In vitro interactions between a potyvirus-encoded, genome-linked protein and RNA-dependent RNA polymerase

John Fellers,† Jinrong Wan, Yiling Hong,‡ Glenn B. Collins and Arthur G. Hunt

Department of Agronomy, University of Kentucky, Lexington, KY 40546-0091, USA

Recent studies have shown that potyvirus VPg/proteinases and RNA-dependent RNA polymerases are capable of protein–protein interactions in yeast cells. We have extended these studies in vitro. We found that tobacco vein mottling virus (TVMV) VPg is retained on glutathione–Sepharose matrices if co-incubated with a glutathione S-transferase (GST)–NIb fusion protein, but not with GST, which is suggestive of a direct physical interaction between these two proteins. However, a mutation in the VPg (Y1860S) that eliminates virus infectivity and the interaction in yeast cells had little effect on the in vitro interaction. We also found that the TVMV VPg and Nla proteins are capable of stimulating the polymerase activity of the NIb protein. Since this stimulatory activity is retained when the proteinase domain of the Nla is removed, we conclude that the VPg is the moiety responsible for the stimulation of polymerase activity. As with the interaction revealed by co-purification, the Y1860S mutation had little or no effect on the stimulation of polymerase activity. Moreover, the VPg was able to stimulate a mutant NIb with an altered 'GDD' motif. Our studies thus provide two lines of evidence indicative of in vitro interactions between the TVMV VPg and NIb proteins.

Introduction

Potyviruses are the largest family of plant viruses and belong to the Potyviridae super family. The potyvirus genome is a positive-sense, single-stranded RNA, typically of about 10,000 nucleotides. This RNA encodes one large polyprotein that is processed by three virus-encoded proteinases to yield between seven and eleven polypeptides (Riechmann et al., 1992). Several potyvirus-encoded proteins have been implicated in replication in genetic studies (Lain et al., 1990; Riechmann et al., 1992; Eagles et al., 1994; Klein et al., 1994; Restrepo-Hartwig & Carrington, 1994; Martin et al., 1995; Verchot & Carrington, 1995; Kasschau & Carrington, 1995; Mahajan et al., 1996). Among these are the so-called CI, 6K, Nla and NIb proteins. These proteins are analogous, in terms of gene order and amino acid sequence, to the poliovirus 2C, 3A, 3B, 3C and 3D proteins, respectively (Domier et al., 1987).

These parallels suggest similarities in function as well. Such a supposition has been borne out in the case of the genome-linked proteins (or VPg; Shahabuddin et al., 1988; Murphy et al., 1990, 1991), proteinases (Carrington & Dougherty, 1987; Dougherty & Carrington, 1988; Hellmann et al., 1988), RNA-dependent helicases (Lain et al., 1990) and RNA-dependent RNA polymerases (Hong & Hunt, 1996).

The possible functions of potyvirus-encoded proteins in replication imply interactions, either direct or indirect, between these proteins and the virus-encoded RNA-dependent RNA polymerase, or NIb protein. This hypothesis has been tested in the case of the Nla and NIb proteins of two potyviruses, tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV). Li et al. (1997) have reported an interaction, detected in yeast cells using a two-hybrid assay, between the proteinase domain of the TEV Nla and the TEV NIb protein. Hong et al. (1995) described interactions, again using a two-hybrid assay, between the TVMV Nla and NIb proteins. Interestingly, this interaction was diminished by mutations in the VPg domain of Nla, suggesting a possible difference between the studies carried out with these two viruses.

In this report, we describe in vitro studies intended to explore in more detail the interaction between the TVMV Nla and NIb proteins. Using proteins purified from E. coli engineered to express the appropriate polypeptide, we have
found that the VPg domain of the TVMV Nla protein is capable of a physical interaction with the Nlb protein. Moreover, the Nla and VPg polypeptides are capable of stimulating the inherent RNA polymerase activity of the Nlb protein. This latter property is similar to that of the poliovirus 3AB protein, which contains the VPg of this virus and is a stimulatory co-factor for RNA synthesis catalysed by 3Dpol (Lama et al., 1994, 1995; Molla et al., 1994; Plotch & Palant, 1995). Our studies suggest that a plant virus VPg can play an active role in viral RNA replication, which is in addition to its role as the genome-linked protein.

Methods

- **Recombinant DNA manipulations.** The TVMV Nla, VPg and mutant VPg coding regions were isolated from plasmid subclones by PCR using oligonucleotides (Table 1) that incorporated 5’ ScaI and 3’ HindIII restriction sites for subsequent cloning. The conditions for PCR were as described previously (Hong et al., 1995). PCR products were cloned into the expression vector pQE30 (Qiagen; Fig. 1). Since the exact location of VPg cleavage from the C-terminal protease is not known, a site was chosen based on amino acid sequence homology with other TVMV Nla proteinase processing sites; this site corresponds to amino acid 1979 of the TVMV polyprotein (Domier et al., 1986). The Y1860S mutant VPg coding region was amplified from a plasmid containing the mutant gene (Hong et al., 1995; Murphy et al., 1996).

- **Expression and purification of His-tagged proteins.** Plasmids were introduced into E. coli strain M15 ( lac−, recA1, strain M15 (Nalr, Strr, lac−, ara−, gal−, mtl−, F′ [ recA1, uvrD] pK18M SucI)] which contains the pREP repressor plasmid (Qiagen). Overnight cultures were used to inoculate 200 ml cultures of bacterial cells at a ratio of 1:20. These cultures were incubated at 37 °C until the cell density reached A660 0.7–0.9. IPTG was then added to a final concentration of 1 mM. After 4 h induction at 37 °C, cells were pelleted and resuspended in sonication buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl). Cells were lysed by the addition of lysozyme (1 mg/ml final concentration) to suspended bacterial cells, incubated on ice for 30 min and sonicated. The cell debris was removed by centrifugation at 0, 300 mM NaCl). Cells were lysed by the addition of lysozyme (1 mg/ml final concentration) to suspended bacterial cells, incubated on ice for 30 min and sonicated. The cell debris was removed by centrifugation at 18,000 g. The sonicates were passed over a 1 ml column of Ni2+-agarose (Qiagen) that had been equilibrated with sonication buffer, and washed with 5 volumes of sonication buffer. Ni2+-agarose was then washed with 5 volumes of wash buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol). Histidine-tagged proteins were eluted from the column using a 20 ml linear gradient (0–500 mM) of imidazole in the wash buffer. Fractions containing the protein peak were pooled and dialysed overnight in dialysis buffer (40 mM KCl, 25 mM HEPES–KOH, pH 7.9, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol, 6 mM β-mercaptoethanol). These protein samples were then diluted 1:2 in NEB buffer.

- **Immunoblot analysis of NIa and VPg preparations.** Two micrograms of each protein sample was analysed by SDS-PAGE and the gels examined by immunoblotting with anti-NIa. Lanes: 1, NIa; 2, control extract (made from an E. coli lysate that had been fractionated as described for the histidine-tagged proteins); 3, Y1860S mutant VPg; 4, wild-type VPg; and 5, as in lane 2. The gel on the left was a 10% acrylamide gel, and that on the right, a 15% gel. The positions of the three bands that correspond to the Nla and presumed proteolytic products thereof, as well as a polypeptide with a mobility similar to the VPg (VPg?), are shown to the right of lanes 3, 4 and 5.

Table 1. Oligonucleotides used for cloning of the Nla and VPg coding regions

<table>
<thead>
<tr>
<th>Oligonucleotide sequence (5’ → 3’ orientation)</th>
<th>Corresponding codon in Nla (TMV)*</th>
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<tr>
<td>Nla 5’</td>
<td>TCGGAGCTCGCGCAAGAAGTAGACCGCCA</td>
</tr>
<tr>
<td>Nla 3’</td>
<td>CAGAGGCTTGTGACTGGGACCAAATC</td>
</tr>
<tr>
<td>VPg 3’</td>
<td>CAGAAGCTTTTTGATGGCGACCAATC</td>
</tr>
</tbody>
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* The corresponding codon in Nla is that present immediately preceding the ScaI or HindIII site in the oligonucleotide. The TVMV codon, given in parentheses and taken from Domier et al. (1986), is that present immediately preceding the ScaI or HindIII site in the oligonucleotide.
(dialysis buffer containing 10% glycerol) and loaded onto a 3 ml DEAE-Sepharose column. The DEAE column was washed with 5 column volumes of NEB. Histidine-tagged proteins were eluted using a 16 ml linear gradient (0–1 M) of KCNI in NEB. Fractions containing protein were dialysed overnight in dialysis buffer and evaluated for purity by SDS–PAGE. The identity of authentic Na and VPg was confirmed by determination of the molecular masses of the purified proteins and by immunoblotting using antibodies raised against GST–Na fusion proteins. The concentration of Na or VPg in each preparation was estimated after SDS–PAGE and staining with Coomassie brilliant blue by comparison with known quantities of BSA.

For comparison, extracts from untransformed E. coli cells were prepared using a similar protocol. DEAE fractions that eluted at the same concentrations of KCl as the recombinant Na and VPg were collected and their protein contents assessed; the polypeptide profiles of such extracts were similar to those of the different Na or VPg preparations, with the exception of the recombinant protein.

- **In vitro interactions.** To assay interactions between the VPg and Nb protein, an affinity-based co-purification technique was used. For this, glutathione–Sepharose containing either GST or the GST–Nb fusion protein was prepared as described by Hong & Hunt (1996). However, instead of eluting the GST or GST–Nb with glutathione-containing buffer, 50 µl matrix suspension (containing GST or GST–Nb; the matrix was about 50% of the volume) was added to 15 µg of the appropriate VPg preparation and brought to a final volume of 150 µl in binding buffer (50 mM HEPES, pH 7.3, 10% glycerol, 0.05% Triton X-100 and 0.01% BSA). This suspension was incubated, with gentle agitation, at room temperature for 30 min. The suspension was then collected by centrifugation (500 g for 5 min) and washed as described by Ohno et al. (1995). The matrices were then suspended in 50 µl SDS–PAGE sample buffer; those proteins still associated with the matrices were eluted by boiling and the supernatants were analysed by immunoblotting using anti-Na antiserum (Fellers, 1996).

- **Poly(U) polymerase assays.** RNA polymerase activity was determined as described by Hong & Hunt (1996), using poly(A) as a template and oligo(dT)$_{20}$ as a primer. Unless otherwise stated, reaction mixtures contained 200 ng purified GST–Na or the GST–G2575A mutant protein [prepared as described by Hong & Hunt (1996)] and 0–1.5 µg of the preparations of Na, VPg or the Y1860S mutant VPg. Reactions were carried out for 90 min at 30 °C, which is well within the period during which incorporation is linear.

### Results

#### Production of histidine-tagged Na and VPg

Previous studies have documented interactions between the TVMV-encoded Na and Nb proteins in yeast cells (Hong et al., 1995). The Na protein consists of an N-terminal domain that includes the VPg and a C-terminal domain that contains a site-specific proteinase responsible for processing of the virus-encoded polyprotein [see Fig. 1(a) for an illustration of the domains present in the TVMV Na protein]. Mutations in the VPg domain diminished the Na–Nb interactions, implicating the VPg as an important domain. To explore this further, we elected to study interactions of VPg and Nb in vitro. For these experiments, it was necessary to identify a suitable source of TVMV-encoded Na and/or VPg.

In contrast to the case with some potyviruses, TVMV-infected plants do not accumulate nuclear inclusions and are thus relatively poor sources of purified Na and Nb. Thus, to examine possible roles of the TVMV Na and VPg in replication, it proved necessary to purify these proteins after expression in E. coli. For this purpose, we chose to express the TVMV Na and VPg proteins with N-terminal extensions of six histidine residues (Fig. 1). Poly(U) polymerase assays were used to determine the activities of the recombinant Na and VPg, and the enzymes were then purified by affinity chromatography on Ni$_2^+$–agarose and chromatography on DEAE–Sepharose. At this point, polypeptides with mobilities similar to those predicted for the Na or VPg proteins were among the most prominent polypeptides, consisting of as much as 5% of the total protein in the preparation (not shown). The identity of these as Na or VPg was confirmed by immunoblot analysis (Fig. 1). The Na preparations contained a series of polypeptides of greater than 43 kDa that correspond to the full-length Na protein and to presumed proteolytic products thereof (Fig. 1, lane 1). In addition, a number of smaller polypeptides, specific for cells containing the Na expression vector, were also recognized by the anti-Na antiserum, including one that was similar in size to the recombinant VPg (Fig. 1, lane 1). This observation implies the presence of ‘authentically’ processed VPg in our preparations, but we have not confirmed this.

The VPg preparations, in contrast, contained a set of two polypeptides with very similar mobilities [apparent as a single band in lane 1 of Fig. 1(a) and a doublet in Fig. 2]. These are consistent with that of the expected polypeptide (predicted molecular mass of about 20 kDa). The smaller of these two polypeptides probably represents a proteolytic product of the expected histidine-tagged VPg; if true, this proteolysis would be localized to the C-terminal part of the protein, since the smaller of these is retained on the Ni$_2^+$ affinity matrix. We have not examined this in any more detail.
In a similar manner, we prepared a mutant VPg in which the tyrosine to which the genomic RNA is attached has been changed to a serine (Y1860S; Murphy et al., 1996). This mutation is lethal in the context of a full-length viral genome (Murphy et al., 1996) and also diminishes the interaction between NIa and NIb that is observed in yeast cells (Hong et al., 1995). The quality of this protein preparation was similar to that of the wild-type NIa and VPg preparations (Fig. 1, lane 2), but this band was distinguishable from those bands that were unique to extracts prepared from cells that expressed the VPg-containing extracts, contained a similar spectrum of VPg-related polypeptides was preferentially retained in this study. However, significant retention of the larger of the pair was also observed. A similar result was obtained with the Y1860S mutant (Fig. 2, lane 4). These results demonstrate an in vitro association of the VPg with GST–NIb. However, they differ from studies done in yeast (Hong et al., 1995) in that the in vitro interactions were not affected by a mutation (Y1860S) that diminished the previously noted in vivo interaction.

Similar experiments were attempted with histidine-tagged, affinity-purified NIa. However, this protein associated non-specifically to the glutathione–Sepharose matrix (not shown), thus precluding studies similar to those shown in Fig. 2.

**Stimulation of NIb-dependent RNA polymerase activity by the NIa and VPg proteins**

We next characterized the effects of the NIa and VPg preparations on the RNA polymerase activity of purified NIb. For this, the activity of a GST–NIb fusion protein using poly(A) as a template and oligo(dT) as a primer was measured. As shown in Fig. 3, the addition of the NIa preparation increased the activity observed with a constant amount of GST–NIb protein. The extent of stimulation observed (about 5-fold) was consistently seen with different NIa preparations. Comparable chromatographic fractions prepared from control E. coli cells did not enhance NIb activity (Fig. 3); in fact, a significant inhibition of activity was consistently observed when higher amounts of control extracts were added to polymerase reactions. This inhibition is a likely explanation for the decrease in activity that is sometimes observed at higher NIa concentrations. Other purified proteins, such as GST or BSA, had no stimulatory effect on the activity of GST–NIb (data not shown). In addition, the quantities of NIa tested here possessed a stimulatory effect on the activity of GST–NIb (data not shown).

**In vitro associations of VPg and NIb**

Having arrived at a source of NIa and VPg, we next set out to measure direct interactions between these proteins and purified NIb in vitro. For this, purified GST–NIb (Hong & Hunt, 1996) was adsorbed to glutathione–Sepharose and the resulting matrix was added to the histidine-tagged VPg preparations described above and in Fig. 1. The matrix was then recovered by centrifugation and the associated proteins were analysed by immunoblotting, using antibodies raised against the TVMV NIa protein.

The results of this experiment (Fig. 2) indicate a retention of the VPg in the glutathione matrix that was dependent upon the presence of the NIb protein; retention of the VPg was not observed in the absence of GST–NIb (Fig. 2, lane 2), or when GST–NIb was replaced with GST (Fig. 2, lane 3). Interestingly, the smaller of the pair of VPg-related polypeptides was preferentially retained in this study. However, significant retention of the larger of the pair was also observed. A similar result was obtained with the Y1860S mutant (Fig. 2, lane 4). These results demonstrate an in vitro association of the VPg with GST–NIb. However, they differ from studies done in yeast (Hong et al., 1995) in that the in vitro interactions were not affected by a mutation (Y1860S) that diminished the previously noted in vivo interaction.

Similar experiments were attempted with histidine-tagged, affinity-purified NIa. However, this protein associated non-specifically to the glutathione–Sepharose matrix (not shown), thus precluding studies similar to those shown in Fig. 2.
domain of NIa can act to stimulate the polymerase activity of NIb in vitro.

Effects of specific mutations on the stimulation of polymerase activity by VPg

To obtain information regarding the possible roles of specific domains or functional groups in the stimulation of polymerase by VPg, a mutant VPg and NIb were incorporated into this study. As shown in Fig. 3, preparations containing the Y1860S VPg mutant (Fig. 1) stimulated the activity of the GST–NIb protein much as the wild-type VPg and NIa proteins did. We thus conclude from this result that the stimulation of NIb by VPg is not dependent upon the integrity of the site (Y1860) to which genomic RNA is attached. This property distinguishes the stimulation seen here from the previous NIa–NIb interaction that was observed in yeast cells (Hong et al., 1995).

The TVMV NIb contains a GDD amino acid motif that is common to all RNA-dependent RNA polymerases (Poch et al., 1989). Changing the glycine in this motif (G2575) to alanine reduces the polymerase activity of NIb by 85% (Hong & Hunt, 1996). This mutation, however, has little effect on the interaction of NIa and NIb in yeast cells (Hong et al., 1995). To further study the role of this motif in the functioning of NIb, we compared the ability of NIa to stimulate the mutant NIb with the stimulation of wild-type NIb. Overall activity of the mutant NIb was about 20% of that of the wild-type NIb (Fig. 4a), regardless of the presence or absence of NIa. However, NIa consistently stimulated both the wild-type and mutant NIb proteins to a similar extent (Fig. 4b). Thus, the G2575A mutation does not affect the interaction between VPg and NIb that is responsible for stimulation of polymerase activity.

Discussion

Our experiments show that purified TVMV NIa or VPg can interact, by two criteria, with the TVMV NIb protein in vitro. Firstly, the VPg co-purified with a GST–NIb fusion protein when the latter is isolated using an affinity matrix (Fig. 2). This co-purification required the presence of NIb, which is indicative of a specific interaction. Secondly, the NIa and VPg proteins were observed to stimulate NIb-associated RNA polymerase activity (Figs 3 and 4). Comparable quantities of E. coli proteins, which co-purify with the histidine-tagged NIa and VPg, lack the stimulatory effect seen with NIa- or VPg-containing preparations (Fig. 3). This rules out a coincidental stimulation by one or more E. coli proteins in the NIa and VPg preparations. Purified proteins such as GST and BSA have no stimulatory effect (data not shown), indicating that the stimulation seen with NIa and VPg is not an effect specific for the latter proteins.

There have been a number of recent studies that deal with interactions between potyvirus-encoded NIa and NIb proteins, from which a somewhat contradictory set of conclusions has been drawn. Hong et al. (1995) described interactions between the TVMV-encoded NIa and NIb proteins in yeast cells using a two-hybrid system-based assay. These interactions were diminished by mutations at or near the RNA attachment site in the VPg domain of the NIa protein, but were not affected by a mutation in the so-called GDD (G2575A) motif of the NIb. These authors concluded that the TVMV NIa and NIb proteins interacted through the VPg domain of the NIa. At first glance, this conclusion is corroborated by the present study: we find that the TVMV VPg interacts physically with the NIb in vitro (Fig. 2) and that this VPg (and NIa) stimulates the RNA polymerase activity of the NIb (Figs 3 and 4). Moreover, the GDD motif mutation (G2575A) does not affect the in vitro (Fig.
4) or in vivo (Hong et al., 1995) interactions. However, the in vitro interactions observed are not significantly affected by a mutation (Y1860S) that severely diminishes the Nla–Nlb interaction seen in yeast cells (Figs 2 and 3; Hong et al., 1995). It is possible that this latter mutation affects the isolated VPg differently to the intact Nla, thus leading to the different results obtained in vitro (this study) and in yeast cells (Hong et al., 1995). However, this disparity raises the possibility that different interactions are being measured by these different approaches, and thus that the TVMV Nla and Nlb proteins may be capable of different interactions. It is also possible that the two interactions described in this report are distinct as well, involving different domains and functions of the VPg and Nlb. More extensive mutagenesis studies, especially ones that isolate and characterize mutants impaired in the in vitro interactions, will be needed to address these different possibilities.

Li et al. (1997) have also documented interactions between TEV Nla and Nlb in yeast cells. However, in these studies, the interaction involved the proteinase domain of the Nla, with the isolated VPg domain being unable to elicit a positive interaction in yeast cells. Additionally, clustered mutations involving the GDD motif in the TEV Nlb diminished the Nla–Nlb interaction in this instance. These results seem contradictory to the two-hybrid results obtained with the TVMV Nla and Nlb proteins (Hong et al., 1995) as well as the in vitro studies reported here. However, the studies done with the TEV and TVMV systems need not be mutually exclusive. Li et al. (1997) did not detect a VPg–Nlb interaction in yeast cells using TEV-encoded proteins, but such a negative result does not rule out an interaction. Likewise, the studies of Hong et al. (1995) and the present work do not directly address the matter of interactions between the proteinase domain of the TVMV Nla and Nlb proteins. It is possible that potyvirus-encoded Nla and Nlb proteins are capable of multiple interactions; this raises interesting questions regarding the regulation of action of the viral polymerases and merits further experimental consideration. It is also possible that the TEV and TVMV Nla and Nlb proteins differ in significant ways with regard to interactions. Such a possibility may be reflected in the differences in accumulation patterns of these proteins that are seen in infected plants; the TEV Nla and Nlb proteins accumulate in distinctive nuclear inclusions while the corresponding TVMV proteins do not accumulate to appreciable levels in infected cells. Given the ramifications of the observed interactions, this matter also deserves considerable further study.

The stimulation of polymerase activity by the TVMV Nla and VPg is consistent with reports that potyvirus proteins that contain the poliovirus VPg (e.g. 3AB and 3B) can stimulate the activity of purified poliovirus 3Dpol (Lama et al., 1994, 1995; Molla et al., 1994; Plotch & Palant, 1995). With poliovirus, the 3AB protein, which includes the VPg, is much more effective in stimulating the polymerase activity of 3Dpol than is the VPg by itself (Lama et al., 1994, 1995; Molla et al., 1994; Plotch & Palant, 1995). In contrast, the TVMV VPg is itself an effective activator of Nlb (Fig. 3). We have failed to see similar stimulation by histidine-tagged 6K–VPg (J. F. Fellers & A. G. Hunt, unpublished observations), but this may reflect nothing more than an aggregation of the purified 6K–VPg. Thus, a direct comparison between the poliovirus and TVMV systems is not possible with the data in hand. However, the effectiveness of the TVMV VPg does stand in contrast to its poliovirus counterpart.

As mentioned above, several studies indicate that potyvirus-encoded Nla and Nlb proteins can interact. While some issues remain to be resolved, it is likely that one or more of these interactions reflect physiologically important functions. Perhaps, as suggested by Li et al. (1997), such physical interactions serve to bring the VPg domain of Nla into close proximity with the polymerase active site, thus promoting initiation or a related early event in RNA synthesis. Alternatively, as the results here and those for the poliovirus 3AB protein suggest, the VPg may enhance the polymerase activity of the Nlb protein, perhaps promoting early events in genome replication as a consequence. More likely, however, is a scenario whereby polymerase stimulation and physical proximity of the RNA attachment site to the polymerase active site are both important.

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References


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