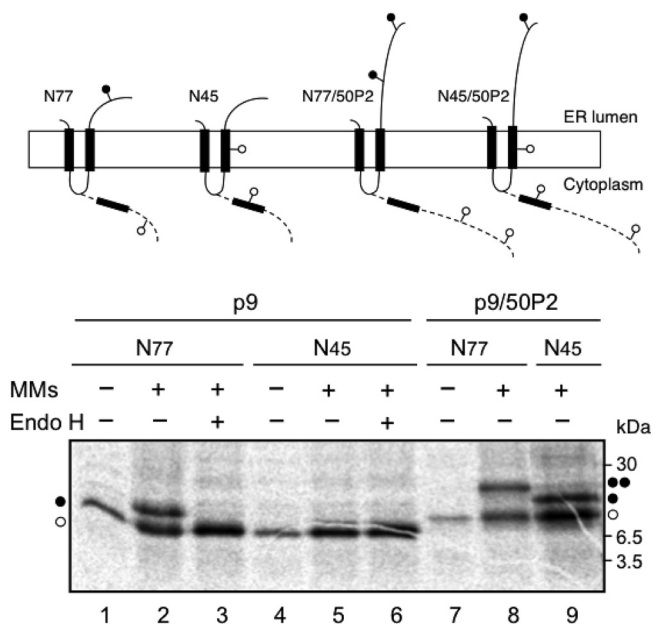


FIG. 4. TCv p9 MP topology. (Top) Schematic representation of the constructs used in the study of p9 topology. The locations of acceptor sites are indicated with a white or a black dot indicating nonglycosylation and glycosylation, respectively. (Bottom) *In vitro* translation in the presence (+) and absence (-) of microsomal membranes (MMs) and endoglycosidase H (Endo H; a glycan-removing enzyme) of samples encoding engineered glycosylation sites either in the soluble C-t domain (N77) (lanes 1 to 3) or in the middle of HR2 (N45) (lanes 3 to 6). Translation of N77 and N45 mutants fused to the first 50 amino acids of the P2 domain from Lep were also included (lanes 7 to 9). Bands of nonglycosylated protein are indicated by a white dot; singly and doubly glycosylated proteins are indicated by one and two black dots, respectively.

All together, these results demonstrate that TCv p9 MP is preferentially a double-spanning membrane protein that orients the N-t of HR1 and the C-t of HR2 toward the lumen of the ER membranes. Nevertheless, the possibility that the HR2 domain could display some dynamic insertion (i.e., a flip into and out of the membrane) should not be ruled out based on previous studies (reviewed in reference 56). Thus, it is possible that HR2 of p9 inserts posttranslationally into the membrane at different stages of TCv infection. Such a property may allow the protein to interact in a regulated and perhaps transient manner with other proteins, such as its partner p8, to anchor proteins or assemblies associated with viral genome transport.

p9 integrates into the ER membrane cotranslationally and interacts with SRP. We have previously reported that the MPs p9 from CarMV (47) and p7B from MNSV (34) are cotranslationally inserted into ER-derived membranes. Since the insertion of proteins through the translocon can be monitored by glycosylation (24), we sought to investigate whether or not p9 from TCv is cotranslationally inserted into the ER membrane by blocking protein synthesis after p9 has been translated in the absence of membranes. As shown in Fig. 5A, p9 N77 was glycosylated when microsomal membranes were added to the translation mixture cotranslationally. But when microsomal membranes were included posttranslationally after translation was inhibited by cycloheximide, the N77 acceptor site was not glycosylated, suggesting that TCv p9 is integrated cotransla-

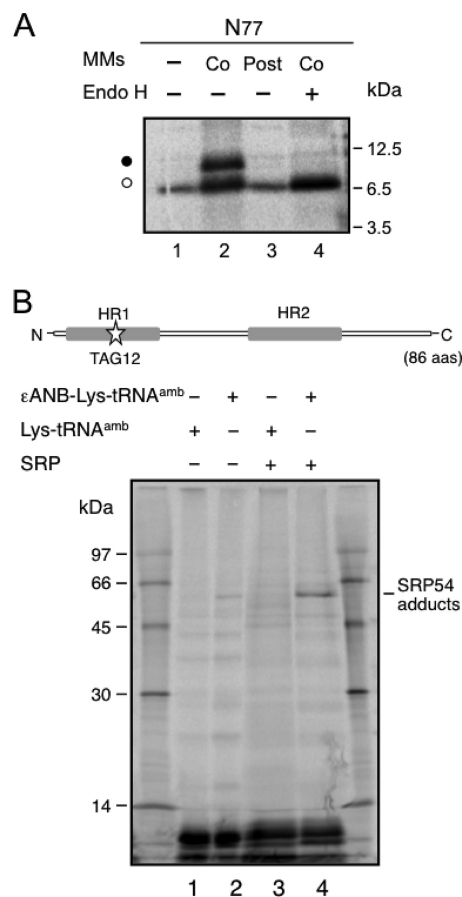


FIG. 5. TCv p9 integrates into the membrane cotranslationally and interacts with SRP. (A) p9 (harboring an engineered glycosylation site at the C-t, position 77) was translated in either the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of microsomal membranes. In lane 3, microsomal membranes were added posttranslationally (after 1 h; Post) and incubation was continued for another 1 h. In lane 4, the p9 construct was translated in the presence of cotranslationally added membranes (Co) and treated later with Endo H. (B) Photo-cross-linking of TCv p9 to SRP. Structural organization of p9 (top). A single photoreactive probe was incorporated by positioning an amber stop codon at position 12. RNCs containing 70-residue radioactive nascent chains were prepared in the presence of unmodified Lys-tRNA^{amb} or photoreactive ϵ ANB-Lys-tRNA^{amb}, as indicated. Only the sample in lane 4 was supplemented with exogenous SRP. aas, amino acids.

tionally through the ER translocon. If true, then p9 would be expected to contain a signal sequence that is recognized by the SRP. This interaction would then target the ribosome-nascent chain complex (RNC) to the translocon. Since p9 lacks a cleavable signal sequence, the first TM segment of the protein presumably acts as a signal sequence and binds to SRP54, the 54-kDa subunit of the SRP that is responsible for signal recognition (27, 30). To investigate this interaction, a previously described photo-cross-linking technique was used (47, 48). Briefly, a photoreactive probe was selectively incorporated, using an amber suppressor aminoacyl-tRNA (ϵ ANB-Lys-tRNA^{amb}), into the middle of the first TM segment to assess its proximity to SRP. p9 translation intermediates were prepared by translating mRNAs that were truncated within the coding region. A translating ribosome halts when it reaches the end of

such an mRNA, but the nascent chain does not dissociate from the tRNA-ribosome complex, because the absence of a stop codon prevents normal termination from occurring.

The amber stop codon was introduced at position 12 of the TCV p9 sequence (p9TAG12), approximately in the middle of the HR1 region. When RNCs with 70-residue nascent chains were photolyzed, a prominent photoadduct was generated only in the presence of added SRP and ϵ ANB-Lys-tRNA^{amb} and not in the presence of unmodified Lys-tRNA^{amb}. This translation mixture lacked microsomal membranes to obtain RNC-SRP intermediates. As shown previously (45, 47), a 70-residue RNC is sufficient to ensure that RH1 is outside the ribosome exit tunnel and accessible for putative SRP binding. After UV illumination, an ~61-kDa photoadduct was formed when SRP and ϵ ANB-Lys-tRNA^{amb} were present (Fig. 5B, lane 4). In those cases where the photoreactive probe was not present or was replaced by unmodified Lys-tRNA^{amb}, no significant adduct was detected. The apparent molecular mass of this photoadduct corresponds to an adduct between SRP54 and the 70-residue nascent chain (27). HR1 is therefore adjacent to the SRP54 subunit of SRP and apparently acts as a signal sequence to target the RNC to the translocon.

p9 inserts into the ER membrane through the translocon.

To dissect the process of membrane integration, we focused on determining whether this viral membrane protein is adjacent to translocon components after targeting, as has been observed previously for its homologue MP from CarMV (47). To identify proteins adjacent to TCV p9 nascent chains during membrane insertion, integration intermediates containing nascent p9 chains of 70 and 110 residues (based on previous data [47]) were prepared using microsomal membranes. The photoactive probe was incorporated by translation of the truncated mRNAs with an amber codon at position 12 (p9TAG12) in the presence of ϵ ANB-Lys-tRNA^{amb}. Since p9 is only 86 amino acids long, it was necessary to elongate its C-t. The 110-residue intermediate was therefore prepared by fusing the P2 domain from Lep at the end of p9's sequence and then obtaining the desired length by PCR using the specific reverse primer prior to transcription. These insertion intermediates were photolyzed, and the nature of the photoadducts was analyzed by immunoprecipitation using affinity-purified antibodies to Sec61 α , the core component of the eukaryotic translocon (52). The 70-residue intermediate contained the p9 nascent chain that had reacted covalently with Sec61 α (Fig. 6, lane 2), but photo-cross-linking to Sec61 α was severely diminished when the 110 intermediate was used (Fig. 6, lane 5). These data confirm that p9 is integrated through the translocon and suggest that RH1 is not tightly bound to Sec61 α since the photo-cross-linking decreases in the longer nascent chain.

Another protein component of the translocon, TRAM (translocating chain-associating membrane protein) has been photo-cross-linked to the homologous protein p9 from CarMV (46, 47). TRAM may be required as a chaperone to increase the efficiency of the insertion of certain TM segments. However, interactions with TRAM vary greatly among different translocon-integrated membrane proteins (10, 19, 20, 36, 38, 45). To probe the interaction between TCV p9 and TRAM, 70- and 110-residue integration intermediates were photoactivated and immunoprecipitated using TRAM antibodies. In these experiments, RH1 was adjacent to TRAM in both the 70-

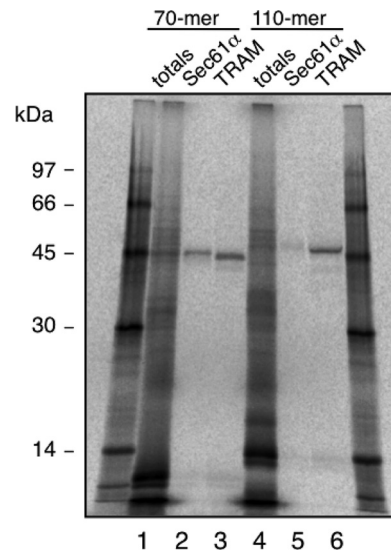


FIG. 6. Photo-cross-linking of TCV p9 to Sec61 α and TRAM. After photolysis in the presence of membranes, an aliquot from each RNC (70 and 110 residues) was removed and directly analyzed by SDS-PAGE to detect and normalize the total radioactive translation products (labeled as totals). The remaining samples were split for IPs with Sec61 α (lanes 2 and 5) and TRAM (lanes 3 and 6) antisera.

110-residue intermediates (Fig. 6, lanes 3 and 6). Thus, the insertion of p9 into the ER membrane takes place through the translocon complex. Furthermore, although the juxtaposition of HR1 and Sec61 α appears to decrease significantly before translation terminates, the HR1 remains in close proximity to TRAM in the translocon until translation is completed.

Proper targeting, insertion, and topology of viral membrane proteins are essential for successful infection of the host cell. These processes have been extensively described for both eukaryotic integral membrane proteins and bacterial inner membrane proteins, but less information is available about the mechanisms used by membrane proteins from viruses to reach the cellular membranes. In fact, the mechanism of plant viral MP biogenesis had been examined only for CarMV p9 MP, where a photo-cross-linking approach showed that the protein is targeted to the ER membrane by SRP, is inserted into the membrane, and is sequentially in close proximity to Sec61 α and TRAM (46, 47).

Using the same approach, we have now mapped the proteinaceous environment of TCV p9 MP and found that this protein inserts into the ER membrane through the translocon and passes by Sec61 α and TRAM. Interestingly, cross-linking to Sec61 α indicates that the first TM segment of TCV p9 is adjacent to the channel at a chain length of 70 residues, but the cross-linking is much reduced at a chain length of 110 residues, when the entire hydrophobic segment of HR2 is out of the ribosome. The simplest interpretation of our results is that HR1 moves from the channel but remains in proximity to the translocon, since the nascent chains strongly cross-link to TRAM at both chain lengths (Fig. 6). The loss of cross-linking to Sec61 α may be explained by the dynamic insertion of HR2 (see above), as has been suggested previously to cause the release of a TM segment from the translocon into the lipid phase of the membrane (9, 20); however, integration can also

be controlled by other translocon components, such as TRAM (7, 19, 36, 45, 46). Although further studies are needed to define the precise role of TRAM, our results indicate that its role in the mechanism of TM integration for double-spanning membrane proteins is much more relevant than appreciated previously and can give more clues to help us understand the mechanism of plant viral transport.

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