Expression of a plant virus non-structural protein in *Saccharomyces cerevisiae* causes membrane proliferation and altered mitochondrial morphology

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Carnation Italian ringspot tombusvirus encodes a protein, referred to as 36K, that possesses a mitochondrial targeting signal and two transmembrane segments which are thought to anchor this protein to the outer membrane of the mitochondrial envelope of infected plant cells. To determine the topology of the virus protein inserted in the cell membrane, as well as the sequence requirements for targeting and insertion, an *in vivo* system was set up in which this could be analysed in the absence of productive virus infection. The 36K protein was expressed in the yeast *Saccharomyces cerevisiae* in native form or fused to the green fluorescent protein. Using a fluorescence microscope, large green-fluorescing cytoplasmic aggregates were visible which stained red when cells were treated with the vital stain MitoTracker, which is specific for mitochondria. These aggregates were shown by electron microscopy to be composed of either mitochondria or membranes. The latter type was particularly abundant for the construct in which the green fluorescent protein was fused at the N terminus of the 36K protein. Immunoelectron microscopy demonstrated that the viral protein is present in the anomalous aggregates and Western blot analysis of protein extracts showed 36K to be resistant to alkaline, urea or salt extraction, a property of integral membrane proteins.

Introduction

Tombusviruses are small, plus-strand RNA viruses belonging to the genus *Tombusvirus* in the family *Tombusviridae*. They contain a single copy of a plus-sense RNA genome of ca. 4700 nt that has five ORFs. Following infection, the viral genome of tombusviruses is transcribed into non-structural proteins of 33 or 36 kDa (33K and 36K proteins, respectively), depending on the species, and the corresponding readthrough products of 92K or 95K. Both the shorter protein and the complete readthrough product are essential for virus replication and were shown to contain the eight conserved motifs (PI–PVIII) of RNA-dependent RNA polymerases of supergroup II of the positive-strand RNA viruses (Koonin, 1991; Russo *et al.*, 1994). The capsid protein and other non-structural proteins are synthesized from subgenomic RNAs later in the virus infection cycle (Russo *et al.*, 1994).

Carnation Italian ringspot virus (CIRV) is one of several tombusviruses which have been studied (Di Franco *et al.*, 1984; Rubino *et al.*, 1995). In particular, early cytopathological investigations have shown that CIRV-infected cells contain vesiculated structures (multivesicular bodies, MVB) composed primarily of a large number of vesicles derived from the proliferation of the outer membrane of mitochondria (Di Franco *et al.*, 1984). CIRV-induced MVBs are similar to structures induced by other tombusviruses except that these latter structures develop from proliferation of the limiting membrane of peroxisomes (Russo *et al.*, 1987). Whichever organelle gives rise to MVB, vesicles are thought to be the site of virus replication (Rubino & Russo, 1998). Recently, it was proposed that the CIRV 36K protein contains a signal that directs the virus replicase to mitochondria, where it is anchored to the outer lamella of the limiting membrane by two transmembrane segments (Rubino & Russo, 1998).

It is an open question how proliferation of the mitochondrial membrane takes place in CIRV-infected cells, i.e. if and how the 36K protein is involved in the generation of the
membranous vesicles. A good model system for analysis of membrane proliferation and vesicle transport is represented by the yeast *Saccharomyces cerevisiae* (Wright et al., 1988; Kaiser & Scheckman, 1990; Pryer et al., 1992). This prompted us to use yeast cells to express the CIRV 36K protein to allow study of its cytological effects in the absence of a productive virus infection.

### Methods

**Cloning of the wild-type CIRV 36K protein gene.** DNA encoding the 36K protein was obtained from a full-length cDNA clone of the genomic RNA of CIRV in pUC18 (Burgyan et al., 1990). Firstly, the context around the AUG start codon was mutated to an NcoI site by site-directed mutagenesis using the *Pfu* DNA polymerase in the QuikChange Site-Directed Mutagenesis kit (Stratagene). The full-length clone was then digested with NcoI and Smal (present only in the vector linker), treated with Klenow enzyme and religated. This eliminated the 5’ non-translated sequence from the full-length clone. The resulting clone was then digested with *Eco*RI (at the 3’ terminal downstream of the stop codon of the 36K protein) and SwaI (at the 3’ end of the viral sequence) and religated. The resulting clone contained essentially only the CIRV 36K gene (clone p36K).

**Construction of GFP+36K plasmids.** To construct a vector expressing the 36K protein fused to the green fluorescent protein (GFP), a clone of a yeast codon-optimized form of the GFP (*pyEGFP*) gene in pUC19 was used (Cormack et al., 1997). The coding sequence for the 36K protein was fused to either the 5’ or 3’ end of the GFP gene. For the first construct (p36K-GFP), a HindIII restriction site was engineered at the stop codon of the 36K gene in the clone p36K by site-directed mutagenesis. The fragment containing the sequence between the HindIII sites in the linker and at the end of the viral gene was purified and ligated into the HindIII site of clone pyEGFP. A clone containing 36K and GFP in the correct orientation was chosen by sequencing. This contained four additional amino acids between the 36K and GFP protein sequences encoded by the nucleotides between the HindIII site and the start codon of GFP.

To produce a plasmid containing the 36K gene positioned at the 3’ end of the GFP sequence, the clone *pyEGFP* was digested with *Pst*I, which cuts 21 nt upstream of the protein stop codon, treated with T4 DNA polymerase to generate a blunt end and then digested with *Hind*III (in the linker, upstream from the GFP start codon); the clone p36K was first digested with NcoI, made blunt-ended with Klenow enzyme and digested with *Hind*III (in the linker, upstream from the 36K start codon). Finally, the *Hind*III-*Pst*I fragment from *pyEGFP* was cloned into p36K to produce pGFP-36K. In this clone, 7 aa were deleted in the 3’ terminal region of GFP.

** Yeast transformation. Plasmids p36K, *pyEGFP*, p36K-GFP and pGFP-36K were digested with *Hind*III and *Eco*RI (present only at the ends of the vector linker sequence), and the resulting fragments were ligated into the plasmid pYES2 containing the galactose-activated GAL1 promoter (Invitrogen) digested with the same restriction enzymes. Clones in vector pYES2 maintain the same nomenclature as those in pUC18.

For expression, the *S. cerevisiae* strain YP499 (MATa, ura3-52, lys2-801, ade2-101, trp1-1delta63, his3-delta200, leu2-delta1; Sikorski & Hieter, 1989) was used throughout. Transformation was done using frozen cells. Briefly, the yeast was cultured on YPD (1% yeast extract, 2% bacteropeptone, 2% dextrose, pH 4.8–5.0) plates at 30 °C for 2–3 days to form a continuous layer. Cells were then scraped from one plate with a spatula, transferred to an Eppendorf tube containing 1 ml TES (1 M sorbitol, 10 mM Tris–HCl, 3% ethylene glycol, pH 8.3), pelleted by centrifugation (6000 r.p.m. for 30 s) and washed three times with TES. The washed cell pellet was resuspended in 750 µl TES to which 45 µl DMSO was added. The mixture was then divided into 100 µl aliquots in 1.5 ml Eppendorf tubes. These were snap-frozen in liquid nitrogen for a few seconds and stored at −70 °C for at least 30 min. About 1 µg DNA in 20–30 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) was pipetted on top of the frozen cells which were then allowed to thaw for a few seconds at room temperature. The tubes were then incubated at 37 °C for 5 min with gentle shaking every 30 s. One milliliter of TPEG buffer (200 mM Tris–HCl, 40% PEG 1000, pH 8.3) was added and the cells were incubated for 1 h at 30 °C on a rotary agitator (150 r.p.m.). Finally, the cells were sedimented and resuspended in 300 µl TN buffer (10 mM Tris–HCl, 150 mM NaCl, pH 8.3). The cell suspension (200 µl) was spread on minimal selective medium (SM) plates containing 0.67% Difco yeast nitrogen base without amino acids, 2% dextrose, amino acids at 40 µg/ml, adenine at 10 µg/ml, 2% agar and no uracil. Red colonies were streaked and maintained on SM plates.

**Culture of transformants and Western blot analysis.** A single colony was collected, inoculated in 10 ml SM containing 2% dextrose and incubated for 24 h at 30 °C at 150 r.p.m. One milliliter of this culture was used to inoculate 10 ml SM containing 3% glycerol and 0.1% dextrose, followed by incubation for a further 24 h. Finally, 1 ml of this second culture was used to inoculate 10 ml YP (1% yeast extract, 2% dextrose, pH 4.8–5.0) containing 3% glycerol and 2% galactose and the mixture was incubated for 16 h.

Cells were converted to spheroplasts using the protocol of Ausbel et al. (1995) except that it was adapted to a smaller scale by using 5 ml of a 10 ml culture (A600 of 0.6) and by performing washings, resuspension and treatment with Zymolyase (20 U/ml) in a volume of 500 µl. To extract proteins, the final spheroplast pellet was resuspended in 1 ml lysis buffer (50 mM Tris–HCl, pH 7.4, 15 mM MgCl2, 10 mM KCl, 0.3 M sorbitol, 0.1% β-2-mercaptoethanol, 5 µg/ml leupeptin, 2 µg/ml aprotinin) and incubated for 2 min at room temperature. The cell lysate was centrifuged at 500 g for 3 min at 4 °C to remove large debris and intact cells, and the supernatant was centrifuged at 12,000 g for 15 min at 4 °C, saving the pellet (P12) and supernatant (S12). The P12 pellet was examined as it was or after treatment with one of the following reagents for 30 min on ice: 100 mM Na2CO3 (pH 11.5), 4 M urea or 1 M KCl (Schaad et al., 1997). After treatment, the solution was centrifuged at 12,000 g for 15 min at 4 °C to generate a supernatant and a pellet fraction. Immunoblot analysis was performed as previously described (Rubino et al., 1995) using an enhanced chemiluminescent assay (Amersham).

**Light and electron microscopy.** Microscopic observations of cells expressing GFP were routinely done with a Nikon ECLIPSE E800 epifluorescence microscope equipped with FITC filters (excitation 450–490, dichroic minor 505, band pass 520). Photography was with a Nikon ECLIPSE E800 epifluorescence microscope equipped with GFP(R)-Long Pass (excitation 460–500, dichroic minor 505, long pass 510) or GFP(R)-Band Pass (excitation 460–500, dichroic minor 505, band pass 510–560). To stain cells with MitoTracker (Molecular Probes), cells were incubated with 0.5 µM MitoTracker, washed three times with PBS (10 mM potassium phosphate buffer, 0.14 M NaCl, pH 7.4) and observed using a TRITC filter set (excitation 510–560, dichroic minor 575, band pass 590). Cells were immobilized on glass slides by mixing 5 µl cell suspension with 5 µl 1% low-melting-point agarose maintained at 45 °C.
and covering the mixture with a cover-slip. Images were collected with a Power HAD 3CCD videocamera (Sony), using Visiolab b 200 (Biocom) and processed with Adobe Photoshop.

For electron microscopy, cells from a galactose-induced 10 ml liquid culture were sedimented, fixed with 4% (v/v) glutaraldehyde, post-fixed with 4% (w/v) potassium permanganate and embedded in Spurr’s resin as described (Yaffe, 1995). For immunogold labelling, cells were fixed and embedded in LR White resin (Wright & Rine, 1989). The primary anti-36K or pre-immune sera were used at a dilution of 1/1000, and the gold (10 µm)-labelled goat anti-rabbit antiserum (Amersham) at 1/20.

**Results**

**Growth of transformed yeast**

Yeast cells transformed with pyEGFP, p36K, p36K-GFP and pGFP-36K grew well on agar plates containing dextrose as the carbon source, similarly to cells transformed with the vector only. Growth on solid medium containing galactose as the sole carbon source was poorer but still significant except for transformants containing pGFP-36K, where single colonies remained very small or were barely visible (Fig. 1). In contrast, no difference was noticed among transformants grown in liquid culture containing glycerol, regardless of whether synthesis of recombinant proteins was induced by galactose. For instance, cell density, measured as $A_{600}$ of all recombinant yeast cells in liquid medium containing galactose was approximately 0.3, 0.6 and 1.0 after 9, 18 and 24 h, respectively. However, microscopic observations (see below) showed that the number of non-fluorescent cells in the samples containing p36K-GFP- and pGFP-36K-transformed yeasts were much higher in the liquid culture (ca. 70%) than in solid media (ca. 40%), indicating segregation and positive selection of cells that had lost the plasmid or the insert.

**Immunoblot analysis**

To test whether fused and unfused CIRV 36K proteins were expressed in yeast cells and interacted with membranes,
**Light microscopy**

Bright field microscopic observations of transformed yeast cells growing in the presence of galactose, either in solid or liquid medium, showed that cells had size and shape identical to untransformed cells, with a number of them in the process of division. Under the imaging conditions for visualization of GFP, all cells transformed with pYEGFP, but only a portion of cells transformed with p36K-GFP or pGFP-36K were fluorescent. yEGFP-expressing cells showed green fluorescence distributed throughout the cytoplasm and in the nucleoplasm, but not in the vacuoles (Fig. 3 a). When the same cells were stained with a mitochondrial-specific dye, MitoTracker, globular structures interpreted as mitochondria were visible above the pale red background (Fig. 3 f). Conversely, in cells expressing GFP fused to the 5′ terminus of the 36K protein (pGFP-36K), green fluorescence was restricted to defined regions of the cytoplasm. These were either punctate structures 1–3 µm in size (Fig. 3 b) or large patches comprising most of the cytoplasm (Fig. 3 c). The same areas stained red when cells were treated with MitoTracker showing that they might be constituted in part by mitochondria (Fig. 3 g, h). In the case of cells expressing GFP fused to the 3′ terminus of the 36K protein (p36K-GFP), localization of fluorescence usually involved a defined part of the cytoplasm (Fig. 3 d, e) with some evidence of localization in defined structures possibly representing mitochondria (Fig. 3 d). Vital staining with MitoTracker supported this interpretation (Fig. 3 i, l). Nuclei and vacuoles of all types of cells treated with MitoTracker appeared free of dye.

**Electron microscopy**

Preliminary observations of transformed and non-transformed yeast cells fixed with glutaraldehyde and post-fixed with osmium tetroxide showed very electron-dense cytoplasm
Expression of CIRV 36K protein in yeast

Fig. 5. Electron microscopic analysis of *S. cerevisiae* cells transformed with p36K (a, b), p36K-GFP (c, d) and pGFP-36K (e, f).

(a) The upper row (a, c and e) shows the accumulations of mitochondria less evident in the case of pGFP-36K (e) where the membrane accumulation is more conspicuous. The lower row (b, d and f) shows details of membrane accumulations. Cells were glutaraldehyde-permanganate fixed. Bar, 200 nm.

Prominent features of cells transformed with vector only were a nucleus surrounded by a double membrane interrupted by several pores, a vacuole containing very electron-dense material and several strands of endoplasmic reticulum. The plasmalemma lined a cell wall in which one or more scars indicated where daughter cells separated during budding. Profiles of mitochondria were rounded or elongated and were regularly distributed in the cytoplasm, never showing a preferential site of accumulation. Up to 10–12 mitochondrial profiles were counted per section. Cristae lay mostly perpendicular to the mitochondrial surface (Fig. 4a). Cells transformed with pyEGFP showed no difference compared to those transformed with the vector only (Fig. 4b).

Cells transformed with DNA encoding unfused CIRV 36K or this protein fused to the N terminus of GFP (p36K-GFP) exhibited profoundly altered mitochondria with respect to both number and morphology. Groups of twenty or more mitochondrial profiles could be counted in a cell section. They were of variable shape and size and contained poorly developed cristae or no cristae at all (Fig. 5a, c). Intermingled with the mitochondria and, in some cases, in apposition to them, were strands of membranous material (Fig. 5b, d). Cells transformed with the construct consisting of the 36K protein fused to the C terminus of GFP (pGFP-36K) displayed stacks of irregularly arranged membranes often surrounding what appeared to be abnormal mitochondria with very few or no cristae (Fig. 5e, f). Although the abnormal membranes were often very close to or were in apposition to the nuclear membrane, there was no evidence of continuity between the two types of membranes.

To analyse the involvement of the CIRV 36K protein with the abnormal membrane accumulations and altered mitochondria, cells were examined by indirect immunoelectron microscopy. Sections of transformed yeast cells were incubated...
Fig. 6. Immunoelectron microscopic analysis of *S. cerevisiae* cells transformed with pGFP-36K. Cells were glutaraldehyde–formaldehyde fixed. (a) Primary antibodies against the 36K protein. (b) Pre-immune serum. Bar, 200 nm.

Table 1. Distribution of the CIRV 36K protein in yeast cells transformed with different constructs

Values given represent the average number of gold particles in various areas of yeast cells treated with the anti-36K antiserum (AS) or pre-immune serum (Pr). Ten to fifteen cell profiles were counted for each construct.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Membrane</th>
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<th>Vacuole</th>
<th>Cytoplasm*</th>
<th>Wall</th>
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<td>5 2</td>
<td>4 4</td>
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<td>pYES2</td>
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* Areas not containing accumulations of membranes.

either with a polyclonal antiserum reactive against the 36K protein or a pre-immune serum followed by incubation with gold-conjugated secondary antibody. In both cases, label was present on the cell wall (Fig. 6a, b). This was a result of the formation of aldehyde groups following the reaction of carbohydrate residues with periodate, which is only partially prevented by treatment with ammonium chloride (van Tuinen & Riezman, 1987; van Tuinen, 1996). In fact, the same pattern of labelling of the wall was also found in cell sections of yeast cells transformed with vector only (not shown; Table 1). If the chemical reactivity of the cell wall is not considered, the amount of label in sections treated with the immune antiserum was higher than in those treated with the pre-immune antiserum (Table 1). For instance, in the case of the pGFP-36K-transformed cells, an average of 50 gold particles was counted on membranes in single sections, i.e. about ten times as much as on the controls treated with pre-immune serum (Fig. 6a, b). Labelling was particularly frequent in the cytoplasmic areas containing abnormal membranes and mitochondria. Some gold particles were also present on the vacuoles and occasionally also on the nucleus, as in sections treated with pre-immune antiserum. Virtually no particles were observed on the cells treated with secondary antiserum only (not shown).

Analysis of cells transformed either with unfused 36K or 36K-GFP did not show significant differences between the effects of the two constructs with regard to the accumulation of gold label. There was less labelling than in the case of the expression of the pGFP-36K construct, but the levels were still
higher than the controls (average 30 particles vs 5; Table 1). Conversely, the distribution of gold particles was comparable to that observed with GFP-36K, extending in particular over the areas involved in the alterations (not shown).

Discussion

The yeast S. cerevisiae is now widely used as a host either for the replication of viral genomes or for the expression of specific virus proteins (see for instance, Janda & Ahlquist, 1993; Barco & Carrasco, 1995). In this paper, we examined the possibility of expressing the protein encoded by ORF 1 of CIRV in S. cerevisiae and have carried out experiments to determine whether its expression provoked a modification of yeast mitochondria. Western blot analysis showed that the viral protein is correctly expressed in yeast cells conserving the same electrophoretic mobility and immunological properties as the form expressed in infected plants. Moreover, cell extract fractionation and treatments of cell extract fractions with alkali, urea or salt confirmed that the CIRV 36K protein behaves as an integral membrane protein in yeast as in plant cells (Rubino & Russo, 1998). Fusion of 36K with GFP modified only its size and left its immunoreactivity unaltered, permitting analysis of its localization by fluorescence and immuno-electron microscopy.

The subcellular distribution of fluorescence in cells expressing unfused GFP or 36K-fused GFP is very distinct. Cells expressing unfused GFP show equal distribution of fluorescence throughout the cytoplasm and nucleus. Given its small size (ca. 27 kDa), GFP probably enters the nucleoplasm by diffusion through the nuclear pores. Conversely, it is excluded from vacuoles, as it lacks the specific sorting signal possessed by yeast proteins targeted to this organelle (Banta et al., 1988). When fused to the CIRV 36K protein, GFP acquired a strong localization signal, since fluorescence in p36K-GFP and pGFP-36K cells was not diffuse, but associated with cytoplasmic structures. Under the electron microscope, these structures appeared distinct, depending upon whether they were induced by the construct having the viral protein at the N or the C terminus. With p36K-GFP, the abnormal structures were composed of accumulations of mitochondria and a few membranous strands, whereas with pGFP-36K, membranous strands were much more abundant than mitochondria. Structures induced by the unfused 36K were identical to those elicited by p36K-GFP. No definitive explanation can now be provided concerning the significance of these structures. One possibility could be that massive membrane flow is activated by the 36K protein leading to an increase in the number of mitochondria. It is worth noting that transformation of yeast cells with the DNA encoding the cymbidium ringspot tombusvirus (CymRSV) 33K protein (known to induce the formation of MVBs from peroxisomes; Burgyan et al., 1996) fused to GFP did not lead to any particular localization of the reporter protein (unpublished results). Since under the growing conditions we have used, peroxisomes are not detectable in yeast, it can be deduced that no localization of GFP fused to a viral protein takes place in the absence of the targeted organelle. These findings suggest that the localization of the GFP fused to CIRV 36K is specifically due to interactions with mitochondria. The different behaviour of the p36K-GFP and pGFP-36K constructs may be ascribed to the fact that, in the latter construct, the targeting signal, usually located towards the N terminus of the protein, is obscured by the presence of GFP upstream of it. The formation of true MVBs similar to those formed in CIRV-infected plant cells was not really expected since the expression of the ORF 1-encoded protein is not the only factor leading to the synthesis of MVBs. In fact, transgenic plant cells constitutively expressing the CymRSV 33K protein do not contain peroxisome-derived MBVs, but only proliferating membranes and extremely altered peroxisomes, where the 33K protein accumulates, and no alterations of any other organelle (Bleve-Zacheo et al., 1997). Therefore, we believe that the constitutive or transient expression of tombusvirus ORF 1-encoded proteins may be useful to study the interactions of this protein with cell organelles, including the analysis of host cell factors composing the active replicase, of which the CIRV 36K and CymRSV 33K are part.

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References


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