Update section

Short communication

Inhibitory effects of human cystatin C on plum pox potyvirus proteases

Juan Antonio García¹, María Teresa Cervera¹, José Luis Riechmann¹ and Carlos López-Otín²

¹Centro de Biología Molecular (CSIC-UAM)-Centro Nacional de Biotecnología, Campus de la Universidad Autónoma de Madrid, 28049 Madrid, Spain; ²Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, Oviedo, Spain

Received 15 July 1992; accepted in revised form 24 March 1993

Key words: plum pox potyvirus, protease, human cystatin C, inhibition

Abstract

The effect of different protease inhibitors on the proteolytic processing of the plum pox potyvirus (PPV) polyprotein has been analyzed. Human cystatin C, an inhibitor of cysteine proteases, interfered with the autoprocessing of the viral papain-like cysteine protease HCPro. Unexpectedly, it also had an inhibitory effect on the autocatalytic cleavage of the Nla protease which, although it has a Cys residue in its active center, has been described as structurally related to serine proteases. Other protease inhibitors tested had no effect on any of the cleavage events analyzed.

Proteolytic processing of polyproteins constitutes a largely employed mechanism in gene expression of plant and animal RNA viruses. In particular, plant potyviruses express their genome through a unique polyprotein which is processed by three virus-encoded proteases (Fig. 1; for a review see [26]). Proteinases P1 [24, 32] and HCPro [5] cleave at their respective carboxy ends, while protease Nla cleaves at seven sites characterized by conserved heptapeptides that differ in their sus-

ceptibility to be processed inter- or intramolecularly (*trans* or *cis*), as well as in the reaction profiles [7–10, 12–14, 17, 20]. Based on sequence analysis and site-directed mutagenesis, P1 has been proposed to be a serine protease extensively divergent from cellular proteases of this type [32], while HCPro was identified as a papain-like cysteine protease [19, 25]. On the other hand, Nla belongs to a group of viral proteases related to chymotrypsin-like cellular serine proteases that

Fig. 1. Proteolytic processing of the potyviral protein. Cleavage events are represented by arrowed marks. The protein products identified in potyviral infections and those whose existence has been postulated relying on the characterization of cleavage sites are indicated in the lower part of the map. Since the Nla protein is a major product in infected cells, both the complete protein and its two domains, VPg and protease (Pro), which can be separated by a proteolytic cleavage (dashed line), are indicated in the map.

Fig. 2. Effect of human cystatin C on PPV HCPro and Nla proteolytic activities. A and B. Fluorography of the translation products synthesized in a rabbit reticulocyte lysate system (Promega, used according to supplier's instructions) in the presence of 35 S-methionine. Reactions were performed in the absence (lanes a) or in the presence (lanes b and c) of human cystatin C at the concentrations (μ g/ml) indicated on the top of the lanes. The templates were synthetic RNAs obtained by *in vitro* transcription (as previously described [13]) of plasmids pGG5S6N, (linearized with *Pvu* II and *Dra* III, panel A) and pGGNla (with *Pvu* II and *Hind* III, panel B). These plasmids contain PPV cDNA sequences cloned in the pGEM 3 vector (Promega) under the control of a truncated T7 promoter [28]. The PPV cDNA inserts include the 5'-untranslated region (with the first, non-functional AUG of the ORF mutated [27] and with the context of the initiation codon changed to create a *Nco* I restriction site) followed by different PPV cDNA sequences: nt 151–3632 (cistrons P1, HCPro, P3 and part of 6K₁) in pGG5S6N, and from nt 5715 to the end (cistrons Nla, Nib and CP, and 3'-untranslated region) in pGGNla (numbering of the sequence is according to Laín *et al.* [23]). *Dra* III and *Hind* III restriction sites employed to linearize the plasmids were at PPV nt 2904 and 8020 of pGG5S6N and pGGNla, respectively. Maps indicating the PPV proteins encoded by the template RNAs (asterisks indicate truncated proteins) as well as the molecular weights of the translation products and those of the polypeptides expected from the proteolytic events (indicated by arrows), are shown at the bottom of the figure. The dotted line at the P1-HCPro junction of the panel B map indicates that this site is not processed in reticulocyte lysate, which was the *in vitro* translation system

have a cysteine residue substituting for the catalytic serine [2, 18].

Given the high specificity of the viral proteases, they can be considered as putative targets for anti-viral therapy, and protease inhibitors as potential tools for these actions [16, 22, 29]. Inhibitors of protein nature could be specially useful because of the possibility to use gene transfer techniques to induce their endogenous synthesis and to modify them by site-directed mutagenesis. The analysis of the sensitivity to different protease inhibitors will also give important information in the characterization of the proteolytic activities that participate in the viral life cycle. The action of several protease inhibitors on *trans* processing by the tobacco etch virus (TEV) Nla protease has been previously studied [11]. In this paper we describe the effect of human cystatin C and other protease inhibitors (leupeptin and aprotinin) on the cleavage activities of the plum pox potyvirus (PPV) HCPro and Nla proteins. An *in vitro* transcription/translation system was used for these experiments. Plasmids containing cDNA fragments of the PPV genome encoding the proteases were linearized with restriction endonucleases and *in vitro* transcribed from a phage T7 promoter. The RNA transcripts were used as templates in a rabbit reticulocyte lysate (RR) *in vitro* translation system, where autocatalytic (*cis*) processing took place.

In vitro translation in a RR system of a synthetic transcript encoding the complete P1 and HCPro proteins and 153 amino acids of the P3 protein (the full-length translation product has a molecular mass of 106.4 kDa) was used to study the autocatalytic processing of HCPro at its carboxy terminus. Only a major band, with an electrophoretic mobility similar to that expected for the P1-HCPro protein (87.6 kDa), was detected (Fig. 2A, lane a), suggesting that efficient processing at the HCPro C-end took place. As previously reported for other potyviruses [24, 32], cleavage between P1 and HCPro did not occur in the RR

employed in this experiment. Human cystatin C purified according to Abrahamson *et al.* [1] was a generous gift from Dr A. Gruub and Dr M. Abrahamson.

system. The same processing pattern was obtained when the *in vitro* translation was performed in the presence of 30 $\mu\text{g/ml}$ of human cystatin C (Fig. 2A, lane b). However, in the presence of the inhibitor at 300 $\mu\text{g/ml}$, a protein of the size of the intact translation product was detected and the intensity of the band with the mobility corresponding to the P1-HCPro protein was much lower, indicating that proteolytic processing was inhibited. Densitometric analysis, taking into account the number of methionine residues in each protein, showed that about 50% of the precursor was not processed. Leupeptin and aprotinin (Boehringer Mannheim) at 400 $\mu\text{g/ml}$ had no appreciable effect on the processing by the HCPro protease (not shown).

The Nla protein has been shown to perform *cis* and *trans* proteolytic cleavages [4, 6, 12, 15]. We have studied the effect of protease inhibitors in the autocatalytic processing at the Nla-Nlb junction. *In vitro* translation in a RR system of a synthetic RNA coding for Nla and 333 amino acids of the Nlb protein resulted in the appearance of two major polypeptides with the size corresponding to the products of processing of the encoded polyprotein at the Nla-Nlb junction (Fig. 2B, lane a). In the presence of human cystatin C at 300 $\mu\text{g/ml}$, a protein with the mobility expected for the intact translation product was detected (Fig. 2B, lane c), indicating that some inhibition of the cleavage was occurring. Densitometric analysis showed that about 13% of the protein was not processed in the presence of the inhibitor. No alteration on the efficiency of translation in the RR system, at any of the concentrations of cystatin employed, was observed. Cystatin at a lower dosis (30 $\mu\text{g/ml}$, Fig. 2B, lane b), or aprotinin or leupeptin at 400 $\mu\text{g/ml}$ (not shown), did not affect the processing. No inhibitory effect of cystatin, leupeptin or aprotinin on the *trans* cleavage at the Nlb-CP site, assayed by incubation of *in vitro* translation products with extracts of *Escherichia coli* expressing the Nla protease [13], could be detected (not shown).

The lack of significant inhibitory effects of aprotinin (inhibitor of serine proteases) and leupeptin (inhibitor of serine and cysteine proteases)

on the *trans* cleavage by the potyviral Nla protease at the Nlb-CP junction has been previously explained on the basis of the particular specificity of the Nla recognition sites [11]. With the results presented in this paper, this reasoning can now be extended to the *cis* processing activity of the Nla protease and of the cysteine protease HCPro. Interestingly, human cystatin C, a known inhibitor of papain-like thiol proteases, efficiently interfered with the autoprocessing of the HCPro protease, indicating a close relationship between this protein and the cellular cysteine proteases. The case of the Nla protease is more complex. Based on sequence analysis it has been described that, although it contains in the active center a cysteine residue, this protease is much more related to serine proteases than to the cysteine ones [2, 18]. Since the mechanism of action of cystatins involves interactions with particular structural features of thiol proteases but does not produce a non-specific blockage of the active cysteine [30], it seemed unlikely that human cystatin C has an effect on Nla activities. Surprisingly, the experiments reported here indicate that the autocatalytic processing of the PPV Nla protease was little, but clearly, interfered by this inhibitor. This result could suggest for the Nla-type proteases an evolutionary position more equidistant between serine and cysteine protease than that currently accepted. However, due to the low level of inhibition and the high dosis of inhibitor required to produce the effect, this conclusion should be considered only as tentative. On the other hand, preliminary results using a wheat germ *in vitro* translation system seemed to indicate that P1 protease autoprocessing was not affected by either of the protease inhibitors employed in the experiments described in this paper (not shown), in agreement with its previous characterization as an atypical serine protease [24, 31, 32].

The inhibitory effects of human cystatin C on two highly specific proteases of PPV open the possibility to use it in strategies designed to interfere with potyvirus infections. Human cystatin C and rabbit cystatin have been shown to drastically reduce the replication of herpes simplex virus [3] and picornaviruses [21, 22]. Although

the proteolytic processing of potyvirus polyprotein likely consists of a highly regulated series of events and could be disturbed without completely abolishing the protease activity, the high dosis of cystatin required to observe an inhibitory effect suggests that it probably could not be actually used as an antipotyviral agent. However, it is probable that further studies on structure-function relationships and site-directed mutagenesis could allow the design of highly specific inhibitors of the potyviral proteases with low collateral effects on the cellular physiology.

Acknowledgements

We are grateful to Dr A. Grubb and Dr M. Abrahamson for kindly providing us with purified human cystatin C. This work was supported by Grants BIO92-0187 from CICYT, C168/90 from Comunidad de Madrid and by an institutional grant from Fundación Ramón Areces. M.T.C. and J.L.R. are recipients of fellowships from Plan Nacional de Formación del Personal Investigador and CSIC, respectively.

References

1. Abrahamson M, Dalbøge H, Olafsson I, Carlsen S, Grubb A: Efficient production of native, biologically active human cystatin C by *Escherichia coli*. *FEBS Lett* 236: 14–18 (1988).
2. Bazan JF, Fletterick RJ: Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: Structural and functional implications. *Proc Natl Acad Sci USA* 85: 7872–7876 (1988).
3. Björck L, Grubb A, Kjellén L: Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus. *J Virol* 64: 941–943 (1990).
4. Carrington JC, Cary SM, Dougherty WG: Mutational analysis of tobacco etch virus polyprotein processing: *cis* and *trans* proteolytic activities of polyproteins containing the 49-kilodalton proteinase. *J Virol* 62: 2313–2320 (1988).
5. Carrington JC, Cary SM, Parks TD, Dougherty WG: A second proteinase encoded by a plant potyvirus genome. *EMBO J* 8: 365–370 (1989).
6. Carrington JC, Dougherty WG: Processing of the tobacco etch virus 49K protease requires autoproteolysis. *Virology* 160: 355–362 (1987).
7. Carrington JC, Dougherty WG: Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. *J Virol* 61: 2540–2548 (1987).
8. Carrington JC, Dougherty WG: A viral cleavage site cassette: identification of amino acid sequences required for tobacco etch virus polyprotein processing. *Proc Natl Acad Sci USA* 85: 3391–3395 (1988).
9. Dougherty WG, Parks TD: Molecular genetic and biochemical evidence for the involvement of the heptapeptide cleavage sequence in determining the reaction profile at two tobacco etch virus cleavage sites in cell-free assays. *Virology* 172: 145–155 (1989).
10. Dougherty WG, Parks TD: Post-translational processing of the tobacco etch virus 49-kDa small nuclear inclusion polyprotein: identification of an internal cleavage site and delimitation of VPg and proteinase domains. *Virology* 183: 449–456 (1991).
11. Dougherty WG, Parks TD, Cary SM, Bazan JF, Fletterick RJ: Characterization of the catalytic residues of the tobacco etch virus 49-kDa proteinase. *Virology* 172: 302–310 (1989).
12. García JA, Laín S, Cervera MT, Riechmann JL, Martín MT: Mutational analysis of plum pox potyvirus polyprotein processing by the Nla protease in *Escherichia coli*. *J Gen Virol* 71: 2773–2779 (1990).
13. García JA, Martín MT, Cervera MT, Riechmann JL: Proteolytic processing of the plum pox potyvirus polyprotein by the Nla protease at a novel cleavage site. *Virology* 188: 697–703 (1992).
14. García JA, Riechmann JL, Laín S: Artificial cleavage site recognized by plum pox potyvirus protease in *Escherichia coli*. *J Virol* 63: 2457–2460 (1989).
15. García JA, Riechmann JL, Laín S: Proteolytic activity of the plum pox potyvirus Nla-like protein in *Escherichia coli*. *Virology* 170: 362–369 (1989).
16. García JA, Riechmann JL, Martín MT, Laín S: Proteolytic activity of the plum pox potyvirus Nla-like protein on excess of natural and artificial substrates in *Escherichia coli*. *FEBS Lett* 256: 269–273 (1989).
17. Ghabrial SA, Smith HA, Parks TD, Dougherty WG: Molecular genetic analyses of the soybean mosaic virus Nla protease. *J Gen Virol* 71: 1921–1927 (1990).
18. Gorbalenya AE, Donchenko AP, Blinov VM, Koonin EV: Cysteine proteases of positive strand RNA viruses and chymotrypsin-like serine proteases. A distinct protein superfamily with a common structural fold. *FEBS Lett* 243: 103–114 (1989).
19. Gorbalenya AE, Koonin E, Lai MM-C: Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, α - and coronaviruses. *FEBS Lett* 288: 201–205 (1991).
20. Hellmann GM, Shaw JG, Rhoads RE: *In vitro* analysis of tobacco vein mottling virus Nla cistron: Evidence for a virus encoded protease. *Virology* 163: 554–562 (1988).

21. Korant BD, Brzin J, Turk V: Cystatin, a protein inhibitor of cysteine proteases alters viral protein cleavages in infected human cells. *Biochem Biophys Res Commun* 127: 1072–1076 (1985).
22. Korant BD, Towatari T, Ivanoff L, Petteway S Jr, Brzin J, Lenarcic B, Turk V: Viral therapy: prospects for protease inhibitors. *J Cell Biochem* 32: 91–95 (1986).
23. Lain S, Riechmann JL, García JA: The complete nucleotide sequence of plum pox potyvirus RNA. *Virus Res* 13: 157–172 (1989).
24. Mavankal G, Rhoads R: *In vitro* cleavage at or near the N-terminus of the helper component protein in the tobacco vein mottling virus polyprotein. *Virology* 185: 721–731 (1991).
25. Oh CS, Carrington JC: Identification of essential residues in potyvirus proteinase HC-Pro by site-directed mutagenesis. *Virology* 173: 692–699 (1989).
26. Riechmann JL, Lain S, García JA: Highlights and prospects of potyvirus molecular biology. *J Gen Virol* 73: 1–16 (1992).
27. Riechmann JL, Lain S, García JA: Identification of the initiation codon of plum pox potyvirus genomic RNA. *Virology* 185: 544–552 (1991).
28. Riechmann JL, Lain S, García JA: Infectious *in vitro* transcripts from a plum pox potyvirus cDNA clone. *Virology* 177: 710–716 (1990).
29. Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Kröhn A, Lambert RW, Merret JH, Mills JS, Parkes KEB, Redshaw S, Ritchie AJ, Taylor DL, Thomas GJ, Machin PJ: Rational design of peptide-based HIV proteinase inhibitors. *Science* 248: 358–361 (1990).
30. Turk V, Bode W: The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett* 285: 213–219 (1991).
31. Verchot J, Herndon KL, Carrington JC: Mutational analysis of the tobacco etch potyviral 35-kDa proteinase: Identification of essential residues and requirements for autoproteolysis. *Virology* 190: 298–306 (1992).
32. Verchot J, Koonin EV, Carrington JC: The 35-kDa protein from the N-terminus of a potyviral polyprotein functions as a third virus-encoded proteinase. *Virology* 185: 527–535 (1991).