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Proteolytic processing of potyviral proteins and polyprotein processing intermediates in insect and plant cells

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Processing of the polyprotein encoded by *Potato virus A* (PVA; genus *Potyvirus*) was studied using expression of the complete PVA polyprotein or its mutants from recombinant baculoviruses in insect cells. The time-course of polyprotein processing by the main viral proteinase (NIaPro) was examined with the pulse–chase method. The sites at the P3/6K1, CI-6K2 and VPg/NIaPro junctions were processed slowly, in contrast to other proteolytic cleavage sites which were processed at a high rate. The CI-6K2 polyprotein was observed in the baculovirus system and in infected plant cells. In both cell types the majority of CI-6K2 was found in the membrane fraction, in contrast to fully processed CI. Deletion of the genomic region encoding the 6K1 protein prevented proper proteolytic separation of P3 from CI, but did not affect processing of VPg, NIaPro, NIb or CP from the polyprotein. The 6K2-encoding sequence could be removed without any detectable effect on polyprotein processing. However, deletion of either the 6K1 or 6K2 protein-encoding regions rendered PVA non-infectious. Mutations at the 6K2/VPg cleavage site reduced virus infectivity in plants, but had a less pronounced, albeit detectable, effect on proteolytic processing in the baculovirus system. The results of this study indicate that NIaPro catalyses proteolytic cleavages preferentially *in cis*, and that the 6K1/CI and NIb/CP sites can also be processed *in trans*. Both 6K peptides are indispensable for virus replication, and proteolytic separation of the 6K2 protein from the adjacent proteins by NIaPro is important for the rate of virus replication and movement.

Introduction

Potato virus A (PVA) is a member of the genus *Potyvirus* (family *Potyviridae*), the largest genus of plant viruses. The positive-sense genomic RNA of PVA isolate B11 is 9565 nucleotides long and contains a single open reading frame (ORF) encoding a polyprotein of 3059 amino acids (Puurand *et al.*, 1994). The polyprotein is proteolytically processed in infected cells into up to ten mature proteins by three virus-encoded proteinases (Fig. 1; Riechmann *et al.*, 1992). The P1 proteinase and the helper component-proteinase (HC-Pro) cleave only their respective C termini autocatalytically (Carrington *et al.*, 1989; Verchot *et al.*, 1991). A proteinase domain (NIaPro) at the C terminus of nuclear inclusion protein a (NIa) catalyses cleavage at the remaining proteolytic sites (Carrington & Dougherty, 1987a, b; Carrington *et al.*, 1988; García *et al.*, 1990).

Several potyvirus-encoded proteins are known to, or have been suggested to, participate in the formation of the potyvirus replication complex. The cylindrical inclusion protein (CI) is an RNA helicase and NTPase (Fernandez *et al.*, 1995). The viral genome-linked protein (VPg), processed from the N-proximal half of NIa by NIaPro, is covalently bound to the 5′-end of potyvirus RNA (Murphy *et al.*, 1990; Oruetxebarria *et al.*, 2001). VPg is necessary for infectivity and may function as a primer for minus-strand RNA synthesis (Murphy *et al.*, 1990, 1996; Schaad *et al.*, 1996). Nuclear inclusion protein b (NIb) is an RNA-dependent RNA-polymerase (Hong & Hunt, 1996). Domains of NIa, NIaPro (Darós *et al.*, 1999; Li *et al.*, 1997) and VPg (Hong *et al.*, 1995; Fellers *et al.*, 1998; Guo *et al.*, 2001) can interact with NIb and, hence, may participate in the direction of NIb to the replication complex. The proteins putatively involved in the potyvirus replication complex (CI, VPg, NIaPro and NIb) bind RNA (Merits *et al.*, 1998) and interact with the

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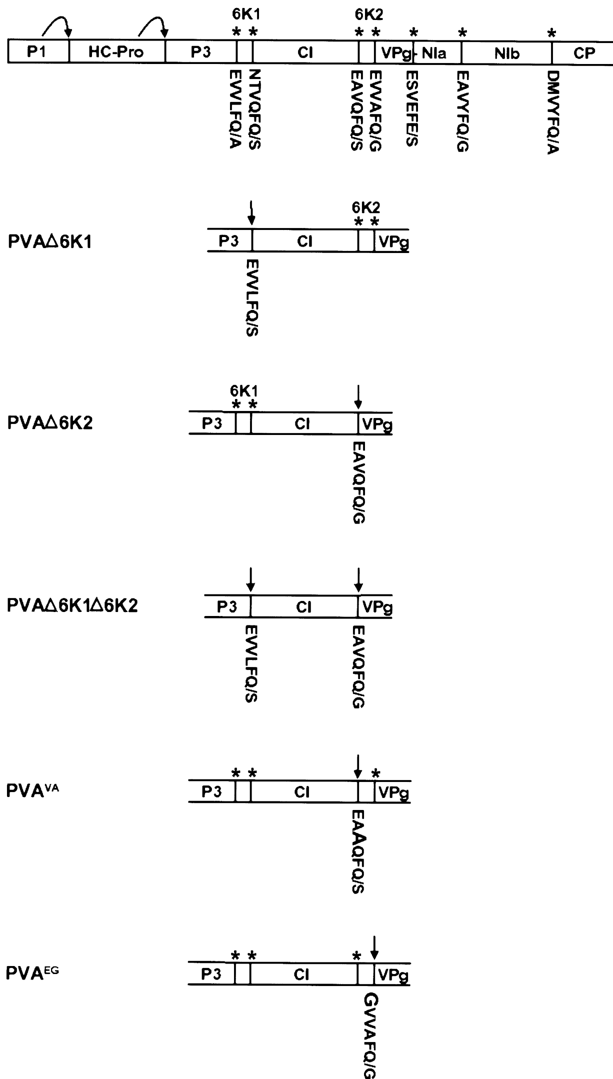

Fig. 1. Organization of proteins and schematic presentation of proteolytic processing in the PVA polyprotein. Autoproteolytic cleavage sites for P1 and HC-Pro are shown with arrows, cleavage sites for NIaPro are marked with an asterisk (*) and their sequences in the PVA polyprotein are indicated. In the P3-VPg fragments of mutant polyproteins PVAΔ6K1, PVAΔ6K2, PVAΔ6K1Δ6K2, PVA^{VA} and PVA^{EG}; recombinant cleavage sites for NIaPro are indicated with straight arrows and mutated residues are in bold.

non-structural proteins P1 and P3 (Merits *et al.*, 1999). In addition, potyviral coat protein (CP) can interact with NIa and NIb (Hong *et al.*, 1995). The P3 protein has no known enzymatic activity but contains a putative transmembrane domain and may be involved in anchoring the replication complex to membranes (Rodríguez-Cerezo & Shaw, 1991; Klein *et al.*, 1994; Martin *et al.*, 1995). The domains for the small 6K1 and 6K2 peptides in the polyprotein flank the CI domain. Mutations that prevent processing of the 6K1 peptide from the P3 protein, or the 6K2 peptide from CI, are not lethal to *Tobacco etch virus* (TEV) (Restrepo-Hartwig & Carrington, 1994) and *Plum pox virus* (PPV) (Riechmann *et al.*, 1995). It has been hypothesized that the 6K2 peptide may function as a membrane anchor for CI and VPg during TEV replication (Restrepo-Hartwig & Carrington, 1994; Schaad *et al.*, 1996, 1997). Subsequently, the replication complex may be released from the membrane by catalysis of cleavage at the polyprotein proteolytic site between 6K2 and VPg (Schaad *et al.*, 1996, 1997).

Since potyviruses contain only a single ORF encoding a polyprotein, rather than independent ORFs (genes) for each of the ten proteins, regulation of protein function might occur, at least in part, through regulation of polyprotein processing. This is supported by findings that resistance to potyviruses can be increased in transgenic plants by expression of cysteine proteinase inhibitors (Gutierrez-Campos *et al.*, 1999). In this study we set out to investigate the time-course of polyprotein processing catalysed by NIaPro, the main potyviral proteinase. Since it appeared that deletion of 6K1 or 6K2 abolished infectivity of PVA in plants, the studies were done by expressing the PVA polyprotein in recombinant baculovirus-infected insect cells. This choice was motivated partly because both the N-terminal proteinases of potyviruses, P1 and HC-Pro, are proteolytically active in insect cells (Thornbury *et al.*, 1993). The baculovirus–insect cell system has potential advantages compared to *in vitro* translation systems. It enables monitoring of proteolytic processing events in a real time-course using pulse–chase experiments. Also, *in vitro* translation systems may not produce functionally active proteinases in sufficient amounts to perform all proteolysis events *in trans*. Our data show that the 6K2 peptide of PVA is linked to CI rather than to NIa or VPg both in insect cells and in infected plant cells, and that modification of the cleavage site between 6K2 and VPg debilitates virus infectivity. The data also indicate that the 6K1 peptide has an impact on the processing of the potyviral polyprotein and that there are large differences in the NIaPro-mediated cleavage efficiencies at the different cleavage sites in the polyprotein.

Methods

■ **Site-specific mutagenesis.** The infectious cDNA clone of PVA isolate B11 (pPVA; Puurand *et al.*, 1996) was used as template for the site-specific mutagenesis reactions. The mutant clones $pPVA^{EG}$ and $pPVA^{VA}$, containing amino acid substitutions (underlined) <u>E</u>VVAFQ/G → <u>G</u>VVAFQ/G at the proteolytic site between the 6K2 protein and the VPg and EA<u>V</u>QFQ/S → EA<u>A</u>QFQ/S at the proteolytic site between CI protein and 6K2, were made using appropriate primers and the QuikChange Site-directed Mutagenesis system (Stratagene) essentially as described by Rajamäki & Valkonen (1999). Other site-specific mutagenesis reactions were done using appropriate primers and the ExSite PCR-based mutagenesis system (Stratagene) according the manufacturer's protocol. The mutant clones of the PVA cDNA lacking coding sequences for 6K1, 6K2 or both 6K peptides are designated pPVAΔ6K1, pPVAΔ6K2 and pPVAΔ6K1Δ6K2, respectively. All constructs produced by site-specific mutagenesis were verified by sequencing.

■ **Inoculation of plants and detection of PVA.** The plasmids containing the PVA cDNA constructs pPVA, pPVAΔ6K1, pPVAΔ6K2,

pPVAΔ6K1Δ6K2, $pPVA^{VA}$ and $pPVA^{EG}$ placed behind the T7 promoter were linearized and subsequently transcribed using T7 RNA polymerase (Puurand *et al.*, 1996). Transcripts were coated on gold particles (4·0 μg RNA/1 mg gold) and inoculated into leaves of intact test plants by microprojectile bombardment using a hand-held device (Helios Gene Gun, Bio-Rad) and bombardment parameters optimized as previously described (Hämäläinen *et al.*, 2000). Inoculation was done by two shots on to one lower leaf of a 4-week-old tobacco plant (*Nicotiana tabacum* L. cv. Samsun nn), or *Nicandra physaloides* var. 'Black Pod'. Each cDNA construct was also inoculated to fully expanded leaves of 4-week-old cuttings of the potato clone 'A6', a hypersensitive indicator host of PVA (Hämäläinen *et al.*, 2000). The plants were grown under constant conditions in a growth chamber (in Uppsala, 8·8 m^2; Weiss Umweltstechnik, Germany). The photoperiod was 18 h (250 μmol/s/m^2), the temperature was 19/17 °C (day/night) and the relative humidity was 40%.

Viruses were detected by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using the monoclonal antibody (mAb) 58/0, raised to the CP of PVA (Adgen, UK) (Browning *et al.*, 1995; Rajamäki *et al.*, 1998). Leaf discs (diam. 20 mm) containing the entire bombarded area were sampled from tobacco and *N. physaloides* plants 14 days after bombardment [post-infection (p.i.)]. Leaf discs from the upper non-inoculated leaves were sampled at 21 days p.i. The samples were weighed, and ground in ELISA sample buffer at 1 g/3 ml. Two aliquots (100 μl each) were transferred to two wells of a microtitre plate coated with mAb 58/0. Known amounts of purified PVA isolate B11 were included as a standard. Absorbance was measured at 405 nm with a Benchmark microtitre plate reader using Microplate Manager software (Bio-Rad). Viruses from the upper non-inoculated leaves which tested positive in ELISA were checked by RT–PCR using virus-specific primers, and the mutated region was verified by sequencing.

■ **Preparation and electroporation of protoplasts.** Protoplasts were prepared from leaves of young tobacco plants (variety SR1) and inoculated with the PVA RNA transcripts by electroporation (Luciano *et al.*, 1987; Andrejeva *et al.*, 1999). Each batch of 1×10^6 protoplasts was electroporated with 5–10 μg of viral RNA transcripts. Protoplasts were incubated for 72 h under dim light at 22 °C and analysed by DAS-ELISA.

■ **Construction and propagation of recombinant baculoviruses expressing the PVA polyprotein.** The Bac-to-Bac recombinant baculovirus system (Gibco BRL) was used according to the manufacturer's protocols. The PVA genome was excised with *Sph*I and *Kpn*I from pPVA, pPVAΔ6K1, pPVAΔ6K2, pPVAΔ6K1Δ6K2, $pPVA^{VA}$ and $pPVA^{EG}$ and cloned into pFastBac1 (Gibco BRL) digested with the same enzymes. The recombinant baculoviruses expressing these constructs are designated BacPVA, BacPVAΔ6K1, BacPVAΔ6K2, BacΔ6K1Δ6K2, $BacPVA^{VA}$ and $BacPVA^{EG}$, respectively. The PVA genes (P3, CI, CI-6K2, VPg, NIa, NIaPro, NIb and CP) were PCR-amplified using the corresponding primers. The amplified fragments were cloned into pGEM-T (Promega), sequenced and cloned into pFastBac1 digested with *Eco*RV and *Sal*I (for CI and CI-6K2), or *Bam*HI and *Sal*I (for P3, VPg, NIa, NIaPro, NIb and CP). The recombinant baculoviruses containing these constructs are designated BacCI, BacCI-6K2, BacP3, BacVPg, BacNIa, BacNIaPro, BacNIb and BacCP, respectively.

Virus stocks were amplified and titrated using an Sf9 insect cell culture (Gibco BRL) and Sf-900 II culture media (Gibco BRL) containing foetal calf serum (10%) and gentamycin (50 mg/l). However, for polyprotein and protein expression and pulse–chase experiments, High Five cells (BTI-TN-5B1-4) (Invitrogen) and High Five medium (Invitrogen) were used. Optimal expression time was determined to be 40–48 h post-infection (p.i.) at 27 °C at m.o.i. 10. These conditions were used in all experiments.

■ **Detection of PVA proteins and proteolytic processing products of the PVA polyprotein.** About 5×10^6 High Five cells per Petri dish (diam. 6 cm) were infected with each recombinant baculovirus at m.o.i. 10. Cells were harvested at 40 h p.i., pelleted by centrifugation (10 min at 500 ***g***) and lysed by adding hot Laemmli sample buffer (Laemmli, 1970) to the pellet. Samples were boiled for 2 min, diluted with Laemmli buffer and analysed by SDS–PAGE (using extracts from 5000 cells per packet of a minigel) and Western blotting (Bio-Rad apparatus). The expressed viral proteins were detected using rabbit polyclonal antisera raised to the different PVA proteins (Merits *et al.*, 1999) followed by visualization by ECL (Amersham Pharmacia).

■ **Pulse–chase experiments on the recombinant baculovirus expression system.** About 2×10^6 High Five cells per Petri dish (diam. 3 cm) were infected with each recombinant baculovirus at m.o.i. 10 at 27 °C. The original medium was changed to a methionine-free Grace's medium at 40 h p.i. A pulse of 20 min was given after 1 h of incubation (500 μCi/ml [^{35}S]methionine; Amersham Pharmacia). Samples were collected immediately after the pulse or after chasing for 2 h with High Five medium containing a 10-fold excess of unlabelled methionine. Samples were collected, immunoprecipitated with rabbit polyclonal antisera raised against PVA P3, CI, VPg, NIaPro, NIb or CP essentially as described by Peränen *et al.* (1988) and subjected to SDS–PAGE. The gels were dried and exposed to the image plate. Data were analysed using a BAS-1500 apparatus (Fujifilm) and TINA program, version 2.09c (Raytest Isotopenme*ß*geräte GmbH). All precursors were identified by immunoprecipitation with antibodies against all their domains (mature proteins), except 6K1 and 6K2. In the case of P3-NIb it means that this precursor was identified by immunoprecipitation with anti-P3, -CI, -VPg, -NIaPro and -NIb antibodies and immunoprecipitation reactions were run in the same gel. Quantification of data was always done on several gels from several independent labelling experiments.

■ **Subcellular localization and membrane association of PVA proteins in infected plants and insect cells.** Systemically infected leaves of tobacco (SR1) and leaves from mock-inoculated tobacco plants were collected at 9 days p.i and homogenized in a buffer containing 50 mM Tris–HCl (pH 7·4), 15 mM $MgCl_2$, 120 mM KCl, 0·1% *β*-mercaptoethanol, 1 μM pepstatin and 0·1 mM PMSF (Osman & Buck, 1996).

For the membrane flotation assay of the baculovirus-expressed PVA polyproteins, about 5×10^6 High Five cells per Petri dish (diam. 6 cm) were infected with BacPVA at m.o.i. 10. Cells were collected by centrifugation (10 min at 500 ***g***) at 40 h p.i., frozen at −20 °C, thawed, resuspended (in 20 mM HEPES 7·5, 0·25 M sucrose, 1 mM EDTA and 1 mM PMSF) and disrupted by pressing the solution repeatedly through a syringe. The nuclei were pelleted from the cell lysate at 2000 ***g*** for 5 min.

Flotation analysis of the supernatant and the homogenates prepared from PVA- or mock-infected plant material was done as follows. The supernatant was mixed with 67% sucrose to give a final concentration of 60% sucrose. The sample (1·5 ml) was first laid over 67% sucrose (0·5ml), then overlaid with 50% and 5% sucrose (8·0 ml and 1·0 ml, respectively), and finally centrifuged in a Beckman SW 41 rotor at 35000 r.p.m. for 8 h at 4 °C. All sucrose solutions were prepared in a buffer containing 100 mM NaCl and 50 mM Tris–HCl, pH 7·5. Fractions were collected from the bottom of the tube and analysed by Western blotting with polyclonal antibodies to CI as described earlier.

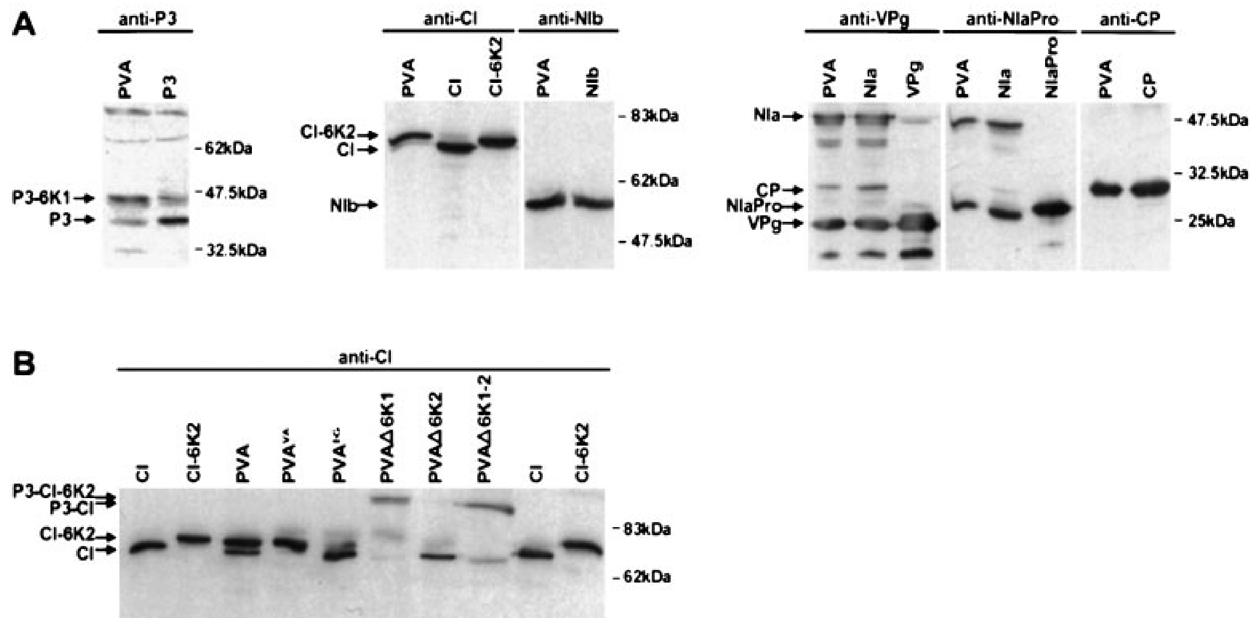

Fig. 2. Western blotting of the proteins produced by recombinant baculoviruses. Lysates from 500 infected cells were loaded per lane. Proteins were separated by SDS–PAGE in 12% gels, Western blotted and detected using antibodies to PVA proteins. Blots were visualized using the ECL procedure. Molecular masses of New England Biolabs Broad Range molecular mass standards are indicated to the right. Positions of PVA proteins and polyproteins are marked with arrows to the left. (A) Comparison of proteolytic products from the PVA polyprotein-expressing baculovirus BacPVA (PVA), and the products expressed by recombinant baculoviruses BacP3 (P3), BacCI (CI), BacCI-6K2 (CI-6K2), BacVPg (VPg), BacNIa (NIa), BacNIaPro (NIaPro), BacNIb (NIb) and BacCP (CP). (B) Comparison of CI-containing proteolytic products from mutated PVA polyprotein-expressing baculoviruses BacPVA^{VA} (PVA^{VA}), BacPVA^{EG} (PVA^{EG}), BacPVAΔ6K1 (PVAΔ6K1), BacPVAΔ6K2 (PVAΔ6K2) and BacPVAΔ6K1Δ6K2 (PVAΔ6K1-2) with corresponding products of BacPVA (PVA), BacCI (CI) and BacCI-6K2 (CI-6K2).

Results

Processing of the PVA polyprotein into mature proteins proceeds via intermediates in insect cells

Western analysis of the proteolytic products derived from the wild-type PVA polyprotein in insect cells revealed fully processed PVA-encoded proteins P3, CI, VPg, NIaPro, NIb and CP (for schematic overview, see Fig. 1). The sizes of the mature proteins were similar to the corresponding proteins expressed and purified from *E. coli* (Merits *et al*., 1998, 1999) or expressed as individual proteins in the baculovirus system (Fig. 2A). However, three rather stable and several short-lived polyprotein processing intermediates were also observed.

Detection of a putative P3-6K1 polyprotein with Western blotting was difficult, since the anti-P3 antiserum also recognized insect protein(s) with mobility corresponding to the expected mobility of the P3-6K1 polyprotein (Fig. 2A). However, immunoprecipitation with anti-P3 antibodies clearly revealed the presence of a P3-6K1 polyprotein (Fig. 3A), whereas only relatively small quantities of fully processed P3 protein were detected (Figs 2A, 3A).

Only a faint band corresponding to the expected size (71 kDa) of the fully processed CI was detected with anti-CI antibodies, whereas the major product had an electrophoretic mobility corresponding to ca. 80 kDa (Fig. 2A, B). We therefore assume that CI was linked with one of the two 6K proteins (see below). The putative CI-6K protein was stable since its relative amount (compared to the other proteins and the fully processed CI) remained similar during longer expression experiments (24–120 h p.i., data not shown).

The anti-VPg and anti-NIaPro antisera both recognized a product with an electrophoretic mobility of approximately 50 kDa. This product co-migrated with a protein produced by the recombinant baculovirus BacNIa and, hence, corresponded to the full-length, non-processed NIa protein (Fig. 2A). The NIa appeared as a double band (Figs 2A, 3C, D). The lower molecular mass band was probably due to partial self-cleavage at the C terminus of the NIaPro, as shown for *Turnip mosaic virus* (Kim *et al*., 1995, 1996) and TEV (Parks *et al*., 1995).

P3-6K1, CI-6K2 and NIa (VPg-NIaPro) are relatively stable polyproteins in insect cells

The time-course of the proteolytic processing of PVA polyprotein was analysed using a pulse–chase method. After a pulse of 20 min, the majority of the NIb and CP were already found fully processed from the polyprotein (Fig. 3E, F). However, unprocessed precursor proteins were also detected, which decreased in amounts in the chased samples. They included P3-6K1-CI-6K2-NIa-NIb (designated P3-NIb in Fig. 3A, E), P3-6K1-CI-6K2 (Fig. 3A, B), CI-6K2-NIa-NIb (desig-

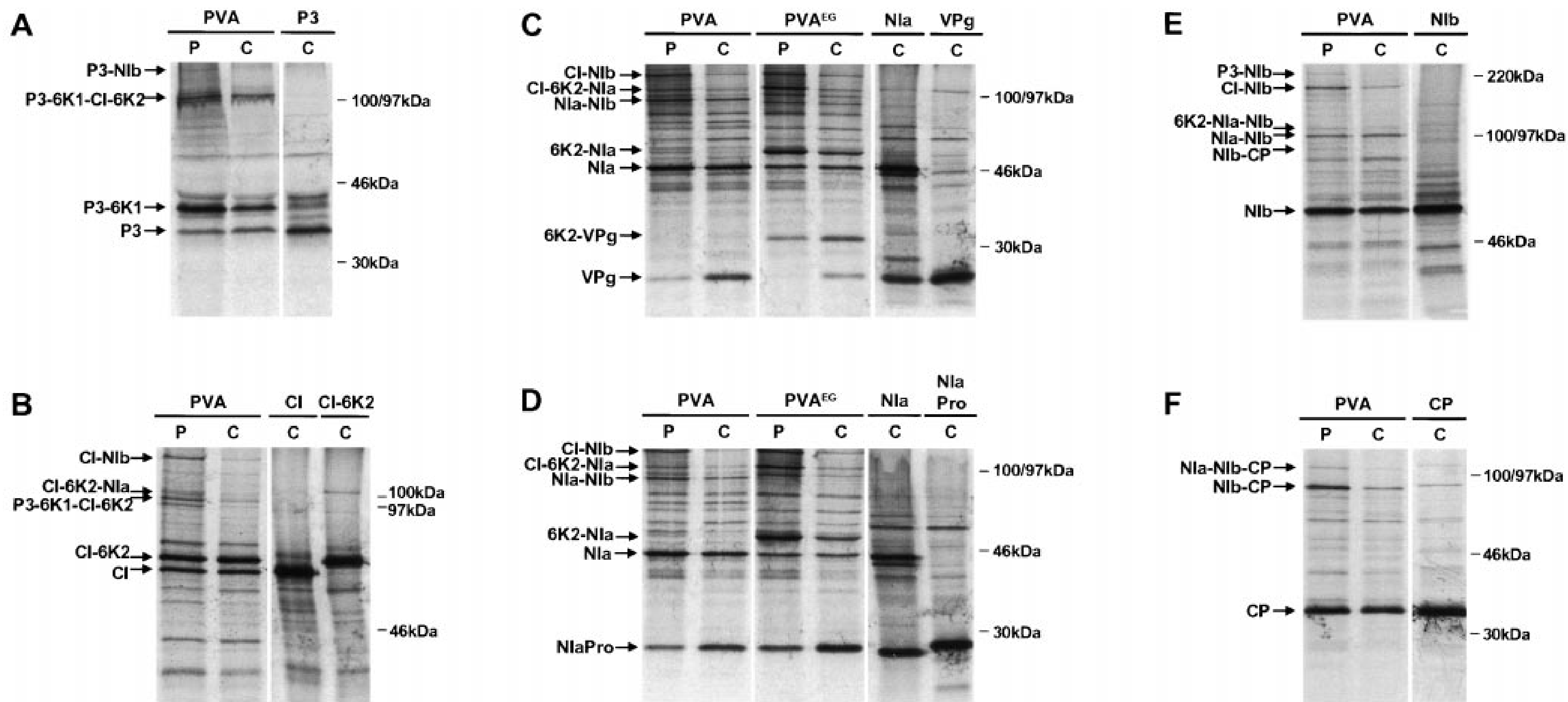

Fig. 3. Pulse–chase experiments on insect cells infected with recombinant baculoviruses expressing PVA proteins. The proteins expressed by BacPVA (PVA) and BacPVA^{EG} (PVA^{EG}) were metabolically labelled with [^{35}S]methionine, immunoprecipitated with anti-P3 (A), anti-CI (B), anti-VPg (C), anti-NIaPro (D), anti-NIb (E) or anti-CP (F) antibodies and subjected to SDS–PAGE. Similarly treated chase samples from proteins expressed by BacP3 (P3), BacCI (CI), BacCI-6K2 (CI-6K2), BacVPg (VPg), BacNIa (NIa), BacNIaPro (NIaPro), BacNIb (NIb) and BacCP (CP) were used as controls on corresponding panels. Gels were fixed, dried and exposed to an image plate. P corresponds to pulse samples (20 min) and C to chase samples (2 h). Arrows at the left indicate the positions of the processed proteins, stable polyproteins and identified polyprotein processing intermediates. Molecular mass standard positions are indicated on the right. P3-NIb means P3-6K1-CI-6K2-VPg-NIaPro-NIb and CI-NIb means CI-6K2-VPg-NIaPro-NIb.

Table 1. Infectivity of the deletion and cleavage site mutants of PVA

Construct	Response in 'A6'†	Infection as detected by ELISA*				Infectivity in tobacco protoplasts at 3 days p.i. (A_{405})‡
		N. tabacum		*N. physaloides*		
		IL	SL	IL	SL	
pPVA	NLL	8/8	8/8	3/3	3/3	0·49
pPVAΔ6K1	NS	0/5	0/5	NT	NT	0·02
pPVAΔ6K2	NS	0/5	0/5	NT	NT	0·01
pPVAΔ6K1Δ6K2	NS	0/5	0/5	NT	NT	0·01
pPVA^{VA}	NS	0/5	0/5	0/10	0/10	0·12
pPVA^{EG}	NS	2/7	1/7§	0/13	0/13	0·26
Mock-inoculated	NS					0·02

* Number of plants infected as tested by ELISA on inoculated leaves (IL) at 14 days p.i. and systemically infected (SL) leaves at 21 days p.i. NT, Not tested.
† NLL, 7–30 necrotic lesions per shot leaf area; NS, no symptoms. A total of 16 leaves (32 shots) per virus construct was tested.
‡ ELISA values 3 times higher than those of mock-inoculated protoplasts were considered to indicate virus multiplication.
§ Progeny of virus isolated from systemic leaves had wild-type sequence at the 6K2/VPg cleavage site, indicating that revertant virus was responsible for systemic movement.

nated CI-NIb in Fig. 3B–E), CI-6K2-NIa (Fig. 3B–D), NIa-NIb (Fig. 3C–E), NIa-NIb-CP (Fig. 3F) and NIb-CP (Fig. 3E, F).

The rate of proteolytic turnover of each polyprotein processing intermediate was determined. The intensity of bands corresponding to NIb-CP decreased by a factor of 9–11 during the 90 min chase (Fig. 3E). The precursors CI-6K2-NIa and CI-6K2-NIa-NIb, which contain multiple NIaPro cleavage sites, decreased by a factor of 7–12 within the same time. The processing rate of the precursors NIa-NIb and P3-6K1-CI-6K2 was slower and the intensities of bands decreased by a factor of only 1·5–3 after the 90 min chase. These data also indicate that processing of P3-6K1-CI-6K2 and NIb-CP polyproteins must have continued *in trans* as they do not contain NIaPro.

Deletion of 6K1 or 6K2 affects maturation of the P3 or CI proteins in insect cells and abolishes infectivity in plants

Deletion of the genomic region encoding the 6K1 protein (mutant BacPVAΔ6K1) affected the proteolytic processing of P3 and CI (Fig. 2B) while the only effect of deletion of the genomic region encoding the 6K2 protein (mutant BacPVAΔ6K2) was disappearance of CI-6K polyprotein. Neither of these deletions affected processing of the VPg, NIaPro, NIb or CP from the polyprotein (data not shown). Data from Western analysis indicated that expression of the polyprotein from BacPVAΔ6K2 resulted in a fully processed CI, and no product corresponding to the expected size of the CI-6K2 polyprotein was observed (Fig. 2B). These results revealed that the 6K peptide associated with CI expressed from the wild-type polyprotein is the 6K2 protein.

Expression of the polyprotein from BacPVAΔ6K1 produced traces of the fully processed CI (71 kDa). However, most of the CI obtained from BacPVAΔ6K1 was detected as a product with an electrophoretic mobility corresponding to ca. 120 kDa (Fig. 2B). A product of 120 kDa was also detected in BacPVA-infected cells with antibodies raised to the P3 protein and it was immunoprecipitated with the anti-CI and anti-P3 antibodies (data not shown). Therefore it probably corresponds to a polyprotein including the P3, the CI and probably also the 6K2 protein. A minor product with a slightly lower molecular mass detected with these antisera is assumed to represent a P3-CI polyprotein without 6K2 (Fig. 2B).

Deletion of both 6K-protein encoding regions (mutant BacPVAΔ6K1Δ6K2) resulted in combination of the effects observed with BacPVAΔ6K1 and BacPVAΔ6K2. A fully processed CI was detected as a minor product, while most of CI was found in a product with a molecular mass of approximately 110 kDa, representing the expected polyprotein of P3 and CI (Fig. 2B).

The deletion mutants pPVAΔ6K1, pPVAΔ6K2 and pPVAΔ6K1Δ6K2 showed no infectivity in inoculated plants or protoplasts (Table 1). These results indicate that both 6K proteins, and not only 6K2 (Klein *et al.*, 1994; Restrepo-Hartwig & Carrington, 1994), are important for the replication of PVA.

CI-6K2 is a membrane-bound protein both in PVA-infected plants and in insect cells

In the systemically infected plants, virus infection is not synchronous in different cells and tissues and the potyviral

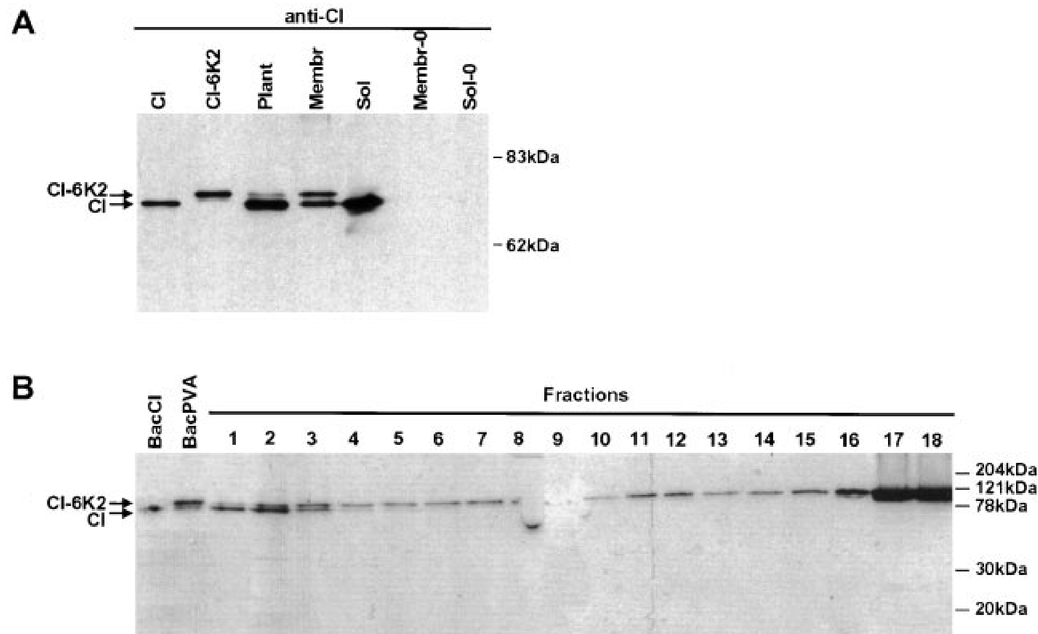

Fig. 4. CI-6K2 is a membrane-bound polyprotein in PVA-infected plants and in insect cells. (A) Distribution of the PVA-encoded proteins CI and CI-6K2 in PVA-infected plant extracts. Membrane (Membr) and soluble (Sol) fractions of PVA-infected and mock-inoculated plants (Membr-0 and Sol-0) were collected and subjected to SDS–PAGE. Extracts from insect cells, infected with BacCI and BacCI-6K2 (designated CI and CI-6K2, respectively), were used as controls. Lane 'Plant' on each panel corresponds to unfractionated extract from a PVA-infected plant. Distribution of PVA CI and CI-6K2 was analysed using Western blotting with antibodies against CI. Arrows to the left indicate the positions of detected PVA proteins and polyproteins. New England Biolabs prestained molecular mass standards are shown on the right. (B) Distribution between membrane and soluble fractions of CI and CI-6K2 in insect cells. Flotation gradient from cells infected by BacPVA was drop-divided into 18 fractions. Equal amounts of fractions were analysed by SDS–PAGE followed by Western blotting using antibodies to CI and ECL procedure. Arrows to the left indicate positions of detected PVA proteins and polyproteins. Lane 18, top of the gradient (floated membranes); lane 1, bottom of the gradient; lanes BacCI and BacPVA, non-fractionated proteins from insect cells infected by BacCI and BacPVA, respectively. Bio-Rad kaleidoscopic protein molecular mass standards are shown on the right.

proteins are expected to exist in several differently processed and localized forms (Roberts *et al.*, 1998). The PVA-infected tobacco plants (but not mock-inoculated plants) contained two bands, corresponding to the expected sizes of CI and CI-6K2 as detected with the anti-CI antibodies (Fig. 4A), as previously reported for plants infected with TEV (Restrepo-Hartwig & Carrington, 1994). Using the membrane flotation assay it was found that the CI-6K2 was localized almost exclusively in membrane fractions from infected plants, whereas the majority of the fully processed CI was found in the soluble fraction (Fig. 4A). These results suggest that the CI-6K2 polyprotein probably is mostly bound to membranes and is not to a significant extent involved in formation of cytoplasmic inclusions.

To reveal whether the CI-6K2 polyprotein is also membrane-bound in insect cells, extracts of the High Five cells infected by BacPVA were floated on sucrose gradients and the distribution of recombinant proteins in the gradient was analysed by SDS–PAGE and Western blotting. The non-processed CI-6K2 polyprotein produced from the wild-type PVA polyprotein (BacPVA) was almost exclusively found in the membrane fraction (Fig. 4B), similar to that observed for CI-6K2 from infected plants.

Point mutations at proteolytic cleavage sites flanking 6K2 affect polyprotein processing and infectivity in plants

It has been hypothesized that the 6K2 peptide may serve as a membrane anchor for the putative virus replication complex via a covalent linkage with VPg, which is thought to be a part of the replication complex (Schaad *et al.*, 1996, 1997). However, in the BacPVA-infected insect cells, no protein corresponding to 6K2-VPg was detected, in contrast to the CI-6K2 which represented a stable major polyprotein (Fig. 2A). To study the cleavages at the 6K2 boundaries in more detail, two point mutations were introduced into the PVA genome. The proteolytic cleavage site between CI and 6K2 was mutated (underlined) from EAVQFQ/S to EAAQFQ/S to yield constructs designated pPVA^{VA} and BacPVA^{VA} (Fig. 1). This mutation completely abolished the cleavage at the mutated site (Fig. 2B) in the baculovirus expression system but had no effect on cleavages at other sites (data not shown), similar to the results on TEV obtained in *in vitro* translation systems (Carrington *et al.*, 1988).

Another mutation introduced near the cleavage site between 6K2 and VPg (EVVAFQ/S to GVVAFQ/S), yielding

constructs $pPVA^{EG}$ and $BacPVA^{EG}$, slowed down but did not prevent proteolytic cleavage between 6K2 and VPg (Fig. 3C, D). Hence, significant amounts of relatively stable polyproteins 6K2-NIa and 6K2-VPg (Fig. 3C, D) were detected and they were slowly processed into NIaPro and VPg (Fig. 3C, D). On the other hand, the mutation significantly enhanced cleavage between CI and 6K2 (Fig. 2B). These data are supported by previous studies suggesting that cleavages at both sites flanking the 6K2 protein are processed by NIaPro preferentially in *cis* (Carrington *et al*., 1988).

$pPVA^{EG}$ infected tobacco protoplasts, but at a lower rate than the wild-type PVA (Table 1). Also, two of the seven inoculated tobacco plants accumulated detectable amounts of CP antigen in the leaf areas inoculated by particle bombardment, and one of these two plants was systemically infected, albeit at a slow rate (Table 1). However, in the virus-positive systemic leaves, analysis of virus progeny by RT–PCR, cloning and sequencing revealed only the wild-type sequence of pPVA, indicating reversion of the point mutation introduced to $pPVA^{EG}$. Taken together, the data suggested that the amino acid substitution E to G at the 6K2/VPg cleavage site slowed down virus replication and inhibited virus movement. The other virus mutant, $pPVA^{VA}$, replicated in protoplasts to only a very low level and caused no detectable infection in tobacco plants (Table 1). This is similar to results obtained with a mutant of TEV in which the cleavage site between CI and 6K2 was modified (Restrepo-Hartwig & Carrington, 1994). Collectively, the data indicate that the 6K peptides are indispensable for virus replication and their proteolytic separation from the adjacent proteins by NIaPro is important for the rate of virus replication and movement.

Discussion

In this study, we set out to examine the proteolytic processing of the potyviral polyprotein by the viral proteinase NIaPro, which is responsible for cleavages at most of the proteolytic sites of the polyprotein. Data available on the proteolytic processing of the potyviral polyproteins are largely from *in vitro* translation systems or from *E. coli*-based expression systems (Carrington & Dougherty, 1987a, b; Carrington *et al*., 1988; García *et al*., 1992; Kim *et al*., 1995; Parks *et al*., 1995). Studies on such processes in plants *in vivo* are hampered (Carrington *et al*., 1993; Schaad *et al*., 1996) by the fact that mutations in the proteolytic cleavage sites often debilitate potyvirus replication (Carrington *et al*., 1993; Restrepo-Hartwig & Carrington, 1994; Schaad *et al*., 1996; this study) and mutations in the active site of NIaPro cannot be complemented *in trans* (Schaad *et al*., 1996). Potyviral infection in plants is unsynchronized, which hampers detailed studies of proteolytic processes that occur quickly. Therefore, in this study, the course of proteolytic cleavages catalysed by NIaPro was investigated using an efficient and easy-to-use system where the potyviral polyproteins were expressed from baculovirus vectors in eukaryotic (insect) cells. The amounts of recombinant PVA proteins (relative to the total protein content) in insect cells after 40 h p.i. were ca. 5- to 10-fold lower than in systemically infected tobacco leaves at 10 days p.i. (data not shown). Therefore, the baculovirus system was unlikely to suffer from problems of protein overexpression. Proper processing of the N-terminal part of the potyviral polyprotein by the viral proteinases P1 and HC-Pro has previously been shown to occur in the baculovirus system (Thornbury *et al*., 1993) but NIaPro-mediated processing of the polyprotein in a baculovirus system has not been studied.

The NIaPro has *cis* and *trans* proteolytic activities (Carrington *et al*., 1988) and different cleavage sites are processed with different efficiencies depending on their consensus sequences. The neighbouring sequence motifs and contexts have an impact (Carrington *et al*., 1993; Riechmann *et al*., 1995; Schaad *et al*., 1996). However, the sequential order of the proteolytic cleavage events carried out by the NIaPro has not been resolved. In this study, the complete PVA polyprotein was never detected, suggesting that polyprotein processing starts co-translationally. However, a number of proteolytic processing intermediates (P3-6K1-CI-6K2-NIa-NIb, P3-6K1-CI-6K2, CI-6K2-VPg-NIaPro-NIb, CI-6K2-VPg-NIaPro, VPg-NIaPro-NIb, VPg-NIaPro-NIb-CP and NIb-CP) appeared in pulse samples. Most of these intermediates had relatively short half-lives, but since they were detected after a 20 min pulse and some even after a 2 h chase, they might play some role in the potyvirus replication cycle, as described for non-structural polyproteins of alphaviruses (Lemm *et al*., 1994; Shirako & Strauss, 1994). Most proteolytic sites (6K1/CI, 6K2/VPg, NIaPro/NIb and NIb/CP) within the polyproteins were quickly processed by the NIaPro. The site between NIb and CP in the NIb-CP polyprotein had a fast turnover rate despite the fact that it was processed by NIaPro *in trans*. However, there were obvious *cis*-preferences for cleavage by NIaPro. Although our results provide information about cleavage preferences and relative processing rates of NIaPro, additional experiments are needed to reveal the full sequential order of potyvirus polyprotein processing.

Three proteolytic sites were processed slowly, and the corresponding polyproteins were readily detected by Western analysis. The proteolytic sites between P3 and 6K1, CI and 6K2, and the internal proteolytic site of NIa were processed at a low rate, as confirmed by the pulse–chase experiments. The partial or slow processing of the proteolytic site between P3 and 6K1 in the PVA polyprotein is similar to a previous report on PPV (García *et al*., 1992; Riechmann *et al*., 1995). Making the heptapeptide sequence at the cleavage site similar to the efficiently cleaved NIb/CP junction does not enhance cleavage in PPV, which suggests that a sequence and/or conformational context outside the conserved heptapeptide sequence is modulating the cleavage reaction catalysed by the NIaPro (García *et al*., 1992). No specific function has been reported for the 6K1 peptide or for the P3-6K1 polyprotein during the

potyviral infection cycle, and the significance of slow processing therefore remains obscure. Mutations preventing the cleavage at the proteolytic site between P3 and 6K1 do not abolish infectivity (Riechmann *et al*., 1995). However, our data show that deletion of the 6K1-encoding sequence prevented proteolytic cleavage between P3 and CI. This is a novel finding and shows that the 6K1 peptide is functionally important. Possibly it might function as a spacer between P3 and CI, having an impact on the proteolytic processing of the two larger proteins from the polyprotein.

The CI and 6K2 proteins are involved in cell-to-cell movement of virus through plasmodesmata and/or intracellular translocation of the viral RNA (Carrington *et al*., 1998; Roberts *et al*., 1998; Rajamäki & Valkonen, 1999). At the infection frontier in plant tissues, the CI protein is transiently associated with the plasmodesmata (Carrington *et al*., 1998; Roberts *et al*., 1998). The 6K2 protein may mediate intra- and/or intercellular movement of potyviruses (Rajamäki & Valkonen, 1999) analogous to the endoplasmic reticulum (ER)-bound 30K movement protein of *Tobacco mosaic virus* (genus *Tobamovirus*) (Heinlein *et al*., 1998; Reichel & Beachy, 1998). Therefore, it is interesting that the mutants PVA^{EG} and PVA^{VA}, in which processing of the proteolytic site between CI and 6K2 proteins was modified, replicated in protoplasts but were debilitated in their infectivity in plants. These data suggest that separation of CI and 6K2 is biologically important, but further studies are needed to determine whether the main effects are manifested via intra- or intercellular virus transport.

Efficient processing of the cleavage site between CI and 6K2 was observed only if proteolysis of the site between 6K2 and VPg was prevented, which indicates that the cleavage between CI and 6K2 takes place in *cis*, as proposed earlier for TEV (Carrington *et al*., 1988). Mutations at the 6K2/VPg cleavage site reduced virus infectivity, slowing down PVA multiplication and preventing movement, which indicates a requirement for a delicate balance in cleavages at the 6K2 boundaries. The suboptimal consensus cleavage sequence for NIaPro between the VPg and NIaPro domains of NIa (Carrington *et al*., 1993; Puurand *et al*., 1994; Schaad *et al*., 1996) results in a relatively slow processing of the site, which is required for efficient genome amplification (Schaad *et al*., 1996).

Our data provide evidence that the stable processing intermediates P3-6K1, CI-6K2 and NIa (VPg-NIaPro) play a role during the virus infection cycle. The 6K2 protein is the only potyviral protein reported to have specific affinity to the ER (Schaad *et al*., 1996). Therefore, it has been hypothesized that the 6K2 protein anchors the putative virus replication complex to the ER-derived sites for replication as a polyprotein with NIa (Restrepo-Hartwig & Carrington, 1994; Schaad *et al*., 1996, 1997) which, in turn, binds viral RNA (Murphy *et al*., 1990; Carrington *et al*., 1993). The replication complex might then be released from the membrane by a cleavage at the 6K2/VPg junction. However, no traces of a 6K2-VPg or a 6K2-NIa polyprotein were detected in insect cells expressing the PVA polyprotein, nor have they been detected in PVA-infected plant tissues. These data suggest that such polyproteins either do not exist or have a very short half-life (less than 5 min). In contrast, CI-6K2 represents a relatively stable membrane-bound polyprotein in insect and plant cells. It is therefore possible that there are differences in the membrane-binding mechanisms for the replication complexes, or the 6K2-associated functions are mediated differently between PVA and TEV. This would not be surprising considering some other known differences between these viruses. For example, the VPg of PVA binds RNA (Merits *et al*., 1998), in contrast to the VPg of TEV (Darós & Carrington, 1997). Furthermore, the VPg of PVA is bound to the viral RNA in virions only in the processed form (Oruetxebarria *et al*., 2001), whereas both VPg and the unprocessed NIa are detected in the virions of TEV (Murphy *et al*., 1990; Carrington *et al*., 1993).

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References

Andrejeva, J., Puurand, Ü., Merits, A., Rabenstein, F., Järvekülg, L. & Valkonen, J. P. T. (1999). Potyvirus helper component-proteinase and coat protein (CP) have coordinated functions in virus–host interaction and the same CP motifs affects virus transmissions and accumulation. *Journal of General Virology* **80**, 1133–1139.

Browning, I. A., Burns, R., George, E. L. & Darling, M. (1995). Development and evaluation of ELISA assays incorporating monoclonal antibodies for detection of potato A potyvirus. *EPPO Bulletin* **25**, 259–268.

Carrington, J. C. & Dougherty, W. G. (1987a). Small nuclear inclusion protein encoded by plant potyvirus genome is a protease. *Journal of Virology* **61**, 2540–2548.

Carrington, J. C. & Dougherty, W. G. (1987b). Processing of the tobacco etch virus 49K proteinase requires autoproteolysis. *Virology* **160**, 355–362.

Carrington, J. C., Cary, S. M. & Dougherty, W. G. (1988). Mutational analysis of tobacco etch virus polyprotein processing: *cis* and *trans* proteolytic activities of polyproteins containing the 49-kilodalton proteinase. *Journal of Virology* **62**, 2313–2320.

Carrington, J. C., Cary, S. M., Parks, T. D. & Dougherty, W. G. (1989). A second proteinase encoded by a plant potyvirus genome. *EMBO Journal* **8**, 365–370.

Carrington, J. C., Haldeman, R., Dolja, V. V. & Restrepo-Hartwig, M. A. (1993). Internal cleavage and trans-proteolytic activities of the VPg-proteinase (NIa) of tobacco etch potyvirus *in vivo*. *Journal of Virology* **67**, 6995–7000.

Carrington, J. C., Jensen, P. E. & Schaad, M. C. (1998). Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. *Plant Journal* **14**, 393–400.

Darós, J. A. & Carrington, J. C. (1997). RNA binding activity of NIa proteinase of tobacco etch potyvirus. *Virology* **237**, 327–336.

Darós, J. A., Schaad, M. C. & Carrington, J. C. (1999). Functional analysis of the interaction between VPg-proteinase (NIa) and RNA polymerase (NIb) of tobacco etch potyvirus, using conditional and suppressor mutants. *Journal of Virology* **73**, 8732–8740.

Fellers, J., Wan, J., Hong, Y., Collins, G. B. & Hunt, A. G. (1998). *In vitro* interactions between a potyvirus-encoded, genome-linked protein and RNA-dependent RNA polymerase. *Journal of General Virology* **79**, 2043–2049.

Fernandez, A., Lain, S. & García, J. A. (1995). RNA helicase activity of the plum pox potyvirus CI protein expressed in *Escherichia coli*. Mapping of an RNA binding domain. *Nucleic Acids Research* **23**, 327–332.

García, J. A., Lain, S., Cervera, M. T., Riechmann, J. L. & Martin, M. T. (1990). Mutational analysis of plum pox potyvirus polyprotein processing by the NIa protease in *Escherichia coli*. *Journal of General Virology* **71**, 2773–2779.

García, J. A., Martin, M. T., Cervera, M. T. & Riechmann, J. L. (1992). Proteolytic processing of the plum pox potyvirus polyprotein by the NIa protease at novel cleavage site. *Virology* **188**, 697–703.

Guo, D., Rajamäki, M. L., Saarma, M. & Valkonen, J. P. (2001). Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *Journal of General Virology* **82**, 935–939.

Gutierrez-Campos, R., Torres-Acosta, J. A., Saucedo-Arias, L. J. & Gomez-Lim, M. A. (1999). The use of cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. *Nature Biotechnology* **17**, 1223–1226.

Hämäläinen, J. H., Kekarainen, T., Gebhardt, C., Watanabe, K. N. & Valkonen, J. P. T. (2000). Recessive and dominant resistance interfere with the vascular transport of *Potato virus A* in diploid potatoes. *Molecular Plant–Microbe Interactions* **13**, 402–412.

Heinlein, M., Padgett, H. S., Gens, J. S., Pickard, B. G., Casper, S. J., Epel, B. L. & Beachy, R. N. (1998). Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmatic reticulum and microtubules during infection. *Plant Cell* **10**, 1107–1120.

Hong, Y. & Hunt, A. G. (1996). RNA polymerase activity catalysed by potyvirus encoded RNA-dependent-RNA-polymerase. *Virology* **226**, 146–151.

Hong, Y., Levay, K., Murphy, J. K., Klein, P. G., Shaw, J. G. & Hunt, A. G. (1995). A potyvirus polymerase interacts with the viral coat protein and VPg in yeast cells. *Virology* **214**, 159–166.

Kim, D. H., Park, Y. S., Kim, S. S., Lew, J., Nam, H. G. & Choi, K. Y. (1995). Expression, purification, and identification of a novel self-cleavage site of the NIa C-terminal 27 kDa protease of turnip mosaic potyvirus C5. *Virology* **213**, 517–525.

Kim, D. H., Hwang, D. C., Kang, B. H., Lew, J., Han, J., Song, B. D. & Choi, K. Y. (1996). Effects of internal cleavages and mutations in the C-terminal region of NIa proteinase of turnip mosaic potyvirus on the catalytic activity. *Virology* **226**, 183–190.

Klein, P. G., Klein, R. R., Rodríguez-Cerezo, E., Hunt, A. G. & Shaw, J. G. (1994). Mutational analysis of the tobacco vein mottling virus genome. *Virology* **204**, 759–769.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lemm, J. A., Rümenapf, T., Strauss, E. G., Strauss, J. H. & Rice, C. M. (1994). Polypeptide requirements for assembly of functional Sindbis virus replication complex: a model for temporal regulation of minus and plus-strand RNA synthesis. *EMBO Journal* **13**, 2925–2934.

Li, X. H., Valdez, P., Olvera, R. E. & Carrington, J. C. (1997). Functions of the tobacco etch virus RNA polymerase (NIb): subcellular transport and protein–protein interactions with VPg/proteinase (NIa). *Journal of Virology* **71**, 1598–1607.

Luciano, C. S., Rhoads, R. E. & Shaw, J. G. (1987). Synthesis of potyviral RNA and proteins in tobacco mesophyll protoplasts inoculated by electroporation. *Plant Science* **51**, 295–303.

Martin, M. T., Cervera, M. T., García, J. A. & Bonay, P. (1995). Properties of the active plum pox potyvirus RNA polymerase complex in defined glycerol gradient fractions. *Virus Research* **37**, 127–137.

Merits, A., Guo, D. & Saarma, M. (1998). VPg, coat protein and five non-structural proteins of potato A potyvirus bind RNA in a sequence unspecific manner. *Journal of General Virology* **79**, 3123–3127.

Merits, A., Guo, D., Järvekülg, L. & Saarma, M. (1999). Biochemical and genetic evidence for interactions between potato A potyvirus-encoded proteins P1 and P3 and proteins of the putative replication complex. *Virology* **263**, 15–22.

Murphy, J. F., Rhoads, R. E., Hunt, A. G. & Shaw, J. G. (1990). The VPg of tobacco etch virus RNA is the 49 kDa proteinase or the N-terminal 24 kDa part of the proteinase. *Virology* **178**, 285–288.

Murphy, J. F., Klein, P. G., Hunt, A. G. & Shaw, J. G. (1996). Replacement of the tyrosine residue that links a potyviral VPg to the viral RNA is lethal. *Virology* **220**, 535–538.

Oruetxebarria, I., Guo, D., Merits, A., Mäkinen, K., Saarma, M. & Valkonen, J. P. T. (2001). Identification of the genome-linked protein in virions of potato virus A, with comparison to other members in genus *Potyvirus*. *Virus Research* **73**, 103–112.

Osman, T. A. & Buck, K. W. (1996). Complete replication *in vitro* of tobacco mosaic virus RNA by a template-dependent, membrane-bound RNA polymerase. *Journal of Virology* **70**, 6227–6234.

Parks, T. D., Howard, E. D., Wolpert, T. J., Arp, D. J. & Dougherty, W. G. (1995). Expression and purification of a recombinant tobacco etch virus NIa proteinase: biochemical analysis of the full-length and a naturally occurring truncated proteinase form. *Virology* **210**, 194–201.

Peränen, J., Takkinen, K., Kalkkinen, N. & Kääriäinen, L. (1988). Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *Journal of General Virology* **69**, 2165–2178.

Puurand, Ü., Mäkinen, K., Paulin, L. & Saarma, M. (1994). The nucleotide sequence of potato virus A genomic RNA and its sequence similarities with other potyviruses. *Journal of General Virology* **75**, 457–461.

Puurand, Ü., Valkonen, J. P. T., Mäkinen, K., Rabenstein, F. & Saarma, M. (1996). Infectious *in vitro* transcripts from cloned cDNA of the potato A potyvirus. *Virus Research* **40**, 135–140.

Rajamäki, M. L. & Valkonen, J. P. T. (1999). The 6K2 protein and the VPg of *Potato virus A* are determinants of systemic infection in *Nicandra physaloides*. *Molecular Plant–Microbe Interactions* **12**, 1074–1081.

Rajamäki, M., Merits, A., Rabenstein, F., Andrejeva, J., Paulin, L., Kekarainen, T., Kreuze, J. F., Forster, R. L. S. & Valkonen, J. P. T. (1998). Biological, serological, and molecular differences among isolates of potato A potyvirus. *Phytopathology* **88**, 311–321.

Reichel, C. & Beachy, R. N. (1998). Tobacco mosaic virus infection induces severe morphological changes of endoplasmatic reticulum. *Proceedings of the National Academy of Sciences, USA* **95**, 11169–11174.

Restrepo-Hartwig, M. A. & Carrington, J. C. (1994). The tobacco etch potyvirus 6-kilodalton protein is membrane associated and involved in virus replication. *Journal of Virology* **68**, 2388–2397.

Riechmann, J. L., Lain, S. & García, J. A. (1992). Highlights and

prospects of potyvirus molecular biology. *Journal of General Virology* **73**, 1–16.

Riechmann, J. L., Cervera, M. T. & García, J. A. (1995). Processing of the plum pox virus polyprotein at the P3-6K1 junction is not required for virus viability. *Journal of General Virology* **76**, 951–956.

Roberts, I. M., Wang, D., Findlay, K. & Maule, A. J. (1998). Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (Cis) shows that CI protein acts transiently in aiding virus movement. *Virology* **245**, 173–181.

Rodríguez-Cerezo, E. & Shaw, J. G. (1991). Two newly detected nonstructural proteins in potyvirus-infected cells. *Virology* **185**, 572–579.

Schaad, M. C., Haldeman-Cahill, R., Cronin, S. & Carrington, J. C. (1996). Analysis of the VPg-proteinase (NIa) encoded by tobacco etch potyvirus: effects of mutations on subcellular transport, proteolytic processing, and genome amplification. *Journal of Virology* **70**, 7039–7048.

Schaad, M. C., Jensen, P. E. & Carrington, J. C. (1997). Formation of plant RNA virus replication complexes on membranes: role of an endoplasmatic reticulum-targeted viral protein. *EMBO Journal* **16**, 4049–4059.

Shirako, Y. & Strauss, J. H. (1994). Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP2 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *Journal of Virology* **68**, 1874–1885.

Thornbury, D. W., van den Heuvel, J. F., Lesnaw, J. A. & Pirone, T. P. (1993). Expression of potyvirus proteins in insect cells infected with recombinant baculovirus. *Journal of General Virology* **74**, 2731–2735.

Verchot, J., Koonin, E. V. & Carrington, J. C. (1991). The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase. *Virology* **185**, 527–535.

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