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Membrane Targeting Sequences in Tombusvirus Infections

Luisa Rubino[1] and Marcello Russo

Dipartimento di Protezione delle Piante, Università degli Studi, and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Bari, Italy

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Infection of *Nicotiana benthamiana* cells with cymbidium ringspot (CymRSV) and carnation Italian ringspot (CIRV) viruses results in the formation of conspicuous membranous bodies [multivesicular bodies (MVBs)], which develop from modified peroxisomes or mitochondria, respectively. The organelle targeting signal is located in the proteins of 33 kDa (CymRSV) or 36 kDa (CIRV) encoded by ORF 1, which contain an N-terminal hydrophilic portion followed by two predicted hydrophobic transmembrane segments. Biochemical analysis showed that the 33- and 36-kDa proteins are integral membrane proteins. By exchanging small portions of the ORF 1 sequence between the infectious full-length clones of the two viruses, hybrid constructs were obtained of which the *in vitro* synthesized RNA was inoculated to *N. benthamiana* plants and protoplasts. The structure of infectious clones suggested that both the N-terminal hydrophilic region and the transmembrane segments of the ORF 1-encoded proteins specify which organelle is involved in the synthesis of MVBs. Mutational analysis of the CIRV 36-kDa protein also suggested the presence of an internal mitochondrial targeting sequence similar to that found in several normal host proteins that are synthesized in the cytoplasm and transported to mitochondria. The CymRSV 33-kDa protein did not contain the obvious consensus signals thought to be characteristic of proteins targeted to peroxisomes, and an mitochondrial targeting sequence motif was not evident.

INTRODUCTION

Cymbidium ringspot (CymRSV) and carnation Italian ringspot (CIRV) viruses are species in the genus *Tombusvirus* (family Tombusviridae). Tombusviruses are icosahedral viruses containing a single copy of a messenger single-stranded RNA genome of ~4700 nucleotides (nt) that has five open reading frames (ORFs) (Russo *et al.*, 1994). The genomic RNA functions as an mRNA for translation of 33-kDa [33K (CymRSV)] or 36-kDa [36K (CIRV)] proteins and readthrough proteins of 92 kDa (92K) or 95 kDa (95K), respectively. These proteins contain the eight conserved motifs (PI–PVIII) of RNA-dependent RNA polymerases characteristic of supergroup II of the positive-strand RNA viruses (Koonin, 1991). The 41-kDa coat protein (CP) is centrally located and is translated from a subgenomic RNA of 2.1 kb. Two nested genes are located near the 3′ end of the genome and are translated into two proteins of 22 kDa (22K) and 19 kDa (19K) from a second subgenomic RNA of 0.9 kb. The 22K protein is required for cell-to-cell movement and contains a sequence motif conserved in the "30K superfamily" of movement proteins (Mushegian and Koonin, 1993), whereas the 19K protein influences the severity of symptoms in infected plants (Scholthof *et al.,* 1995a).

Cytopathological studies of CymRSV and CIRV infections have identified vesiculated structures [multivesicular bodies (MVBs)], which are made up of a main body surrounded by many spherical-to-ovoid vesicles 80–150 nm in diameter, resulting from proliferation of the limiting membrane of peroxisomes (CymRSV) or mitochondria (CIRV) (Russo *et al.,* 1983; Di Franco *et al.,* 1984). Earlier evidence that the peripheral vesicles of MVBs are the site of tombusvirus replication derived from observations that they (1) appear in infected cells before virus particles (Appiano *et al.,* 1981), (2) contain fine fibrillar material consisting of double-stranded RNA (Di Franco *et al.,* 1984; Russo *et al.,* 1983), and (3) incorporate [^{3}H]uridine (Appiano *et al.*, 1986). More recently, it was shown that the proteins of the replicase complex are associated with membranous cell components sedimenting at 30,000*g* (Lupo *et al.,* 1994; Scholthof *et al.,* 1995b). Furthermore, the 33K protein of CymRSV was identified by immunogold labelling at the periphery of modified peroxisomes of infected cells or uninfected transgenic cells expressing the 33K (before readthrough) or the 33K/92K (replicase) proteins (Bleve-Zacheo *et al.,* 1997).

Burgyan *et al*. (1996) used full-length hybrid infectious clones of CymRSV and CIRV and showed that a sequence as short as ~600 nucleotides in ORF 1 contained the determinants for the formation of MVBs from peroxisomes or mitochondria and that the 5′ untranslated leader sequence had no targeting function. We have now analyzed the protein sequence encoded by this region in the two viruses by mutagenesis to determine the signals

[1] To whom reprint requests should be addressed at Via Amendola 165/A, 70126 Bari, Italy. Fax: 39-80-5442911. E-mail: csvvlr02@area.ba.cnr.it.

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for the localization of the replication complex to mitochondrial or peroxisomal membranes.

RESULTS AND DISCUSSION

CIRV 36K and CymRSV 33K are integral membrane proteins

Pairwise amino acid (aa) sequence comparison of the CIRV 36K and CymRSV 33K proteins had shown that the C-terminal region (composed of about one third of the amino acid residues) is 90% identical in the two proteins, whereas the remaining two thirds in the N-terminal part are only 59% identical (Fig. 1). This region in both proteins contains two central hydrophobic segments (Burgyan *et al.*, 1996). Analysis of the CIRV 36K and CymRSV 33K N-terminal regions using an improved method of protein structure prediction (Rost *et al.,* 1995) suggests the presence of two transmembrane domains in each protein. Domain I spans aa 86–103 in CymRSV and aa 102–119 in CIRV, whereas domain II encompasses aa 136–153 in CymRSV and aa 169–186 in CIRV. The two domains are connected by a loop on the inner side of the organelle limiting membrane, whereas both the N- and C-termini are predicted to lie in the cytoplasm. Figure 2 shows the hypothetical arrangement of the CIRV 36K and CymRSV 33K proteins in the vesicles formed by invagination of the mitochondrial and peroxisomal membranes, respectively, and open to cytoplasm.

The possible insertion of the CIRV 36K and CymRSV 33K proteins in cytoplasmic membranes was assessed by verifying the acquired resistance to alkaline, urea, or salt extractions, a property typical of integral membrane proteins. The proteins either were not or were only partly removed from membrane pellets after treatment with 0.1 M Na_2CO_3, pH 11.5, 4 M urea, or 1 M KCl, thus indicating that they have one or more regions stably inserted in the membranes (Fig. 3).

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                                                  *
  1 MEGLKAESTTTVGINCNFQQTVMTETGNKLMF.......GSLPSSLERPV 43
                              ||  .:|::       |.:: :::|.|
  1 .......................METIKRLIWPKKEIFVGDFAIGVNRTV 27

                 ▼
 44 AKRNYYRTLSDWILTYTKTGKIEDTHDNAWSYVEDLVKTDFNAKYDWLAK 93
    :  : :. ::  :| | :|||| . |. ..:| :|:||| .||::|: |
 28 PV.DVFQLVCRVVLRYMRTGKIECESDSLSKFVVELLKTDCAAKWEWFMK 76

Asc I                  Mlu I
 94 RGVARDYRTAVGLAIIPGIPAIVANTRIVTGTCEVARVAVKLPKALNMLS 143
    |  : || .::::|.|| :| : ..|.: ....:|  .:.
 77 RRQGGDYIVPLSIATIPLMPLLSCTTSVRAVSVGVLGCGF.......... 116

                    Sph I                        Spl I
144 RFTHASVKVPYFYPRVDMSARGMLIGLAAAAALVPICAGIAYATLPREKL 193
       | .:|  .||:.:. :|:|: |||: ||.||||  .||||||||||
117 .....SSNIP..IPRLSVPRKGLLLRLAAGLALAPICALAVYATLPREKL 159

                                  Sac I
194 SVFRLKSEVRVHMEDEKEATDLLLVEPAREL 224
    ||::|:.|.|.|||||||||| |:||.||||
160 SVYKLRTEARTHMEDEKEATDCLVVESAREL 190
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FIG. 1 Alignment of the aa sequences of the N-terminal regions of CIRV 36K and CymRSV 33K proteins up to restriction site *Sac*I in the corresponding cDNA clones. Restriction sites used for preparation of hybrid constructs are shown. The 14 residues between the arrowheads constitute the putative mitochondrial sequence; the serine residue (*) was mutated either to aspartic acid or to arginine to produce clones CIRV/asp and CIRV/arg, respectively. Transmembrane segments are overlined and underlined.

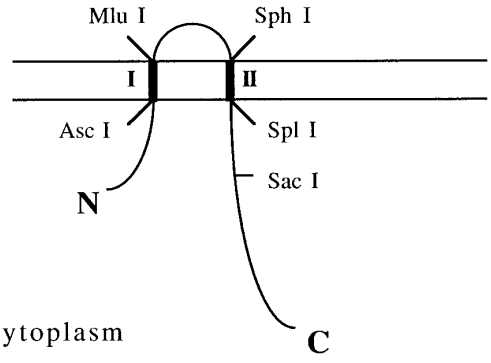

FIG. 2. Predicted model for the insertion of CIRV 36K and CymRSV 33K proteins in the outer membrane of modified mitochondria or the single membrane of modified peroxisomes, respectively. The N- and C-termini are both localized in the cytoplasmic portion of the vesicles. The C-terminus represents the C-termini of the 36K and 33K proteins as well as the C-termini of the fusion protein representing the complete replicase. The thick lines (I and II) represent the transmembrane segments. The drawing is to an approximate scale; therefore, restriction sites, although not located at identical positions in CIRV and CymRSV, are shown in the same approximate positions.

Infectivity and cytopathology of hybrid CIRV-CymRSV clones

On the basis of the hypothetical model of Fig. 2, we constructed hybrid CIRV-CymRSV clones containing 33K and 36K protein exchanges. Four domains were used for these exchanges: (1) a leader sequence up to the first transmembrane segment, (2) the first and (3) second transmembrane, and (4) the remainder of the protein sequence downstream of the second transmembrane domain. Some of these areas in the cDNA clones are delineated by natural restriction sites, whereas others were introduced by site-directed mutagenesis. An *Asc*I site was introduced in both the CymRSV and CIRV sequences, which caused one (Gln to Ala) and two (Gly-Val to Arg-Ala) aa changes in the 33K and 36K protein sequences, 7 and 5 aa upstream of transmembrane I, respectively. A mutation to introduce a *Mlu*I site in the CIRV sequence had no effect in the encoded protein, but a similar mutation in the CymRSV cDNA elicited a change (Ser to Arg) in the last aa of the transmembrane I domain. Two additional restriction sites were introduced in the 33K protein coding sequence: *Sph*I, which caused one aa substitution (Leu to Met) 4 aa upstream of transmembrane II, and *Spl*I, which changed aa 15 (Val) of transmembrane II to Ala. Mutant CymRSV and CIRV clones were infectious and elicited cytopathological features identical to the respective wild types (wt). Therefore, they were used as sources for the construction of

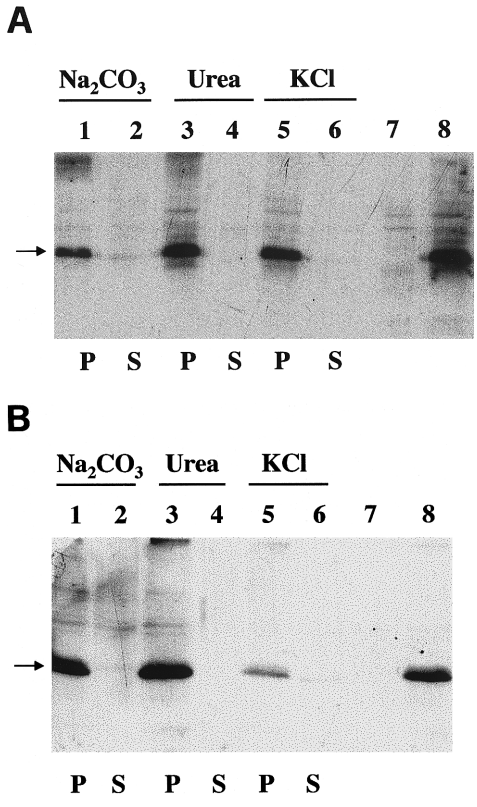

FIG. 3. Immunoblot analysis of 33K (A) and 36K (B) proteins. The P30 fractions were extracted in 0.1 M Na_2CO_3 (lanes 1 and 2), 4 M urea (lanes 3 and 4), or 1 M KCl (lanes 5 and 6) and centrifuged at 30,000*g* to yield supernatant (S) and pellet (P) fractions. Lanes 7 and 8 contain total P30 fraction from uninfected and infected plants, respectively. Arrows point to 33K (A) and 36K (B) positions.

hybrid clones in which one or more portions of the N-terminal region were exchanged.

As shown in Table 1, 5 of the 10 hybrids failed to infect *N. benthamiana* plants. We were unable to determine whether the lack of biological activity of these clones was due to structural instabilities of the hybrid 33K/36K protein or to their inability to be targeted, anchored, or both to mitochondria or peroxisomes. On the other hand, it is possible that structural modifications to the hybrid RNAs interfere with their abilities to be replicated.

Clones CIRV L_{CymRSV}, CIRV $I+II_{CymRSV}$, CymRSV $I+II_{CIRV}$, CIRV II_{CymRSV}, and CymRSV II_{CIRV} were infectious. To rule out that the infections were not due to contamination, progeny were sequenced and shown to correspond to the hybrid constructs used for inoculation. Cytopathological observations of leaf cells infected with *in vitro* synthesized RNA from these clones revealed the presence of MVBs associated with mitochondria and peroxisomes, but the distribution was variable. In cells infected with CIRV L_{CymRSV}, CIRV $I+II_{CymRSV}$, and CIRV II_{CymRSV}, most mitochondria were transformed into MVBs, similar to those in wt CIRV-infected cells. However, only a few peripheral vesicles were present in peroxisomes that resembled the prominent MVBs found in cells infected with wt CymRSV. In contrast, in cells infected with CymRSV $I+II_{CIRV}$ and CymRSV II_{CIRV} transcripts, MVBs originating from peroxisomes were typical of those found in wt CymRSV infections, but mitochondria with peripheral vesiculation were also present. Organellar modifications induced by CIRV II_{CymRSV} and CymRSV II_{CIRV}, representative of the two groups of infectious hybrid clones, are shown in Figs. 4, A and B.

The experiments described above represent a continuation of those reported by Burgyan *et al.* (1996), which revealed that a stretch of ~200 aa of the N-proximal region of ORF 1 of CymRSV and CIRV contained the signals for the association of the viral replication complex with either peroxisomes or mitochondria. Our current attempts to localize precisely the targeting signals in the 33K and 36K proteins by construction of hybrid CymRSV and CIRV full-length clones were only partially successful. In fact, the region containing the 5′-terminal 609 and 628 nt (i.e., up to the site *Spl*I in the cDNA clones) in the CymRSV and CIRV sequences, respectively, constitutes a unit block whose integrity we have been unable to manipulate extensively without altering

TABLE 1

Infectivity of Hybrid CIRV/CymRSV Clones and Origin of MVBs from Mitochondria (M) or Peroxisomes (P)

Leader	I	II	C-Terminus	Infectivity	Designation	Origin of MVB
CIRV	CIRV	CIRV	CIRV	+	CIRV wt	M
CymRSV	CymRSV	CymRSV	CymRSV	+	CymRSV wt	P
CymRSV	CIRV	CIRV	CIRV	+	CIRV L_{CymRSV}	P + M
CIRV	CymRSV	CymRSV	CymRSV	−	CymRSV L_{CIRV}	
CIRV	CymRSV	CymRSV	CIRV	+	CIRV $I+II_{CymRSV}$	M + P
CymRSV	CIRV	CIRV	CymRSV	+	CymRSV $I+II_{CIRV}$	P + M
CIRV	CymRSV	CIRV	CIRV	−	CIRV I_{CymRSV}	
CymRSV	CIRV	CymRSV	CymRSV	−	CymRSV I_{CIRV}	
CIRV	CIRV	CymRSV	CIRV	+	CIRV II_{CymRSV}	M + P
CymRSV	CymRSV	CIRV	CymRSV	+	CymRSV II_{CIRV}	P + M
CymRSV	CymRSV	CIRV	CIRV	−	CIRV L + I_{CymRSV}	
CIRV	CIRV	CymRSV	CymRSV	−	CymRSV L + I_{CIRV}	

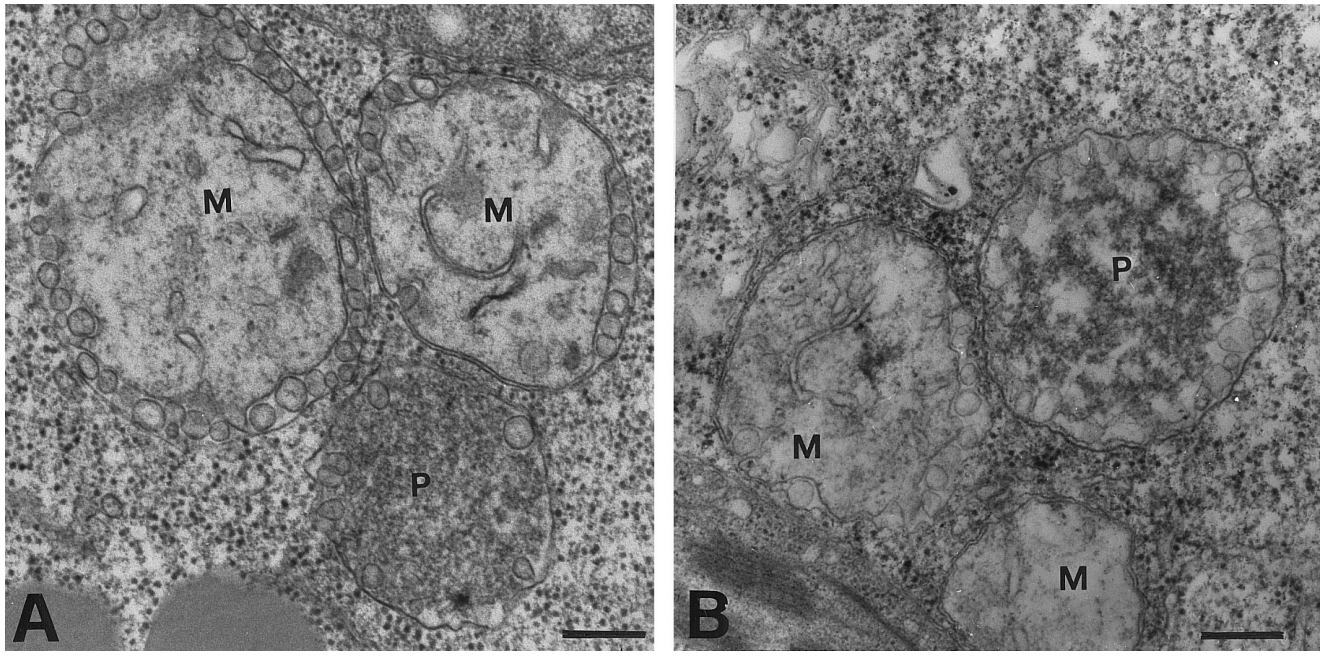

FIG. 4. Electron micrographs of *N. benthamiana* cells infected with hybrid constructs CIRV II_{CymRSV} (A) and CymRSV II_{CIRV} (B) showing vesiculated mitochondria (M) and peroxisomes (P) in the same cell. Magnification bar, 200 nm.

the targeting properties of CymRSV and CIRV ORF 1-encoded proteins or abolishing the biological activity of the hybrid constructs.

Observations of cells infected with viable hybrid clones seem to indicate that targeting of the wt 33K or 36K proteins to peroxisomal or mitochondrial membranes is determined by both the leader sequences and the transmembrane segments. This is not surprising because it is known that besides anchoring soluble segments to a membrane, transmembrane segments of integral membrane proteins contain the information for targeting proteins to several cell membrane types (McBride *et al.*, 1992; Nguyen *et al.,* 1993; Swift and Machamer, 1991).

Putative targeting signals in the 36K and 33K proteins

As noted above, the CymRSV 33K protein is characterized by the presence of the heptapeptide PKKEIFV absent in the corresponding area of the CIRV 36K protein. Further downstream in the latter sequence is a stretch of 15 aa (positions 134–148) (Fig. 1) that are absent in the 33K protein. Two additional mutant clones were prepared: CIRV+7aa, in which the heptapeptide PKKEIFV was inserted between aa 32 and 33 in the 36K wt CIRV sequence, and clone CIRVΔ15, in which the stretch of 15 aa was deleted. Cells infected with either these constructs produced MVBs similar to those elicited by wt CIRV (not shown), indicating that these sequences have no targeting functions.

The search for membrane targeting sequences was therefore carried out elsewhere in the 36K and 33K protein sequences, taking into account the information available on the transport to diverse cellular compartments of proteins synthesized in the cytoplasm (Rusch and Kendall, 1995; Whelan and Glaser, 1997).

The N-terminal regions of nuclear-encoded mitochondrial proteins do not have specific primary sequences, but most of them are poor in or devoid of aspartic acid, glutamic acid, valine, and isoleucine, whereas they are relatively rich in arginine, serine, and leucine. In addition, these sequences can potentially form amphiphilic helices with basic and hydrophilic amino acids forming a polar face and hydrophobic residues forming the nonpolar opposite face (von Heijne, 1986). Most mitochondrial targeting sequences (MTS) form amphipathic helices with a value of the hydrophobic moment (μH, calculated as in Eisenberg *et al.,* 1984) of $>$7.3 and a value of the hydrophobicity of the nonpolar face of $>$4.5 (von Heijne, 1986). A search for a MTS in the N-terminal regions of the CIRV 36K protein was performed using the computer program MitoProt (Claros, 1995). Amphipathic helices with minimum values of μH and hydrophobicity of the nonpolar face of $>$5.0 and $>$3.9, respectively, represent putative MTS, whereas amphipathic helices with values of $>$7.3 and $>$4.5 are considered certain MTS. No such signal was found in the extreme N-terminal region of the 36K protein, due to the presence of the two closely located acidic residues (Asp at positions 2 and 5). However, a strong suggestion that a MTS may reside downstream of the methionine at position 23 is given by the use of CIRV mutant clone C80 (Burgyan *et al.,* 1996), in which the translation of the ORF 1 was made

to start at this second methionine. Electron microscopic observations of cells infected with this clone had in fact revealed that MVBs originated from mitochondria exactly as in wt infections. We therefore extended the analysis to this region and found that a stretch of 14 residues (FGSLPSSLERPVAK, position 32–45 in the complete 36K sequence) forms an amphipatic α-helix that satisfies the conditions for an MTS signal by having a μH of 9.59 and a nonpolar face hydrophobicity of 6.47.

To evaluate this prediction, two mutants were prepared within the putative 36K MTS: the serine residue at position 34 was mutated to aspartic acid (AGU to GAU) or arginine (AGU to AGG) to give clones CIRV/asp and CIRV/arg, respectively. Cytopathological analysis of cells infected with CIRV/asp transcripts revealed the presence of some vesiculated peroxisomes in addition to MVBs that clearly originated from mitochondria. This observation was comparable to the multidirected targeting in cells infected with hybrid clones CIRV $I+II_{CymRSV}$ and CIRV II_{CymRSV}, where MVBs originated mainly, but not exclusively, from mitochondria. By contrast, cells infected with clone CIRV/arg were indistinguishable from wt infections. Sequence analysis demonstrated that the mutations were maintained in the progeny RNA from both mutants. This may indicate that replacing one serine residue with an acid residue (Asp) modifies the functionality of the MTS, whereas substitution of the same amino acid with another positively charged (Arg) does not alter the target of the 36K protein. This hypothesis is in agreement with the suggested optimal composition of nuclear-encoded mitochondrial proteins (von Heijne, 1986).

The position of the MTS in the 36K protein downstream of the methionine at position 23 raises the question whether the functional product of ORF 1 is indeed a protein starting at this second methionine, having a size of ~34K. Although previous Western blot analysis (Burgyan *et al.,* 1996; Rubino *et al*., 1995) did not support this hypothesis, an experiment was carried out to confirm that translation of ORF 1 does indeed begin at the first methionine codon. To do so, the AUG codon at position 23 was mutated to ACU coding for valine, obtaining clone CIRV/val. Western blot analysis showed the presence of a protein of ~34K in C80 infections and a protein of 36K in CIRV/val infections, identical to wt (not shown). Electron microscopic analysis of CIRV/val-infected tissue again indicated the presence of MVBs originating from mitochondria and the absence of vesiculated peroxisomes (not shown). These results then suggest that the MTS in the CIRV 36K protein is not located at the extreme N-terminal region. It appears to be located internally and to be active in the otherwise unfavourable enviroment of the N-terminal 22 aa. In conclusion, these data seem to support a model in which CIRV 36K protein is directed in part to mitochondria by a matrix-targeting signal located in the N-terminal region (aa 32–45). However, the transmembrane segments located downstream stop translocation at the outer membrane of the mitochondrial envelope.

As reported in the previous section, clone CymRSV L_{CIRV}, in which the MTS-containing CIRV sequence is present, was not infectious. However, because it was of interest to analyze the targeting properties of the CIRV MTS in the CymRSV context, attempts were made to restore the infectivity of this clone by using a CIRV leader sequence more similar to that of CymRSV. To do so, three additional clones were prepared: (1) CymRSV $L_{CIRVC80}$, in which the AUG start codon of wt CIRV was changed to AUC so the protein would start with the second methionine codon at aa position 23; (2) CymRSV $L_{CIRV+7aa}$, in which the sequence PKKEIFV of CymRSV was inserted in the sequence of CIRV between aa 32 and 33; and (3) CymRSV $L_{CIRVC80+7aa}$, in which both mutations were inserted in the CIRV 36K protein sequence. None of these clones were infectious. Therefore, attempts to exchange CIRV leader sequences with corresponding regions of CymRSV were discontinued.

Two pathways are known for the translocation of proteins from the cytoplasm, where they are synthesized, to the peroxisomal matrix. Each of these pathways requires the presence of a specific peroxisomal targeting sequence (PTS). Most peroxisomal matrix proteins have a PTS1 signal consisting of the C-terminal tripeptide serine-lysine-leucine (SKL) with minor variants where the serine can be substituted by either alanine or cysteine (AKL or CKL) or lysine can be replaced by either arginine or hystidine (SRL or SHL). Other peroxisomal proteins have the N-terminal PTS2 signal that consists of the nonapeptide (R/K) (L/V/I) $(X)_5$ (H/Q) (L/A) (Gould *et al.*, 1990; Rachubinski and Subramani, 1995). These signals and similar motifs are not present in the CymRSV 33K protein sequence.

As reported above, the insertion of the heptapeptide PKKEIFV between aa 32 and 33 in the CIRV 36K sequence did not modify the mitochondrial targeting signal of this protein. This same oligopeptide was deleted in the CymRSV full-length clone, obtaining clone CymRSV-7aa. Plants inoculated with this clone did not show either local or systemic symptoms, whereas protoplasts became infected but yielded an amount of progeny RNA about one-tenth of the amount detected with wt infection. At this time, we are unable to say which factor is affected in the replication of mutant CymRSV-7aa. Given the result obtained with the corresponding clone CIRV+7aa, which did not suggest any targeting activity of the heptapeptide PKKEIFV, the reason for the poor infectivity of clone CymRSV-7aa was not investigated further.

This study contributes to an understanding of factors involved in the development of a membranous environment in which viral replication takes place. The most interesting outcome of these experiments was the possible identification of a structural motif in a protein of CIRV, the only tombusvirus that elicits the formation of

MVBs from mitochondria rather than peroxisomes. This confirms experimentally the finding relative to the presence of a putative MTS signal identified in the protein encoded by ORF 1 of three members of the related genus *Carmovirus* that elicit transformation of mitochondria into MVBs (Ciuffreda *et al.*, 1998).

MATERIALS AND METHODS

Fractionation and analysis of cell membranes

Cell membranes were isolated, fractionated, and treated according to Schaad *et al.* (1997). Briefly, 1 g of uninoculated or systemically infected leaf tissue was collected 5–7 days after inoculation and ground in 4 ml of buffer (50 mM Tris–HCl, pH 7.4, 15 mM $MgCl_2$, 10 mM KCl, 20% glycerol, 0.1% β-mercaptoethanol, 5 μg/ml leupeptin, and 2 μg/ml aprotinin). Intact cells, plastids, nuclei, and large debris were removed by centrifugation at 3000*g* for 10 min. The supernatant was centrifuged at 30,000*g* for 30 min, and the membrane-containing pellets (P30) were examined as such or after treatment with Na_2CO_3, pH 11.5, or extraction with 4 M urea or 1 M KCl. After these treatments, samples were again centrifugated at 30,000*g* for 30 min. The pellet (P) and supernatant (S) fractions were subjected to immunoblot analysis using an anti-CymRSV 33K serum (Lupo *et al.*, 1994) known to also react with the heterologous CIRV 36K (Rubino *et al.,* 1995) using an enhanced chemiluminiscent assay (ECL; Amersham).

Construction and inoculation of recombinant clones

Site-directed mutagenesis was done according to Kunkel *et al*. (1987) with a Mutagenesis Kit (BioRad) according to the manufacturer's instructions, except that *Escherichia coli* strains RZ1032 and TG1 were used as *ung*$^-$ and *ung*$^+$ hosts, respectively, or using the high fidelity termostable *Pfu* DNA polymerase in the QuikChange Site-Directed Mutagenesis kit (Stratagene). Mutations were introduced in restriction fragments, which were completely sequenced before subcloning back to the full-length clone. Mutagenic oligonucleotides were generally 20–40 nt long, with the looped-out region or the mismatched nucleotides located in the center.

Hybrids were prepared from constructs containing the CymRSV or CIRV cDNAs downstream of the phage T7 promoter (Burgyan *et al.,* 1996; Dalmay *et al.*, 1993). To exchange different segments between the two genomes, natural common restriction sites were used and others were engineered by site-directed mutagenesis (Kunkel *et al.,* 1987). RNA transcripts were diluted with an equal volume of inoculation buffer containing 1% celite and 1% bentonite (Heaton *et al.,* 1989) and inoculated to *N. benthamiana* plants. Protoplasts were prepared from leaf tissue and transfected as described previously (Dalmay *et al.*, 1993; Nagy and Maliga 1976).

RNA was extracted from inoculated plants and protoplasts and analysed by Northern blot hybridization as described previously (Dalmay *et al.,* 1993). To examine the sequence of progeny RNA, cDNAs were prepared using an oligonucleotide complementary to a sequence downstream of the region of interest. This region was PCR-amplified, and the products were cloned and sequenced.

Electron microscopy

Leaf tissue fragments from systemically infected leaves of plants at 5–7 days postinoculation were excised and fixed with 4% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2. Samples were postfixed with 1% osmium tetroxide, stained overnight in 0.5% uranyl acetate in distilled water, dehydrated in a graded ethanol series, and embedded in Spurr's medium. Sections were stained with uranyl acetate and lead citrate and viewed with a Philips 201C or 400T electron microscope.

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