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Evidence for Two Nonoverlapping Functional Domains in the Potato Virus X 25K Movement Protein

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To study subdomain organization of the potato virus X (PVX) movement protein (MP) encoded by the first gene in the triple gene block (TGB), we mutated the 25-kDa TGBp1 protein. The N-terminal deletion of the helicase motifs I, IA, and II resulted in loss of the ATPase activity and RNA binding. A frameshift mutation truncating the C-terminal motifs V and VI gave rise to increase of the TGBp1 ATPase activity and had little effect on RNA binding *in vitro*. Fusions of the green fluorescent protein with 25-kDa MP and its derivative lacking motifs V–VI exhibited similar fluorescence patterns in epidermal cells of *Nicotiana benthamiana* leaves. Cell-to-cell movement of the 25K-deficient PVX genome was not complemented by the TGBp1 of *Plantago asiatica* mosaic potexvirus (PlAMV) but was efficiently complemented by a chimeric TGBp1 consisting of the N-terminal part of PlAMV protein (motifs I–IV) and the PVX-specific C-terminal part (motifs V–VI). These results suggest that NTP hydrolysis, RNA binding, and targeting to the specific cellular compartment(s) are associated with the N-terminal domain of the TGBp1 including the helicase motifs I–IV and that the C-terminal domain is involved in specific interactions with other virus proteins. © 1999 Academic Press

INTRODUCTION

Intercellular transport of nucleic acids and proteins in plants involves specialized channels, the plasmodesmata (PD) (reviewed in Mezitt and Lucas, 1996; Ding, 1997; Ghoshroy *et al.*, 1997; MacLean *et al.*, 1997). Plant viruses recruit the plasmodesmal pathway for short- and long-distance movement that is potentiated by virus-coded movement proteins (MPs) (reviewed in Atabekov and Taliansky, 1990; Carrington *et al.*, 1996; Gilbertson and Lucas, 1996; Ghoshroy *et al.*, 1997).

Many virus genomes code for a single MP with sequences falling into several distinct phylogenetic groups (Mushegian and Koonin, 1993). Biochemical studies and subcellular localization of the dissimilar MPs revealed several common features. For instance, the MPs of tobacco mosaic virus (TMV), red clover necrotic mosaic virus (RCNMV), and cucumber mosaic virus (CMV) were shown to bind nucleic acids and GTP, to be targeted to PD, to modify PD by increasing their size exclusion limit, and to traffick nucleic acids through PD (reviewed in Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997).

In a number of phylogenetically distant virus groups, a distinct transport gene module, the triple gene block (TGB), was found (Morozov *et al.*, 1989). The TGB codes for three MPs referred to as TGBp1, TGBp2, and TGBp3 (Solovyev *et al.*, 1996). All of them were found to be necessary for virus cell-to-cell movement (Petty and Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Angell *et al.*, 1996; Herzog *et al.*, 1998). The TGBp2 and TGBp3 proteins possessing regions of highly hydrophobic sequences (Morozov *et al.*, 1987) are able to associate with membranes (Niesbach-Klosgen *et al.*, 1990; Morozov *et al.*, 1990, 1991; Donald *et al.*, 1993) and cell walls (Hefferon *et al.*, 1997). The TGBp1 proteins contain NTPase/helicase sequence motifs required for virus movement *in vivo* (Gorbalenya and Koonin, 1989; Donald *et al.*, 1995; Bleykasten *et al.*, 1996) and possess ATP/GTPase and RNA-binding activities *in vitro* (Rouleau *et al.*, 1994; Donald *et al.*, 1995, 1997; Bleykasten *et al.*, 1996; Kalinina *et al.*, 1996; Lough *et al.*, 1998). It should be noted that in contrast to the TGBp1 of foxtail mosaic potexvirus (Rouleau *et al.*, 1994), the RNA binding ability of the PVX 25K MP was very low at physiological conditions (Kalinina *et al.*, 1996; Karpova *et al.*, 1997). The TGBp1 protein is able to increase the PD size exclusion limit and move through PD to adjacent cells (Angell *et al.*, 1996; Lough *et al.*, 1998). Thus some functional properties of the TGBp1 proteins resemble those of the TMV, RCNMV, and CMV MPs.

TGBp1 represents the superfamily 1 helicase, which contains seven conserved amino acid sequence motifs (reviewed in Gorbalenya and Koonin, 1993; Kadare and Haenni, 1997). In contrast to the numerous DNA helicases of the superfamily 1, the helicase activity of the

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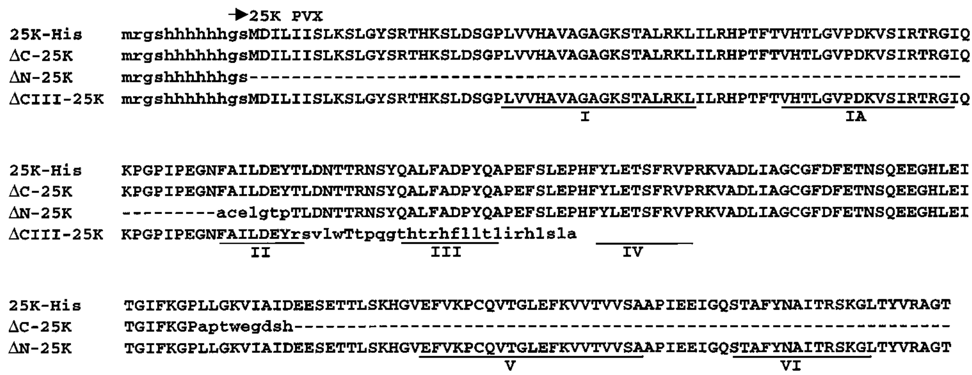

FIG. 1. Sequences of the PVX 25K MP and its deletion mutants. Positions of helicase motifs are indicated by underlining and Roman numerals. Lower case letters indicate artificial sequences present at the ends of superexpressed wt and mutated TGBp1 proteins.

superfamily 1 NTPase/helicases of plus-RNA viruses has only been demonstrated for alphaviruses (de Cedron *et al.,* 1999). Experimental determination of the protein tertiary structure, mutagenesis, and computer predictions showed that superfamily 1 DNA helicases comprise two distinct spatial and functional domains with each domain having two subdomains. The subdomain 1A including motifs I–IV is responsible for the NTPase activity, whereas the subdomain 2A (motifs V and VI) may contribute to conformational changes related to the coupling of ATPase activity and DNA binding (Gorbalenya *et al.,* 1989; Koonin and Rudd, 1996; Subramanya *et al.,* 1996; Korolev *et al.,* 1997; Hall *et al.,* 1998).

In this study, deletion mutations were introduced into the potato virus X (PVX) 25K TGBp1 to dissect the activities of the N-terminal (motifs I–IV) and C-terminal (motifs V–VI) portions of the protein. Our findings suggested that the PVX TGBp1 region including the helicase motifs V–VI is dispensable for NTP hydrolysis, RNA binding, and the MP targeting to specific cellular compartment(s) and could be involved in specific interactions with other virus proteins.

RESULTS

Deletion of the helicase motifs V and VI of the PVX TGBp1 does not abolish NTPase and RNA-binding activities

To study the role of the putative structural domains in the PVX 25K TGBp1 in the NTPase and RNA-binding activities, we constructed several frameshift and deletion derivatives of the protein lacking the N-terminal (ΔN-25K) or the C-terminal regions (ΔC-25K and ΔCIII-25K) (Fig. 1). The mutated proteins and the wild-type (wt) PVX 25K protein were expressed in *Escherichia coli* as fusions with N-terminal 6xHis tag, purified to near homogeneity (Fig. 3 and data not shown), and used for biochemical tests.

The mutant protein ΔN-25K, in which motifs I, IA, and II were deleted, exhibited no ATPase activity (Fig. 2). This result is consistent with the view that this portion of the helicase protein is critical for binding and hydrolysis of NTPs (Gorbalenya and Koonin, 1993; Subramanya *et al.,* 1996; Korolev *et al.,* 1997). Furthermore deletion of a region including the motifs III–VI and a small part of motif II (mutant ΔCIII-25K) greatly reduced ATPase activity of the PVX TGBp1 (Fig. 2). Unexpectedly, deletion of the helicase motifs V and VI in the ΔC-25K mutant did not result in decreased ATPase activity. Moreover the ATPase activity of this mutated protein was >2.5-fold higher than that of the wt 25K protein (Fig. 2). GTPase and UTPase activities of the ΔC-25K mutant were also about twofold higher than that of the wt 25K protein (data not shown).

The NTPase activity of the PVX 25K protein could be stimulated by ssRNA (Kalinina *et al.,* 1996; Fig. 2). In the mutant protein ΔN-25K with no detectable ATPase activity, ssRNA had no stimulating effect, whereas with both other deletion mutants (ΔC-25K and ΔCIII-25K),

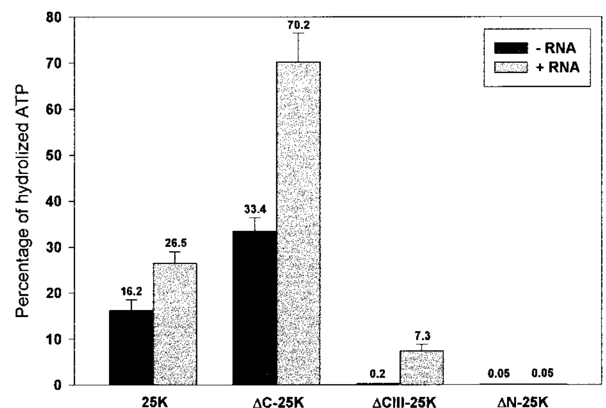

FIG. 2. The ATPase activities of 25K protein and its mutant forms. Standart ATPase assays were performed with 1 μCi of [γ-^{32}P]ATP and the percentage of hydrolysed ATP was quantified. The hydrolysis of ATP by 500 ng of each protein is shown in the absence (dark bars) and in the presence (light bars) of RNA (1μg of TMV RNA). The data presented are from five independent experiments. The mean values are shown above each bar.

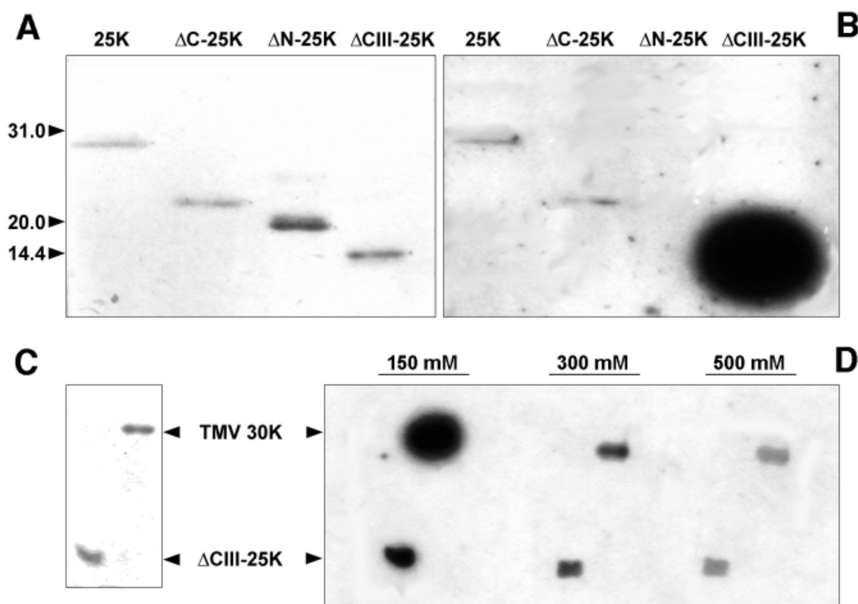

FIG. 3. RNA binding activity of the 25K protein and its mutant forms in Northwestern binding assay. (A) Ponceau S staining of the transferred 25K, ΔC-25K, ΔN-25K, and ΔCIII-25K proteins. The purified proteins (1 μg) were subjected to 15% SDS–PAGE and transferred to nitrocellulose. (B) the same gel as in (A) tested for RNA binding activity of the 25K protein and its mutants. Following incubations in 6 M urea and the renaturation buffer without NaCl, the blot was hybridized with a [^{32}P]-labeled RNA probe, washed, dried, and exposed to an X-ray film. The positions of the protein markers and their sizes in kilodaltons are shown at the left. (C) Ponceau S staining of the transferred the ΔCIII-25K and the TMV 30K proteins. (D) RNA binding activities of the ΔCIII-25K protein and the 30K movement protein of TMV at different concentrations of NaCl in the renaturation buffer. NaCl concentrations are shown above the panel. The positions of the ΔCIII-25K protein and the 30K protein of TMV are indicated between (C) and (D).

the ssRNA strongly stimulated ATPase activity (Fig. 2). Interestingly, increase of the enzymatic activity in the presence of ssRNA was more pronounced in the C-terminally truncated mutants than in the wt protein (Fig. 2).

The PVX 25K protein has been shown to bind single-stranded but not double-stranded nucleic acids *in vitro*, though the complex of the 25K protein with ssRNA was rather nonstable and could be detected only at low ionic strength (Kalinina *et al.,* 1996). To examine the possible correlation of the RNA-binding with the ATPase activity, we tested the RNA binding ability of the C-terminally truncated 25K protein (ΔC-25K mutant). The mutated protein retaining ATPase activity was also able to bind ssRNA similarly to wt 25K protein, whereas the N-terminally truncated ΔN-25K mutant had no detectable RNA binding activity (Figs. 3A and 3B). Therefore both ATPase and RNA-binding activities are associated with the PVX 25K protein N-terminal region, which includes helicase motifs I–IV. Unexpectedly, the mutant ΔCIII-25K exhibiting a low level of ATP hydrolysis but the highest level of ATPase stimulation by RNA (Fig. 2) can form stable complexes with RNA even in 0.5 M NaCl, showing the RNA binding ability similar to that of the 30K MP of TMV (Figs. 3C and 3D). These findings suggest that RNA binding domain (or at least its major part) of the PVX TGBp1 is located in the region including N-terminal helicase motifs I, IA, and II.

Subcellular distribution of the PVX TGBp1 and its C-terminally truncated mutant

Assuming that the C-terminal region of the PVX 25K protein is dispensable for its ATPase and RNA binding activities *in vitro*, we examined the role of this domain in the subcellular distribution of the protein in plant cells. Two fusions of the wt 25K protein with green fluorescence protein (GFP) were constructed. The GFP sequence was fused to the N terminus of the 25K protein (GFP-25K) or to its C terminus (25K-GFP). In preliminary experiments, we tested the functional competence of the GFP fusions by *trans*-complementation of the PVX genome containing a frameshift mutation in the 25K protein gene, pPVX.GUS-Bsp, as described by Morozov *et al.* (1997). The cell-to-cell movement of this movement-deficient PVX mutant was restored by cobombardment of plant leaves with the 35S promoter-driven 25K-GFP gene construct (pRT-25K-GFP) but not by the plasmid pRT-GFP-25K expressing the GFP-25K fusion protein (Figs. 4C and 4D). Therefore for subsequent experiments, we used the wt 25K-GFP fusion and its C-terminal deletion mutant ΔC-25K-GFP lacking the helicase motifs V and VI (Fig. 1).

Fluorescent and confocal laser scanning microscopy of the *Nicotiana benthamiana* leaves bombarded with constructs pRT-25K-GFP and pRT-ΔC-25K-GFP revealed similar fluorescence patterns in epidermal cells (Figs. 4G and 4H). Punctate fluorescence was located within or

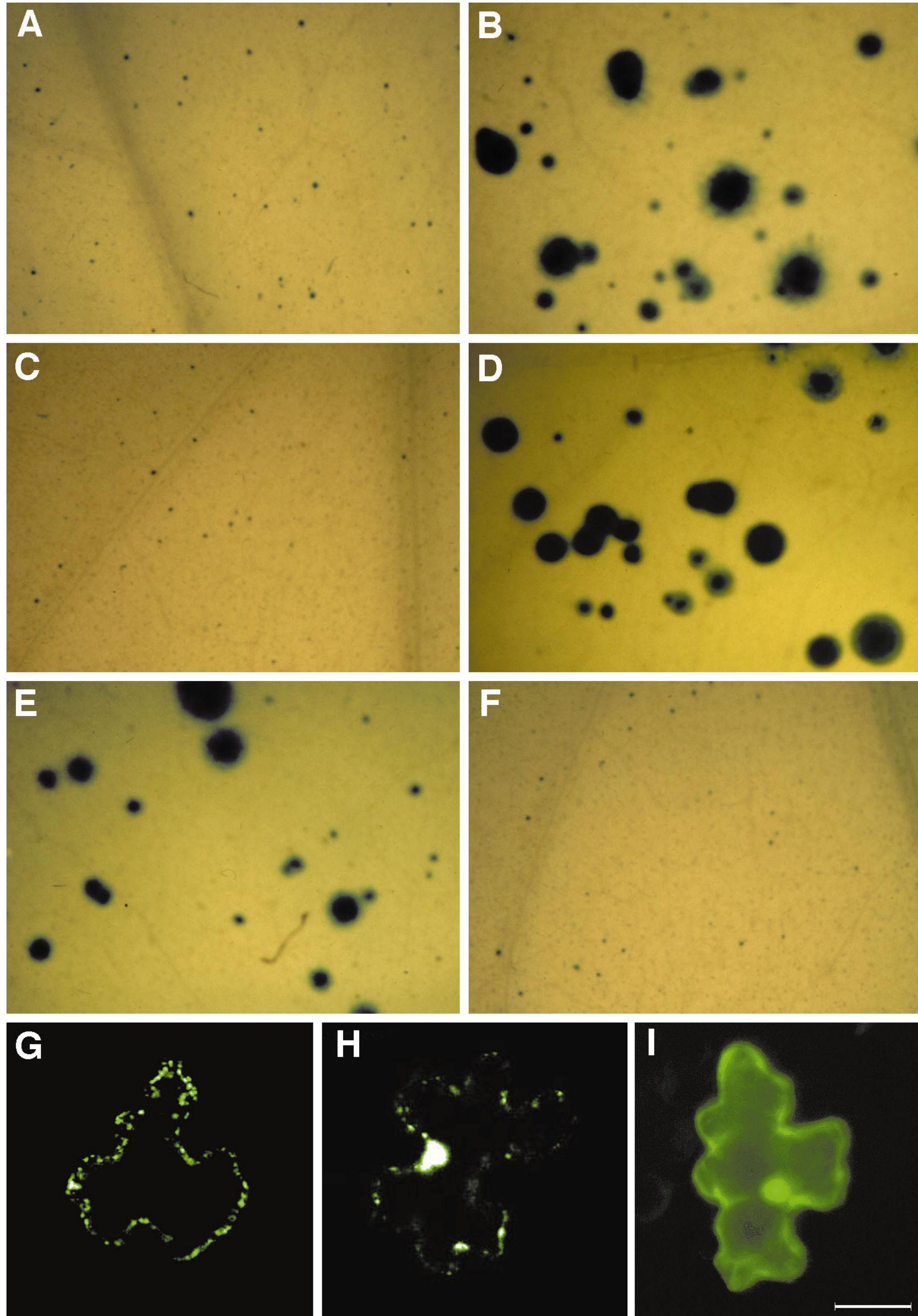

FIG. 4. Particle bombardment of the *N. benthamiana* leaves with TGBp1 constructs. (A–F) complementation of cell-to-cell movement of the PVX genome containing a frameshift mutation in the 25K protein gene, PVX.GUS-Bsp, by different TGBp1 constructs. Following histochemical detection of GUS activity, leaves were were destained with 70% ethanol and photographed at equal magnification. Each experiment was repeated 6–10 times. The presented images show leaf areas of 12 × 8.8 mm. (A) bombardment of the leaf with pPVX.GUS-Bsp [mean size of spots = 58.6 ± 12.1 (SE) μm]. Cobombardment of pPVX.GUS-Bsp with pRT-PVX.25K (B) (mean = 522.7 ± 28.4 μm), pRT-GFP-25K (C) (mean = 55.4 ± 24.8 μm), pRT-25K-GFP (D) (mean = 496.9 ± 36.8 μm), pRT-PIAP25K (E) (mean = 488.4 ± 32.1 μm), or pRT-PIAMV-25K (F) (mean = 56.2 ± 20.5 μm). (G–I) distribution of the GFP fluorescence in the epidermal cells of the leaves bombarded with pRT-25K-GFP (G), pRT-ΔC-25K-GFP (H), or pCK-GFP-S65C (I). (G) and (H) are the optical sections of the typical cells taken with confocal laser scanning microscope. The bar in (I) represents 50 μm for (G and H).

adjacent to the cell wall. In control experiments with pCK-GFP-S65C producing nonfused GFP, no cell-wall association or other specific targeting of fluorescence was observed in the epidermal cells (Fig. 4I) where distribution of fluorescence was typical for free GFP in plant cells (Reichel *et al.,* 1996). The ability of the 25K-GFP fusion protein to complement cell-to-cell movement of PVX.GUS-Bsp suggested that it retains subcellular distribution properties resulting from the intracellular protein targeting inherent to the wt PVX 25K TGBp1. It should be noted that subcellular distribution of fluorescence was similar in the cells of leaves bombarded with pRT-25K-GFP (Fig. 4G) and cobombarded with the mixture of pPVX.GUS-Bsp and pRT-25K-GFP plasmids (data not shown), suggesting that intracellular targeting of the PVX 25K protein was not seriously affected by the other virus products. Thus deletion of the 25K MP C-terminal domain containing the helicase motifs V and VI did not influence the subcellular distribution of the protein.

TGBp1 specificity in complementation experiments

Previously we reported that the cell-to-cell movement of the movement-deficient PVX.GUS-Bsp mutant could be restored by cobombardment with pRT-PVX.25K expressing homologous PVX 25K TGBp1 gene (Morozov *et al.,* 1997). In this study, we tested the ability of a heterologous TGBp1 protein of *Plantago asiatica* mosaic potexvirus (PlAMV) to complement cell-to-cell movement of PVX.GUS-Bsp. A plasmid was constructed with the PlAMV TGBp1 gene placed under control of the 35S promoter, and cobombardment of this construct with pPVX.GUS-Bsp was carried out. The PlAMV TGBp1 protein is similar in sequence to the PVX 25K (Solovyev *et al.,* 1994). However, the cell-to-cell movement of the PVX.GUS-Bsp could not be complemented by the PlAMV TGBp1 protein (Figs. 4B and 4F).

To further test the compatibility of the TGB proteins in the related potexviruses, a *Bsp*120I site was engineered in the PlAMV TGBp1 gene at the position precisely corresponding to that in the homologous PVX gene, and a PlAMV analogue of the PVX ΔC-25K mutant was obtained (mutant PlAMV-ΔC-25K). Similarly to the PVX ΔC-25K, this mutant of the PlAMV TGBp1 protein was unable to complement PVX.GUS-Bsp movement (data not shown). Using the *Bsp*120I site as a junction point, we constructed a chimeric TGBp1 consisting of the PlAMV-specific N-terminal region with the helicase motifs I–IV and the PVX C-terminal portion containing the motifs V and VI (Fig. 1). This hybrid protein complemented cell-to-cell movement of PVX.GUS-Bsp at a level comparable with the PVX 25K protein (Fig. 4E). Thus replacement of the PlAMV TGBp1 C-terminal region by that of PVX rendered protein ability to complement the TGBp1-deficient PVX genome.

DISCUSSION

The well-studied plant virus MPs, e.g., those of TMV and RCNMV, were shown to comprise a set of functional domains involved in binding nucleic acids, targeting to PD, increasing PD size exclusion limit, and trafficking nucleic acids through PD (Giesman-Cookmeyer and Lommel, 1993; Lekkerkerker *et al.,* 1996; Kahn *et al.,* 1998; for review, see Carrington *et al.,* 1996; Gilbertson and Lucas, 1996; Ghoshroy *et al.,* 1997). In this study, we demonstrate that the 25K MP of PVX, the TGBp1 protein, consists of at least two functional domains.

The structural data have been presented recently showing that the DNA helicases of superfamily 1, which are similar to plant virus TGBp1 NTPase/helicases (Gorbalenya and Koonin, 1989), consist of four spatial subdomains. The subdomain 1A contains the conserved motifs I–IV, and the subdomain 2A includes motifs V–VI (Subramanya *et al.,* 1996; Korolev *et al.,* 1997). Protein sequence alignment of the PVX TGBp1 with two well-characterized DNA helicases (Rep and PcrA) showed that the subdomains 1B and 2B of the latter proteins (located in the protein sequence between motifs IA–II and IV–V, respectively) are almost precisely deleted in the TGBp1 (data not shown). Assuming probable similarity of subdomain organization between TGBp1 and Rep and PcrA, we assayed several deletion variants of the PVX TGBp1 to study contribution of the two putative structural domains to its activities. The observations that the mutations in motifs I, IA, and II of the TGBp1 proteins inhibited both their ATPase activity and virus cell-to-cell movement (Donald *et al.,* 1995, 1997; Bleykasten *et al.,* 1996) are consistent with the hypothesis that the cell-to-cell movement of viral genomes is an energy-dependent process (for review, see Carrington *et al.,* 1996; Ghoshroy *et al.,* 1997). In addition to the TGB, involvement of the virus-coded NTPases was demonstrated for a number of other virus transport systems (Agranovsky *et al.,* 1997, 1998; Carrington *et al.,* 1998; Roberts *et al.,* 1998). There are at least three stages where ATP hydrolysis may be important: first, intracellular transport of MP and/or virus-specific ribonucleoproteins (RNPs) to the cell wall (to PD or to a compartment in the vicinity of PD) through the cytoskeleton and the endomembrane system; second, increasing the PD size-exclusion limit; third, trafficking of proteins and RNPs through PD that uses a combination of protein/RNA unfolding and microchannel dilation (for references, see Ghoshroy *et al.,* 1997; Heinlein *et al.,* 1998; Kahn *et al.,* 1998; Kragler *et al.,* 1998; Reichel and Beachy, 1998). Amazingly, the plant viruses involving ATPases in the cell-to-cell movement possess the largest genomic RNAs. For example, no MP-associated ATPase activity was found in the monopartite plus-RNA plant viruses with the genome length of ca. 4 kb (as in the genera *Tombusvirus* and *Umbravirus*) to 8.2 kb (as in genus *Vitivirus*), whereas movement-related ATPases

were found in the monopartite viruses with the genomes of ca. 6 kb (genus *Potexvirus*), or 10 kb (genus *Potyvirus*), to 20 kb (genus *Closterovirus*) (for review, see Morozov and Solovyev, 1999). It was proposed by Ryabov *et al.* (1999) that single-gene coded MPs of RNA viruses may be adapted to transport the genomes of a certain size only by analogy with MPs of DNA-containing geminiviruses (Rojas *et al.,* 1998). The putative cell enzymes that couple energy from nucleoside triphosphate hydrolysis to PD microchannels dilation and RNA translocation through them might be adapted to transport RNAs not exceeding the average size of cell mRNAs (≤2–3 kb in length as a rule). Taking into consideration these facts, we hypothesize that plant viruses with the large RNA genomes have evolved own energy-transducing proteins with abilities of enhanced ATP turnover related to high processivity of translocation along RNA chain, which are specifically adapted to move viral RNAs from cell to cell. A clue to the role of the TGBp1 NTPase in these processes was provided recently by Lough *et al.* (1998). It was shown that the potexvirus TGBp1 can increase the PD size-exclusion limit in the absence of other viral proteins and traffick RNA to the adjacent cells with the assistance of two smaller TGB proteins and CP. Importantly, the mutation of the helicase motif I significantly inhibits these activities. These data taken together with the results presented in this paper suggest that the NTP binding/hydrolysis by TGBp1 is important for modification of PD structure and/or transport to (and through) PD. Deletion of the C-terminal domain (motifs V and VI) did not impair the NTPase and RNA-binding activities of the 25K MP (Figs. 2, 3A, and 3B), which implies that these functions are associated with the N-terminal domain (motifs I–IV). These findings were strengthened further by the observation that the 25K MP fragment including motifs I, IA, and II exhibited, unlike the wt protein, very strong RNA binding comparable with that of the TMV MP (Figs. 3C and 3D). It is conceivable that removal of the C-terminal part of the 25K MP (motifs III–VI) alters the conformation of the protein molecule and allows strong RNA binding. These data are in agreement with the results of Kadare *et al.* (1996) showing that the putative helicase domain in replicative 206K protein of turnip yellow mosaic virus with deleted motifs III–VI can bind RNA more efficiently than the isolated full-length domain. We presume that interactions of the PVX TGBp1 with other viral or cell proteins may result in conformational changes causing exposition of the N-terminal RNA binding site and more pronounced RNA binding ability. Such transient changes may explain the differences in the efficiency of RNA binding between TGBp1 of potexviruses observed *in vitro* (see Rouleau *et al.,* 1994; Kalinina *et al.,* 1996).

Using the translational fusions of the GFP to the PVX TGBp1 protein and its truncated derivative lacking the C-terminal sequence with NTPase motifs V–VI, we demonstrated that such deletion did not influence intracellular distribution of the TGBp1 protein. This observation is in line with the finding that motif VI was dispensable for trafficking the white clover mosaic potexvirus TGBp1 between cells (Lough *et al.,* 1998). All these data support the idea that intra- and intercellular trafficking of this protein is a function of the N-terminal protein domain (Lough *et al.,* 1998).

The TGBp1 proteins of PVX and PlAMV were shown to be similar in their sequences (Solovyev *et al.,* 1994; Wong *et al.,* 1998). However, our results showed that the PlAMV TGBp1 was unable to complement cell-to-cell movement of the PVX genome deficient in TGBp1 in the experiments on transient complementation of the movement-deficient GUS gene-tagged PVX upon cobombardment with PlAMV TGBp1 gene (Figs. 4B and 4F). Similarly, the mutation in the PVX TGBp1 could not be complemented by the distantly related TGBp1 proteins of hordeiviruses (O. N. Fedorkin, unpublished data). These observations suggested that specific interactions between the TGBp1 protein and other virus proteins could be involved in virus movement. To further test the phenomenon of the interviral TGBp1 MP compatibility, we constructed the chimeric TGBp1 protein with PlAMV-specific N-terminal portion (motifs I–IV) and PVX-specific C-terminal domain (motifs V–VI). In contrast to the PlAMV TGBp1, the chimeric protein was able to complement the mutated PVX TGBp1 as efficiently as the homologous PVX TGBp1 protein (Fig. 4E). Presumably, the C-terminal region (motifs V and VI) is involved in specific interactions of the TGBp1 with other virus protein(s). The TGBp2 and TGBp3 proteins seemed to be plausible candidates for interactions with the TGBp1 because recent genetic evidences suggested such interactions between the TGB proteins (Lauber *et al.,* 1998; Solovyev *et al.,* 1999). It is tempting to speculate that interactions of the aforementioned proteins in PD or a cell compartment in the vicinity of PD might result in transducing the allosteric effects from the C-terminal domain to the enzymatic N-terminal domain and leading to significant increase of RNA binding and ATP hydrolysis. This suggestion is in agreement with the proposed role of motif VI in DNA helicases (Korolev *et al.,* 1997; Hall *et al.,* 1998) and the effects of motif VI deletion on the potexvirus TGBp1 functioning in the transient cell-to-cell RNA transport system (Lough *et al.,* 1998).

These results suggest that the potexvirus TGBp1 proteins consist of at least two functional domains corresponding to the spatially divided structural subdomains found in the DNA helicases of superfamily I. The N-terminal domain determines the protein enzymatic functions involved in intra- and intercellular trafficking, whereas the C-terminal domain presumably plays a structural role in interactions with other virus products.

METHODS

Construction of the potexvirus TGBp1 mutant and chimeric forms

All recombinant DNA procedures were carried out by standard methods (Sambrook *et al.,* 1989). *E. coli* strains DH5α and M15 [pREP4] were used for cloning of recombinant constructs and for protein superexpression, respectively. The expression vector pQ25 carrying the gene of the PVX 25K protein with a six-histidine tag at the amino terminus was described previously (Kalinina *et al.,* 1996). To obtain pQ25ΔC, the plasmid pQ25 was linearized by *Bsp*120I at the site corresponding to the position 4945 in the complete nucleotide sequence of PVX (Skryabin *et al.,* 1988), blunted by Klenow fragment, and religated. To obtain pQ25IIIC, the plasmid pQ25 was digested by *Sna*I at the site corresponding to the position 4734 in the complete nucleotide sequence of PVX (Skryabin *et al.,* 1988) and *Hin*dIII located in pQ25 downstream of the PVX sequence, then the plasmid was blunted by Klenow fragment, and religated. To construct the pQ25ΔN, the plasmid pQ25 was digested by *Sna*I and *Hin*dIII, and the resulting DNA fragment was inserted into pQE30 plasmid (QIAGEN) digested by *Sma*I and *Hin*dIII.

For construction of the 25K fusions, a gene of red-shifted GFP mutant from pCK-GFP-S65C plasmid (Reichel *et al.,* 1996) was used. The PVX 25K protein gene was PCR amplified with primers 5′-ATTT**GAATTC**CATGGATATTCTCATCATTAG and 5′-CGGT*CTCGAG*ATGTCCCTGCGCGGACATATG using as the template the plasmid pRT-PVX.25K (Morozov *et al.,* 1997). The 5′-terminal primer for PVX 25K gene contained *Eco*RI site (shown in bold), and the 3′-terminal primer contained *Xho*I site (italic) replacing the termination codon. The *gfp* gene was excised from pCK-GFP-S65C by *Nco*I-*Bam*HI digestion and cloned into similarly digested pRT100 (Töpfer *et al.,* 1987) to give pRT-GFP. To construct pRT-25K-GFP, pRT101 digested with *Eco*RI and *Bam*HI was ligated with *Xho*I-*Bam*HI fragment excised from pRT-GFP and with *Eco*RI-*Xho*I-digested 25K PCR product. To construct pRT-GFP-25K, the plasmid pRT-GFP was modified to replace the terminator codon of the GFP gene by the sequence GGATCCATGG containing *Bam*HI and *Nco*I restriction sites. Following digestion of the resulting plasmid with *Nco*I, the fragment containing the GFP gene was isolated and cloned into *Nco*I-digested plasmid pRT-PVX.25K (Morozov *et al.,* 1997). To obtain pRT-ΔC-25K-GFP, pRT-25K-GFP recombinant plasmid was completely digested with *Bsp*120I and religated.

The PlAMV 25K protein gene was amplified with primers 5′-GG**CCATGG**ACTCCATTATCAACGCA and 5′-CTT*TCTAGA*GTCAGAGGGTGGGGTGAGGT containing *Nco*I site (shown in bold) and *Xba*I site (italic), and the resulting PCR product was digested with *Nco*I and *Xba*I and cloned into similarly digested pRT100 (Töpfer *et al.,* 1987) to give the plasmid pRT-PlAMV-25K. To obtain plasmid pRT-PlAMV-25K-Bsp, the *Bsp*120I restriction site was engineered at a positions of the PlAMV 25K protein gene corresponding to the positions 4701–4706 in the PlAMV genome (Solovyev *et al.,* 1994). This restriction site precisely matched the *Bsp*120I site in the PVX 25K gene sequence. To construct pRT-PlAMV-ΔC-25K, the plasmid pRT-PlAMV-25K-Bsp was completely digested by *Bsp*120I, blunted by Klenow fragment, and religated. To obtain the plasmid pRT-PlAP25K with the chimeric TGBp1 gene, *Bsp*120I-*Xba*I-fragment from pRT-PVX.25K (Morozov *et al.,* 1997) was cloned into similarly digested pRT-PlAMV-25K-Bsp to replace the PlAMV sequence by that of PVX.

Expression and purification of the potexvirus TGBp1 and mutant forms

E. coli strain M15 transformed with the recombinant vectors was grown at 37°C in liquid culture until an OD_{600} of 0.8–0.9 was reached. Expression of the proteins was induced with 1 mM IPTG followed by growth for 2–4 h at 37°C. The purification of recombinant proteins from cultures followed a general procedure described by the manufacturer (QIAGEN) for denaturing Ni–NTA chromatography.

ATPase and RNA binding assays

ATPase assays were carried out as described previously (Kalinina *et al.,* 1996). In brief, in a final volume of 10 μl reaction mixtures contained 10 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 5 μM ATP, 1 μCi [γ-^{32}P]ATP, and 0.5 μg of protein. When indicated, the TMV RNA was included in the reaction mixture at the concentration of 100 μg/ml. Reactions were incubated for 1 h at 37°C and stopped by the addition of EDTA to a final concentration of 20 mM. To estimate the ATPase activity, unreacted ATP was precipitated by addition of 300 μl of 7.5% activated charcoal in 50 mM HCl/5 mM H_3PO_4; the mixtures were vortexed and allowed to stand for 5 min, then charcoal was centrifuged in a microcentrifuge for 10 min, and half of supernatant was analyzed by Cherenkov's counting.

RNA-binding assays were performed with filter-bound proteins as described previously (Kalinina *et al.,* 1996). The purified recombinant proteins were separated by electrophoresis in 15% SDS–PAGE and electroblotted onto nitrocellulose membranes. Membranes were washed twice for 30 min with 6 M urea containing 0.1% Tween 20. Membrane-bound proteins were renatured in buffer R (20 mM Tris–HCl, pH 7.5, 0.2 g/l BSA, 0.2 g/l Ficoll, 0,2 g/l polyvinylpyrrolidone) for 1 h with two to three changes of buffer. Following renaturation, the membranes were incubated at room temperature with ^{32}P-labeled RNA transcript (1–2 $\times$ 10^6 dpm/ml) in 2–5 ml of buffer R during 1 h. Membranes were washed with buffer R three to four

times for 30 min, dried, and autoradiographed. Labeled single-stranded RNA was synthesized by T7 RNA-polymerase in the presence of [α-^{32}P]UTP from linearized pXT7–25 template (Morozov *et al.,* 1990).

Particle bombardment

Particle bombardment was performed using flying disk method with a high-pressure helium-based apparatus PDS-1000 (Bio-Rad) as described in Morozov *et al.* (1997). Replication and movement of PVX.GUS was monitored by histochemical detection of GUS expression (Jefferson, 1987). Samples were infiltrated in the colorimetric GUS substrate modified to limit the diffusion of the intermediate products of the reaction (De Block and Debrouwer, 1992). After incubation overnight at 37°C, the leaves were fixed in 70% ethanol and examined by light microscopy. GFP fluorescence was detected using Zeiss Axioscope 20 fluorescence microscope (excitation filter BP 450–490, chromatic beam splitter FT 510, and long pass emission filter LP 520 or band pass filter HQ 535/50x) or Bio-Rad MRC-1024 confocal laser scanning imaging system with excitation light of 488 nm produced by krypton/argon laser tuned to 15 mW and filters UBHS and E2.

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