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, Nlb 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 Nlb/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 & 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, 1996; Schaad et al., 1996). Nuclear inclusion protein b (Nlb) 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 Nlb and, hence, may participate in the direction of Nlb to the replication complex. The proteins putatively involved in the potyvirus replication complex (CI, VPg, NIaPro and Nlb) bind RNA (Merits et al., 1998) and interact with the...
non-structural proteins P1 and P3 (Merits et al., 1999). In addition, potyviral coat protein (CP) can interact with Nla and Nlb (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 NlaPro, 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 proteases 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 Nla 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 NlaPro-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^6K1 and pPVA^6K2, containing amino acid substitutions (underlined) \( \text{EVVAFQ/G} \rightarrow \text{GVVAFQ/G} \) at the proteolytic site between the 6K2 protein and the VPg and EVVAFQ/G \( \rightarrow \text{EAQVQFS} \rightarrow \text{EAQVQFS} \) at the polyprotein site between CI protein and 6K2, were made using appropriate primers and the QuickChange 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 to the manufacturer’s protocol. The mutant clones of the PVA cDNA lacking coding sequences for 6K1, 6K2 or both 6K peptides are designated pPVA^6K1, pPVA^6K2 and pPVA^6K16K2, 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, pPVA^6K1, pPVA^6K2,
The PVA genome was excised with manufacturer’s protocols. The PVA genome was excised with the T7 promoter and subsequently transcribed using T7 RNA polymerase (Puurand et al., 1996). Transcripts were coated on gold particles (40 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, 8–8, 8–8 cm) 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., 1995; Rajamäki et al., 1998). Leaf discs (diam. 20 mm) containing the entire bombarded area were sampled from tobacco and Nicotiana 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 (Luciano et al., 1987; Andrejeva et al., 1999). Each batch of 1 × 10⁵ 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 ELISA-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 Spod and KpnI from pPVA, pPVAΔ6K1, pPVAΔ6K2, pPVAΔ6K1Δ6K2, pPVAΔV and pPVAΔE and cloned into pFastBac1 (Gibco BRL) digested with the same enzymes. The recombinant baculoviruses expressing these constructs are designated BacPVA, BacPVAΔ6K1, BacPVAΔ6K2, BacΔ6K1Δ6K2, BacPVAΔV and BacPVAΔE, respectively. The PVA genes (P3, CI, CI-6K2, VPg, Nla, NlaPro, Nlb 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 EcoRV and SalI (for CI and CI-6K2), or BamHI and SalI (for P3, VPg, Nla, NlaPro, Nlb and CP). The recombinant baculoviruses containing these constructs are designated BacCI, BacCI-6K2, BacP3, BacVPg, BacNla, BacNlaPro, BacNlb and BacCP, respectively.

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.

Pulse–chase experiments on the recombinant baculovirus expression system. About 2 × 10⁸ High Five cells per Petri dish (diam. 3 cm) were infected with each recombinant baculovirus at m.o.i. 10 at 27 °C. The optimal 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 [35S]methionine; Amersham Pharmacia). Samples were collected immediately after the pulse or after chasing for 2 h. Samples were collected immediately after the pulse or after chasing for 2 h. Samples were collected immediately after the pulse or after chasing for 2 h. 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 Isotopenmeßgeräte GmbH). All precursors were identified by immunoprecipitation with antibodies against all their domains (mature proteins), except εK1 and εK2. In the case of P3-Nlb it means that this precursor was identified by immunoprecipitation with anti-P3-, -CI, -VPg, -NlaPro and -Nlb 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 MgCl₂, 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 × 10⁶ 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 mM 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 layered over 67% sucrose (0.5ml), then overlaid with 50% and 5% sucrose (0.5 ml and 1.0 ml, respectively), and finally centrifuged in a Beckman SW 41 rotor at 35,000 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.

NlaPro-mediated processing of PVA polyprotein. About 5 × 10⁶ 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 antiserum raised to the different PVA proteins (Merits et al., 1999) followed by visualization by ECL (Amersham Pharmacia).
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, NLaPro, Nlb 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-NLaPro 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 BacNla and, hence, corresponded to the full-length, non-processed Nla protein (Fig. 2A). The Nla 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 NlaPro, as shown for Turnip mosaic virus (Kim et al., 1995, 1996) and TEV (Parks et al., 1995).

P3-6K1, CI-6K2 and Nla (VPg-NLaPro) 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 Nlb 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-Nla-Nlb (designated P3-Nlb in Fig. 3A, E), P3-6K1-CI-6K2 (Fig. 3A, B), CI-6K2-Nla-Nlb (desig-
Fig. 3. Pulse–chase experiments on insect cells infected with recombinant baculoviruses expressing PVA proteins. The proteins expressed by BacPVA (PVA) and BacPVA*^G (PVA*^G) were metabolically labelled with [35S]methionine, immunoprecipitated with anti-P3 (A), anti-CI (B), anti-VPg (C), anti-NlaPro (D), anti-Nlb (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), BacNla (Nla), BacNlaPro (NlaPro), BacNlb (Nlb) 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-Nlb means P3-6K1-CI-6K2-VPg-NlaPro-Nlb and CI-Nlb means CI-6K2-VPg-NlaPro-Nlb.
nated CI-Nlb in Fig. 3B–E). CI-6K2-Nla (Fig. 3B–D), Nla-Nlb (Fig. 3C–E), Nla-Nlb-CP (Fig. 3F) and Nlb-CP (Fig. 3E, F).

The rate of proteolytic turnover of each polyprotein processing intermediate was determined. The intensity of bands corresponding to Nlb-CP decreased by a factor of 9–11 during the 90 min chase (Fig. 2B). The precursors CI-6K2-Nla and CI-6K2-Nla-Nlb, which contain multiple NlaPro cleavage sites, decreased by a factor of 7–12 within the same time. The processing rate of the precursors Nla-Nlb 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 Nlb-CP polyproteins must have continued in trans as they do not contain NlaPro.

### 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, NlaPro, Nlb 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 polypeptide 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 poylviral

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

<table>
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<tr>
<th>Construct</th>
<th>Response in <em>A6</em></th>
<th><em>N. tabacum</em></th>
<th><em>N. physaloides</em></th>
<th>Infectivity in tobacco protoplasts at 3 days p.i. (A~00)</th>
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<td>IL</td>
<td>SL</td>
<td>IL</td>
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<tr>
<td>pPVA</td>
<td>NLL</td>
<td>8/8</td>
<td>8/8</td>
<td>3/3</td>
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<tr>
<td>pPVAΔ6K1</td>
<td>NS</td>
<td>0/5</td>
<td>0/5</td>
<td>NT</td>
</tr>
<tr>
<td>pPVAΔ6K2</td>
<td>NS</td>
<td>0/5</td>
<td>0/5</td>
<td>NT</td>
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<tr>
<td>pPVAΔ6K1Δ6K2</td>
<td>NS</td>
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<td>0/5</td>
<td>0/5</td>
<td>0/10</td>
</tr>
<tr>
<td>pPVAΔ6K1Δ6K2</td>
<td>NS</td>
<td>2/7</td>
<td>1/7§</td>
<td>0/13</td>
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<tr>
<td>Mock-inoculated</td>
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* 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 shoot 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.
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 EA\_VQFQ\_Sto\_EAOQFQ\_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<sup>EG</sup> and BacPVA<sup>EG</sup>, slowed down but did not prevent proteolytic cleavage between 6K2 and VPg (Fig. 3C, D). Hence, significant amounts of relatively stable polyproteins 6K2-Nla and 6K2-VPg (Fig. 3C, D) were detected and they were slowly processed into NlaPro 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 NlaPro preferentially in cis (Carrington et al., 1988).

pPVA<sup>EG</sup> 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<sup>EG</sup>. 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<sup>VEG</sup>, 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 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 NlaPro 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 NlaPro, 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 NlaPro 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 NlaPro 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 NlaPro-mediated processing of the polyprotein in a baculovirus system has not been studied.

The NlaPro 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 NlaPro 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-Cl-6K2-Nla-Nlb, P3-6K1-Cl-6K2, Cl-6K2-VPg-NlaPro-Nlb, Cl-6K2-VPg-NlaPro, VPg-NlaPro-Nlb, VPg-NlaPro-Nlb-CP and Nlb-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/Cl, 6K2/VPg, NlaPro/Nlb and Nlb/CP) within the polyproteins were quickly processed by the NlaPro. The site between Nlb and CP in the Nlb-CP polyprotein had a fast turnover rate despite the fact that it was processed by NlaPro *in trans*. However, there were obvious *cis*-preferences for cleavage by NlaPro. Although our results provide information about cleavage preferences and relative processing rates of NlaPro, 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, Cl and 6K2, and the internal proteolytic site of Nla 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 Nlb/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 NlaPro (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 (Rieckmann 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 intracellular 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<sup>EG</sup> and PVA<sup>ΔA</sup>, 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 NlaPro between the VPg and NlaPro domains of Nla (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 Nla (VPg-NlaPro) 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 Nla (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-Nla 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 Nla are detected in the virions of TEV (Murphy et al., 1990; Carrington et al., 1993).

Financial support from EU INCO COPERNICUS (grant ERBIC15CT970900), Estonian Science foundation (grant N 4224), the Academy of Finland (grant 34529), and SJFR (grant 320667/97) is gratefully acknowledged. M.S. is a fellow of Biocentrum Helsinki.

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Received 6 November 2001; Accepted 14 January 2002