Molecular Cloning of Two Caspase-Like Genes from the Hard Tick *Haemaphysalis longicornis*

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ABSTRACT. We identified two caspase-like genes from the midgut cDNA library of the hard tick, *Haemaphysalis longicornis*. Sequence analysis showed that these cDNAs encoded homologues of caspase-2 and caspase-8 that were categorized as apoptosis initiators. The *H. longicornis* caspase-2 (Hlcaspase-2) cDNA encodes 340 amino acid residues with a predicted molecular weight (Mw) of 38.5 kDa. Another cDNA identified as the *H. longicornis* caspase-8 (Hlcaspase-8) encodes 306 amino acid residues with an estimated Mw of 35.3 kDa. A catalytic active site was highly conserved in Hlcaspase-8 but not in Hlcaspase-2. RT-PCR analysis showed that both Hlcaspase-2 and Hlcaspase-8 were expressed in tick midgut and salivary glands. This is the first report of the molecular cloning of apoptosis-related genes in the tick.

KEY WORDS: apoptosis, caspase, *Haemaphysalis longicornis*.

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The hard tick, *Haemaphysalis longicornis*, is distributed mainly in East Asia and Australia [6, 7], where it transmits several pathogens, such as *Babesia* and *Theileria* protozoa, causing important diseases of domestic animals [20]. The economic injury caused by these tick-borne diseases on the cattle industry is the serious matter in various parts of the world. Various methods have been employed to suppress tick-borne diseases, including the application of biological control agents and chemical acaricides to control the tick vector population. However, the development of resistance to acaricides [26] and increases in legislation to combat the detrimental effects of acaricide residues on the environment [23] has emphasized the need to develop alternative methods for tick control. Elucidating the parasite-carrying mechanism of *H. longicornis* ticks is essential for the control of tick-borne diseases. Apoptosis plays a central role in the immune defense system of all multi-cellular organisms. Infection of the pathogen induces apoptosis of the host cell, chromatin condensation, DNA fragmentation, and blebbing of the plasma membrane, then, cells are phagocytosed by macrophages together with the pathogens [18]. In the malaria vector, the anopheline mosquito, it is reported that the invasion of the malaria parasite, *Plasmodium* spp., into the midgut epithelial cells triggers a series of toxic reactions that lead to apoptotic cell death [8, 9]. The invaded epithelial cells undergo apoptosis and are expelled toward the midgut lumen; as a result, the parasite has a time limit to escape unharmed to the basal side of the epithelium [15, 22]. Furthermore, several studies have shown that the invasion of malaria parasites into the anopheline midgut cells results in the activation of caspases and apoptosis [1, 3, 28]. The caspases belong to the cysteinyl aspartate-specific proteinase and acts as the central molecules of the initiation and execution of apoptosis [21, 25]. Currently, at least 14 caspases have been identified in mammals, and they are categorized into two types, apoptotic initiators, which include caspase-2 and -8, and apoptotic executioners, which include caspase-3 and -7 [24]. Caspases consist of large and small subunits (caspase family domains p20 and p10, respectively) and an extended N-terminal prodomain, and they also contain a conserved active site, QACXG, for the cleavage of the peptide bond C-terminal to the aspartate residues. All caspases are synthesized in cells as catalytically inactive zymogens and undergo proteolytic activation in the process of apoptosis. The activation of executioner caspases is performed by initiator caspases through cleavage at specific internal aspartate residues that separate the pro-domain and two subunits [11, 14], whereas initiator caspases are autoactivated as a result of death signal transduction [11, 17]. The caspases are highly conserved and have been identified in a wide range of metazoaons, from insects to mammals, but no homologous caspase genes have been identified to date in ticks. In the present report, the identification of two genes encoding caspase-like proteins from the midgut cDNA library of the hard tick *H. longicornis* was performed and the role of these proteins from the perspective of parasite control associated with apoptosis was discussed.

Two cDNA clones encoding caspase homologues were found in an expressed sequence tag (EST) database constructed from an *H. longicornis* (parthenogenetic Okayama strain) midgut cDNA library made from partially fed (4 days post-attachment) ticks [27]. Plasmids containing an insert encoding the estimated caspase gene were extracted from the corresponding clones, and the inserts were sequenced using the big dye terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA, U.S.A.) with the plasmid-specific primer pGCAPI-2 (5'-ACTGCTCCTCAGTGATGTT-3')
the cDNA targeted primers Hlcasp2 (5'-CCAAACTTTTC-3') and Hlcasp8 (5'-CATGTCGTCGAAAGAGAGGAAGA-3'), which were designed on the basis of the cDNA derived product sequences. The nucleotide sequences and deduced amino acid sequences are shown in Fig. 1. BLASTX analysis showed that these two cDNAs encoded the homologues of caspase-2 and caspase-8, respectively. The H. longicornis caspase-2 (Hlcaspase-2) cDNA had a 1023 bp-long open reading frame (ORF) encoding 340 amino acid residues with an estimated molecular weight (Mw) and theoretical isoelectric point (pl) of 35.3 kDa and 6.57. The deduced amino acid sequence showed 35% similarity with chicken caspase-2 (from DDBJ/ENBL/GenBank™ database, accession number AAC29881) and 34% with human caspase-2 (AAO25653). The H. longicornis caspase-8 (Hlcaspase-8) cDNA also has a 921 bp-long ORF encoding 306 amino acid residues corresponding to Mw 38.5 kDa and pl 5.9, and a similarity of 31% with catfish caspase-8 (AA137512) and 28% with human caspase-8 (AAD24962). The sequence data of Hlcaspase-2 and -8 has been submitted to the GenBank database under accession number DG666174 and DG666369, respectively. A domain structure analysis showed that Hlcaspase-2 had two potential N-glycosylation sites at amino acid residues 63–66 and 69–72 (Fig. 1A) and Hlcaspase-8 had three sites at 157–160, 193–196, and 273–276 (Fig. 1B). Furthermore, both proteins had two subunits, the caspase family domains p20 (large subunit) and p10 (small subunit), similarly to vertebrate caspases (Fig. 2A). The alignments of the deduced amino acid sequences of the large subunits of caspase-2 and caspase-8 including a cysteine active site are shown in Fig. 2B and 2C. As shown in these alignments, the large subunit of both proteins shared high sequence similarity with vertebrate caspases. However, in Hlcaspase-2, the most important active center sequence (QACRG) encompassing the catalytic cysteine residue was substituted (Fig. 2B). This result suggests that Hlcaspase-2 has no cysteine protease activity. It is conceivable that Hlcaspase-2 acts as a caspase analogue without enzymatic activity. In contrast, Hlcaspase-8 had a completely conserved cysteine active site sequence, QACRG (Fig. 2C).

Another structural characteristic of Hlcaspase-2 and -8 is the lack of a long amino-terminal pro-domain. Caspase-2 and caspase-8 are categorized as apoptosis initiators. Formerly known vertebrate initiator caspases have a long N-terminal prodomain that is more than 90 amino acids long, whereas the executioner caspases contain 20–30 residues in their pro-domain sequence [19]. Initiator caspases have a specific regulatory domain, such as CARD or DED (death effector domain) in the N-terminal pro-domain and interact with other molecules acting in the apoptotic signal transduction through these regulatory domains [5, 12]. Our sequence analysis suggested that the N-terminal pro-domain of Hlcaspase-2 was less than 20 amino acids long and that of Hlcaspase-8 was also less than 55 residues long (Fig. 2A). Furthermore, no regulatory domains were found in the pro-domain of either Hlcaspase-2 or -8. Although these two proteins were identified as homologues of caspase-2 and caspase-8 by BLASTX analysis, their structures were similar to those of executioner caspases rather than to those of initiator caspases. Otherwise, there is a possibility that the regulation of caspases during apoptotic signal transduction in the tick is completely different from the known pathways in vertebrate apoptosis.

To determine the expression profiles of Hlcaspases, RT-PCR analysis with total RNA samples from different tick organs, such as midgut, salivary glands, and ovary, was performed. Unfed, partially fed (1–4 days post-attachment to the rabbit), and engorged adult female ticks were dissected, and their organs were collected. Total RNA was extracted from the midgut, salivary glands, and ovary using TRI® reagent (Sigma-Aldrich Corporation, St. Louis, MO, U.S.A.) in accordance with the manufacturer’s protocol. RT-PCR was performed using a One-step RNA PCR kit (Takara, shiga, Japan) with one set of gene-specific primers: for Hlcaspase-2, sense primer: 5’-ATGCTGGAGTCCGT-TCCGCCC-3’ and anti-sense primer: 5’-CTACCAATC-CTTCACCCA-3’; for Hlcaspase-8, sense primer: 5’-ATGGGCAGAAAAGGAATCGCTA-3’ and anti-sense primer: 5’-CTAAAGGGGCGGAGTCTTAAAA-3’. The reverse transcription reaction was carried out at 50°C for 30 min, and PCR was repeated for 30 cycles under the following conditions: 30 sec of denaturation at 94°C, 30 sec of primer annealing at 60°C, and 1 min of elongation at 72°C. Positive control RT-PCR reactions were carried out using tick actin primers [27]. The results are shown in Fig. 3. In the midgut, both Hlcaspase-2 and -8 expressed ubiquitously, but they showed up-regulation during the initial phase of the blood-sucking period (1–4 days post-attachment) and then decreased in the engorged tick (Fig. 3A). Densitometric analysis of PCR products showed that the expression level of Hlcaspase-8 mRNA was higher than that of Hlcaspase-2 and significantly up-regulated in the partially fed ticks (Fig. 4). It has been known that tick midgut epithelial cells are frequently renewed during the blood-sucking period [2]. Digestive cells are expelled toward the midgut lumen and finally lysed in the lumen during this process [2]. Given apoptosis, it is reasonable that Hlcaspases are highly expressed in the midgut of partially fed ticks. In the salivary glands, Hlcaspases showed low-level expression only in the initial phase of the blood-sucking period (1–3 days post-attachment) (Fig. 3B). It has been suggested from morphological evidence that tick salivary glands also undergo degeneration induced by apoptotic cell death [16]. However, this event is limited shortly after completing the blood meal. Therefore, it can be thought that expression of apoptosis related molecules in the salivary glands is not constant and that Hlcaspases might express ahead of the apoptotic cell death of salivary glands. On the other hand, the ovary showed no expression of Hlcaspases in the unfed and blood-sucking ticks (Fig. 3C). It is estimated that apoptotic cell death does not occur in this period. In fact, it was observed that the tick ovary became enlarged during the blood-sucking period.
Fig. 1. Nucleotide and deduced amino acid sequences of *H. longicornis* caspase cDNAs. (A) *H. longicornis* caspase-2 (Hlcaspase-2) cDNA contains a 1023 bp-long ORF encoding 340 amino acid residues. (B) *H. longicornis* caspase-8 (Hlcaspase-8) cDNA has a 921 bp-long ORF encoding 306 amino acid residues. Potential glycosylation residues (N) are indicated in bold letters. A conserved cysteine active site (QACXG) is underlined.
In this study, expression profiling of two Hlcaspases was performed without parasite infection. The most interesting point is how the expression of caspases is regulated by parasite invasion in the midgut and salivary glands. Hlcaspase-2 is presumed a caspase analogue without enzymatic activity because of the amino acid substitution in the catalytic cysteine active site (Fig. 2B). It has been reported that some pathogens inhibit host cell apoptosis through the expression of antiapoptotic factors in order to increase their chances of survival [4]. Interestingly, Trypanosoma parasites that cause Chagas’ disease employ the host antiapoptotic protein, c-FLIP, for the inhibition of apoptosis [10]. c-FLIP was identified as a homologue of FLICE (caspase-8) [13]. FLICE changes to the active form and proteolytically activates the executioner caspases [11, 14, 17]. However, since c-FLIP has no protease activity, downstream caspases can-
not be activated, and the apoptosis signal transduction is interrupted [13]. It is expected that Hlcaspase-2 also acts as an apoptosis inhibitory molecule and plays an important role in the control of parasite invasion in midgut cells.

In conclusion, this is the first report of the molecular cloning of apoptosis-related genes in Chelicerata. Two genes encoding caspase-like proteins, Hlcaspase-2 and Hlcaspase-8, were expressed in tick midgut and salivary glands. Sequence analysis showed that a catalytic cysteine active site was conserved in Hlcaspase-8 but not in Hlcaspase-2. Furthermore, these caspases showed a structural variation from the vertebrate caspase-2 and -8. Since Hlcaspase-2 and -8 lack a long amino-terminal pro-domain that includes an essential regulatory domain for the apoptosis initiator caspases, their function and behavior in the apoptosis signaling cascade probably differ from those of known initiator caspases. It needs more detailed examination to elucidate the involvement of Hlcaspases in tick apoptosis.

Apoptosis is an important key event for the control of pathogen invasion in a host cell. From this point of view, future investigations of tick caspases related to parasite infection will provide important information for the suppression of tick-bone diseases.

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