HcPro, a multifunctional protein encoded by a plant RNA virus, targets the 20S proteasome and affects its enzymic activities

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Received 14 April 2005
Accepted 4 June 2005

The proteasome is a multicatalytic complex involved in many cellular processes in eukaryotes, such as protein and RNA turnover, cell division, signal transduction, transcription and translation. Intracellular pathogens are targets of its enzymic activities, and a number of animal viruses are known to interfere with these activities. The first evidence that a plant virus protein, the helper component-proteinase (HcPro) of Lettuce mosaic virus (LMV; genus Potyvirus), interferes with the 20S proteasome ribonuclease is reported here. LMV infection caused an aggregation of the 20S proteasome to high-molecular mass structures in vivo, and specific binding of HcPro to the proteasome was confirmed in vitro using two different approaches. HcPro inhibited the 20S endonuclease activity in vitro, while its proteolytic activities were unchanged or slightly stimulated. This ability of HcPro, a pathogenicity regulator of potyviruses, to interfere with some of the catalytic functions of the 20S proteasome suggests the existence of a novel type of defence and counter-defence interplay in the course of interaction between potyviruses and their hosts.

INTRODUCTION

The 20S proteasome is a protein complex present in all eukaryotes (Dahlmann et al., 1991). Its barrel-shaped structure consists of a stack of four rings made up of seven α-subunits for the two outer rings and seven β-subunits for the two inner rings (Unno et al., 2002) with at least four distinct peptidase activities. The 20S core associates with a 19S regulatory complex to form the 26S particle, which degrades ubiquitinated proteins in an ATP-dependent fashion (Ciechanover & Schwartz, 1998). Alternatively, an 11S regulator associates with the 20S core and modulates antigen presentation in animals (Rechsteiner et al., 2000).

The proteasome plays a central role in many cellular processes (Coux et al., 1996). It can also interfere in vitro with protein synthesis from viral RNAs and mRNAs from virus-infected cells (Homma et al., 1994), in relation to an intrinsic proteasome-associated endonuclease activity targeting viral RNAs and some cellular mRNAs containing tRNA-like structures (Gautier-Bert et al., 2003). The presence of residual fragments of the RNA substrate in purified proteasome as well as the reconstruction in vitro of complexes between proteasome and RNA substrates confirmed the 20S endonuclease activity (Ballut et al., 2003; Gautier-Bert et al., 2003). Recently, the 20S endonuclease activity was found to be targeted specifically against the RNA genome of two plant viruses, Tobacco mosaic virus (TMV) and Lettuce mosaic virus (LMV), suggesting that this 20S RNase could have an antiviral function in vivo (Ballut et al., 2003).

The 20S proteolytic activities are involved in the specific turnover of several viral proteins in mammals (Hu et al., 1999; Reinstein et al., 2000) and plants (Drugeot & Jupin, 2002; Karsies et al., 2002; Reichel & Beachy, 2000). In turn, some viruses have developed strategies to interfere with this proteolytic machinery through interaction with the 20S core or the 19S regulatory complex (Apcher et al., 2003; Seeger et al., 1997; Zhang et al., 2000). However, to date there is no description of a plant virus interfering with the
proteasome protease, and no description of a virus protein interfering with the 20S endonuclease activity, despite its probable role in constitutive antiviral defence.

The RNA genome of plant viruses in the genus Potyvirus (family Potyviridae) is translated into a polyprotein that is further processed by three virus-encoded proteinases (Carrington et al., 1990). One of these proteinases, HcPro, is a multifunctional protein (Maia et al., 1996): as a strictly cis-acting protease it is responsible for its self-cleavage from the polyprotein precursor (Carrington & Herndon, 1992) and, besides, it is also involved in a number of infectious processes as diverse as aphid transmission (Govier et al., 1977), cell-to-cell (Rojas et al., 1997) and long-distance movement (Saenz et al., 2002), suppression of gene silencing (Llave et al., 2000), synergism between co-infecting viruses (Pruss et al., 1997) and symptom development (Redondo et al., 2001). Some, but not all, of these biological functions might be related, for instance the inhibition of silencing-based host defence and the implication in virus synergism and as a virulence determinant. LMV HcPro is a 52 kDa protein with two structural domains (Plisson et al., 2003). The proteinase is C-terminal and in several potyviruses the N-terminal one-fifth of HcPro, included in the largest structural domain, is required for aphid transmission but dispensable for replication (Dolja et al., 2000). HcPro binds RNA in a sequence non-specific manner (Maia & Bernardi, 1996). In addition, it interacts with various virus-encoded proteins such as capsid protein (CP) (Roudet-Tavert et al., 2002), P1 (Merits et al., 1999) and VPg (Yamboo et al., 2003), as well as with itself (Urcuqui-Inchina et al., 1999), perhaps in relation with its ability to oligomerize in vivo (Thornbury et al., 1985) and in vitro (Plisson et al., 2003). HcPro also interacts with various host proteins (Guo et al., 2003), including a calmodulin-related protein involved in gene silencing (Anandalakshmi et al., 2000). Since LMV RNA was shown to be a target of the proteasome RNase activity in an earlier report (Ballut et al., 2003), it was hypothesized that this RNase activity could have an antiviral function in vivo. In this scenario, an inhibitory effect by viral proteins could be anticipated, and more specifically of proteins involved in virus pathogenicity, such as HcPro. Therefore, the effect of LMV infection on the proteasome was investigated and, more specifically, the possibility that HcPro interacts physically with the proteasome 20S core was evaluated, as well as the possible effects of such an interaction on the 20S enzymic activities, both proteasine and RNase.

**METHODS**

### 20S proteasome purification

Cauliflower was homogenized and post-ribosomal pellets were prepared as described previously (Kremp et al., 1986). Core proteasome (20S) was isolated by fast protein liquid chromatography (FPLC) from these pellets (Schleiphecke et al., 1991).

### Production of recombinant LMV HcPro

Three different forms of recombinant HcPro were produced from plants infected with recombinant LMV constructs. In *his*HcPro (Plisson et al., 2003), a polyhistidine tag (*His*<sub>6</sub>) was inserted between HcPro aa 3 and 4. In *his*HcPro, aa 4–102 were replaced with *His*<sub>6</sub>. In *strept*HcPro, a Strept-tag II (Voss & Skerra, 1997) followed by a thrombin cleavage site replaced the *His*<sub>6</sub> of *his*HcPro.

Pea or lettuce plantlets were inoculated as described previously (German-Retana et al., 2000). Leaves were harvested 2–3 weeks after inoculation and ground in 2 vols of ST buffer (100 mM Tris pH 8.0, 20 mM MgSO<sub>4</sub>, 500 mM EGTA) with 500 mM NaCl, 0.2% Na<sub>2</sub>SO<sub>4</sub> and 0.1% polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth and one layer of miracloth, and centrifuged (60 min at 10000 g). To concentrate the soluble fraction, differential precipitation with 20% followed by 40% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was carried out and the pellet was resuspended in ST with 0.5 or 1 M NaCl and 1 mM Pefabloc. Such partially purified HcPro was stored at −70 °C and centrifuged (5 min at 5000 g) after thawing, prior to further HcPro purification.

For *strept*HcPro, the supernatant was applied on a phenyl– Sepharose column (Sigma) at room temperature. The column was washed with 5 vols ST with 1 M NaCl. HcPro was eluted with 5 vols ST and concentrated by precipitation with 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The pellets were resuspended in ST with 100 mM NaCl, and applied onto a 1 ml streptactin column (IBA). After rinsing with 5 ml ST with 100 mM NaCl, *strept*HcPro was eluted with 5 ml ST with 100 mM NaCl and 10 mM desthiobiotin.

For *His*<sub>6</sub>-tagged HcPro, the supernatant was mixed with 1 ml Ni-NTA resin and 10% methanol was added. After 30 min incubation on ice, the resin was rinsed twice for 30 min in a batch procedure with 50 ml ST with 500 mM NaCl and 10% methanol, followed by two washes with 50 ml ST with 100 mM NaCl and the resin was applied on a column. The proteins eluted with 5 ml ST with 100 mM NaCl and 500 mM imidazole were precipitated with 60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and resuspended in ST with 100 mM NaCl.

### Protein separation by gel filtration

Infected leaves were homogenized in 20 mM Tris/HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 100 μM EDTA pH 7.4, 50 mM KCl, 6% sucrose, and filtered through four layers of cheesecloth. The homogenate was centrifuged for 10 min at 4000 r.p.m. (Sorvall SS34 rotor) and the supernatant was further centrifuged for 1 h at 15 000 r.p.m. (Sorvall SS34 rotor) and then again for 2 h at 42 000 r.p.m. (Beckman Ti45 rotor) on a 30% sucrose cushion.

The supernatant was loaded onto a gel filtration column (Suprose 6; Amersham). The proteins from 1 ml fractions were precipitated with 10% trichloroacetic acid and separated by 12-5% SDS-PAGE (Laemmli, 1970) for analysis. After electrophoresis, the protein fractions were analysed for the presence of 20S proteasome by Western blotting using polyclonal rabbit antibodies raised against 20S proteasome purified from cauliflower. When these antibodies were tested against proteasomes from calf liver, cauliflower or sunflower in preliminary Western blot experiments, a positive reaction was observed only with the plant proteasome. When these antibodies were tested in Western blot against a post-ribosomal supernatant from pea, lettuce or sunflower, the only positive signal observed was against subunits from the 20S proteasome.

### In vitro analysis of protein interactions on Ni-NTA agarose

Purified *his*HcPro (150 μg) was retained (30 min at room temperature with shaking) in HC buffer (100 mM Tris/HCl pH 8.0, 100 mM NaCl, 2 mM EDTA) on Ni-NTA agarose beads (200 μl...
previously equilibrated in HC). After washing excess protein with HC, the beads were equilibrated in TBK240 (20 mM Tris/HCl pH 7-4, 2.5 mM MgCl2, 3.5 mM DTT, 240 mM KCl) and incubated with shaking overnight at 4°C with 150 μg of 20S proteasome. After washing with TBK240 to eliminate excess 20S proteasome, hisHcPro and the proteins retained were eluted with 250 mM imidazole and analysed by SDS-PAGE.

**Biacore analysis.** Surface plasmon resonance (SPR) allows real-time analysis of molecular interactions (Pollard-Knight et al., 1990). SPR was performed at 25°C using a Biacore 3000 (Biacore AB). Purified 20S or hisHcPro were covalently immobilized on carboxymethylated dextran sensor chips (CM5) via their amino groups (EDC/NHS activation) according to the manufacturer’s instructions. A control surface was prepared with the same treatment (EDC/NHS activation) according to the manufacturer’s instructions. The surface densities of hisHcPro and 20S proteasome were 7041 RU and 6548 RU (a resonance unit, RU, is molar structure of the 20S core). The assays (30 min at 37°C) were carried out with 10 μM casein. The fluorescent products were visualized under UV after adding casein. The fluorescent products were visualized under UV after.

**In vitro transcription.** A full-length TMV cDNA cloned in pGEM-4Z (Promega) was obtained from Dr W.O. Dawson (Lake Alfred, Florida). Transcription with the T7 RNA polymerase (Ribomax Large-Scale RNA Production Systems-T7; Promega) yielded TMV RNA.

**Peptidase and proteinase assays.** The chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolase activities of 20S were measured by quantitating methylcoumarin amide (MCA) released from peptides substrates (Dahlmann et al., 1991). Fluorescence was measured with a spectrophuorimeter (JY3D; Jobin-Yvon Instruments) at 442 nm; excitation was at 352 nm. Briefly, 20S proteasome was incubated (15 min at 37°C) in the presence or absence of hisHcPro prior to addition of 200 μM substrate. A 1:2 (20S:hisHcPro) molar ratio was used, in agreement with the symmetrical structure of the 20S core. The assays (30 min at 37°C) were carried out with 200 μM substrate in 100 μl 250 mM Tris/HCl, 10 mM DTT (pH 8.5).

The 20S proteolytic activity was assayed by measuring the breakdown of FITC-labelled casein. The assays (30 min at 37°C) were carried out with 10 μM casein in 50 mM Tris/HCl pH 7-4. Purified 20S proteasome (15 μg) was incubated (15 min at 37°C) in the presence or absence of hisHcPro at the same ratio as above, prior to adding casein. The fluorescent products were visualized under UV after 12.5% SDS-PAGE. The degradation rate was deduced from the band intensities using ‘Molecular Analyst’ (Bio-Rad). In control experiments, the same assays were performed in the same conditions in the presence of hisHcPro without 20S proteasome.

**RNase assay.** This procedure was described in detail previously (Ballut et al., 2003). Briefly, TMV RNA (6 μg) was incubated (30 min at 37°C) in 200 μl TBK240 with 20 μg 20S proteasome previously incubated (15 min at 37°C) or not with purified hisHcPro at the same molar ratio as above. The products were analysed by gel filtration on a Superose 6 column equilibrated with TBK240. The RNA degradation rate was deduced from the difference in absorbance with the control.

**RESULTS**

**LMV infection induced an aggregation of the 20S proteasome in vivo**

To examine the potential interaction of the 20S core proteasome with some LMV proteins, subcellular fractionation from infected pea leaves was first performed. Post-ribosomal supernatants prepared from infected plants were analysed by gel filtration followed by Western blotting (Fig. 1). The proteasome 20S from non-infected plants eluted in fractions 13–14, corresponding to their expected molecular mass of about 650 kDa (Fig. 1b). Although some proteasome could be detected in the same fractions from LMV-infected plants, most of it eluted in fractions 7–9, corresponding to molecular mass ~2000 kDa. This shift suggested that the 20S proteasome from infected plants is associated in high-order complexes absent from healthy plants, and possibly containing other factors of host or viral origin.

**The proteasome associates with immobilized hisHcPro**

The proteasome is known to interact with different viral proteins in other systems. Therefore, the hypothesis that,
in infected plants, the proteasome could also interact with virus-encoded proteins was tested. Among the proteins encoded by LMV, HcPro plays a central role in the infection. To investigate the possibility that HcPro could be directly involved in aggregation of the 20S proteasome core, the possibility that HcPro and the proteasome could interact physically was first assayed.

Purified 20S proteasome was applied to hisHcPro immobilized onto Ni-NTA agarose beads. After washing with TBK240, the proteins were eluted with imidazole and analysed by SDS-PAGE. The 20S proteasome and hisHcPro co-eluted from the column (Fig. 2). The 20S proteasome was not retained in the absence of hisHcPro and no co-elution was detected for thyroglobulin, a protein approximately the same size as the 20S complex. These results suggest a specific interaction in vitro between hisHcPro and 20S proteasome.

**Evaluation of the binding constants**

Using the Biacore technology, an interaction was observed between immobilized hisHcPro and 20S proteasome (Fig. 3a), confirming the above observation. In a reverse assay, the binding of various concentrations of hisHcPro to immobilized proteasome was measured (Fig. 3b). The resulting sensograms were fitted using a global fitting model (Bia-evaluation 3.2) to determine the rate and equilibrium dissociation constants (Table 1). Two other HcPro constructs differing in their N-terminal structure were also injected onto immobilized 20S proteasome: strepHcPro differs by the nature of the N-terminal tag and hisΔHcPro has a large but viable N-terminal deletion. The equilibrium dissociation constants (K_D) obtained for hisHcPro and strepHcPro were in the same range (Table 1), although slightly higher for strepHcPro, in relation to a higher dissociation rate. This suggested that the nature of the N-terminal tag of HcPro does not greatly affect its interaction with the proteasome, and therefore confirmed that the interaction between the proteasome and hisHcPro was not with the His6 tag. The K_D for hisΔHcPro was 6·55 nM; this 2·2-fold increase compared with hisHcPro was in relation with a higher association rate.

**Fig. 2.** Interaction of 20S proteasome with hisHcPro immobilized on Ni-NTA agarose. The interaction was studied by Ni-NTA agarose affinity chromatography. Washed and eluted proteins were analysed by SDS-PAGE and visualized by Coomassie blue staining. In this particular experiment, 150 μg hisHcPro (arrow) was loaded on Ni-NTA agarose in ST with 100 mM NaCl. Excess HcPro was washed with ST (lanes 1–3). The column was then equilibrated in TBK240 (lanes 4–5) and proteasome (150 μg, upper panel, bracket) or bovine thyroglobulin (150 μg; Sigma-Aldrich; lower panel, bracket) was added and the samples were incubated overnight at 4°C. Excess protein was washed out with TBK240 (lanes 6–8). To elute bound proteins, the beads were finally treated with 250 mM imidazole (lanes 9–11). Purified HcPro (lane 12) and proteasome (lane 13) were electrophoresed separately as controls, and the position of protein molecular mass standards are shown.

**Fig. 3.** Biacore analysis of the proteasome–HcPro interaction. (a) Specificity of the association between the 20S proteasome and immobilized hisHcPro. Proteasome (90 μl, 72 nM) (i) or a control protein (glutathion S-transferase) (ii) were injected simultaneously onto an empty control surface or onto the hisHcPro-coupled measuring surface (flow rate: 50 μl min⁻¹). (b) Interaction between hisHcPro and 20S. Increasing concentrations of hisHcPro (18, 36 and 72 nM in HBS) were injected onto an empty control surface or onto immobilized proteasome at 50 μl min⁻¹. For all the sensograms, injection times were 180 s and dissociation times were 400 s. The control values were subtracted from the sensograms shown.
**Table 1.** Affinity constants of the interaction between the 20S proteasome core and three N-terminal tagged HcPro constructs, evaluated by fitting the curves shown in Fig. 3 with model curves \((K_a, \text{association rate constant}; K_d, \text{dissociation rate constant}; \text{and } K_D, \text{equilibrium dissociation constant}; K_D = K_d/K_a)\).

<table>
<thead>
<tr>
<th>Protein</th>
<th>(K_a \times 10^{-4} (M^{-1} \text{ s}^{-1}))</th>
<th>(K_d \times 10^{4} (s^{-1}))</th>
<th>(K_D (\text{nM}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HisHcPro</td>
<td>3.40 ± 0.10</td>
<td>5.03 ± 0.02</td>
<td>14.79 ± 0.04</td>
</tr>
<tr>
<td>StrepHcPro</td>
<td>4.66 ± 0.32</td>
<td>12.10 ± 0.01</td>
<td>25.96 ± 0.03</td>
</tr>
<tr>
<td>His∆HcPro</td>
<td>12.90 ± 0.03</td>
<td>8.45 ± 0.09</td>
<td>6.55 ± 0.01</td>
</tr>
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**HcPro effect on the 20S proteasome peptidase and proteolytic activities**

The effect of hisHcPro on the chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolase activities of the 20S proteasome was analysed using fluorogenic peptides. A slight but significant activation of the two former activities was observed in the presence of hisHcPro, but no effect on the latter was detected (Fig. 4a). To confirm these results using an entire protein as substrate, the effect of hisHcPro on the proteasome 20S-mediated degradation of FITC-labelled casein was analysed. A significant stimulation of proteolysis was observed in the presence of hisHcPro (Fig. 4b). When hisHcPro was incubated with the fluorogenic peptides or with FITC-labelled casein in control experiments, no proteolysis was observed (data not shown), indicating that the increase in proteolytic activity was not related to an endogenous activity of hisHcPro.

**HcPro inhibits the proteasome RNase activity**

After incubation with 20S proteasome, TMV RNA digestion products were analysed by gel filtration (Fig. 5). Approximately 80% of the substrate TMV RNA was degraded, as evaluated by the comparison of the RNA absorbance peaks before and after incubation with purified proteasome. However, when proteasome was previously incubated with hisHcPro, RNA degradation was largely abolished. In a control experiment, incubation of TMV RNA with hisHcPro alone, or with an unrelated protein (glutathione S-transferase, purified from recombinant *Escherichia coli*), did not result in any degradation (Fig. 5d and e). Together, these results showed that hisHcPro protected RNA against the 20S proteasome RNase activity.

**DISCUSSION**

The proteasome is a complex structure, carrying several enzymic activities (peptidase and endonuclease). Given their nucleoprotein nature, RNA viruses are possible elicitors and targets of both of these activities and indeed, there are several reports on the targeting of virus proteins or RNA by the proteasome. In particular, the 20S endonuclease activity was reported to specifically degrade the RNA genome of two plant viruses including a potyvirus, LMV. To understand better the effect of the proteasome on the potyvirus cycle and vice versa, we investigated more specifically its relationships with HcPro, a multifunctional potyvirus-encoded protein (Maia *et al*., 1996). Several of its known functions have been studied in detail by various groups: aphid transmission, self-proteolysis, suppression of RNA silencing, enhancement of virus replication and effect on symptom development (Anandalakshmi *et al*., 1998; Blanc *et al*., 1998; Brigneti *et al*., 1998; Carrington & Herndon, 1992; Kasschau & Carrington, 1998; Kasschau *et al*., 2003; Mallory *et al*., 2002; Pruss *et al*., 1997). In this report, we present the first evidence that, in addition, HcPro...
interacts with the 20S proteasome core and modulates its enzymic activities.

Affinity chromatography and SPR independently revealed that HcPro binds specifically to the proteasome 20S complex \textit{in vitro}. In addition, SPR indicated that the binding was strong and not significantly affected by the nature of the N-terminal tag sequence of HcPro. The deletion of the N-terminal region of HcPro increased the association rate without affecting as much the dissociation rate, suggesting that the interaction surface is located in the remaining part of HcPro, and is partly blocked or masked by the N-terminal portion of the protein. In HcPro, the N terminus (aa 1–100 deleted in ΔHcPro) appears structurally independent of the rest of the molecule, which is made up of two major structural domains: one spanning approximately from aa 100 to 230 and the other comprising the C-terminal ca. 150 aa (Plisson \textit{et al.}, 2003). The HcPro N terminus is strictly required for aphid transmission but dispensable for the other known functions of HcPro, including gene silencing inhibition and viral accumulation (Dolja \textit{et al.}, 1993; German-Retana \textit{et al.}, 2000; Plisson \textit{et al.}, 2003). It now also appears to be dispensable for interaction with the proteasome.

Human immunodeficiency virus-1 (HIV-1) Tat associates with seven \(\alpha\) and \(\beta\) 20S proteasome-subunits (Apcher \textit{et al.}, 2003). Similarly, binding of HcPro to several proteasome sites could explain the drastic proteasome mobility shift observed upon viral infection. This shift, from 650 to about 2000 kDa (a sedimentation range of about 30S), could possibly be related to an association of the proteasome with several copies of HcPro. Alternatively, other cellular or viral proteins, specifically expressed upon viral infection, could also bind to the proteasome or to the proteasome–HcPro complex. Finally, proteasome aggregation in the presence of HcPro, possibly in relation with the ability of the latter to oligomerize (Plisson \textit{et al.}, 2003; Thornbury \textit{et al.}, 1985), could also cause the observed shift through the formation of a high molecular mass network.

In several models, virus-encoded proteins interfere with the proteasome enzymic activities. Generally, this concerns the proteolytic activity of the 20S complex in relation to its 19S and 11S regulators. HIV-1 Tat inhibits both the binding of the 11S regulator to the 20S core and the 20S peptidase activities (Apcher \textit{et al.}, 2003; Seeger \textit{et al.}, 1997). In plants, a role of the ubiquitin degradation pathway and therefore of the proteasome has been demonstrated for the turnover of several RNA virus proteins (Drugeon & Jupin, 2002; Hazelwood & Zaitlin, 1990; Hericourt \textit{et al.}, 2000; Jockusch & Wiegang, 2003). In this context, the stimulation of the proteasome 20S proteolytic activity in the presence of HcPro suggests that potyviruses could modulate the proteasome-based protein degradation pathway for their own purposes. The hypothesis that the observed stimulation of the protease activity is related to the endogenous activity of HcPro is unlikely since the HcPro proteinase is inactive in trans (Carrington \textit{et al.}, 1989), and in addition no endogenous activity of \(\text{hisHcPro}\) was observed in control experiments in this study. A role of the stimulation of the 20S protease activity through the virus-encoded protein HcPro could be, for example, to increase the turnover of host proteins involved in defence and/or of some of the virus proteins necessary in the earlier stages of its cycle. This would directly favour virus accumulation, and could also interfere with the cell cycle. The interaction of Clink, a plant DNA virus protein, with SKP1, a component of the ubiquitin-based protein degradation pathway, was tentatively associated with the virus ability to alter the cell cycle (Aronson \textit{et al.}, 2000).

Another characteristic of the interaction between HcPro and the proteasome with no equivalent in other systems described so far is the inhibition of the 20S proteasome RNase activity in the presence of HcPro. The mode of action of this inhibition is still unclear, since the experimental data presented here do not discriminate between a direct inhibition by binding to an RNase subunit of the proteasome and protection of the RNA target through the RNA-binding.
ability of HcPro (Maia & Bernardi, 1996). However, the
demonstration of an interaction between HcPro and the
proteasome as well as the need for prior incubation of
HcPro with the proteasome for the RNase assays to detect an
inhibition argues for a direct effect of HcPro on the RNase
activity rather than through protection of the template.

Taken together, the association of the proteasome with an
RNase activity specifically aimed at virus genomic RNAs
(Ballut et al., 2003) and the ability of HcPro to target pro-
tease and interfere with this RNase activity, clearly open
perspectives regarding the functions of HcPro during viral
infection. Indeed, this suggests that the proteasome RNase
activity could be involved in a novel plant anti-viral defence
system that potyviruses have evolved to counteract. The
proteasome RNase activity interferes in mammals and
plants with the translation of viral RNAs with a 5′
structure and no 3′ polyadenylate, like TMV, by preventing
the formation of initiation complexes (Homma et al., 1994).
It also interferes with the translation of viral RNAs with
no 5′ cap but with a 3′ polyadenylate, like Cowpea mosaic
virus (Petit et al., 2000). Therefore, neither the 5′ cap struc-
ture nor the 3′ polyadenylate of plant viral RNAs are
required for mRNA recognition and translation inhibition.
Proteasome interference with the formation of initiation
complexes could be mediated by direct interactions between
proteins of these two complexes (Dunand-Sauthier et al.,
2002) or from template mRNA cleavage by the proteasome
endonuclease, resulting in RNAs unable to circularize and
therefore to promote assembly of a functional translation
initiation complex (Wells et al., 1998). Therefore, the inhi-
bition of the proteasome RNase by HcPro could be directly
linked to keeping high levels of viral RNA translation.

In addition, the proteasome endonuclease could also target
virus RNA outside the translation context. Inhibition of
the proteasome 20S RNase by HcPro is reminiscent of the
ability of HcPro to inhibit RNA silencing, a plant antiviral
defence (Voinnet et al., 2001). While the proteasome as a struc-
ture and RNA silencing as a mechanism are two conserved
features among eukaryotes, several lines of evidence suggest
that the proteasome RNase activity is probably not directly
related to RNA silencing. One of the most striking features of
RNA silencing is a sequence-specificity related to previous
exposure to a sequence identical to at least a portion of the
target (Tang et al., 2003), while the proteasome RNase
specificity depends on intrinsic properties of the target RNA
molecules, such as the presence of secondary structures,
rather than being acquired (Gautier-Bert et al., 2003). The
selective degradation of viral RNAs by the 20S complex
could represent an alternative pathway, parallel to RNA
silencing and reinforcing the cellular antiviral defence in
plants.

Such a scheme would be essentially similar to the dual
antimicrobial defence of vertebrates, where an innate and
an adaptive immunity pathway cross-talk to ensure both
an early broad-spectrum protection and a finer-tuned but

Similarly, plants could have two pathways of antiviral
defence, both based on RNA breakdown. The first com-
ponent of this dual strategy would be innate and target
non-host RNAs in a specific but broad-spectrum fashion,
mediated by the proteasome RNase activity. The second
component, adaptive and based on RNA silencing, would
result in a somewhat delayed but fine-tuned degradation
of the virus genome and include a systemic trigger. In this
scenario, HcPro would be a dual-function pathogenicity
effector inhibiting both pathways and therefore effectively
linking them. Further experiments are clearly needed to
investigate the potential contribution of the proteasome to
the plant antiviral defence in vivo, to understand the role of
the interference of HcPro with the proteasome enzymic
activities during the infection process, and to establish
whether this is a specificity of the potyvirus system or a
feature also found in other plant RNA viruses.

ACKNOWLEDGEMENTS

This work was supported by the Plant Health and Plant Genetics
departments of INRA, the French Ministry of Research and the
Regional Councils of Aquitaine and Auvergne. We thank Dr W. O.
Dawson for providing a TMV cDNA clone, M. Sibaud for technical
assistance and Dr S. German-Retana and the other researchers from
the IPV group in Bordeaux and from UMR 1095 in Clermont-Ferrand
for useful discussions.

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