Topogenesis in membranes of the NTB–VPg protein of *Tomato ringspot nepovirus*: definition of the C-terminal transmembrane domain

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INTRODUCTION

Replication of the genome of positive-strand RNA plant viruses involves the formation of a replication complex composed of several viral proteins and host plant proteins, associated with specific intracellular membranes (Buck, 1996). The replication complexes of picornaviruses and several plant picorna-like viruses (including potyviruses, comoviruses and nepoviruses) have been shown to be associated with membranes having properties of the endoplasmic reticulum (ER) (Carette et al., 2000; Han & Sanfaçon, 2003; Ritzenthaler et al., 2002; Schaad et al., 1997; Suhy et al., 2000). Virus infection results in a massive modification and proliferation of the ER membranes, and de novo lipid synthesis has been shown to be required for the replication of *Poliovirus* (PV), *Cowpea mosaic virus* (CPMV, genus *Comovirus*), and *Grapevine fanleaf nepovirus* (GFLV) (Carette et al., 2000; Guinea & Carrasco, 1990; Ritzenthaler et al., 2002).

Specialized viral proteins are thought to play a role as membrane anchors, either by directly associating with intracellular membranes as integral membrane proteins or by interacting with membrane proteins from the host. The viral proteins that act as membrane anchors may in turn interact with other viral proteins (such as the RNA-dependent RNA polymerase, Pol) to redirect them towards the membranes and induce the formation of the replication complexes. In picorna-like viruses, mature proteins as well as intermediate precursor proteins are released from large polyproteins by virus-encoded proteinases. The membrane anchors may therefore associate with membranes as mature proteins or as larger polyprotein precursors. For example, in PV, 3AB is a membrane-associated intermediate polyprotein that forms a complex with 3CD (proteinase–polymerase) and with the viral RNA (Porter, 1993; Xiang et al., 1997).

*Tomato ringspot virus* (*ToRSV*, genus *Nepovirus*, family *Comoviridae*) has a bipartite, single-stranded, positive-sense RNA genome (Mayo & Robinson, 1996; Sanfaçon, 1995). Each molecule of RNA is covalently linked to a protein at its 5′ end (VPg), is polyadenylated at its 3′ end and encodes one large polyprotein. The RNA1-encoded polyprotein (P1)
contains the domains for proteins likely to be involved in replication including the putative NTP-binding protein (NTB), the VPg, a 3C-like proteinase (Pro) and the Pol (Fig. 1a; Rott et al., 1995). The NTB protein contains a hydrophobic region at its C terminus that has characteristics of a transmembrane domain. In infected plants, ToRSV proteins containing NTB (including two predominant proteins: the NTB–VPg polypeptide and the mature NTB) are associated as integral membrane proteins with membranes derived from the ER and they also co-fractionate with the ToRSV replication activity (Han & Sanfaçon, 2003). Based on these results, we have proposed a model in which NTB–VPg (or another protein containing NTB) may serve as a membrane anchor for the ToRSV replication complex. In addition, proteinase K protection experiments have revealed that a fragment of the membrane-associated proteins containing the VPg and probably the hydrophobic domain at the C terminus of NTB is embedded in the membranes while the middle portion of the NTB domain is exposed to the cytoplasmic face (Han & Sanfaçon, 2003). This result suggests that NTB–VPg is a transmembrane protein with the VPg domain present on the luminal face of the membranes. This is surprising since VPg is assumed to be present on the cytoplasmic face of the membrane to serve as a possible primer for RNA replication, as has been suggested for PV (Paul et al., 1998). One possible explanation is that alternative topologies of the NTB–VPg protein are present in infected plants, but they would not have been detected in the previous study with the available antibodies. Alternatively, other precursors containing the VPg domain in a cytoplasmic orientation (for example, VPg–Pro–Pol) may be present in infected plants.

In this study, we have further analysed the association of NTB–VPg with membranes using an in vitro assay and commercially available canine microsomal membranes that consist predominantly of ER-derived membranes. Using site-directed mutagenesis and the glycosylation mapping technique, we have examined the orientation of the C-terminal region of NTB–VPg (including the VPg domain) in the membranes and defined the functional regions of the transmembrane domain at the C terminus of NTB.

**METHODS**

**Plasmid constructions.** Plasmid pT7-cNTB-VPg (pT7-cNV, containing ToRSV RNA1 nt 2948–3794) has been described previously (Wang & Sanfaçon, 2000b). Plasmid pT7-NV was obtained by amplifying a fragment of ToRSV RNA1 (nt 1959–1977) using cDNA clone pMR10 (Rott et al., 1995) as a template, primers W025 (5’-ACGAACCAGCTGGCGAATGGTGGGCGG-3’, complementary to ToRSV RNA1 nt 3794–3778 and containing a SacI site [underlined]) and W030 (5’-ACGCCATGTTCCCTCTGAGTATCATGA-3’, corresponding to ToRSV RNA1 nt 1959–1977 and containing an Ncol site) and Pfu polymerase (Stratagene). The amplified fragment was digested with Ncol and SacI and inserted into the corresponding sites of vector pCITE-4a (+) (Novagen). Plasmid pT7-cNV3 was constructed in a similar manner using primers W025 and 91 (5’-CTGCCACCATGAGGATGGTGGTTGAAAGCGATG-3’, corresponding to ToRSV RNA1 nt 3385–3404) and containing an Ncol site. Deletion of regions of the NTB domain and mutation of specific amino acids were achieved by amplification reactions using primers containing the desired mutation, plasmid pT7-cNV as a template and Pfu polymerase (Fisher & Pei, 1997). The amplified products were self-ligated to produce the various mutated versions of pT7-cNV. Presence of the mutation was verified by sequencing using the ABI PRISM Dye Terminator cycle sequencing reaction kit (Applied Biosystems) and an ABI PRISM 310 Genetik Analyser (Perkin Elmer).

**In vitro transcription and translation.** Approximately 0.2 μg each plasmid DNA (in a 6 μl reaction) was translated at 30°C for 2 h using the TNT coupled transcription/translation rabbit reticulocyte system (Promega) in the presence of [35S]methionine with or without canine microsomal membranes (Promega; 0.6 μl membranes was added to the reaction unless otherwise indicated). The reactions were arrested by the addition of 2 × protein loading buffer (PLB) and separated by SDS-PAGE (Laemmli, 1970). Immunoprecipitations of the translation products were performed as previously described (Hans & Sanfaçon, 1995).

**Deglycosylation assays.** In vitro translation products (13 μl reactions) were supplemented with 4–4 μl x PLB and the proteins were denatured at 95°C for 5 min. The boiled reactions were diluted with 9 vols deglycosylation buffer (200 mM potassium phosphate buffer, pH 7.0; 25 mM EDTA and 1 % Triton X-100). Fifty mU N-glycosidase F (Roche) was added to a 20 μl aliquot of the solution and incubated at 30°C overnight.

**Computer-assisted analysis of protein topology and of the putative signal peptide cleavage site.** Prediction of transmembrane helices was performed using the following programs, which are available on the internet: PHDhtm (Rost et al., 1996), Sosui (Hirokawa et al., 1998), Tmpred (Hofmann & Stoffel, 1993), TopPred2 (von Heijne, 1992), TMHMM (Sonnhammer et al., 1998), HMMTOP (Tusnady & Simon, 1998) and MEMSAT2 (Jones et al., 1994). Putative signal peptide cleavage sites were predicted using the SignalP prediction program available on the internet (Nielsen et al., 1997). The SignalP program is trained to analyse signal peptide cleavage sites present at the N terminus of the protein and will not recognize such sites at the C terminus of proteins. However, it was recently shown that a signal peptide can recognize cleavage sites with similar sequences when located at the C terminus of proteins (Nilsson et al., 2002). Therefore, a small region of the C-terminal portion of the NTB–VPg protein, including the hydrophobic domain at the C terminus of NTB, the sequences downstream of this domain including the VPg and 5–20 amino acids upstream of the hydrophobic domain, was used in the analysis with the SignalP program. Prediction of signal peptide cleavage sites was similar, whether a short (5 aa) or a larger (20 aa) region upstream of the hydrophobic domain was included in the analysis.

**RESULTS**

**Computer-assisted prediction of putative transmembrane helices in the NTB–VPg polypeptide**

Two hydrophobic regions were previously identified in the ToRSV NTB protein (Fig. 1a) (Han & Sanfaçon, 2003). A putative amphipathic helix was identified in the N-terminal hydrophobic region, while the C-terminal hydrophobic region was predicted to contain one or two transmembrane helices (Han & Sanfaçon, 2003). We have now used a number of programs available on the internet to predict
Fig. 1. Analysis of possible transmembrane helices in the NTB–VPg polyprotein. (a) Schematic representation of NTB–VPg. The ToRSV RNA1-encoded polyprotein (P1) is shown at the top of the figure. Vertical lines indicate the cleavage sites recognized by the ToRSV proteinase, and the putative functions of the mature proteins are indicated (NTB, putative nucleoside triphosphate-binding protein; VPg, viral genome-linked protein; Pro, proteinase; Pol, RNA-dependent RNA polymerase). The coding region for the NTB–VPg polyprotein is shown below the P1 polyprotein. The hatched box represents the VPg coding region and the two shaded areas represent hydrophobic regions in the NTB domain. A consensus N-glycosylation site is shown (symbol Y above the VPg domain). The cleavage site recognized by the ToRSV proteinase between the NTB and VPg domains is indicated by an open arrow. (b) Prediction of transmembrane helices in the hydrophobic region at the C terminus of NTB. Possible transmembrane helices were predicted using prediction programs available on the internet. The deduced amino acid sequence of the hydrophobic region at the C terminus of NTB is shown at the top, with letters in bold representing regions of the putative transmembrane helices that were common to all predictions. Below this sequence, the transmembrane helices predicted by each individual program are shown. Scores for the first and second putative transmembrane helices were as follows: program Tmpred, 2698 for the first helix and 1151 for the second helix; program MEMSAT 2, 265 for the first helix and 208 for the second helix (scores for each predicted transmembrane helix were not provided for the HMMTOP program). In the Sosui program, the first putative transmembrane helix was predicted to be a primary transmembrane helix while the second putative transmembrane helix was predicted to be a secondary transmembrane helix. The dotted lines represent amino acids in the sequence that were predicted not to traverse the membrane. (c) Models for the topological orientation of the NTB–VPg protein in the membranes. The NTB and VPg domains are represented by the continuous and dotted lines, respectively. Black boxes indicate the putative transmembrane helices predicted in (b). The symbol Y representing the N-glycosylation site in the VPg domain is shown in black when glycosylation is predicted to occur [i.e. when it is oriented towards the luminal face of the membranes, (1) and (3)] and in white when glycosylation is not predicted (2). As mentioned previously (Han & Sanfaçon, 2003), confidence in the prediction for topology 1 was 60% with the TMHMM program. A previously identified putative amphipathic helix at the N terminus of the protein (Han & Sanfaçon, 2003) is shown in association with the membranes (grey boxes), although the role of this helix in association of the protein with membranes remains to be determined.
more precisely the location of transmembrane helices in the C-terminal region of the NTB protein (Fig. 1b). Two putative transmembrane helices were predicted. The first helix was implied with a high degree of confidence by all the programs, although prediction of its exact length and position varied from one program to another. A consensus region (LLLVLAAVILILFF) was determined, which consisted of the amino acids present in all the predictions. The second helix was predicted to traverse the membrane by some (but not all) programs. The degree of confidence in the prediction of this second putative transmembrane helix was lower (see legend of Fig. 1b). Similarly, analysis of the C-terminal region of NTB from three other nepoviruses (GFLV, Grapevine chrome mosaic nepovirus and Tomato black ring nepovirus) revealed the presence of two adjacent putative transmembrane helices separated by a few amino acids (data not shown). As with ToRSV, only the first transmembrane helix was predicted with a high degree of confidence by all the programs.

The implied topology for the C terminus of NTB–VPg was that of a transmembrane protein with the VPg in the lumen. In this model (topology 1, Fig. 1c), only the first putative transmembrane helix is traversing the membrane while the second hydrophobic stretch is located on the luminal side of the membrane. Alternative topologies were also predicted for the C terminus of NTB–VPg by the various programs. One of these predicted topologies (topology 2, Fig. 1c) implied a hairpin structure with the two putative transmembrane helices traversing the membrane resulting in a cytoplasmic orientation of the VPg. Finally, it is also possible that both hydrophobic regions traverse the membrane jointly, forming a large transmembrane domain with the first transmembrane helix perpendicular to the membranes and the second transmembrane helix parallel to the membranes (topology 3, Fig. 1c). This has been suggested for long transmembrane helices with a positive mismatch (Monne & von Heijne, 2001). Our previous results, using proteinase K protection assays of membrane fractions purified from infected plants, suggested a luminal orientation for the VPg consistent with topology 1 or 3. However, our results did not exclude the possibility that a proportion of the NTB–VPg molecules adopts topology 2 in which the VPg is oriented in the cytoplasm (Han & Sanfaçon, 2003).

The C-terminal region of NTB is sufficient to constitute an efficient transmembrane domain and translocate the VPg into the lumen

To test whether the C-terminal NTB hydrophobic domain was sufficient to promote the luminal orientation of the VPg, truncated mutants with increasing deletions of the N-terminal region of NTB were generated (constructs pT7-cNV and pT7-cNV3, Fig. 2a). In vitro translations were performed using the TNT coupled transcription/translation system in the presence of [35S]methionine and the translation products were separated by SDS-PAGE. A protein of approximately 70 kDa was synthesized, which corresponded to the calculated molecular mass for the entire NTB–VPg precursor (Fig. 2b, lane 1). Additional smaller proteins were also synthesized, which probably corresponded to translation initiation events at internal AUG codons. Addition of canine microsomal membranes to the in vitro transcription/translation of plasmid pT7-NV resulted in the production of a new protein (with an apparent molecular mass of 73 kDa) in addition to the 70 kDa protein (Fig. 2b, lane 2). The relative concentration of the 73 kDa protein varied between different batches of membranes and was influenced by the concentration of membranes used in the translation reactions. Treatment of the translation products with N-glycosidase F resulted in the disappearance of the 73 kDa protein, indicating that this protein was a glycosylated form of the NTB–VPg protein (data not shown). Recognition of the VPg glycosylation site suggested that NTB–VPg associates with microsomal membranes in vitro resulting in the translocation of the VPg into the lumen.

NTB–VPg associates with microsomal membranes in vitro, resulting in a luminal orientation of the VPg domain

To test the membrane association and topology of NTB–VPg in the absence of other viral proteins, we used canine microsomal membranes and a rabbit reticulocyte in vitro translation assay. N-Glycosylation sites can be used as convenient markers to identify segments of a membrane-associated protein that are located in the lumen. N-glycosylation sites are characterized by the sequence NX(S/T) (Kasturi et al., 1995). Examination of the deduced amino acid sequence of NTB–VPg revealed the presence of a consensus N-glycosylation site in the VPg domain (sequence NMT, starting at position 1228 of the deduced ToRSV amino acid sequence; numbering according to Rott et al., 1995). No other glycosylation sites were identified in NTB–VPg.

The coding region for NTB–VPg was inserted in the pCITE vector (plasmid pT7-NV, Fig. 2a). In vitro translations were performed using the TNT coupled transcription/translation system in the presence of [35S]methionine and the translation products were separated by SDS-PAGE. A protein of approximately 70 kDa was synthesized, which corresponded to the calculated molecular mass for the entire NTB–VPg precursor (Fig. 2b, lane 1). Additional smaller proteins were also synthesized, which probably corresponded to translation initiation events at internal AUG codons. Addition of canine microsomal membranes to the in vitro transcription/translation of plasmid pT7-NV resulted in the production of a new protein (with an apparent molecular mass of 73 kDa) in addition to the 70 kDa protein (Fig. 2b, lane 2). The relative concentration of the 73 kDa protein varied between different batches of membranes and was influenced by the concentration of membranes used in the translation reactions. Treatment of the translation products with N-glycosidase F resulted in the disappearance of the 73 kDa protein, indicating that this protein was a glycosylated form of the NTB–VPg protein (data not shown). Recognition of the VPg glycosylation site suggested that NTB–VPg associates with microsomal membranes in vitro resulting in the translocation of the VPg into the lumen.
Fig. 2. The C-terminal region of NTB–VPg is sufficient to allow membrane association and modification of the protein. (a) Schematic representation of the ToRSV-derived wild-type and truncated NTB–VPg proteins. The proteins synthesized in the in vitro translation experiments are shown. The dotted lines represent amino acids from the vector fused in-frame with the ToRSV-derived proteins. The expected molecular mass of each protein is indicated. The amino acids from the ToRSV sequence present in each protein are indicated. The shaded areas represent hydrophobic regions in the NTB domain and the hatched box indicates the VPg domain (with the consensus N-glycosylation site shown by the symbol Y). The open arrow indicates the cleavage site recognized by the ToRSV proteinase between the NTB and VPg domains, while the black arrowhead indicates the position of a predicted signal peptidase cleavage site. (b) Modification of wild-type and truncated NTB–VPg by enzymes associated with microsomal membranes in vitro. Coupled in vitro transcription/translation reactions were performed in the presence (+) or absence (−) of microsomal membranes (MM), as indicated above each lane. Where indicated, the translation products were treated with N-glycosidase F (N-glycoF) as described in the Methods. Translation products were separated by SDS-PAGE (10% polyacrylamide for NTB–VPg, 12% polyacrylamide for cNTB–VPg and 16.5% polyacrylamide for cNV3). The positions of the molecular mass markers (kDa) are indicated on the side of each panel. The asterisks beside the bands on the gels indicate the unprocessed glycosylated form of each protein, while the dots indicate the processing products of each protein. (c) In vitro translation of a mutant derivative of cNTB–VPg containing a point mutation in the VPg glycosylation sequence. Lanes 1–12, coupled in vitro transcription/translation of plasmids pT7-cNV (wild-type, WT) and pT7-cNV (T1229/A) (containing a mutation of the N-glycosylation site in the VPg domain) were performed in the presence (+) or absence (−) of microsomal membranes (MM) as indicated above each lane. Translations were performed for 15, 30, 60 or 120 min as indicated and arrested by the addition of 2 × PLB. The translation products were separated by 15% SDS-PAGE. Lanes 13–16, coupled in vitro transcription/translation of plasmid pT7-cNV (T1229/A) was performed in the presence (+) or absence (−) of microsomal membranes (MM). The translation products were either directly separated by SDS-PAGE (Transl., lanes 13 and 14) or immunoprecipitated with anti-VPg antibodies (IP VPg Abs, lanes 15 and 16) prior to separation by 16.5% SDS-PAGE. The migration of molecular mass standards is shown on the left side of the gel. The asterisk indicates the glycosylated, unprocessed form of the protein while the dots indicate the cleavage products.

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acids at the C terminus of the NTB protein contain an efficient transmembrane domain that directs the translocation of the VPg into the lumen.

**Truncated NTB–VPg proteins are processed by a membrane-associated protease at a position upstream of the junction between the NTB and VPg domains**

The nature of the small proteins produced during translation of plasmid pT7-cNV and pT7-cNV3 in the presence of membranes was further investigated. We postulated that these proteins might be processing products released through the action of a membrane-associated protease such as a signal peptidase. Two fragments were observed for the wild-type cNV synthesized in the presence of membranes: a 30-5 kDa fragment and a 9 kDa fragment (Fig. 2b, lane 4, and Fig. 2c, lanes 2–4; the small fragment is not shown in Fig. 2b). Two fragments were also released from the glycosylation mutant of cNV (cNV T1229/A) in the presence of membranes but the size of the smaller fragment was reduced (the 9 kDa protein observed for the wild-type protein was replaced by a 5-5 kDa fragment in the glyco-xylation mutant, while the 30-5 kDa fragment was observed for both the wild-type protein and the glycosylation mutant; Fig. 2c, lanes 8–10). This result suggested that the 9 kDa fragment present in the translation products from the wild-type construct was a glycosylated form of the 5-5 kDa fragment observed for the glycosylation mutant. Thus, this fragment probably included the VPg domain, which contains the glycosylation site. To test this directly, we conducted immunoprecipitations of the translation products of cNV using antibodies raised against the VPg domain, which have been previously described (Wang et al., 1999). Because these antibodies cannot recognize the VPg domain when it is glycosylated (data not shown), the immunoprecipitation experiments were conducted using the translation products from the cNV (T1229/A) mutant (Fig. 2c, lanes 13–16). The anti-VPg antibodies could immunoprecipitate the 36 kDa unprocessed cNV and the 5-5 kDa processing product but not the 30 kDa processing product, confirming that the 5-5 kDa fragment contains the VPg domain. Time-course studies revealed that glyco-sylation of cNV (appearance of the 39 kDa protein) occurred very rapidly (Fig. 2c, lane 1), while the processing products (30-5 and 9 kDa proteins) were released at a later time (Fig. 2c, lanes 2–4). The size of the small processing product suggested that the cleavage occurred at a position 1–2 kDa upstream of the junction between the NTB and VPg domain (VPg has a calculated molecular mass of 3 kDa plus 1.6 kDa derived from amino acids from the vector sequence fused in-frame to the VPg). A similar processing event was observed in the translation products of construct cNV3 in the presence of membranes. The glycosylated form of cNV3, which has a molecular mass of 18-5 kDa, was cleaved to release two fragments of approximately 9 kDa, which migrated as a diffuse band in the gel (Fig. 2b, lane 7). After deglycosylation of the translation products, the two processing products were readily separated into a 5-5 kDa fragment (corresponding to the small processing fragment observed in the translation products of cNV (T1229/A) and probably containing the deglycosylated VPg domain) and a 9-5 kDa fragment (probably containing the NTB domain). The detection of similar processed products in the translation products of plasmid pT7-NV was masked by the presence of multiple background bands, probably due to internal initiation events and possibly also due to degradation of the large translation product. Therefore, we could not conclusively determine whether similar processing products were also released from the full-length protein.

Signal peptidase cleavage sites are characterized by a net positive charge at their N terminus followed by a stretch of hydrophobic residues and small non-polar residues at the −1 and −3 positions (Nielsen et al., 1997). These parameters have been shown to apply to signal peptidase cleavage sites present either at the N or C terminus of proteins (Nilsson et al., 2002). The SignalP program, which is designed to predict signal peptidase cleavage sites using these conserved features, implied a putative signal pepti-dase cleavage site at the C terminus of the NTB protein. Several possible positions for the cleavage sites were predicted by the program, with the most likely cleavage site predicted to be between amino acids Ala1191 and Ile1192 (Fig. 3). This prediction corresponded to the observed size of the cleaved products. Unfortunately, the exact position of the cleavage site could not be confirmed experimentally as the amounts of the small processing product released were too low to allow the determination of its N-terminal amino acid sequence using Edman degradation. Interestingly, examination of the deduced amino acid sequence of the NTB–VPg domains of other nepoviruses with the SignalP program also implied the presence of a putative signal peptidase cleavage site at a similar position (Fig. 3).

**Definition of the transmembrane domain at the C terminus of NTB**

As mentioned above, the transmembrane domain located at the C terminus of the NTB domain consists of two possible transmembrane helices (see Fig. 1). To determine the role of each of these regions in defining the transmembrane domain, a new series of deletion mutants was generated using plasmid pT7-cNV as a template. As above, glycosylation in the VPg domain was used as a marker for the translocation of the VPg domain in the lumen and thus for the presence of an active transmembrane domain. Derivative ΔTM1 contained a deletion of the entire C-terminal region of NTB including the two putative transmembrane helices (Fig. 4a). Mutants ΔTM2 and ΔTM3 consisted of a deletion of 11 hydrophobic amino acids in the consensus region of the first and second putative transmembrane helices, respectively. The trans-membrane prediction programs described above were used to analyse the amino acid sequence of each mutant. As expected, cNVΔTM1 was predicted to be a soluble protein. cNVΔTM2 was also predicted to be a soluble
protein by all the programs, indicating that the second putative transmembrane helix was not predicted in the absence of the first region of hydrophobic amino acids. In contrast, cNV\(\text{TM3}\) was predicted to be a transmembrane protein. The results of the in vitro membrane association assays are shown in Fig. 4(b). As above, glycosylation was observed by the appearance of a slightly larger protein in the translation products, when translation was conducted in the presence of microsomal membranes. The nature of this protein was confirmed by a deglycosylation assay (Fig. 4c). As expected, mutant \(\text{cNV}\) as a template (T1229/A mutant), we introduced a new glycosylation site at the junction of the NTB–VPg domain (this is the cleavage site recognized by the viral proteinase (NTB–VPg junction). GCMV, Grapevine chrome mosaic nepovirus; TBRV, Tomato black ring nepovirus; GFLV, Grapevine fanleaf nepovirus.

### DISCUSSION

Our analysis of the association of NTB–VPg with canine microsomal membranes in vitro revealed that NTB–VPg has the ability to associate with membranes in the absence of other viral proteins. This result is consistent with our recent finding that NTB–VPg is an integral membrane protein in plants (Han & Sanfaçon, 2003) and provides
further support for the idea that proteins containing the NTB domain may act as membrane anchors for the replication complex. Of course, this does not exclude the possibility that other viral proteins may also interact with membranes and could play a role in the membrane targeting of the replication complex. Indeed, in the related CPMV, both the 66 kDa (NTB–VPg) and 32 kDa proteins have been shown to associate with and modify membranes of the ER (Carette et al., 2002).

Modification of NTB–VPg by membrane-associated enzymes was observed in vitro. While the detection of these modifications in vitro provided useful information on the topology of the protein in the membrane, it is not clear at this point whether these modifications also occur in vivo. The detection of glycosylation or signal peptidase processing on the entire NTB–VPg protein in infected plants is technically difficult, as the modified forms of the protein are expected to migrate very close to the unmodified forms of NTB–VPg and NTB. The glycosylation site present in the VPg domain is conserved in the deduced amino acid sequence obtained from the analysis of cDNA clones or fragments from several independent isolates of ToRSV (Wang & Sanfaçon, 2000a). However, direct amino acid sequencing of the VPg associated with genomic RNA purified from virus particles revealed the presence of a single amino acid change in the sequence, which would result in the destruction of the glycosylation site (deduced

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**Fig. 4.** Definition of functional regions within the transmembrane domain of the NTB protein. (a) Deletions of regions of the hydrophobic domain at the C terminus of NTB. The amino acid sequence (shown for ToRSV aa 1167–1213) represents the hydrophobic domain at the C terminus of NTB with the consensus sequence for the two putative transmembrane helices identified in Fig. 1(b) shown in bold. The open arrow above the sequence indicates the junction between the NTB and VPg domains (i.e. the cleavage site recognized by the viral proteinase) and the arrowhead indicates the position of a predicted signal peptidase cleavage site. Amino acids deleted in each mutant are shown by dotted lines. (b) Modification of mutated cNV proteins by enzymes associated with microsomal membranes in vitro. Coupled in vitro transcription/translation reactions resulting in synthesis of the proteins were performed in the absence (–) or presence of 0.2 or 0.6 μl microsomal membranes (MM), as indicated above each lane. Translation products were separated by 12% SDS-PAGE. The asterisks indicate the glycosylated form of the proteins, while the dots indicate the 30–5 kDa processed product. (c) Deglycosylation assays of modified cNV proteins. Coupled in vitro transcription/translation reactions were performed in the absence (–) or presence (+) of 0.6 μl microsomal membranes (MM). Where indicated, the translation products were treated with N-glycosidase F (N-glycoF) as described in the Methods. Translation products were separated by SDS-PAGE (12% polyacrylamide).
amino acid T\textsuperscript{1229} was replaced by an A; Wang \textit{et al.}, 1999). We do not have an explanation for this discrepancy. It is therefore not clear whether the possible VPg glycosylation site in ToRSV is functional \textit{in vivo}. A putative glycosylation site was found in the amino acid sequence of the VPg domain of GFLV, but not in that of other nepoviruses (data not shown). Putative signal peptidase cleavage sites were identified in the region between the transmembrane domain at the C terminus of NTB and the VPg domain of several nepoviruses by the SignalP program (Fig. 3). Although we cannot eliminate the possibility that the putative signal peptidase cleavage site in ToRSV is a cryptic cleavage site that was exposed in the truncated proteins by deletion of N-terminal regions of the NTB, it is noteworthy that signal peptidase cleavage sites that are slowly processed post-translationally (similar to the one described here for cNTB–VPg) have been shown to play an important role in the polyprotein processing of structural proteins of other viruses (for example, flaviviruses; Amberg & Rice, 1999; Lee \textit{et al.}, 2000). Further work will be necessary to determine whether the putative signal peptidase cleavage site plays a similar role in the processing of nepovirus non-structural polyproteins \textit{in vivo}.

Computer-assisted prediction of the putative transmembrane domain at the C-terminal region of NTB of ToRSV and of three other nepoviruses implied the presence of a predominant transmembrane helix followed by a closely spaced secondary transmembrane helix. The results of these predictions suggested that the most likely topology was that of a tail-anchored protein with only the first transmembrane helix traversing the membrane. Several lines of experimental evidence suggested that this predicted topology is the predominant topology of the truncated NTB–VPg molecules (cNV) that associate with membranes \textit{in vitro}. First, the results of our deletion study suggested that only the first putative transmembrane helix traverses the membrane efficiently. Secondly, a glycosylation sequence placed in proximity to the second putative transmembrane helix was glycosylated efficiently, suggesting that this region was on the luminal side of the membrane and excluding the possibility that this region was part of a long transmembrane helix (Fig. 5). Thirdly, deletion of the second putative transmembrane helix did not result in a drastic increase in the glycosylation efficiency compared with the wild-type protein (Fig. 4), a result that would have been expected if a large proportion of the wild-type
molecules adopted a hairpin structure resulting in a cytoplasmic orientation of the VPg domain. Translocation of the VPg domain in the lumen of the membranes was also observed for the full-length NTB–VPg in vitro (as evidenced by glycosylation of the VPg domain, Fig. 2) and in infected plants (Han & Sanfaçon, 2003). However, our results do not exclude the possibility that sequences present in the N-terminal region of the NTB domain may also play a role in the membrane-association of NTB–VPg and may influence the efficiency of translocation of the C-terminal transmembrane domain in the membranes.

The VPg domain present on the luminal face of the membranes (detected in vivo and in vitro) is unlikely to participate actively in the replication of the genome, a process that presumably takes place on the cytoplasmic face of the membranes. Further experiments will be necessary to determine the role (if any) of the VPg domain in the lumen.

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