Potyviral HC-Pro: a multifunctional protein

Ivan G. Maia, Anne-Lise Haenni and Françoise Bernardi

Institut Jacques Monod, 2 place Jussieu – Tour 43, 75251 Paris Cedex 05, France

Introduction

The genus Potyvirus, family Potyviridae, is the largest genus of plant viruses with 180 members or possible members (Brunt, 1992). Potyviruses are flexuous filamentous particles which contain a single-stranded RNA genome of positive polarity possessing a covalently linked 5’-terminal viral protein (VPg) and a 3’-terminal poly(A) tail (reviewed in Riechmann et al., 1992). They are transmitted from plant to plant by aphids in a non-persistent manner, and this process is dependent on the presence of two virus-encoded proteins (reviewed in Pirone, 1991). One of these, the helper component-proteinase (HC-Pro) has attracted renewed attention during the last few years due to its multifunctionality and to it being implicated in different steps of the potyvirus life cycle. The properties, as well as the established and postulated functions of this protein, are reviewed.

Historical overview

The first indications of a requirement for a specific protein in the potyvirus transmission process arose from studies on aphid transmissibility of co-infecting viruses. Potato potyvirus C (PVC) and potato aucuba mosaic potexvirus, two naturally aphid non-transmissible viruses, were shown to be transmitted by aphids from plants co-infected with an aphid-transmissible potyvirus such as potato virus A or Y (Kassanis, 1961). Restoration of transmissibility of these non-transmissible potyviruses was also observed when aphids first probed or fed on plants infected with an aphid-transmissible potyvirus, but not when aphids probed healthy plants or plants infected with the non-transmissible virus alone (Kassanis & Govier, 1971a, b). Surprisingly, aphids that had first acquired purified virus and then probed on extracts of infected plants to acquire the helper factor were unable to promote transmission (Govier & Kassanis, 1974b).

The first attempts to purify and characterize the helper component (HC) from infected plants were made by Govier et al. (1977). The results obtained constituted an important step in demonstrating the proteinaceous nature of HC. A 75 kDa polypeptide was specifically immunoprecipitated from cell-free translation products programmed by the genome of tobacco vein mottling virus (TVMV) using antibodies against a partially purified preparation of HC from TVMV-infected tobacco plants (Hellmann et al., 1983). This 75 kDa polypeptide was mapped to the N terminus of the TVMV polyprotein (Hellmann et al., 1986) and found to be processed in a wheat-germ translation system to two shorter products (of 30 kDa and 51 kDa for pepper mottle potyvirus; De Mejia et al., 1985) which are now known to correspond to the P1 and HC proteins, respectively (Fig. 1a). Immunoprecipitation assays also provided evidence that the amorphous inclusions detected in the cytoplasm of cells infected with certain potyviruses were antigenically related to HC (De Mejia et al., 1985). Moreover, using a TVMV HC antiserum in immuno-absorption chromatography assays, Thornbury & Pirone (1983) demonstrated that loss of HC activity was associated with the removal of a 53 kDa or 58 kDa polypeptide (as detected by SDS-PAGE), respectively from active fractions of TVMV or potato virus Y (PVY) HC preparations from infected plants. This approach successfully identified the monomeric form of HC as a 53 kDa to 58 kDa protein since, in a subsequent assay under non-denaturing conditions, biological activity was correlated with a protein with an apparent size of 100 to 150 kDa (Thornbury et al., 1985). This led to the widely accepted assumption that the biologically active form of HC is a homodimer.

Proteolytic activity

The first indication that a proteolytic activity was associated with HC was provided by Carrington et al. (1989a). Using deletion analyses and clustered point mutations of Cys residues, a proteolytically active domain could be mapped in the C-terminal half of the tobacco etch potyvirus (TEV) HC.
Autocatalytic cleavage by HC-Pro was further demonstrated in vivo using transgenic plants expressing a polyprotein corresponding to the TEV proteins P1, HC-Pro and part of P3 (Carrington et al., 1990), and was also shown to occur efficiently in insect cells bearing a construct containing the N-terminal three cistrons of the TVMV polyprotein (Thornbury et al., 1993).

Potyvirus genomes containing mutations that compromise HC-Pro proteolytic processing by HC-Pro are not infectious in protoplasts or plants (Klein et al., 1994; Kasschau & Carrington, 1995). The generation, in TEV mutants, of a heterologous cleavage site (recognized by the potyviral Nla proteinase) between the inactive HC-Pro and P3 (Fig. 1a) failed to restore infectivity, suggesting that a proteolytically active HC-Pro is required in cis to maintain virus viability (Kasschau & Carrington, 1995). The fact that this amplification-defective mutant could not be rescued in trans by functional HC-Pro expressed in transgenic plants further supported this assumption.

**Aphid transmission activity**

**Genetic studies**

Potyvirus isolates that are defective in aphid transmission due to alterations in HC-Pro have been described (Kassanis & Govier, 1971b; Thornbury et al., 1990; Lecoq et al., 1991). The first attempt to unravel the reason for this defect at the molecular level was undertaken with an HC-defective strain of PVY designated PVC. In this case, the non-transmissibility of PVC was not due to the absence of HC-Pro in the infected cells.
Nevertheless, this protein was unable to mediate aphid mutagenesis experiments. They observed that, although any demonstration that the Lys 51 → Glu substitution within the N transmission (Thornbury, Pro (Fig. 2) also impaired transmission activity and in some residue such as Arg at this position was permissive. Further-gene from PVY and PVC revealed that the degree of identity transmission-defective strains, the same Lys residue as de-

Since a co-migrating polypeptide antigenically related to PVY HC-Pro could be detected in extracts of PVC-infected plants. Nevertheless, this protein was unable to mediate aphid transmission (Thornbury et al., 1990).

Comparisons of the nucleotide sequence of the HC-Pro gene from PVY and PVC revealed that the degree of identity between these two strains was 92% resulting in 24 amino acid differences. Further comparisons of the deduced amino acid sequences with those of other known potyvirus HC-Pro sequences revealed that only two amino acid substitutions were specifically related to the non-functionality of PVY HC-Pro: a Lys50 → Glu and an Ile225 → Val (Thornbury et al., 1990). A definitive step in confirming these point mutations in HC-Pro activity was provided by site-directed mutagenesis studies using an infectious TVMV cDNA. These experiments demonstrated that the Lys51 → Glu substitution within the N terminus of TVMV HC-Pro (Figs 1b and 2), and not the Ile226 → Val substitution, was responsible for the defect in transmission activity (Atreya et al., 1992).

Atreya & Pirone (1993) confirmed the importance of the Lys → Glu substitution by performing several site-directed mutagenesis experiments. They observed that, although any replacement of the essential Lys residue was deleterious for aphid transmission of the TVMV HC-Pro, a highly basic residue such as Arg at this position was permissive. Furthermore, they showed that changing some of the conserved Cys residues and a His residue within the N terminus of HC-Pro (Fig. 2) also impaired transmission activity and in some cases rendered the virus non-viable. Further investigations of the nucleotide and deduced amino acid sequences coding for the HC-Pro of mechanically inoculated PVY-Fr, PVY-0 and zucchini yellow mosaic virus (ZYMV) demonstrated that, in transmission-defective strains, the same Lys residue as described above for PVC was substituted by Glu in PVY-Fr and ZYMV, and by Asn in PVY-0 (Canto et al., 1995; Grumet et al., 1992; Granier et al., 1993; Legavre et al., 1996). Recently an additional mutation, Gly → Glu in the Cys-rich region, was also shown to result in loss of aphid transmissibility (Canto et al., 1995).

A central, unresolved question is how mutation of the Lys or Gln residues affects the biological activity of HC-Pro. Atreya et al. (1992) first suggested that the Lys residue might participate in an ionic interaction required for the formation of biologically active HC-Pro dimers. It should also be noted that in the N terminus of all potyviral HC-Pro proteins the arrangement of the Cys residues and of the His residue is strongly conserved (Figs 1b and 2). It was postulated that this region probably forms a ‘zinc finger-like’ motif which would play structural and/or functional roles (Robaglia et al., 1989) essential for transmission. Nevertheless, these studies defined the N-terminal region of HC-Pro as the domain involved in mediating aphid transmission of potyviruses. The sequence data have provided sufficient information to assume that mutation of the Lys residue is a general feature of transmission-defective HC-Pro proteins.

In a recent report a Thr339 → Ala substitution within the invariant sequence, Pro-Thr-Lys (termed the PTK box; Figs 1b and 3b), located in the C-terminal half of the HC-Pro of a transmission-deficient strain of ZYMV was also observed (Granier et al., 1993; Huet et al., 1994). Here, the important Lys82 residue (in the N-terminal half of the protein) remained unchanged. The correlation of the Thr → Ala substitution with loss of aphid transmissibility was confirmed by constructing a recombinant genome in which a cDNA fragment containing the mutated PAK box was inserted into the genome of an aphid transmissible isolate of ZYMV in place of the corresponding PTK box (Huet et al., 1994). This led to almost total loss of HC-Pro activity in aphid transmission experiments.
Consequently, alterations in the PTK box are also critical for the biological vector activity of HC-Pro, either because of a direct effect on this domain or because of an indirect effect on protein conformation. It remains to be established whether complementation of the Thr → Ala (in the PTK box) and Lys → Glu HC-Pro mutations can occur when the mutant viruses are co-infected.

Mode of action

As mentioned above, potyviruses are transmitted by aphids in a non-persistent manner (i.e. retained for only short periods within the vector) by a mechanism that is totally dependent on the presence of HC-Pro. Aphids must acquire HC-Pro prior to, or simultaneously with, virus to promote successful transmission (Govier & Kassanis, 1974 b; Raccah & Pirone, 1984). In addition, the HC-Pro of a given potyvirus can functionally mediate transmission of other, but not all, members of the genus Potyvirus, suggesting some degree of specificity (Pirone, 1981; Sako & Ogata, 1981; Lecoq & Pitrat, 1985).

Govier & Kassanis (1974 b) first suggested that the HC-Pro component might be responsible for the binding of virus particles to specific sites in the aphid mouthparts in a manner that would enable subsequent release of the particles. HC-Pro was further predicted to be implicated in this selective binding mechanism on the basis of differential localization of 125I-labelled virions in the food tract of aphids that had acquired active or inactive HC-Pro, respectively (Berger & Pirone, 1986). The virions were retained principally in the distal one-third of the stylets and this was dependent on the presence of active HC-Pro (Wang et al., 1996). Evidence for the direct involvement of HC-Pro in virus attachment was provided by Ammar et al. (1994). Using transmission electron microscopy and immunogold labelling, HC-Pro was co-localized with virions and with the epicuticle of the maxillary food canal and foregut of aphids that had fed on a mixture of purified virus and HC-Pro. In contrast, no virus-like particles could be detected in these regions when aphids had acquired the virus alone. These results firmly support the model whereby HC-Pro serves as a link between the virus and the aphid stylet.

Although these ultrastructural experiments provide strong evidence for a binding activity as the mode of action of HC-Pro during the transmission process, little is known about the molecular mechanism involved. This mechanism implies that HC-Pro might, via its N-terminal domain and/or the PTK box, specifically recognize a binding site in the virus particle and another in the aphid stylet.

In the case of potyviruses, a conserved three amino acid motif, Asp-Ala-Gly (termed the DAG motif), located near the N terminus of the potyviral coat protein (CP) and exposed on the surface of virus particles, has been shown to play a key role in aphid transmission (Atreya et al., 1990, 1991; Gal-On et al., 1992). The exposed DAG motif present in the potyviral CP probably constitutes a putative site for HC-Pro recognition during aphid transmission. Such a possibility was suggested by Harrison & Robinson (1988) in their hypothetical transmission model. On the other hand, it cannot be excluded that HC-Pro acts indirectly by mediating an interaction between the CP DAG motif and the aphid mouthparts. Indeed, Salomon & Bernardi (1995) have recently shown that prefeeding aphids with the N terminus of the CP of maize dwarf mosaic potyvirus expressed in E. coli inhibited aphid transmission of the virus, suggesting direct interaction between the N terminus of the CP and the aphid stylets.

Although there is considerable support from mutagenesis studies for direct involvement of the DAG motif in determining potyvirus transmissibility (reviewed in Pirone, 1991), biochemical evidence for interaction between HC-Pro and virion is still lacking.

Involvement of HC-Pro in virus replication and symptom expression

The identification of amino acid residues in HC-Pro involved in aphid transmission has also provided insight into the possible involvement of this protein in potyvirus replication and symptom expression in infected plants. Thus, Atreya et al. (1992) observed that accumulation of TVMV RNA carrying the artificially introduced Lys substitution (see above) within the N terminus of HC-Pro was reduced, and that the symptoms were greatly attenuated, compared to wild-type. Moreover, virulence was rescued in viruses in which Glu had naturally reverted to the wild-type Lys, 5 to 6 weeks after inoculation. Subsequently, this point
mutation was associated with a reduced level of virus accumulation in a poorly aphid transmissible (PAT) strain of PVY obtained after repeated mechanical inoculations (Legavre et al., 1996). In an additional report, Klein et al. (1994) described a replication-defective mutant of TVMV containing a four amino acid insertion in the N-terminal region of HC-Pro. Replacement mutagenesis of three out of four Cys residues in this region (Fig. 2) was deleterious not only for aphid transmission as previously mentioned, but also for TVMV virulence and symptom expression (Atreya & Pirone, 1993). It has been proposed that these Cys residues are essential for virus viability since TVMV deletion mutants lacking the Cys-rich motif were non-infectious (Atreya & Pirone, 1993). Conversely, however, TEV genomes containing spontaneous N-terminal deletions in HC-Pro were viable even though a reduced level of viral RNA and protein was observed (Dolja et al., 1993). These data also argue for a role of HC-Pro in virus replication, although in the case of TEV the N terminus of HC-Pro was dispensable for virus viability.

There is evidence from a recent report that virus replication may also be affected by a mutation at another site in HC-Pro: replication of TEV was greatly diminished (to less than 1% of wild-type level) by replacement of a conserved tripeptide (Ile251-Gly-Asn by Arg-Pro-Ala) located in the core region of HC-Pro. In addition, no trans-stimulatory effect on the replication of this TEV HC-Pro-defective mutant could be detected in protoplasts from transgenic plants expressing functional HC-Pro (Cronin et al., 1995). It is clear that there are as yet insufficient data to propose a molecular mechanism for the impairment of HC-Pro in potyvirus replication. The fact that HC-Pro also possesses a sequence non-specific RNA binding activity (Maia & Bernardi, 1996) suggests that HC-Pro probably interacts with viral RNA during the replication process. However, additional work is required to support this hypothesis.

**Involvement of HC-Pro in potyvirus movement**

The first experimental evidence for a possible role of HC-Pro in potyvirus movement was provided by Klein et al. (1994), who showed that a four amino acid insertion in HC-Pro resulted in a movement-defective TVMV which, nevertheless, retained the ability to replicate in protoplasts. Cronin et al. (1995) have gained more insight into this process using three TEV-beta-glucuronidase (GUS) chimeric transcripts containing mutations in the coding region of HC-Pro. This led to the identification of a mutant deficient in systemic movement due to replacement of a conserved Cys-Cys-Cys box in the central region of HC-Pro (Figs 1 b and 3 a) by the triplet Arg-Pro-Ala. Whereas replication in protoplasts and cell-to-cell movement was retained, albeit at a reduced rate compared to wild-type, the ability of this mutant to spread systemically was abolished. Examination of the important steps of virus movement within the inoculated leaves by in situ localization of the GUS reporter suggested that the mutant was probably blocked at a late step in the movement pathway, such as trafficking within sieve elements or exit from the vascular tissues. Systemic invasion by this mutant was rescued in transgenic tobacco plants which expressed functional HC-Pro, although a delay of 2 to 3 days was observed. These results confirm a role for HC-Pro in systemic movement.

The CP has also recently been shown to be implicated in cell-to-cell and systemic movement of potyviruses (Dolja et al., 1994, 1995). Involvement of HC-Pro in potyvirus movement could occur by way of its binding to RNA (Maia & Bernardi, 1996) or its putative interaction with the CP.

**Conclusions**

Two domains of HC-Pro involved in aphid transmission have been delineated, one in the N-terminal Cys-rich region, and the other in the central region with the PTK motif. Several hypotheses concerning the role of these two domains have been presented. They may interact inter-molecularly to promote the dimerization (Atreya et al., 1992) reported to be required for biological activity, or intra-molecularly to achieve proper HC-Pro folding. A third possibility can be proposed if the dual binding of HC-Pro to the aphid stylet and to the virus during transmission is considered: each domain could be implicated directly in one of these bindings. This hypothesis could be readily tested by competition experiments between mutated and wild type HC-Pro. Inhibition of transmission would indicate that the mutated HC-Pro is still able to bind to the aphid stylet but has lost the capacity to interact with the virus.

A number of important functions are being assigned to HC-Pro, in addition to its involvement in aphid transmission and polyprotein processing, that provide evidence for its multifunctionality. In spite of significant progress recently made concerning the identification of the functional domains of HC-Pro, little is yet known about the molecular mechanisms underlying most HC-Pro-mediated events. A better understanding of these mechanisms would provide new insights into important aspects of the potyvirus life cycle such as transmission, replication and movement. An essential step in this direction would be the demonstration of an interaction between HC-Pro and the viral CP (or virions). However, to date, biochemical evidence for such interaction is lacking. The recently developed yeast two-hybrid system could provide a promising alternative method to examine the possibility of such an interaction. Finally, further elucidation of such events could lead to the development of novel strategies aimed at preventing virus propagation.

I.G.M. is grateful to CNPq – Brazil for a PhD fellowship. The Institut Jacques Monod is an 'Institut Mixte CNRS – Université Paris VII'.
References


Lecoq, H. & Pitrat, M. (1985). Specificity of the helper-component-
mediated aphid transmission of three potyviruses infecting muskmelon. Phytopathology 75, 890–893.


