Evidence for phosphorylation and ubiquitinylation of the turnip yellow mosaic virus RNA-dependent RNA polymerase domain expressed in a baculovirus–insect cell system

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All RNA viruses known to date encode an RNA-dependent RNA polymerase (RdRp) that is required for replication of the viral genome. We have expressed and purified the turnip yellow mosaic virus (TYMV) RdRp in insect cells using a recombinant baculovirus, either in its native form, or fused to an hexa-histidine tag. Phosphorylation of the protein was demonstrated by labelling experiments *in vivo*, as well as phosphatase treatment of the purified protein *in vitro*. Phospho amino acid analysis and immunoblotting experiments identified serine and threonine residues as being the subject of phosphorylation. Peptide mass mapping using MS analysis of a protein digest revealed that phosphorylation sites are localized within a putative PEST sequence [a sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues] in the N-terminal region of the protein. Using monoclonal antibodies specific for ubiquitin conjugates, we were able to demonstrate that the TYMV RdRp is conjugated to ubiquitin molecules when expressed in insect cells. These observations suggest that the TYMV RdRp may be processed selectively by the ubiquitin/proteasome degradation system upon phosphorylation of the PEST sequence.

Key words: PEST sequence, positive-strand RNA virus, TYMV, viral replication.

INTRODUCTION

Turnip yellow mosaic virus (TYMV), a member of the tymovirus group, is a plant RNA virus that belongs to the 'alpha-like' super-group of viruses [1], which also comprises Sindbis virus. TYMV possesses a monopartite positive-strand genomic RNA of 6.3 kb, which directs the expression of two overlapping non-structural proteins of 69 and 206 kDa [2]. A third open reading frame encodes the 20-kDa coat protein which is expressed from a subgenomic RNA. The longer 206-kDa (206K) protein has considerable amino acid sequence similarities with non-structural putative replication proteins of several positive-strand RNA viruses (reviewed in [3]) and it has been shown to be necessary for TYMV RNA replication [4]. Upon expression *in vitro*, the 206K protein undergoes proteolytic maturation events [5], resulting in the synthesis of N-terminal 140 kDa (140K) and C-terminal 66 kDa (66K) proteins [6,7].

Replication of TYMV occurs in membrane-associated replication complexes [8]. Within these, the genomic plus-strand RNA is transcribed into minus-strand RNA, which in turn can be used as a template for synthesis of progeny genomic and subgenomic plus strands. At least the two viral proteins 140K and 66K appear to be involved in this reaction [9]. While the role in RNA replication of the 140K protein, which carries methyltransferase and NTPase/putative RNA helicase motifs, is less clear, the 66K protein is obviously the key enzyme responsible for synthesis of progeny RNA strands because it contains several motifs considered to be signature sequences for RNA-dependent RNA polymerases (RdRps) [10]. It has been suggested that these motifs may co-operate to form an ordered domain, which constitutes a 'polymerase module' implicated in template interaction and polymerase activity [11]. All RNA viruses known to date encode an RdRp that is required for replication of the viral genome. Some events of TYMV genome replication have been elucidated by mutagenesis of cloned genomes [9] whereas purification of active replication complexes from infected plants [12,13] has allowed the characterization of some of their enzymic properties [13–15]. However, the low amounts of non-structural viral proteins produced during viral infection precluded any biochemical and structural analyses.

Such studies can be greatly facilitated by the use of RdRp purified from recombinant sources. This approach has been successfully used in earlier experiments including poliovirus $3D^{pol}$ [16], tobacco vein mottling potyvirus NIb [17] and hepatitis C virus NS5B protein [18]. Purification and characterization of poliovirus $3D^{pol}$ from different sources showed that recombinant enzyme preparations from bacteria or insect cells are indistinguishable by all measured criteria from the RdRp synthesized in the natural host cell [19].

In order to study the properties of the TYMV RdRp, we report the expression of the TYMV 66K protein (RdRp domain) in insect cells using recombinant baculoviruses. Using a rapid purification procedure by means of an engineered N-terminal hexa-histidine tag, we show that the protein is phosphorylated *in vivo* on serine and threonine residues. Using matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS,

Abbreviations used: MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; Ni-NTA, Ni^{2+}-nitrilotriacetate; RdRp, RNA-dependent RNA polymerase; TYMV, turnip yellow mosaic virus; a.m.u., atomic mass units.

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phosphorylated peptides were localized to a putative PEST region [a region enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) residues]. In addition, higher-molecular-mass products were identified as ubiquitin–66K conjugates. We discuss the possible role of these post-translational modifications in the regulation of the protein's metabolic stability.

EXPERIMENTAL

Plasmid constructions

All DNA manipulations were performed using standard techniques [20,21]. The DNA fragment encoding the TYMV 66K protein (nt 3872–5629 according to [2]) was amplified by PCR using *Pfu* DNA polymerase (Stratagene) and primers 1, 5′-CGGAATTCCATGGGCACCCCCAGCGCATCCCCCACC-CACC-3′, and 3, 5′-CGGGATCCGGTACCCTATTGGACG-TAGTGAAGCAATTC-3′, which provided *Nco*I and *Kpn*I restriction sites (underlined) at the 5′ and 3′ termini of the gene, respectively. The 1766-nt-long *Nco*I–*Kpn*I fragment was cloned in the similarly restricted baculovirus transfer vector p10-119pst (kindly provided by M. Cerutti, Station de Recherches de Pathologie Comparée CNRS-INRA, Saint Christol-lez-Alès, France), a modified version of pGm8022, which allows expression under the control of the late p10 gene promoter [22], to give the construct p17AE. The ATG codon in the engineered *Nco*I site serves as an artificial start codon for translation. In the following, the protein expressed by this construct will be referred to as 66K.

To create the construct p8AF, the same procedure was used with the combination of primers 2, 5′-CGGAATTCCATGG GC(CACCAT)$_3$ACCCCCAGCGCATCCCCCACCCACCGT-TCG-3′ (with the *Nco*I site underlined), and 3. This construct gives rise to a 66K protein with an N-terminal hexa-histidine tag, which will be referred to as His-66K. The transferred PCR fragments were sequenced completely on an ABI Prism 377 DNA sequencer (Applied Biosystems) using a BigDye Terminator Sequencing kit (Applied Biosystems) and specific primers. Due to the engineering of the start codon within an *Nco*I cloning site in the DNA sequence, the N-termini of both recombinant proteins differed from the authentic viral protein by the presence of a methionine followed by a glycine residue instead of the threonine residue at position 1. The amino acid numbering starts at T^{1}PSA … 7, i.e. at the genuine N-terminus of the viral 66K protein [6,7], so that residue positions of recombinant and native 66K proteins correlate.

Expression of 66K and His-66K in insect cells

Recombinant baculoviruses Bac17AE and Bac8AF were generated by co-transfection of *Spodoptera frugiperda* (Sf9) lepidopteran cells with AcSLP10 DNA [22] and the p17AE and p8AF constructs respectively. The growth of Sf9 cell cultures and propagation of recombinant baculoviruses were carried out as described previously [23]. For the expression of 66K or His-66K proteins in insect cells, monolayers of Sf9 cells were infected with recombinant baculovirus Bac17AE or Bac8AF and incubated at 28 °C for 48 to 64 h. When tested, tunicamycin dissolved in DMSO was added to the cell cultures (final concentration, 10 μg/ml) at 7 or 20 h post-infection. As controls, cells were incubated with DMSO alone. The cells were harvested 52 h post-infection and the recombinant proteins were detected as described below.

Purification of His-66K

Bac8AF-infected Sf9 cells were harvested 64 h post-inoculation, pelleted at 500 ***g*** for 5 min, washed with PBS, and resuspended in buffer A (6 M guanidinium hydrochloride/0.1 M sodium phosphate/10 mM Tris/HCl, pH 8) at 10^6 cells/ml. Cells were incubated for 1 h at room temperature and the lysate was centrifuged at 10000 ***g*** for 15 min. Then, 1 ml of a 50 % slurry of Ni^{2+}-nitrilotriacetate (Ni-NTA) Superflow resin (Qiagen) previously equilibrated in buffer A was added to 10 ml of the supernatant and incubated for 45 min at room temperature with occasional shaking. The mixture was then loaded on a 0.5-cm-diameter column, washed with six column volumes of buffer A, three column volumes of buffer B (8 M urea/0.1 M sodium phosphate/10 mM Tris/HCl, pH 8) and three column volumes of buffer C (8 M urea/0.1 M sodium phosphate/10 mM Tris/HCl, pH 6.3). The recombinant protein was eluted with buffer C containing 300 mM imidazole.

Protein gel electrophoresis and staining

Proteins were separated by electrophoresis through SDS/polyacrylamide gels (12.5 or 7 %) according to the procedure described in [24]. After staining with Coomassie Brilliant Blue, the amount of protein was estimated by comparison with known amounts of BSA analysed on the same gel. Alternatively, proteins were detected by reverse staining using imidazole-zinc salts according to [25].

Western-blot analysis

After separation by SDS/PAGE, the proteins were electrotransfered to nitrocellulose BA83 (Schleicher and Schuell) or to PVDF membrane (Millipore). Polyclonal antibodies raised against the C-terminal region of TYMV 66K expressed in *Escherichia coli* as a fusion protein [26] were provided kindly by Dr K. Séron (Institut Jacques Monod, Paris, France) and were used at 1/500 dilution in PBS/5 % dried skimmed milk/0.05 % Tween 20. Antibodies raised against phosphoserine, phosphothreonine and phosphotyrosine were obtained from Zymed and were used at 1/200, 1/200 and 1/400 dilutions, respectively, in 10 mM Tris/HCl, pH 7.5/150 mM NaCl/6 % BSA. Monoclonal antibodies against ubiquitin conjugates (FK2) [27] were provided kindly by Dr R. Haguenauer-Tsapis (Institut Jacques Monod, Paris, France) and were used at 1/1000 dilution in PBS/5 % dried skimmed milk/0.05 % Tween 20. The blots were developed using alkaline phosphatase-conjugated secondary antibodies and Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (NBT-BCIP) as substrates.

Labelling *in vivo*

Sf9 cells (2×10^6 cells in a 25-cm^2 culture flask) were infected with the recombinant baculovirus Bac8AF, and 24 h post-infection the growth medium was replaced with phosphate-free medium containing 25 μCi/ml of [^{32}P]orthophosphate (ICN). After a labelling period of 18 h, the cells were collected and washed with PBS. Total cell proteins were analysed by SDS/PAGE after lysis of 4×10^5 cells in SDS sample buffer. The remaining cells were used to purify the labelled His-66K protein under denaturing conditions as described above.

Phospho amino acid analysis

Phospho amino acid analysis was performed as described in [28] after transfer of ^{32}P-labelled purified His-66K to a PVDF membrane. The protein was subjected to hydrolysis in 200 μl of 6 M HCl for 2 h at 110 °C, after which the supernatant was transferred to a new tube and lyophilized. The pellet was dissolved in 3 μl of water containing 2 μg each of phosphoserine, phosphothreonine and phosphotyrosine, and was spotted on to a

thin-layer cellulose plate (Kodak). The first-dimension ascending chromatography was carried out for 13 h in solvent A (isobutyric acid/0.5 M NH_4OH, 5:3, v/v) followed by 6 h of chromatography in the second dimension in solvent B (2-propanol/HCl/H_2O, 14:3:3, v/v) according to [29]. Phospho amino acids were visualized by spraying with ninhydrin (0.25%, w/v, in acetone) while radioactive molecules were revealed by autoradiography.

Protein phosphatase treatment

Purified His-66K protein (50 ng) was incubated with 400 and 800 units of λ protein phosphatase (New England Biolabs) as recommended by the supplier for 30 min at 30 °C.

Trypsin digestion

Purified His-66K protein (6 μg) was reduced by a treatment with 10 mM dithiothreitol/0.1 M NH_4HCO_3 for 45 min at 56 °C, followed by alkylation with 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 30 min. After electrophoresis on a 7% SDS/polyacrylamide gel and reverse staining, the bands containing the proteins of interest were excised from the gel, and rinsed twice in 1 ml of 50 mM Tris, pH 8/0.3 M glycine/30% acetonitrile and twice in water. Proteins were digested in-gel with sequencing-grade trypsin (Boehringer) according to published procedures [30]. The reaction was stopped by freezing the samples at −20 °C. The supernatant containing tryptic peptides was collected for MS analysis. Further peptide extraction was also performed as described previously [30].

MS analysis

The tryptic peptides were acidified with aqueous trifluoroacetic acid to a final concentration of 0.1% and mixed with a saturated solution of 2,5-dihydroxybenzoic acid (Sigma) in 0.1% aqueous trifluoroacetic acid. The samples were then analysed by MALDI–TOF MS. Ionization was accomplished with a 337-nm beam from a nitrogen laser. Mass spectra of positive ions were recorded in reflectron mode with a single-stage reflectron mass spectrometer (Voyager Elite, PerSeptive Biosystems, Framingham, MA, U.S.A.) equipped with a delayed extraction device. Delayed extraction time was set at 100 ns. Typically, 200–250 shots were averaged for each acquired spectrum. Internal calibration was performed using peptides generated by autoproteolysis of trypsin and corresponding to the protonated peptides 132–142, 56–75 and 76–85 with mono-isotopic mass-to-charge (m/z) ratios of 1153.57, 2163.06 and 2273.16, respectively. Only the mono-isotopic m/z ratio of the protonated peptides are given in the Results section.

RESULTS

Expression and purification of the TYMV 66K protein in insect cells

In order to develop an *in vitro* system for analysis of the viral RdRp domain structure and function, the entire TYMV 66K protein was expressed in insect cells using a recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV). For this purpose, two different recombinant baculoviruses were constructed: Bac17AE directs the expression of the TYMV 66K protein, hereafter called 66K, while Bac8AF allows the expression of an extended version of this protein, hereafter called His-66K, bearing a hexa-histidine affinity tag at the N-terminus. This tag was designed to permit the rapid purification of the protein by affinity chromatography on immobilized metal [31]. *S. frugiperda* (Sf9) lepidopteran cells were infected with the recombinant baculoviruses Bac17AE or Bac8AF, or a control recombinant baculovirus containing TYMV-unrelated sequences [23]. After incubation for 48 h at 28 °C, the total protein content of these cells was analysed by SDS/PAGE (12.5% gel). Coomassie Brilliant Blue staining of this gel (Figure 1A) revealed that a prominent band with a molecular mass of about 69 kDa was present in the protein extracts from Bac17AE- and Bac8AF-infected Sf9 cells (Figure 1A, lanes 2 and 3), but was absent from control extracts (Figure 1A, lane 1). These major proteins were confirmed as being the TYMV 66K protein and its tagged derivative by immunoblot analysis using an antiserum raised against a bacterial fusion protein containing the C-terminal part of the TYMV 66K protein (Figure 1B, lanes 1–3). In addition, a set of bands of lower molecular masses was also visible in the total protein extracts, that may correspond to degradation products of the TYMV 66K protein.

Despite the fact that the His-66K protein synthesized in Bac8AF-infected insect cells appeared mostly insoluble, the use of strong denaturing agents coupled with affinity purification on Ni-NTA resin allowed the rapid purification of significant quantities of this protein. After purification, analysis by SDS/PAGE and Coomassie Brilliant Blue staining, the protein was estimated to be more than 90% pure (Figure 1A, lane 4). Its identity was confirmed by immunoblotting using the anti-66K antiserum (Figure 1B, lane 4). Since the putative degradation products were not retained on Ni-NTA resin, it is likely that the N-terminal hexa-histidine tag was absent from these species.

When the protein extracts from Bac17AE- and Bac8AF-infected cells were analysed on a 7% acrylamide gel, the prominent bands corresponding to 66K and His-66K, as well as the purified fraction of the latter, were resolved into two close bands of similar intensity (Figure 1C, lanes 2–4). Both bands were recognized equally by the anti-66K antiserum (Figure 1D, lanes 2–4). Because there are no other in-frame initiation codons or suppressible stop codons in the surrounding sequences that would allow the expression of a protein product different from the genuine 66K protein, we speculated that the protein species with a shifted electrophoretic mobility may rather correspond to a post-translationally modified version of the TYMV 66K protein.

The TYMV 66K amino acid sequence contains several potential N-glycosylation sites. We therefore investigated the effects of the fungal antibiotic tunicamycin, which inhibits an early step in the N-glycosylation of proteins, on the pattern of expression of His-66K in insect cells. We observed no change in the total amount of recombinant proteins produced, nor a shift in the migration of the proteins synthesized in the presence of tunicamycin relative to the control samples (results not shown). Consequently, the TYMV 66K protein does not appear to be N-glycosylated in insect cells.

Evidence for the phosphorylation of the TYMV 66K protein

The phosphorylation status of the 66K protein was examined by labelling the Bac8AF-infected cells with [^{32}P]orthophosphate for 18 h. Total protein extracts were analysed by SDS/PAGE (12.5% gel), and the labelled proteins were visualized by autoradiography. A radioactive protein of apparent molecular mass 69 kDa was detected in the Bac8AF-infected cell extracts (Figure 2A, lane 2), which was absent in cells infected with the control baculovirus (Figure 2A, lane 1). This labelled protein is likely to correspond to the TYMV His-66K protein because it could be purified from the pool of proteins present in the metabolically

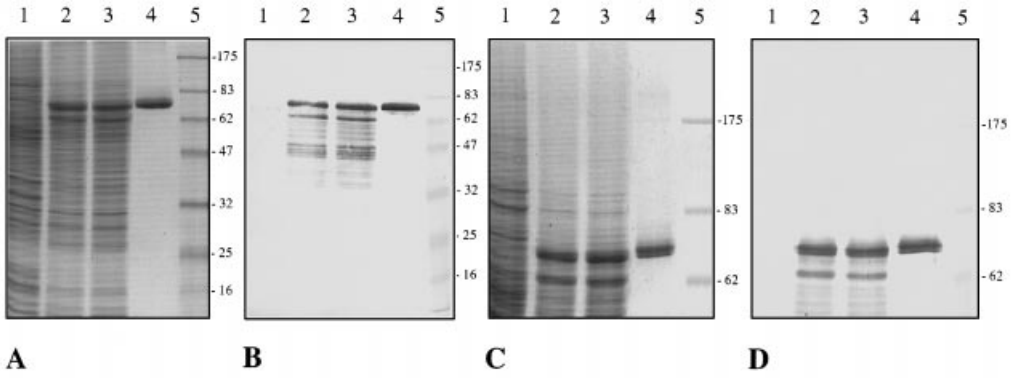

Figure 1 Expression and purification of TYMV 66K in baculovirus-infected insect cells

Total proteins from control baculovirus-infected cells (lanes 1), Bac17AE-infected cells (lanes 2), Bac8AF-infected cells (lanes 3), purified His-66K (lanes 4) and molecular-mass markers (lanes 5) were analysed by SDS/PAGE on 12.5% (**A** and **B**) or 7% (**C** and **D**) gels. The gels were stained with Coomassie Brilliant Blue (**A** and **C**) or electroblotted on to a nitrocellulose filter that was revealed by Western blotting using the anti-66K antiserum (**B** and **D**). The positions of molecular-mass markers are indicated.

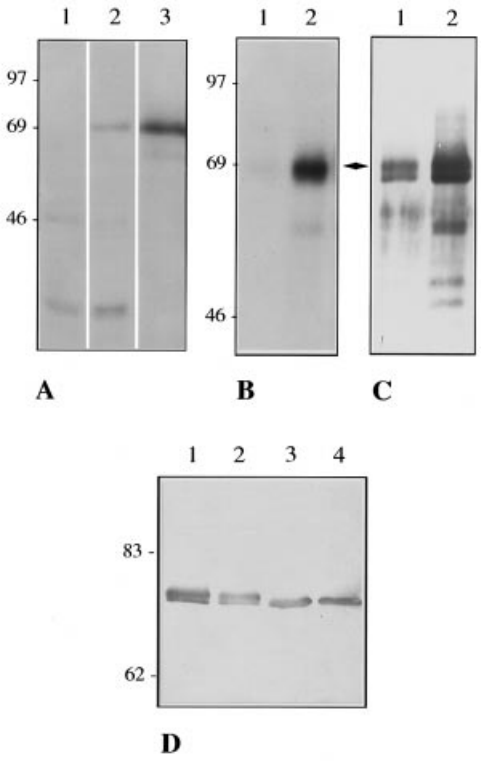

Figure 2 Phosphorylation of TYMV 66K protein expressed in baculovirus-infected insect cells

(**A**) Following metabolic labelling with [^{32}P]orthophosphate, total proteins from control baculovirus-infected cells (lane 1), Bac8AF-infected cells (lane 2) and purified His-66K (lane 3) were analysed by SDS/PAGE (12.5% gel) and revealed by autoradiography. (**B**) Following metabolic labelling with [^{32}P]orthophosphate, 50 and 150 ng of purified His-66K protein (lanes 1 and 2, respectively) were analysed by SDS/PAGE (7% gel), electroblotted on to a nitrocellulose filter and revealed by autoradiography. (**C**) The same blot as in (**B**) was revealed by Western blotting using the anti-66K antiserum. (**D**) Purified His-66K protein (50 ng) was treated with 400 and 800 units of protein phosphatase λ (lanes 3 and 4, respectively). Lanes 1 and 2 contain identical amounts of protein before or after incubation in the reaction buffer in the absence of phosphatase. The proteins were then subjected to SDS/PAGE followed by immunoblotting with the anti-66K antiserum. The positions of molecular-mass markers are indicated.

labelled cells by affinity chromatography on Ni-NTA resin under denaturing conditions (Figure 2A, lane 3).

To investigate whether both bands of the doublet were similarly labelled, different amounts of the purified labelled His-66K protein were separated on a 7% acrylamide gel, followed by transfer to a nitrocellulose membrane. The membrane was first subjected to autoradiography (Figure 2B), and was then probed with the antiserum raised against the 66K protein (Figure 2C). Careful alignment of the signal from the anti-66K antibody with the autoradiograph of the same blot revealed that most of the radioactivity was incorporated in the upper band of the doublet, which therefore corresponded to the phosphorylated form of the TYMV 66K protein. Accordingly, treatment of purified His-66K protein with protein phosphatase λ resulted in the disappearance of the shifted band (Figure 2D, lanes 3 and 4), whereas control incubation of the protein in the absence of phosphatase had no effect on its mobility (Figure 2D, lane 2).

Taken together, these experiments demonstrate that the TYMV RdRp domain is produced in insect cells as a phosphoprotein whose phosphorylation reduces its electrophoretic mobility in SDS/PAGE, resulting in an increased apparent molecular mass.

Phosphorylation occurs on serine and threonine residues

Determination of the amino acid residue subjected to phosphorylation was performed by TLC. The ^{32}P metabolically labelled protein His-66K was purified, totally hydrolysed and the resulting phospho amino acids were mixed with standard phospho amino acids and subjected to two-dimensional TLC according to [29]. Figure 3(A) shows that a unique spot that co-migrated with phosphoserine and phosphothreonine species was detected after autoradiography of the TLC plate.

Because these two species were resolved poorly, the identity of the amino acid subjected to phosphorylation was confirmed by performing immunoblot experiments using antibodies directed against each phospho amino acid. Figure 3(B) shows that the upper band was recognized by the antisera directed against phosphoserine and phosphothreonine residues, whereas the band with the faster mobility was recognized by the antiserum raised against phosphoserine. This demonstrates that both serine and threonine residues are the target residues for phosphorylation of

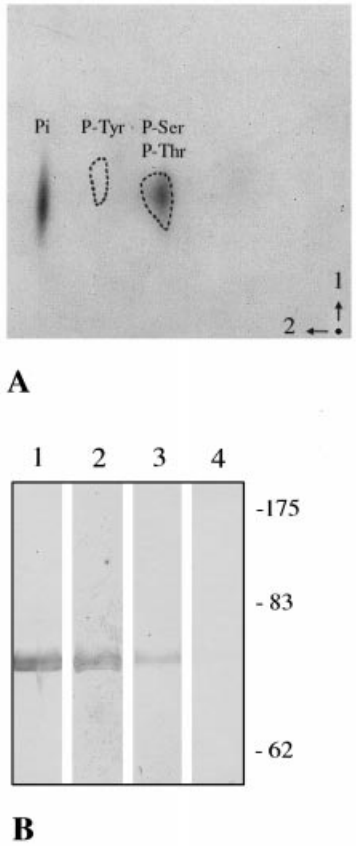

Figure 3 TYMV 66K protein contains both phosphoserine and phosphothreonine residues

(**A**) Purified *in vivo* ^{32}P-labelled His-66K was subjected to phospho amino acid analysis. After hydrolysis of the protein with 6 M HCl, the products were spotted together with standard phospho amino acids and subjected to two-dimensional TLC. The positions of standard phospho amino acid phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), as revealed by ninhydrin reactions, are indicated by dotted circles. First and second dimensions are indicated, as well as the location of free phosphate (Pi). (**B**) Purified His-66K protein was analysed by SDS/PAGE (7% gel) and was revealed by Western blotting using antibodies raised against phosphoserine (lane 2), phosphothreonine (lane 3) and phosphotyrosine (lane 4). Lane 1 was revealed using anti-66K antibodies. The positions of molecular-mass markers are indicated.

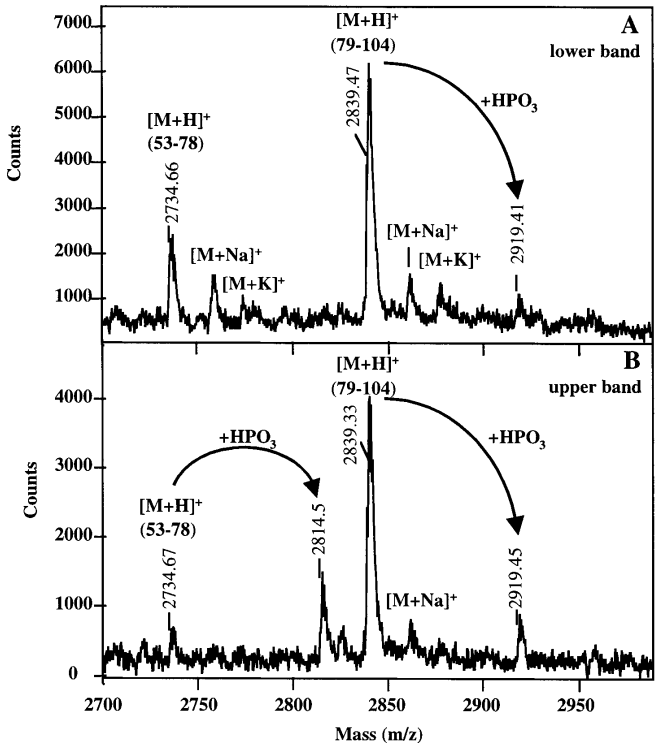

Figure 4 Differential peptide mass mapping of the TYMV 66K lower and upper band tryptic digests: identification of two phosphorylated peptides

Mass spectra obtained by MALDI–TOF MS in the mass range of 2700–3000 a.m.u. of the tryptic peptides generated from the His-66K lower (**A**) and upper (**B**) band digestions. The molecular ions were assigned to amino acid sequences of His-66K protein according to its sequence and the cleavage specificity of trypsin. The amino acid sequences of the peptides corresponding to the molecular ions observed are indicated, as well as their corresponding sodium and potassium adducts ($[M+Na]^+$ and $[M+K]^+$). Amino acid residues were numbered according to the sequence of the native protein. $+HPO_3$ indicates the addition of one phosphate group to each peptide (mass increment of 80 a.m.u.).

the TYMV 66K protein, and suggests that some serine phosphorylation events may not affect the electrophoretic mobility of the protein.

Mapping of the phosphorylated region

To gain some insights into the identification of the phosphorylated sites, we searched for phosphorylated tryptic peptides using MALDI–TOF MS. Purified His-66K was subjected to SDS/PAGE, and after reverse staining, the two bands corresponding to the differently phosphorylated forms were excised from the gel and digested with trypsin. The generated peptides were submitted directly to peptide mass mapping using MALDI–TOF MS (results not shown). The molecular ions were assigned to amino acid sequences of the protein based on the His-66K sequence and cleavage specificity of trypsin. The identified peptides covered 70–80% of the His-66K protein sequence.

Comparison of the mass spectra obtained for each band of the doublet revealed that two peptides clearly displayed a mass increment of 80 atomic mass units (a.m.u.) characteristic of the addition of a single phosphate group (Figure 4). The mass spectrum of the lower band tryptic digest (Figure 4A) revealed the presence of the non-phosphorylated forms of the peptides 53–78 and 79–104 (experimental $[M+H]^+$ values of 2734.66 and 2839.47 a.m.u. respectively), and their corresponding sodium and potassium adducts. In addition, a minor peak with an increase in mass of 80 corresponding to the mono-phosphorylated form of the peptide 79–104 was also observed (experimental $[M+H]^+$ of 2919.41 a.m.u.). In the mass spectrum of the upper band digest (Figure 4B), this mono-phosphorylated form of the peptide 79–104 was still present, with a slightly increased intensity. Moreover, we also observed the mono-phosphorylated form of peptide 53–78 (experimental $[M+H]^+$ of 2814.5 a.m.u.), which was more abundant than its non-phosphorylated form. Both peptides are contiguous in the protein sequence and each encompass four putative phosphorylation sites: Thr-64, Ser-69, Thr-70 and Ser-71 for peptide 53–78 and Ser-80, Ser-90, Ser-102 and Thr-103 for peptide 79–104.

Evidence for ubiquitinylation of the TYMV 66K protein

Several of these putatively phosphorylated residues are contained within the longest sequence of contiguous PEST residues in the protein, which extends from amino acids 56 to 78 (see Discussion).

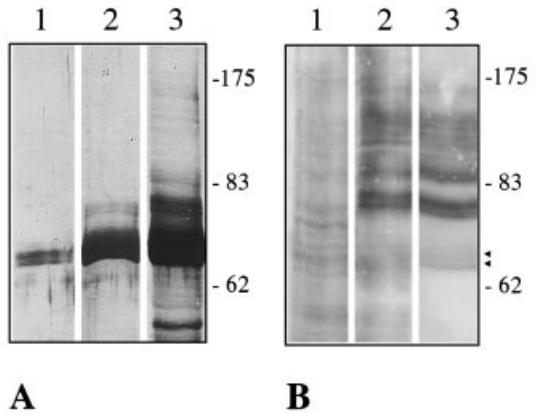

Figure 5 Evidence for the presence of TYMV ubiquitin–66K conjugates

(**A**) Increasing amounts of purified His-66K protein (30, 150 and 500 ng in lanes 1, 2 and 3 respectively) were analysed by SDS/PAGE (7% gel) and were revealed by Western blotting using the anti-66K antiserum. (**B**) Total proteins from control baculovirus-infected cells (lane 1), Bac8AF-infected cells (lane 2) and purified His-66K (lane 3) were analysed by SDS/PAGE (7% gel) and were revealed by Western blotting using the monoclonal antibody FK2 directed against ubiquitin conjugates. The position of the major His-66K doublet is indicated by arrowheads. The positions of molecular-mass markers are indicated.

PEST-rich sequences are potential degradation signals through the ubiquitin/proteasome degradation pathway, and it has been reported that phosphorylation of serine or threonine residues can activate latent PEST sequences. This prompted us to examine whether we could observe ubiquitinylated forms of the 66K protein.

On the conjugation of ubiquitin to a protein substrate, multiple bands are observed in SDS/PAGE, which consist of several molecules of ubiquitin linked to a single molecule of the protein. When increasing amounts of His-66K protein were analysed by Western blotting using the anti-66K antiserum (Figure 5A), a set of larger products of discrete sizes was revealed, which appeared as a ladder of double bands. The size interval between each step of this ladder ($\approx$ 8 kDa) closely matches the molecular mass of ubiquitin, a result consistent with the formation of poly-ubiquitin conjugates.

To ensure that these higher-molecular-mass species were actually covalent complexes between the TYMV 66K protein and ubiquitin, we carried out a Western-blot analysis using a monoclonal antibody (FK2) specific for ubiquitin conjugates [27]. When total proteins from control baculovirus-infected insect cells were analysed with this antibody (Figure 5B, lane 1), a number of proteins were revealed, reflecting the variety of proteins that are ubiquitinylated *in vivo*. However, when total proteins from 8AF-infected cells or purified His-66K protein were analysed similarly (Figure 5B, lanes 2 and 3), the same ladder of additional bands as observed in Figure 5(A) was specifically recognized by the FK2 monoclonal antibody. This recognition was specific because the excess amount of His-66K protein (indicated by arrowheads) gave only background signal. This demonstrates that the TYMV 66K protein is conjugated to ubiquitin in insect cells.

DISCUSSION

The high levels of expression that we obtained by using recombinant baculovirus–insect cell system accomplishes the first step in developing a system for the study *in vitro* of the TYMV RdRp. The observation that the purified protein appeared as a double band with both bands of equivalent intensity, combined with the extensive documentation relative to the ability of the baculovirus expression system to perform the appropriate post-translational modifications to recombinant proteins [32] prompted us to study the TYMV His-66K post-translational modifications. Additional experiments are in progress to characterize its biochemical properties further.

Our results demonstrate that the TYMV RdRp domain expressed in insect cells can be phosphorylated on both serine and threonine residues. This finding implies that phosphorylation may be involved in regulating the RNA polymerase activity of the TYMV 66K protein, as is the case with RNA polymerase II, whose processivity is regulated by such a post-translational modification of its C-terminal domain [33]. Interestingly, the RdRp domain of hepatitis C virus NS5B has also recently been shown to be phosphorylated [34], but its phospho amino acid residues have not yet been characterized. In addition to the RNA polymerase activity, the TYMV 66K protein is likely to participate in numerous other activities and to interact with other viral or cellular proteins in order to accomplish template specificity, chain initiation, regulation of plus- and minus-strand synthesis or membrane association of the replication complex. Conceivably, any of these additional activities or interactions could be affected by modifications of amino acid residues in the RdRp.

In order to determine what role phosphorylation of the 66K protein may have in TYMV replication functions, we were interested in defining more precisely the phosphorylated peptides. MALDI–TOF MS allowed the identification of two contiguous mono-phosphorylated tryptic peptides (amino acids 53–78 and 79–104). Each of these two peptides contains four serine or threonine residues that may constitute possible phosphorylation sites. Analysis of kinase consensus recognition sites [35] revealed that Thr-70 and Ser-102 may constitute target sites for casein kinase II, whereas protein kinase C may use Ser-102 as a substrate. More accurate characterization of phosphorylation sites requiring phosphopeptide purification and sequencing is the subject of further studies.

It should be noted that the MALDI MS analysis does not allow quantification of the amount of peptides present within the mixture, because the intensity of the signal is highly dependent on the nature of the polypeptide and can be significantly affected by a single amino acid change [36]. In particular, it has been reported that phosphopeptides often yield poor response in MALDI MS in positive-ion mode compared with non-phosphorylated peptides, most likely because of the negative charge of the phosphate group [37,38]. These differences in ion detection, which prevent possible quantification of the corresponding biomolecules, are further increased in complex peptide mixtures such as those obtained by tryptic digestion, due to either a 'suppression effect' or low ionization efficiency [37,39]. As a consequence, we cannot exclude the existence of other minor phosphorylated peptides that would have been undetected in the MALDI–TOF mass spectra of either band of the doublet, which may also contribute to the anti-phosphoserine and anti-phosphothreonine antibodies' reactivity.

Some regions of the RdRp domain are highly conserved among plus-strand RNA viruses [40], but the identified phosphorylated peptides are part of the N-terminal sequence, which is more divergent. Amino acid sequence comparison among tymoviral RdRps (Figure 6A) revealed that none of the eight potential phosphorylated residues of TYMV 66K are present at conserved positions, with the exception of residue 80, which consists of either a serine or a threonine in all tymoviral sequences. Despite this lack of sequence conservation among tymoviral

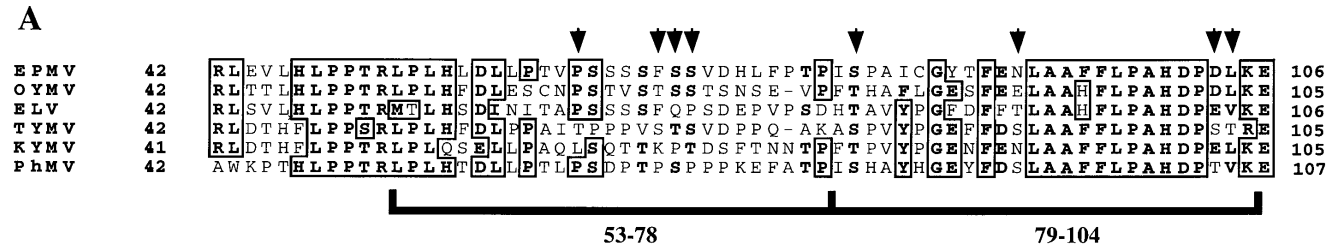

Figure 6 Sequence alignment of tymovirus RdRps

(**A**) The N-terminal region of tymovirus RdRp was aligned using the CLUSTAL W computer program. Conserved amino acids are in bold type and highly conserved ones are boxed. The identified phosphorylated peptide sequences are underlined and the potential phosphorylation sites in TYMV 66K protein are indicated by arrows. (**B**) The PEST sequences identified using the PEST-FIND computer program are shown in bold type. Boxed residues indicate PEST regions displaying high PEST scores [+2.8 for TYMV, +6.7 for ononis yellow mosaic virus (OYMV), +10.3 for erysimum latent virus (ELV) and +14.1 for physalis mottle virus (PhMV)], whereas non-boxed residues indicate PEST regions displaying poor PEST scores [−10 for kennedya yellow mottle virus (KYMV) and −2 for eggplant mosaic virus (EPMV)]. Virus sequences (and accession numbers) used in the alignment were: TYMV (P10358), kennedya yellow mottle virus (P36304), eggplant mosaic virus (P20126), ononis yellow mosaic virus (P20127), erysimum latent virus (P35928) and physalis mottle virus (CAA76071). The numbering refers to amino acid positions in the RdRp protein.

RdRps, we observed that the amino acid composition within the region comprising amino acids 56–84 was rather similar, and displayed a particular enrichment for proline, serine, threonine and acidic residues. This finding led us to the hypothesis that this portion of the protein may consist of a so-called PEST region [41]. Using the PEST-FIND computer program, amino acids 56–78 within the TYMV 66K protein were indeed identified as being a putative PEST sequence (Figure 6B). Interestingly, PEST sequences were invariably identified in this particular region within the different tymoviral RdRp proteins (Figure 6B), although their PEST scores were highly variable (ranking from −10 for kennedya yellow mottle virus RdRp to +14 for physalis mottle virus RdRp).

PEST sequences were first identified as signals for protein instability [42] but it is likely that they function indirectly in degradation. Many PEST sequences are conditional signals, serving principally as sites for phosphorylation, which is necessary for further ubiquitinylation and degradation of some proteins [43–45]. Based on these observations, we propose that phosphate addition to particular Ser and Thr residues in the N-terminal region of TYMV 66K protein may serve for activating a latent PEST signal, thereby controlling the metabolic stability of the protein. In this respect, it should be noted that the TYMV 66K protein has been reported to be unstable when produced by translation *in vitro* in rabbit reticulocyte lysate, resulting in low and variable amounts of products [46]. A truncated protein of 32 kDa comprising the first 240 amino acids behaved similarly [46], whereas an internal deletion of amino acids 72–209 that covers part of the proposed PEST sequence was found to stabilize the protein product [7]. The corresponding protein domains encoded by other tymoviruses were also postulated to be rapidly degraded *in vitro* [47]. These observations support the implication of the PEST region in proteolytic targeting and instability of the tymovirus RdRps.

A considerable body of evidence supports the idea that PEST sequences target proteins for degradation by the ubiquitin/proteasome system. Ubiquitin is a 76-residue protein whose covalent conjugation to lysine residues of other protein substrates is a common means by which eukaryotic cells signal their subsequent degradation by the 26 S proteasome, a multiprotease complex (reviewed in [48]). Using monoclonal antibodies specific for ubiquitin conjugates, we were able to demonstrate that the TYMV 66K protein is conjugated to ubiquitin molecules when expressed in insect cells. These data provide a first step towards proving the involvement of the ubiquitin/proteasome system in TYMV 66K protein processing. Because the ubiquitin conjugates appeared as clearly resolved doublets, they provide evidence that the two forms of 66K can be ubiquitinylated, regardless of the difference in their phosphorylation status. Attempts to identify the ubiquitinylation site(s) by an MS approach have been unsuccessful so far, and therefore directed mutagenesis of lysine residues thought to be implicated in ubiquitin conjugation is the subject of further experiments.

Ubiquitinylation of recombinant proteins produced in the baculovirus–insect cell system has been described previously in a few cases [49,50]. The ubiquitin-dependent proteolysis pathway was reported to be functional in baculovirus-infected insect cells up to $\approx$ 24 h post-infection [50], the lack of ATP in the later stages of infection rendering it non-functional, which may explain our capability of observing stable ubiquitin–66K conjugates. Because some of the components of the ubiquitin/proteasome pathway have been characterized in plants (reviewed in [51]), it is tempting to speculate on the formation of poly-ubiquitin–66K conjugates in virus-infected plant cells, whose degradation may relate to the extremely low levels of 66K protein present during infection. Further studies are required to elucidate whether this is indeed the case.

Interestingly, the involvement of the ubiquitin proteolytic pathway has already been suggested regarding another member of the 'alpha-like' viral super-group: the Sindbis virus RdRp nsP4 protein [52]. It is produced by an endoproteolytic cleavage of the viral precursor polyprotein and, as with other alphavirus RdRps, it bears an N-terminal Tyr residue [53]. It has been reported that, both *in vitro* and *in vivo*, Sindbis nsP4 is degraded

by the ubiquitin-dependent N-end rule pathway [54], a rule that relates the *in vivo* half-life of a protein to the identity of its N-terminal residue [55].

Indirect evidence therefore suggests that selective degradation of viral proteins by the ubiquitin-dependent proteolytic pathway may be a common feature of alpha-like viral RdRps. Whether this reflects a strategy by which the virus regulates the intracellular levels of mature cleavage products or a host defence mechanism aimed at the elimination of viral proteins will be the subject of further studies.

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