Journal of General Virology (2005), **86**, 2595–2603 DOI 10.1099/vir.0.81107-0

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 (Drugeon & 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 proteinase 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.*, 1993; German-Retana *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 (Yambao *et al.*, 2003), as well as with itself (Urcuqui-Inchima *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 proteinase 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 (Schliephacke *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 poly histidine tag (His_6) was inserted between HcPro aa 3 and 4. In *his*ΔHcPro, aa 4–102 were replaced with His_6. In *strep*HcPro, a Strep-tag II (Voss & Skerra, 1997) followed by a thrombin cleavage site replaced the His_6 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 $MgSO_4$, 500 μM EGTA) with 500 mM NaCl, 0·2 % Na_2SO_3 and 0·1 % polyvinylpyrrolidone. The homogenate was filtered through four layers of cheesecloth and one layer of miracloth, and centrifuged (60 min at 100 000 ***g***). To concentrate the soluble fraction, differential precipitation with 20 % followed by 40 % $(NH_4)_2SO_4$ 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 *strep*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 % $(NH_4)_2SO_4$. 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, *strep*HcPro was eluted with 5 ml ST with 100 mM NaCl and 10 mM desthiobiotin.

For His_6-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 % $(NH_4)_2SO_4$ 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 $MgCl_2$, 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 (Superose 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 EGTA) 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 $MgCl_2$, 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, *his*HcPro 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 *his*HcPro 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 but without proteins. The surface densities of *his*HcPro and 20S proteasome were 7041 RU and 6548 RU (a resonance unit, RU, is equivalent to 1 pg protein immobilized per mm^2). The running buffer was HBS-EP (10 mM HEPES pH 7·4, 150 mM NaCl, 3 mM EDTA, 0·005 % P20) with a 50 μl min^{-1} flow. To study the physical interaction between proteasome and HcPro, 50 μg min^{-1} 20S proteasome was simultaneously injected on immobilized *his*HcPro and on a control surface and, conversely, various concentrations of *his*HcPro were simultaneously injected on immobilized 20S proteasome and on a control surface. The same protocol was performed for *his*ΔHcPro and *strep*HcPro. Between each run, the sensor surfaces were regenerated with a pulse of 5 μl 0·03 % SDS.

***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 spectrofluorimeter (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 *his*HcPro prior to addition of 200 μM substrate. A 1 : 2 (20S : *his*HcPro) 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 *his*HcPro 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 *his*HcPro 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 *his*HcPro 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 *his*HcPro

The proteasome is known to interact with different viral proteins in other systems. Therefore, the hypothesis that,

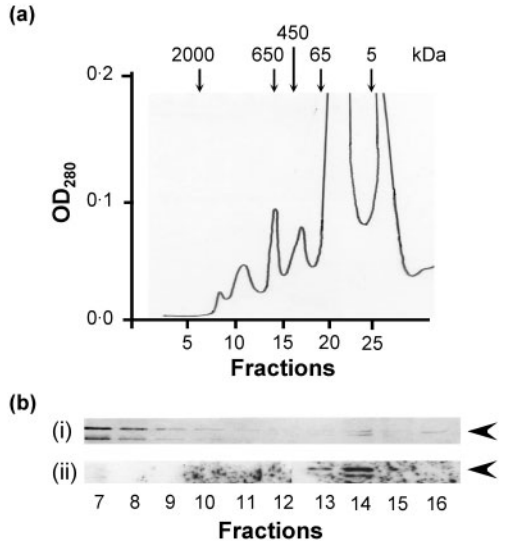

Fig. 1. LMV infection causes a shift in the subcellular localization of the 20S proteasome. Post-ribosomal supernatants of LMV-infected or healthy pea leaves were separated by gel filtration. Filtration was performed at room temperature in TBK240 and elution was at a flow rate of 0·2 ml min^{-1}. Fractions of 1 ml were collected and analysed by SDS-PAGE and Western blotting. (a) Separation of post-ribosomal particles isolated from infected plants. The positions of size markers are indicated above the OD_{280} profile. (b) Proteasome localization in successive subcellular fractions obtained after a gel filtration experiment as shown in (a): (i) in infected tissues and (ii) in healthy tissues. Arrowheads indicate the positions of the proteins revealed with the antibodies.

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 *his*HcPro 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 *his*HcPro co-eluted from the column (Fig. 2). The 20S proteasome was not retained in the absence of *his*HcPro 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 *his*HcPro and 20S proteasome.

Evaluation of the binding constants

Using the Biacore technology, an interaction was observed between immobilized *his*HcPro and 20S proteasome (Fig. 3a), confirming the above observation. In a reverse assay, the binding of various concentrations of *his*HcPro to immobilized proteasome was measured (Fig. 3b). The resulting sensorgrams 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: *strep*HcPro differs by the nature of the N-terminal tag and *his*ΔHcPro has a large but viable N-terminal deletion. The equilibrium dissociation constants (K_D) obtained for *his*HcPro and *strep*HcPro were in the same range (Table 1), although slightly higher for *strep*HcPro, 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 *his*HcPro was not with the His_6 tag. The K_D for *his*ΔHcPro was 6·55 nM; this 2·2-fold increase compared with *his*HcPro was in relation with a higher association rate.

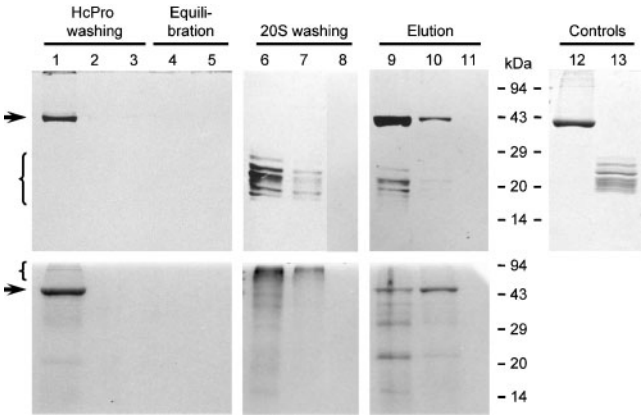

Fig. 2. Interaction of 20S proteasome with *his*HcPro immobilized on Ni-NTA agarose. The interaction was studied by Ni-NTA agarose affinity chromatography. Washed and eluted proteins were analysed by SDS-PAGE and visualized by Coomassie blue staining. In this particular experiment, 150 μg *his*HcPro (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.

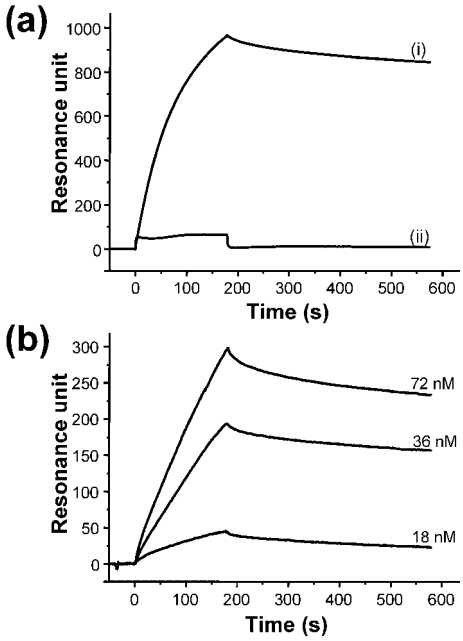

Fig. 3. Biacore analysis of the proteasome–HcPro interaction. (a) Specificity of the association between the 20S proteasome and immobilized *his*HcPro. Proteasome (90 μl, 72 nM) (i) or a control protein (glutathion S-transferase) (ii) were injected simultaneously onto an empty control surface or onto the *his*HcPro-coupled measuring surface (flow rate: 50 μl min^{-1}). (b) Interaction between *his*HcPro and 20S. Increasing concentrations of *his*HcPro (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 sensorgrams, injection times were 180 s and dissociation times were 400 s. The control values were subtracted from the sensorgrams 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, association rate constant; K_d, dissociation rate constant; and K_D, equilibrium dissociation constant; $K_D = K_d/K_a$).

Protein	$K_a \times 10^{-4}$ (M^{-1} s^{-1})	$K_d \times 10^4$ (s^{-1})	K_D (nM)
*His*HcPro	3·40 ± 0·10	5·03 ± 0·02	14·79 ± 0·04
*Strep*HcPro	4·66 ± 0·32	12·10 ± 0·01	25·96 ± 0·03
*His*ΔHcPro	12·90 ± 0·03	8·45 ± 0·09	6·55 ± 0·01

HcPro effect on the 20S proteasome peptidase and proteolytic activities

The effect of *his*HcPro 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 *his*HcPro, but no effect on the latter was detected (Fig. 4a). To confirm these results using an entire protein as substrate, the effect of *his*HcPro on the proteasome 20S-mediated degradation of FITC-labelled casein was analysed. A significant stimulation of proteolysis was observed in the presence of *his*HcPro (Fig. 4b). When *his*HcPro 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 *his*HcPro.

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 *his*HcPro, RNA degradation was largely abolished. In a control experiment, incubation of TMV RNA with *his*HcPro 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 *his*HcPro protected RNA against the 20S proteasome RNase activity.

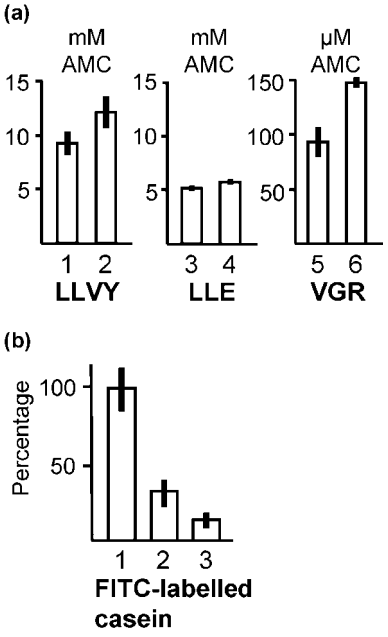

Fig. 4. Effect of *his*HcPro on the 20S proteasome peptidase and proteolytic activities. (a) Effect of *his*HcPro on the 20S peptidase activities. Three fluorogenic substrates were used (Dahlmann *et al.*, 1991): LLVY (succinyl-Leu-Leu-Val-Tyr-MCA; chymotrypsin-like activity); LLE (carbobenzoxy-Leu-Leu-Glu-MCA; peptidylglutamyl-peptides hydrolase activity) and VGR (benzoyl-Val-Gly-Arg-MCA; trypsin-like activity). To test the effect of HcPro on the peptidase activities, proteasome was pre-incubated with HcPro. After incubation with the fluorogenic substrate, the free fluorescence was measured in three independent experiments. The mean values observed and their standard deviations are shown. Lanes 1, 3 and 5: proteasome (10 μg) was incubated with 200 μM of the substrates. Lanes 2, 4 and 6: proteasome (10 μg) was pre-incubated with HcPro and before incubation with 200 μM of the substrates. (b) Effect of *his*HcPro on the proteasome 20S proteolytic activity. FITC-labelled casein (10 μM) was incubated with 15 μg 20S proteasome. After SDS-PAGE, the products were visualized under UV light (254 nm) and the intensity of the bands was evaluated. Three independent experiments were performed; their mean value and standard deviations are shown. Lane 1, incubation in buffer only; lane 2, incubation with 15 μg 20S proteasome; lane 3, incubation with 15 μg 20S proteasome previously incubated with HcPro.

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

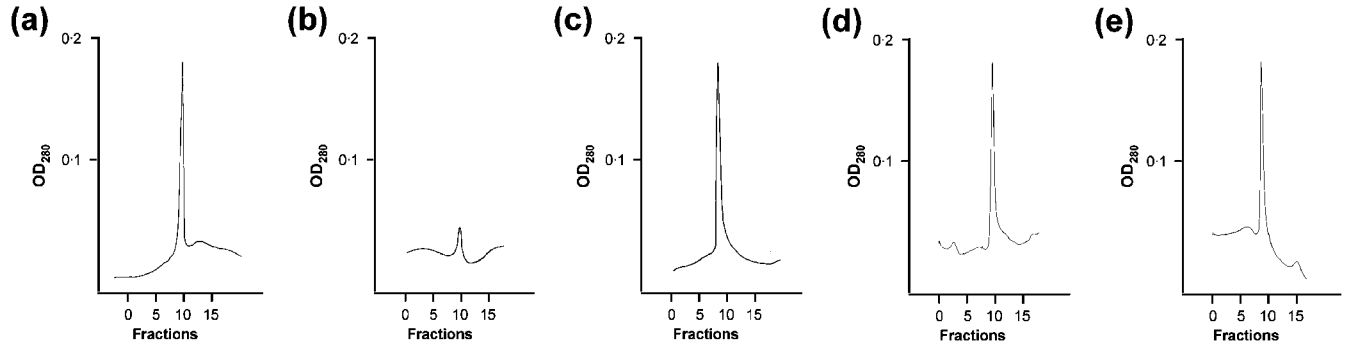

Fig. 5. Effect of *his*HcPro on the breakdown of TMV RNA by 20S proteasome. TMV RNA (6 μg) was incubated in 200 ml TBK240 alone (a), with 20 μg 20S proteasome (b), with 20 μg 20S proteasome previously incubated with *his*HcPro (c) or with 6·6 μg *his*HcPro (d) or with 20 μg glutathione S-transferase (e). The reaction mixture was analysed at 280 nm by gel filtration immediately after incubation.

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 *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 *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 *et al.*, 1993; German-Retana *et al.*, 2000; Plisson *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 α and β 20S proteasome-subunits (Apcher *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 *et al.*, 2003; Thornbury *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 *et al.*, 2003; Seeger *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 *et al.*, 2000; Jockusch & Wiegand, 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 *et al.*, 1989), and in addition no endogenous activity of *his*HcPro 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 *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 proteasome 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′ cap 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 structure 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 inhibition 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, 2001). While the proteasome as a structure 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 delayed response (Janeway, 2001; Yoo & Desiderio, 2003). Similarly, plants could have two pathways of antiviral defence, both based on RNA breakdown. The first component 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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