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Cathepsin L participates in the remodeling of the midgut through dissociation of midgut cells and activation of apoptosis via caspase-1

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ABSTRACT

The larval midgut in holometabolous insects must undergo a remodeling process during metamorphosis to form the pupal-adult midgut. However, the molecular mechanism of larval midgut cell dissociation remains unknown. Here, we show that the expression and activity of *Helicoverpa armigera* cathepsin L (Har-CatL) are high in the midgut at the mid-late stage of the 6th-instar larvae and are responsive to the upstream hormone ecdysone. Immunocytochemistry shows that signals for Har-CatL-like are localized in midgut cells, and an inhibitor experiment demonstrates that Har-CatL functions in the dissociation of midgut epithelial cells. Mechanistically, Har-CatL can cleave pro-caspase-1 into the mature peptide, thereby increasing the activity of caspase-1, which plays a key role in apoptosis, indicating that Har-CatL is also involved in the apoptosis of midgut cells by activating caspase-1. We believe that this is the first report that Har-CatL regulates the dissociation and apoptosis of the larval midgut epithelium for midgut remodeling.

1. Introduction

The insect midgut is an important organ for food digestion, nutrient absorption, and immune defense. In holometabolous insects, the larval midgut must undergo a remodeling process to form the pupal-adult midgut during larval-pupal development. Briefly, the midgut epithelial cells, commonly called columnar and goblet cells, undergo apoptosis and autophagy, and they are finally sloughed from the basement membrane into the lumen as the yellow body in the last larval molt (Hakim et al., 2010; Tettamanti et al., 2007; Uwo et al., 2002). Stem cells near the basement membrane differentiate into new epithelial cells to form the new pupal-adult midgut epithelium, and the yellow body is degraded to permit the recapture of its stored nutrients for pupal-adult development (Baldwin and Hakim, 1991; Hakim et al., 2010). Therefore, the morphological change of the midgut is a crucial physiological event in the insect life cycle. Although morphological change and cell death of the midgut have been studied widely (Hakim et al., 2010), the mechanisms for shedding midgut epithelial cells remain unknown.

Cysteine proteases are a class of ubiquitous lysosomal proteases that are considered to be involved in intracellular and extracellular protein degradation and processing in a number of organisms from bacteria to mammals (Berti and storer, 1995; Chapman et al., 1997; Medina et al., 1988). Among cysteine proteases, cathepsin L (CatL) is a representative protease that is involved in extensive degradation of proteins in human inflammatory diseases as atherosclerosis (Liu et al., 2006b), in *Caenorhabditis elegans* embryogenesis (Hashmi et al., 2002), and in insect embryogenesis, larval molting, and tissue remodeling (Liu et al., 2006a; Lustigman et al., 1996; Wang et al., 2010). Furthermore, lysosomes and cysteine proteases are well known to be involved in the promotion of programmed cell death (PCD) through the cleavage of Bid and the triggering of mitochondrial cytochrome *c* release (Repnik et al., 2012; Stoka et al., 2001) activation of caspase-3 (Zheng et al., 2008).

Steroid hormones are well known to trigger numerous physiological responses, and the typical steroid hormone 20-hydroxyecdysone (20E/ecdysone) in insects plays a central role in development, metamorphosis and tissue remodeling. During the larval-pupal transition, the replacement of the midgut is regulated by ecdysone in *Drosophila*, mosquitoes and lepidopteran insects (Cakouros et al., 2004; Nishiura et al., 2003; Parthasarathy and palli,

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2007). Mutations in the steroid-induced genes *BR-C*, *E93*, *E74A* and *E74B* inhibit destruction of larval midgut (Lee et al., 2002). Furthermore, these ecdysone-induced transcription factors trigger caspase-dependent PCD and autophagic death of midgut cells (Lee et al., 2000; Rusten et al., 2004). The effects of DRONC (initiator caspase) and DRICE (effector caspase) on PCD have been studied extensively in *Drosophila*, indicating that *dronc* and *drice*, upon up-regulation by ecdysone, play key roles in hormone-dependent cell death (Cakouros et al., 2002; Kilpatrick et al., 2005). However, only the activation of caspase-1 was reported in lepidopteran species (Courtiade et al., 2011). The *Spodoptera littoralis* caspase-1 (Sl-caspase-1) is an effector caspase, like caspase-3 in mammals and CED-3 in *C. elegans* (Liu et al., 2005). Thus, caspase-1 plays a crucial role in PCD in lepidopteran species.

In this paper, we examined the expression of cathepsin L in the 6th-instar larval midgut of the cotton bollworm, *Helicoverpa armigera* (Har). *H. armigera* cathepsin L (Har-CatL) is up-regulated by ecdysone at the mid-late stage of the 6th-instar larval midgut. Based on immunocytochemistry, positive signals of Har-CatL-like are present in midgut cells, and high expression of Har-CatL at the mid-late stage of the 6th-instar larvae is consistent with the shedding of midgut epithelial cells from the basement membrane into the lumen. An inhibitor experiment shows that Har-CatL functions in the dissociation of midgut epithelial cells. Further, knockdown of Har-CatL expression *in vitro* suppresses the activity of caspase-1, whereas overexpression of Har-CatL results in increased caspase-1 activity. Furthermore, Har-CatL produced via *in vitro* translation can cleave pro-caspase-1 into the mature peptide, thereby activating caspase-1. These results show that Har-CatL is involved in the dissociation of midgut epithelial cells and that Har-CatL participates in the PCD of midgut cells through activating caspase-1 in insects.

2. Materials and methods

2.1. Insect rearing

H. armigera larvae were reared on an artificial diet at 20 ± 1 °C with a photoperiodic cycle of L14: D10 (light: dark). The developmental stages were synchronized at the fifth larval ecdysis, and larvae remained in the 6th-instar for approximately 8 days under these rearing condition. Larval midguts were dissected in ice-cold 0.75% NaCl and stored at −80 °C until use.

2.2. Cell culture

HzAM1 cells from *Helicoverpa zea,* which is closely related to *H. armigera*, were cultured at 27 °C in Grace's insect medium supplemented with 10% fetal bovine serum (FBS) because no cell line has been established from *H. armigera.* Apoptosis was induced by the addition of 20-hydroxyecdysone (Sigma). Transfection and intracellular localization assays were performed as described previously (Bao et al., 2011).

2.3. RNA extraction and real-time PCR

Total RNA from larval midguts was isolated as reported previously (Lin et al., 2016a). Then, 1 μg of total RNA was reverse transcribed using the Prime Script RT reagent kit (TaKaRa, Dalian, China) in a reaction volume of 20 μl. Real-time quantitative PCR was performed with two primers, Har-CatL-F (5′-GGTACCTGGT GTCGCTGTCGG-3′) and Har-CatL-R (5′-GGCCTTCTCGGTGTC GATGCC-3′), using the SYBR *Premix Ex Taq* II detection system (TaKaRa, Dalian, China). Each PCR reaction, containing 0.4 μl of cDNA, 0.3 μl each of forward and reverse sequence-specific primers (stock 10 μM), 4 μl of H_2O and 5 μl of SYBR *Premix Ex Taq* II (2×), was mixed in a total volume of 10 μl. The PCR conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s. When the temperature reached 80 °C during the elongation phase of each PCR cycle, the fluorescence spectra were recorded.

2.4. Polyclonal antibody generation and western blot analysis

To generate polyclonal antibodies, *H. armigera* caspase-1 cDNA was amplified by PCR with two primers, Acas-1-F (5′-GTTGGACGGTGATGTTCAAG-3′) and Acas-1-R (5′-GCTGGTGATG-CAAGGAACCTGC-3′), according to a report by Yang et al. (2008), and an 847 bp PCR fragment was gel-purified and subcloned into the pMD18-T vector (TaKaRa). The positive clones were selected and used to express caspase-1 protein in BL21 cells by IPTG induction. Caspase-1 was purified using Ni-NTA columns, and recombinant caspase-1 protein was used to generate polyclonal antibodies in rabbits.

Protein from midguts or cells was extracted with ice-cold buffer (PBS, pH 7.0, 4 mM EDTA, 0.2% Triton X-100), and 30–40 μg of midgut protein or 10 μg of cell protein was separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Hybond-P, Millipore). The Har-CatL polyclonal antibody was used at a 1:2000 dilution (Liu et al., 2006a), and Caspase-1 antibody was used at a 1:3000 dilution. The secondary anti-rabbit antibody (Thermo) was used at a 1:3000 dilution, and signals were detected using an ECL system (Thermo).

2.5. Proteolytic activity assay for Har-CatL and caspase-1

Proteolytic activity of Har-CatL was assayed according to Liu et al. (2006a) and Zhang et al. (2013). Briefly, midgut protein (5 μg) was incubated at 37 °C for 10 min with 40 μl of Na_2HPO_4-citrate buffer (pH 4.4), containing 1.25 mM EDTA and 10 mM cysteine to activate the cysteine protease. Then, 5 μl of 1 mM Z-Phe-Arg-MCA (a specific substrate for cathepsins L and B) was incubated at 37 °C for 10 min. Finally, 50 μl of 10% SDS and 1 mL Tris-HCl buffer (pH 9.0) were added to terminate the reaction. Fluorescence was measured using excitation at 360 nm and emission at 460 nm. To inhibit proteolytic activity, the cysteine protease inhibitor E64d (a broad range inhibitor) (Sigma) and CLIK148 (a cathepsin L-specific inhibitor, but did not inhibit other cathepsins) (Katunuma et al., 1999) were used.

Proteolytic activity for caspase-1 was assayed according to the manufacturer's protocol (Promega, USA). Briefly, 5 μg of total protein from HzAM1 cells was incubated with 40 μl Caspase-GloR 3/7 Reagent for 1 h at 25 °C, and the luminescence was monitored using a multifunctional microplate reader (Perkin Elmer 2030).

2.6. Hematoxylin-eosin (HE) staining of tissue sections

Cathepsin inhibitor E64d or solvent (1% DMSO, 5 μl) was injected into 6th-instar larvae on day 5, and larval midguts were dissected on day 7 in ice-cold 0.75% NaCl and fixed in 4% paraformaldehyde overnight at 4 °C. After fixation and two washes in 1 × PBS, the midguts were dehydrated through a graded ethanol series (25, 50, 75, 95 and 100% ethanol in water), infiltrated in paraffin and finally embedded in three successive baths of Paraplast Plus (56 °C, Tyco Healthcare). Paraffin sections were sliced at 10 μm. The histologic sections were dewaxed with dimethylbenzene and gradient-rehydrated. Sections were stained with hematoxylin for 10 min at room temperature. Then, the sections were washed with running water. After these washes, the sections were stained with 0.5% eosin dye solution for 2 min, washed with running water,