

Hlcyst-1 and Hlcyst-2 are Potential Inhibitors of HICPL-A in the Midgut of the Ixodid Tick *Haemaphysalis longicornis*

Kayoko YAMAJI^{1,2,4}), Naotoshi TSUJI^{2,3})*, Takeharu MIYOSHI²), Takeshi HATTA²), M. Abdul ALIM²), ANISUZZAMAN^{2,3}), Shiro KUSHIBIKI^{1,4}) and Kozo FUJISAKI⁵)

¹)Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai, Tsukuba, Ibaraki 305–8572,

²)National Institute of Animal Health, National Agriculture and Food Research Organization, Kannondai, Tsukuba, Ibaraki 305–0856,

³)Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113–8657,

⁴)National Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Ikenodai, Tsukuba, Ibaraki 305–0901 and ⁵)Department of Frontier Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890–0065, Japan

(Received 14 December 2009/Accepted 12 January 2010/Published online in J-STAGE 26 January 2010)

ABSTRACT. Although the actions of cysteine proteases are controlled in part by endogenous tight-binding cysteine protease inhibitors from the cystatin superfamily, regulatory mechanisms used by ticks to control protease activities are unknown. We report here the interaction of 2 endogenous midgut cysteine protease inhibitors, Hlcyst-1 and Hlcyst-2, with an endogenous midgut cysteine protease, HICPL-A in *Haemaphysalis longicornis*. *In vitro* inhibition assays demonstrated that the hydrolytic activity of HICPL-A was inhibited by Hlcyst-1 and Hlcyst-2 in dose dependent manner. Immunofluorescent studies revealed that Hlcyst-1 and Hlcyst-2 are co-localized with HICPL-A in the epithelial cells of the midgut. The hemoglobin degradation activity of HICPL-A was dose-dependently inhibited by Hlcyst-1 and Hlcyst-2. These results strongly indicate that, Hlcyst-1 and Hlcyst-2 are possible inhibitor of HICPL-A and play a key role in regulatory mechanisms of hemoglobin degradation process in ticks.

KEY WORDS: arthropods, enzymes, tick, vector biology.

J. Vet. Med. Sci. 72(5): 599–604, 2010

In ticks, the haematophagous arthropods, development of larva and nymph, and production of eggs by adult are all dependent on the acquisition of nutrients from the host blood-meal including hemoglobin (Hb) from red blood cells. In *Ixodes ricinus*, an ordered pathway of Hb digestion has been revealed where members of at least three different mechanistic classes of enzymes are involved. Aspartic peptidase, cathepsin D supported by cysteine protease cathepsin L [21] and legumain [20], are responsible for primary cleavage of Hb. The production of secondary small fragments of Hb is dominated by the endopeptidase activity of cathepsin B [21]. Exopeptidases act on the peptides released by the action of the endopeptidases activity of cathepsin B and the amino-dipeptidase activity of cathepsin C. Metallopeptidases might participate in the liberation of free amino acids [10]. Midgut proteolytic enzymes including aspartic protease (longepsin) [7], serine carboxypeptidase (HISCP) [17] and legumain (HILgm) [5] have been ascribed to play roles in the degradation of Hb to peptides in the *Haemaphysalis longicornis*. Accordingly, the present data and those available for other tick species suggest that an enzyme cascade for the proteolysis of Hb operates in the midgut of the ticks [10].

Cysteine proteases comprise a group of proteolytic enzymes that cleave peptide bonds by use of a reactive cysteine residue at the catalytic site. One of the families, C1 includes the lysosomal cathepsins B, H, and L, which play a

major role in cell protein turnover following their release from the lysosomes in various pathological processes such as inflammation and tumor invasion [23]. The ixodid tick *H. longicornis* contains two types of Clan CA cysteine peptidases, the cathepsin L-like HICPL-A [24] and the cathepsin B-like longipain [22]. HICPL-A has been characterized from the *H. longicornis*, and this protease was capable of degrading bovine Hb. Consistent with the function in degrading host blood protein, Hb, HICPL-A was expressed mainly in the blood-feeding stages. The most potent physiological regulators of the cathepsin L activity are the inhibitors from the cystatin superfamily [1, 23]. In multicellular organisms, the actions of papain-like cysteine proteases are controlled in part by endogenous tight-binding cysteine protease inhibitors from the cystatin superfamily. The mammalian cystatin homologues in *H. longicornis*, named Hlcyst-1 and Hlcyst-2 for cystatin [26, 28], were shown indeed to be potent and specific inhibitors of several clan CA cathepsins such as mammalian cathepsin L. Hlcyst-1 and Hlcyst-2 were found to be expressed at highest levels in midgut of *H. longicornis* during the blood-feeding stages. Nevertheless, nothing was known about the natural target or biological roles of Hlcyst-1 and Hlcyst-2. One possibility seemed to be that it interacts with and controls activity of endogenous cysteine proteases during the blood-feeding. In this study, we sought to determine whether HICPL-A and endogenous cystatins, Hlcyst-1, Hlcyst-2 and salivary cystatin HISC-1 [25], interact *in vitro*. To make a comparison between Hlcyst-1, Hlcyst-2 and HISC-1 inhibitory potency against their target enzymes purified recombinant cystatins were employed to inhibit the HICPL-A activity *in vitro*. Our

* CORRESPONDENCE TO: TSUJI, N., Laboratory of Parasitic Diseases, National Institute of Animal Health, 3–1–5, Kannondai, Tsukuba, Ibaraki 305–0856, Japan.
e-mail: tsujin@affrc.go.jp

studies suggest that cystatins regulates the endogenous activity of cysteine protease, thus indirectly modulating hemoglobinase activity essential for tick to obtain their nutrients.

MATERIALS AND METHODS

Ticks and animals: *H. longicornis* (Okayama strain) were maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Ibaraki, Japan, on rabbits as described previously [26]. All animals used in this study were acclimatized to the experimental conditions for 2 weeks prior to the experiment. Animal experiments at the NIAH were conducted in accordance with protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 08–021).

Kinetic measurements of peptidase inhibition: Recombinant HICPL-A, Hlcyst-1, Hlcyst-2 and HISC-1 were produced as described earlier [24, 25]. Cathepsin L, B, papain and trypsin were purchased from Sigma (St. Louis, MO, U.S.A.), and cathepsin H was from Calbiochem (San Diego, CA, U.S.A.). Inhibition kinetics were calculated as previously described [25]. To detect the ability of protease inhibitors against recombinant HICPL-A, cathepsin L, B, H, papain and trypsin, hydrolysing activity, the fluorogenic substrates were used. The substrates were Z-Phe-Arg-MCA to measure cathepsin B/L activities, Z-Arg-Arg-MCA to measure cathepsin B, Arg-MCA to measure cathepsin H, and Bz-L-Arg-pNA•HCl to measure papain and trypsin. These synthetic substrates were purchased from Peptide Institute Inc (Osaka, Japan). Enzymes were preincubated with different concentrations of inhibitor for 30 min in assay buffer (100 mM NaAc, pH 5.5, 100 mM NaCl, 1 mM EDTA, 1 mg/ml cysteine, and 0.005% TritonX-100) at 37°C. Fluorogenic peptidyl substrates were added to have a final concentration of 0.25 mM. Fluorogenic assays were monitored by fluorescence spectrophotometry at 380 nm excitation and 460 nm emission (TECAN, Maennedorf, Switzerland). For substrates, K_m and k_{cat} values were obtained by fitting the initial rates to the Michaelis-Menten equation using nonlinear regression analysis (Graph Pad 4.0, CA, U.S.A.).

Inhibition of hemoglobinolytic activity of HICPL-A by *H. longicornis* cystatins: Approximately 5 μ M of Hlcyst-1 and Hlcyst-2 recombinant proteins were used in each reaction. The recombinant Hlcyst-1 and Hlcyst-2 were preincubated with or without 1 μ M HICPL-A for 10 min in citrate-sodium phosphate buffer (pH 4.0) at 37°C. To the reactive mixture 5 μ g of Hb was added and the reactions were continued for 30 min at 37°C before adding SDS loading buffer and boiling. The reaction samples were loaded in each gel lane, resolved by SDS-PAGE using 10–20% Tricine gel (Invitrogen, Carlsbad, CA, U.S.A.), and the gels were stained with CBB.

Quantitative reverse transcription (RT)-PCR: Total RNA was extracted from the midgut of adult ticks using RNeasy Mini Kit (QIAGEN Sciences, Germantown, MA, U.S.A.)

following the manufacturer's instructions. cDNA synthesis from total RNA was performed with a Takara RNA PCR Kit (AMV) Ver3.0 (Takara Bio Inc., Otsu, Japan). The following pairs of primers were used: for Hlcyst-1, forward 5'-CGG AAA AGG TTC GCG AAG AG-3', reverse 5'-CTG CAC AGC TGC AAA GGT TA-3'; for Hlcyst-2, forward 5'-ACC GAC TAC CTT CAC CAT C-3', reverse 5'-CAC GCA GTC GAA TGA AGT GA-3'; for HICPL-A, forward 5'-GCA GCA CAA TAA GGC GTA CA-3', reverse 5'-GAC CAT TTT GGC GAA CTC AT-3' and for Hl β -actin, forward 5'-ATG TGT GAC GAC GAG GTT GCC-3', reverse 5'-AGA GTA GCC ACG CTC GGT GAG-3'. Transcript abundance was measured with a LightCycler 1.5 (Roche Instrument Center AG, Roikreuz, Switzerland) according to the manufacturer's instructions. Real-time RT-PCR was conducted in LightCycler Capillaries (Roche) with a 20 μ l reaction volume containing 4.6 μ l of LightCycler FastStart DNA Master SYBR Green (Roche), 3 mM of MgCl₂, 0.5 μ M of each forward and reverse primer, and cDNA corresponding to 0.2 ng of total RNA. Expression was normalized against β -actin as internal control. The mRNA abundance unit was calculated as the number of mRNA molecules per β -actin mRNA. The data was analyzed by LightCycler Software Version 3.5. The β -actin gene was amplified according to the same procedures and was used as a control.

Hlcyst-1, Hlcyst-2 and HICPL-A polyclonal antisera: The peptides CEVKDADDTVREI (amino acids 9 to 21 of Hlcyst-1) and CDQDPQSSPKY (27 to 37 of Hlcyst-2) were coupled to keyhole BSA. Synthetic peptides were used to raise polyclonal antibodies in BALB/c mice by injection of 20 μ g of the antigen emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, U.S.A.). Whereas, an antiserum against HICPL-A was raised by immunizing mice subcutaneously with 0.2 mg of the recombinant protein emulsified with adjuvant. The antigen administration was repeated at 2 and 4 weeks, and the animals were bled after 6 weeks of first immunization, when antisera were tested by ELISA and Western blotting. Ten micrograms of peptides, recombinant or BSA were diluted in PBS and adsorbed on ELISA 96-well microtiter plates overnight at 4°C. The wells were blocked with PBS, 0.1% Tween 20 (v/v) and 5% non-fat milk for 90 min at 37°C and incubated for 2 hr at 37°C with control PBS or antiserum diluted 1:50 in PBS containing 0.1% of Tween 20 (v/v). The wells were subsequently washed three times with PBS, 0.1% Tween 20 (v/v) and incubated for 1 hr at room temperature with Alkaline Phosphatase-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch, West Grove, U.S.A.) (diluted 1:1,000). After washing as described above, the reaction was developed with o-phenylenediamine (Bio-Rad, CA, U.S.A.) according to manufacturer's instructions and the optical density was determined at 492 nm.

Indirect immunofluorescent microscopy: For endogenous localization of HICPL-A, Hlcyst-1 and Hlcyst-2, midguts were collected from adult ticks at 72 hr of blood-feeding. Midguts were fixed in 4% paraformaldehyde in 0.1 M phos-

phate buffer (pH 7.2) for 6 hr at 4°C, embedded in paraffin and was used to make thin flat sections. The sections on glass slides were blocked with 10% goat serum (MP Bio-medicals, Irvine, CA, U.S.A.) for 30 min at room temperature, washed twice in PBS and then incubated with polyclonal antisera (diluted 1:500) for 24 hr at 4°C. The slides were washed three times with PBS, then incubated with green fluorescence-labeled mouse IgG secondary antibody [Alexa Fluor®488 goat anti-(mouse IgG) (H + L); Invitrogen] for 1 hr at room temperature. The slides were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

RESULTS

Inhibition of HICPL-A activity by *H. longicornis* cystatins: First, we examined whether *H. longicornis* cystatins, HISC-1, Hlcyst-1 and Hlcyst-2 were related to regulate the activity of HICPL-A. Hlcyst-1 and Hlcyst-2 were potent inhibitors of the HICPL-A (Fig. 1), but salivary cystatin HISC-1 did not affect the HICPL-A activity.

To measure the specific inhibitory activity of recombi-

nant *H. longicornis* cystatins, the concentration of Hlcyst-1, Hlcyst-2 and HISC-1 at which 50% inhibition of the activity of the proteolytic enzymes is achieved (IC_{50}) were presented (Table 1). In previous report, the comparison assays of three inhibitors showed a distinct inhibition pattern [25]. Moreover, present study provided experimental evidence that two midgut cystatins distinctly inhibited HICPL-A activity.

Inhibition of hemoglobinolytic activity of HICPL-A by *H. longicornis* cystatins: We attempted to reveal that Hb digestion activity of HICPL-A was regulated by Hlcyst-1 and Hlcyst-2. Inhibition experiments with HICPL-A showed that Hb digestion activity of HICPL-A was potentially inhibited by Hlcyst-1 (Fig. 2, lane 3) and Hlcyst-2 (Fig. 2, lane 5), but was unaffected by salivary cystatin, HISC-1 (Fig. 2, lane 7). These results suggest that among tick proteases, HICPL-A is the principal target of Hlcyst-1 and Hlcyst-2 inhibition.

Comparison of feeding stage-specific expression between HICPL-A and *H. longicornis* cystatins: To examine the comparative expression pattern of HICPL-A, Hlcyst-1 and Hlcyst-2 in midgut during the blood-feeding process, we performed quantitative RT-PCR with total RNA extracted from the unfed and partially fed (24, 48, 72 and 96 hr) adult

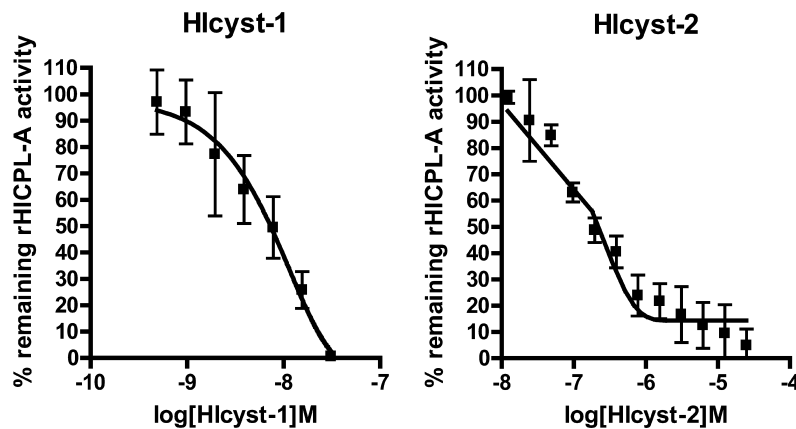


Fig. 1. HICPL-A activity inhibition assay. Inhibition of HICPL-A protease activities by Hlcyst-1 and Hlcyst-2. HICPL-A was incubated with the Z-Phe-Arg-MCA (substrate for cathepsin L/B) in the presence of different concentrations of Hlcyst-1 and Hlcyst-2. Incubation of HICPL-A without Hlcyst-1 and Hlcyst-2 resulted in 100% enzyme activity.

Table 1. Protease inhibition assays

Enzyme	Enzyme conc.	IC ₅₀ ± S.E		
		Hlcyst-1	Hlcyst-2	HISC-1
(cysteine protease)				
HICPL-A	0.1 μM	7.90E-3 μM ± 2.190	2.39E-1 μM ± 8.65	NI
cathepsin L	15–20 pM	2.03E-8 μM ± 1.938	1.70 μM ± 0.490	2.74E-5 μM ± 5.295
cathepsin B	15 mU/ml	9.15E-4 μM ± 1.399	55 μM<	NI
cathepsin H	30 nM	8.4E-3 μM ± 0.450	11.4 μM ± 1.90	NI
papain	50 μg/ml	2.20 μM ± 0.720	17.0 μM ± 1.08	5.30 μM ± 0.670
(serine protease)				
trypsin	20 μg/ml	NI	NI	NI

Repertoire of cysteine proteases tested for inhibition by Hlcyst-1, Hlcyst-2 and HISC-1 and the concentration of inhibitor at which 50% inhibition of the activity of the targeted proteolytic enzyme is achieved (IC_{50}) \pm S.E. Enzyme concentration used in the assays is also given for all their targets. NI, no inhibition.

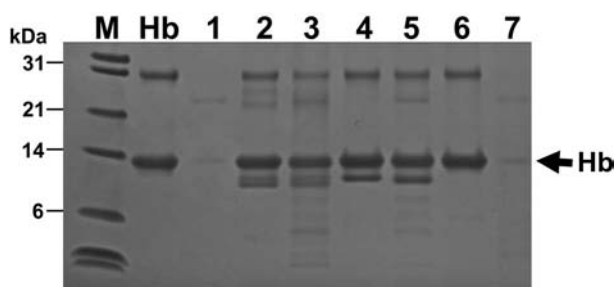


Fig. 2. Inhibition of HICPL-A activity on Hb proteolysis by *H. longicornis* cystatins. The recombinant HICPL-A was incubated in citrate-sodium phosphate buffer (pH 4.0) at 37°C without cystatins (lane 1). HICPL-A and Hb were incubated with Hlcyst-1 (lane 3), Hlcyst-2 (lane 5) and HISC-1 (lane 6). Hb was incubated with Hlcyst-1 (lane 2), Hlcyst-2 (lane 4) and HISC-1 (lane 7) without HICPL-A. M, marker proteins. Hb, hemoglobin was applied.

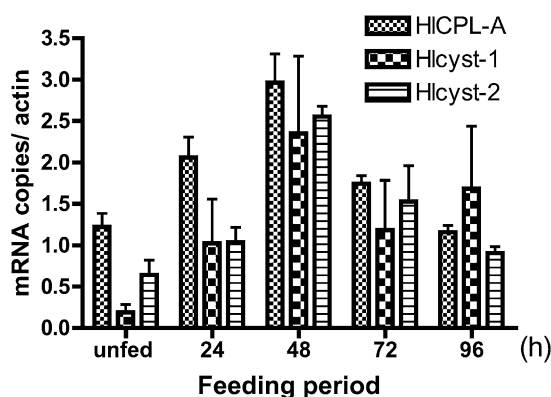


Fig. 3. Expression of the *HICPL-A*, *Hlcyst-1* and *Hlcyst-2* gene during different stages of blood-feeding. Quantitative RT-PCR was carried out with total RNA samples from midguts and salivary glands excised from adult *H. longicornis* at different stages of feeding using the primer sets on described in material and methods. The bars show the mean \pm SD of relative mRNA copy numbers ($n=4$).

ticks (Fig. 3). *Hlcyst-2* and *HICPL-A* transcripts were expressed most strongly at 48 hr of feeding in adult and thereafter their expression decline sharply. Similarly, *Hlcyst-1* transcript was up-regulated by the blood-feeding process.

Co-localization of *Hlcyst-1* and *Hlcyst-2* with *HICPL-A*: The results from the kinetic experiments detailed above showed that cystatin interacts with *HICPL-A*, but to assess whether these interactions occurred in living ticks it was necessary to investigate the respective cellular localizations of each of the proteins. We performed immunohistochemical localization to detect the *HICPL-A*, *Hlcyst-1* and *Hlcyst-2*, by employing fluorescence labeled anti-*HICPL-A*, -*Hlcyst-1* and -*Hlcyst-2*. Punctate patterns of fluorescence were observed for all three antibodies, indicative of midgut epithelial cells localization, with the most intense staining in these tissues. As shown in Fig. 4, some co-localization was

observed between *HICPL-A* and *Hlcyst-1*/*Hlcyst-2* in midgut epithelial cells.

DISCUSSION

Previous studies have shown that cysteine protease and aspartic protease derived from ixodid ticks hydrolyzed host Hb, suggesting that those proteases are involved in Hb digestion cascade in ticks [4,22]. However, regulatory mechanisms of tick cysteine protease activity are yet to be unraveled. The recent papers describe biochemical characterization of several cystatins from the ticks, *Ornithodoros moubata* [9], *Amblyomma americanum* [12], *Ixodes scapularis* [14] and *H. longicornis* [25, 27, 28]. However, there are no reports on how the expression and/or activity of these peptidases are controlled by ticks.

In this study, it was shown that recombinant *Hlcyst-1* potentially inhibited mammalian cathepsin L, cathepsin B, cathepsin H and tick *HICPL-A*. *Hlcyst-2* was also shown to potentially inhibit the activity of *HICPL-A*. Moreover, *Hlcyst-1* and *Hlcyst-2* blocked the Hb degradation by *HICPL-A*, indicating that two cystatins plays a key role in regulatory mechanisms of Hb degradation process. A large number of normal and pathological processes are controlled by the balance between proteinases and their inhibitors. The physiological role of cystatins is believed to be the protection of cells from inappropriate endogenous or exogenous proteolysis by the regulation of cysteine protease. An increase in local proteases and protease inhibitors has been observed in the presence of inflammation, acquired immunodeficiency syndrome, and malignancy [3, 11, 13, 21], that obviously indicate an internal check and balance mechanism between proteases and inhibitors.

Using immunofluorescence microscopy, we showed here that *HICPL-A*, *Hlcyst-1* and *Hlcyst-2* were co-localized in midgut epithelial cells. In addition, *Hlcyst-1* and *Hlcyst-2* were expressed to detectable levels at the different blood-feeding stages, although always at lower concentrations than *HICPL-A*. These results indicated the anatomical close relationship in between *HICPL-A* and *Hlcyst-1* and *Hlcyst-2*, further suggesting the involvement of *Hlcyst-1* and *Hlcyst-2* in the regulation of midgut-derived cysteine protease involved in blood digestion. In mammals, cystatins function as a protection against lysosomal peptidases released occasionally at normal cell death or phagocyte degranulation, or intentionally by proliferating cancer cells or by invading organisms [2]. In addition, endogenous cysteine protease inhibitors have been described in parasites such as *Trypanosoma cruzi* [16], *Leishmania mexicana* [6], *Entamoeba histolytica* [19] and malaria parasites, *Plasmodium falciparum* [18]. These studies showed that Hb degradation occurred in the food vacuole. We hypothesized that endogenous *HICPL-A* was involved in tick lysosomal enzyme of midgut epithelial cells. The epithelial cells of ixodid tick participates in digestion, and ingest Hb and other dissolved components by endocytosis during the feeding stages. The digestion occurs intracellularly in the midgut

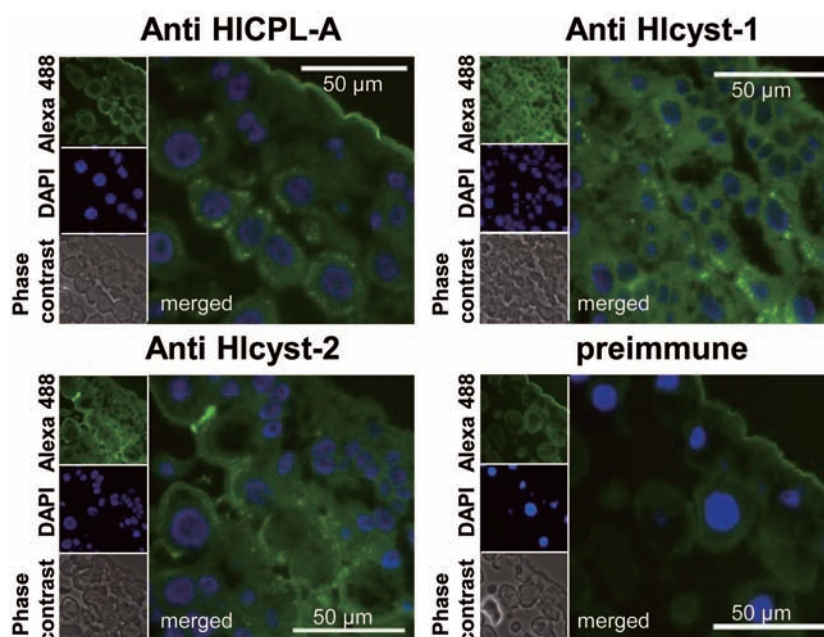


Fig. 4. Co-localization of Hlcyst-1 and Hlcyst-2 with HICPL-A. The endogenous HICPL-A, Hlcyst-1 and Hlcyst-2 were visualized using mouse anti-HICPL-A, -Hlcyst-1 and Hlcyst-2 antibodies (green), and the nuclei of the midgut epithelial cells were stained with DAPI (blue).

epithelium and is accomplished by lysosomal hydrolytic enzymes in vacuoles [8,15]. The results in this study fairly suggest that Hlcyst-1 and Hlcyst-2 modulates the endogenous activity of cysteine protease, involved in the lysosomal Hb digestion network.

ACKNOWLEDGMENTS. This work was supported by Grant-in-Aids (to N.T. and K.F.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by a grant (to N.T. and K.F.) for Promotion of Basic Research Activities for Innovative Biosciences from the Bio-oriented Technology Research Advancement Institution.

REFERENCES

1. Abrahamson, M., Mason, R.W., Hansson, H., Buttler, D.J., Grubb, A. and Ohlsson, K. 1991. Human cystatin C. role of the N-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leucocyte elastase. *Biochem. J.* **273** (Pt 3): 621–626.
2. Abrahamson, M., Alvarez-Fernandez, M. and Nathanson, C.M. 2003. Cystatins. *Biochem. Soc. Symp.* **70**: 179–199.
3. Alavaikko, M., Aine, R., Rinne, A., Järvinen, M., Blanco, G., Apaja-Sarkinen, M. and Hopsu-Havu, V.K. 1985. Behaviour of dendritic reticulum cells possessing immunoreactive acid cysteine-proteinase inhibitor in human lymphoid secondary follicles and in follicular-centre cell lymphomas. *Int. J. Cancer* **35**: 319–325.
4. Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Huang, X., Hatta, T. and Fujisaki, K. 2008. HILgm2, a member of asparaginyl endopeptidases/legumains in the midgut of the ixodid tick *Haemaphysalis longicornis*, is involved in blood-meal digestion. *J. Insect Physiol.* **54**: 573–585.
5. Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Hatta, T. and Fujisaki, K. 2009. Legumains from the hard tick *Haemaphysalis longicornis* play modulatory roles in blood feeding and gut cellular remodelling and impact on embryogenesis. *Int. J. Parasitol.* **39**: 97–107.
6. Besteiro, S., Coombs, G.H. and Mottram, J.C. 2004. A potential role for ICP, a leishmanial inhibitor of cysteine peptidases, in the interaction between host and parasite. *Mol. Microbiol.* **54**: 1224–1236.
7. Boldbaatar, D., Sikalizyo, Sikasunge, C., Battsetseg, B., Xuan, X. and Fujisaki, K. 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. *Insect Biochem. Mol. Biol.* **36**: 25–36.
8. Gough, J.M. and Kemp, D.H. 1995. Acid phosphatase in midgut digestive cells in partially fed females of the cattle tick *Boophilus microplus*. *J. Parasitol.* **81**: 341–349.
9. Grunclová, L., Horn, M., Vancová, M., Sojka, D., Franta, Z., Mares, M. and Kopáček, P. 2006. Two secreted cystatins of the soft tick *Ornithodoros moubata*: differential expression pattern and inhibitory specificity. *Biol. Chem.* **387**: 1635–1644.
10. Horn, M., Nussbaumerová, M., Sanda, M., Kovárová, Z., Srba, J., Franta, Z., Sojka, D., Bogyo, M., Caffrey, C.R., Kopáček, P. and Mares, M. 2009. Hemoglobin digestion in blood-feeding ticks: mapping a multi-peptidase pathway by functional proteomics. *Chem. Biol.* **16**: 1053–1063.
11. Järvinen, M. 1978. Purification and some characteristics of the human epidermal SH-protease inhibitor. *J. Invest. Dermatol.* **71**: 114–118.

12. Karim, S., Miller, N.J., Valenzuela, J., Sauer, J.R. and Mather, T.N. 2005. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. *Biochem. Biophys. Res. Commun.* **334**: 1336–1342.
13. Keilová, H. and Tomásek, V. 1977. Naturally occurring inhibitors of intracellular proteinases. *Acta. Biol. Med. Ger.* **36**: 1873–1881.
14. Kotsyfakis, M., Sá-Nunes, A., Francischetti, I.M., Mather, T.N., Andersen, J.F. and Ribeiro, J.M. 2006. Antiinflammatory and immunosuppressive activity of sialostatin L, a salivary cystatin from the tick *Ixodes scapularis*. *J. Biol. Chem.* **281**: 26298–26307.
15. Lara, F.A., Lins, U., Bechara, G.H. and Oliveira, P.L. 2005. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. *J. Exp. Biol.* **208**(Pt 16): 3093–3101.
16. Monteiro, A.C., Abrahamson, M., Lima, A.P., Vannier-Santos, M.A. and Scharfstein, J. 2001. Identification, characterization, and localization of chagasin, a tight-binding cysteine protease inhibitor in *T. cruzi*. *J. Cell. Sci.* **114**: 3933–3942.
17. Motobu, M., Tsuji, N., Miyoshi, T., Huang, X., Islam, M.K., Alim, M.A. and Fujisaki, K. 2007. Molecular characterization of a blood-induced serine carboxypeptidase from the ixodid tick *Haemaphysalis longicornis*. *FEBS J.* **274**: 3299–3312.
18. Pandey, K.C., Singh, N., Arastu-Kapur, S., Bogyo, M. and Rosenthal, P.J. 2006. Faltstatin, a cysteine protease inhibitor of *Plasmodium falciparum*, facilitates erythrocyte invasion. *PLoS Pathog.* **2**: e117.
19. Rosso, M.N., Dubrana, M.P., Cimbolini, N., Jaubert, S. and Abad, P. 2005. Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Mol. Plant Microbe Interact.* **18**: 615–620.
20. Sojka, D., Hajdusek, O., Dvorák, J., Sajid, M., Franta, Z., Schneider, E.L., Craik, C.S., Vancová, M., Buresová, V., Bogyo, M., Sexton, K.B., McKerrow, J.H., Caffrey, C.R. and Kopáček, P. 2007. IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. *Int. J. Parasitol.* **37**: 713–724.
21. Sojka, D., Franta, Z., Horn, M., Hajdusek, O., Caffrey, C.R., Mares, M. and Kopáček, P. 2008. Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionarily conserved network of aspartic and cysteine peptidases. *Parasit. Vectors* **1**: 7.
22. Tsuji, N., Miyoshi, T., Battsetseg, B., Matsuo, T., Xuan, X. and Fujisaki, K. 2008. A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. *PLoS Pathog.* **4**: e1000062.
23. Turk, V. and Bode, W. 1991. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett.* **285**: 213–219.
24. Yamaji, K., Tsuji, N., Miyoshi, T., Islam, M.K., Hatta, T., Alim, M.A., Anisuzzaman, Takenaka, A. and Fujisaki, K. 2009. Hemoglobinase activity of a cysteine protease from the ixodid tick *Haemaphysalis longicornis*. *Parasitol. Int.* **58**: 232–237.
25. Yamaji, K., Tsuji, N., Miyoshi, T., Islam, M.K., Hatta, T., Alim, M.A., Anisuzzaman, M., Kushibiki, S. and Fujisaki, K. 2009. A salivary cystatin, HISC-1, from the ixodid tick *Haemaphysalis longicornis* play roles in the blood feeding processes. *Parasitol. Res.* **106**: 61–68.
26. You, M., Xuan, X., Tsuji, N., Kamio, T., Taylor, D., Suzuki, N. and Fujisaki, K. 2003. Identification and molecular characterization of a chitinase from the hard tick *Haemaphysalis longicornis*. *J. Biol. Chem.* **278**: 8556–8563.
27. Zhou, J., Ueda, M., Umemiya, R., Battsetseg, B., Boldbaatar, D., Xuan, X. and Fujisaki, K. 2006. A secreted cystatin from the tick *Haemaphysalis longicornis* and its distinct expression patterns in relation to innate immunity. *Insect Biochem. Mol. Biol.* **36**: 527–535.
28. Zhou, J., Liao, M., Ueda, M., Gong, H., Xuan, X. and Fujisaki, K. 2009. Characterization of an intracellular cystatin homolog from the tick *Haemaphysalis longicornis*. *Vet. Parasitol.* **160**: 180–183.