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BmICE-2 is a novel pro-apoptotic caspase involved in apoptosis in the silkworm, *Bombyx mori*

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ABSTRACT

In this study we identified a potential pro-apoptotic caspase gene, *Bombyx mori(B. mori)ICE-2* (*BmICE-2*) which encoded a polypeptide of 284 amino acid residues, including a ^{169}QACRG^{173} sequence which surrounded the catalytic site and contained a p20 and a p10 domain. BmICE-2 expressed in *Escherichia coli* (*E. coli*) exhibited high proteolytic activity for the synthetic human initiator caspase-9 substrates Ac-LEHD-pNA, but little activity towards the effector caspase-3 substrates Ac-DEVD-pNA. When BmICE-2 was transiently expressed in BmN-SWU1 silkworm *B. mori* cells, we found that the high proteolytic activity for Ac-LEHD-pNA triggered caspase-3-like protease activity resulting in spontaneous cleavage and apoptosis in these cells. This effect was not replicated in *Spodoptera frugiperda* 9 cells. In addition, spontaneous cleavage of endogenous BmICE-2 in BmN-SWU1 cells could be induced by actinomycin D. These results suggest that *BmICE-2* may be a novel pro-apoptotic gene with caspase-9 activity which is involved apoptotic processes in BmN-SWU1 silkworm *B. mori* cells.

1. Introduction

Apoptosis is a tightly controlled cell death process leading to elimination of unwanted, damaged or dangerous cells [1]. Diverse apoptotic stimulus trigger apoptosis through intrinsic or extrinsic pathway, which converges on the activation of the cysteine protease (caspase) cascade [2,3]. Caspases are a family of intracellular cysteine proteases that play a central role in cell apoptosis. Apoptotic caspases can be divided into initiator caspases and effector caspases depending on their placement within the apoptotic signal transduction cascade [4,5]. Caspases are normally present in cells as inactive zymogens or bound to apoptotic inhibitors, such as inhibitor of apoptosis protein (IAP) [3,6]. Caspase zymogens comprise three domains, an N-terminal prodomain, a large (p20) domain and a small (p10) catalytic domain [6]. Caspase-9 belongs to the initiator caspases. It is the apical caspase in the cytochrome c pathway for which directly or indirectly activate effector caspases which include caspases-3, -6 and -7 [7,8]. Activated effector caspases cleave intracellular substrates such as lamins and cytokeratins, which are key structure proteins. Therefore caspases are directly implicated in many of the morphological and biochemical features associated with apoptosis, including plasma membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation [6,8,9].

The domesticated silkworm, *Bombyx mori (B. mori)*, is also employed as model system for *lepidopterans* [10]. We previously reported that both intrinsic and extrinsic apoptotic pathways exist in *B. mori* and that the mitochondrial apoptotic pathway might be functional [11,12]. To date, five members of the caspase family have been reported in *B. mori* including two initiators (*BmDredd* and *BmDronc*) and three effectors (*BmCaspase-1*, *BmICE* and *BmCaspase-N*) [11]. *BmDronc* was implicated in caspase-dependent apoptosis [13]. Studies have shown that IAPs are inactivated by specific antagonists that include members of the RHG protein family when cells cannot survive or are condemned to die [14]. The *Drosophila melanogaster* reaper homolog *IBM1* has been shown to function as an RHG protein *B. mori* [15]. Actinomycin D (Act D) is a DNA-damaging agent which binds to DNA and inhibits synthesis of RNA and protein. It has been shown to be a potent inducer of apoptosis in a variety of cells in vitro and in vivo and to induce extensive and rapid apoptosis in insect cells, including *Lepidopteran* cells [16–19]. We previously reported that Act D triggered apoptosis in the BmE-SWU1 *B. mori* cell line [20]. Despite these reports,

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research into the apoptotic mechanisms in silkworms has been limited compared to those in *Drosophila*. BmICE (GeneBank accession: AY88522), BmICE-2 (GeneBank accession: DQ360829) and BmICE-5 (GeneBank accession: DQ360830) contain 275, 284 and 312 amino acids, respectively. According to the 9× silkworm genome database, they each have the same translation and termination sites and belong to the effector caspase subfamily [11]. It has been suggested that *BmICE-2* might be involved in H_2O_2 and UV-induced apoptosis [21], but it was unclear whether *BmICE-2* functioned as an apoptotic gene in *B. mori* cells.

The apoptotic mechanism in *B. mori* remains poorly understood. Therefore we cloned the *BmICE-2* gene and expressed its proteins in *Escherichia coli* cells. The BmICE-2 proteins exhibited high human caspases-9-like protease activity, underwent spontaneous cleavage and triggered apoptosis in BmN-SWU1 *B. mori* cells. Furthermore, Act D-induced apoptosis promoted spontaneous cleavage of endogenous BmICE-2 in these cells. These results suggest that *BmICE-2* may be a novel pro-apoptotic caspase involved in the apoptotic process in BmN-SWU1 silkworm *B. mori* cells.

2. Materials and methods

2.1. Cells, animals and transfection

(*B. mori*) larvae p50T strain (Dazao) were provided by the Institute of Sericulture and Systems Biology, Southwest University, Chongqing, China. They were reared with fresh mulberry leaves in the laboratory at 25 °C under 14 h light/10 h dark cycles. The BmN-SWU1 cell line derived from *B. mori* ovarian tissue [22] and Sf9 cell line derived from *Spodoptera frugiperda* pupal ovarian tissue [23] were maintained at 27 °C in TC-100 (UsBiological) and Grace insect medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories), penicillin G (200 U/ml) and streptomycin sulfate (200 U/ml).

Monolayer cultures of BmN-SWU1 cells (10^6 cells/well) and Sf9 cells (10^6 cells/well) were seeded in 6 well plates (3516, Costar Corning) for 6 h in their respective culture media. The medium was discarded after 8 h and replaced with serum-free media without antibiotics. Cells were transiently transfected using 2 μg of plasmid and 8 μl of liposome (Roche) and incubated for 8 h. The transfection mixtures were replaced with the corresponding culture media.

2.2. Cloning of B. mori ICE-2 (BmICE-2) and plasmid construction

Total RNA was extracted from BmN-SWU1 cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg total RNA by AMV Reverse Transcriptase (Promega) according to the manufacturer's protocol. *BmICE-2* cDNA was amplified by PCR with primers F1 and R1(Table S1) and cloned into PGEM-T-Simple vector (Takara) and sequenced. The *BmICE-2* was cloned into BamHI/Sal I site of pET-28a(+) with primers F2 and R2 (Table S1) to construct pET-28a(+)/BmICE-2 with a His-tag at the C- and N-termini. The *BmICE-2* subcloned into BamHI/EcR I site of PIZ/V5-dsRed vector to construct PIZ/V5-HA-BmICE-2-dsRed with hemagglutinin (HA) tag at the N-terminus with primers F3 and R3 (Table S1) for transient expression of BmICE-2 in BmN-SWU1 and Sf9 cells.

2.3. Peptide antibody production and expression of BmICE-2 in E. coli

The BmICE-2-Peptide (CTYTPKCQETNNK; amino acid residues 255–266 of BmICE-2) coupling KLH carrier protein, termed BmICE-2-Peptide-KLH, was synthesized in an Applied Biosystems 430 large-scale peptide synthesizer using standard Fmoc chemistry. BmICE-2-Peptide-KLH proteins were injected into New Zealand white rabbits for antiserum production (anti-ICE-2) by Zoonbio Biotechnology (China). BmICE-2 was expressed in *E. coli* strain BL21(DE3)pLysS competent cells (Transgene Biotech; China). *E. coli* cells transformed with pET-28a(+)/BmICE-2-His were grown at 37 °C in 500 ml of LB medium containing kanamycin (100 μg/ml) to an absorbance of 0.6 at A600 nm. Protein expression was induced by adding 0.8 mMol/L isopropyl-1-thio-β-galactopyranoside (IPTG) at 16 °C and the cells was cultures for an additional 20 h.

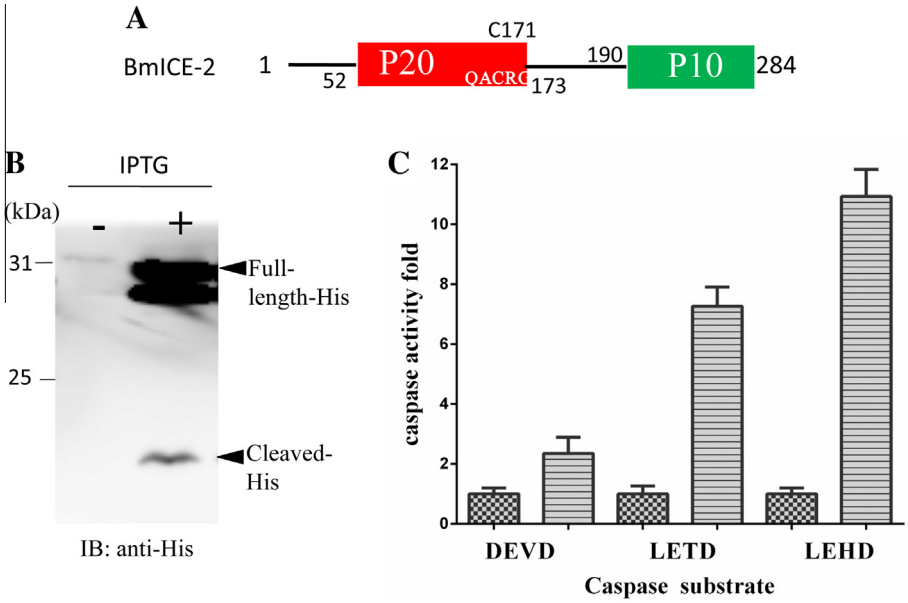

Fig. 1. Predicted protein domains and caspase activity in BmICE-2 expressed in *E. coli*. (A) The caspase p20 and caspase p10 domains were predicted by InterProScan v.4.8. The QACRG protein sequence is in the large subunit. The sequence numbers indicate the start and end of the domains. (B) Immunoblot analysis shows the BmICE-2 expression levels. The arrowheads indicate the full-length and cleaved BmICE-2 proteins. The diagrams on the right show the probable p10 and p20 sites. (C) Fluorometric analysis shows caspase activity with Ac-DEVD-pNA, Ac-LETD-pNA, or Ac-LEHD-pNA synthetic substrates for human caspase-3-8 and -9, respectively. Caspase activities are normalized to those in empty vector cells. The error bars represent standard deviations of the means from three independent.

The *E. coli* cells containing soluble protein of BmICE-2 were stored at −80 °C and prepared the cell lysates as described previously [13].

2.4. Hoechst 33258 staining and double-staining immunofluorescence

Nuclei were stained with Hoechst 33258 (C0003; Beyotime Biotech; China) for indirect immunofluorescence assay (IFA). BmN-SWU1 cells transiently expressing *BmICE-2* or *dsRed* gene, and those that has been treated with 150 ng/ml Act D, were washed with PBS and prepared according to the manufacturer's protocol and examined as previously described [24]. The transfected BmN-SWU1 cells (BmICE-2 or mock dsRed) were cultured for 36 h, 48 h or 72 h, washed once with ice-cold PBS and fixed with a 4% paraformaldehyde for 20 min at room temperature, incubated with Anti-lamin A + C (anti-Lamin) rabbit monoclonal antibody (bs-1839R; Bioss Biotechnology; China) and the proteins were detected by laser confocal microscope as previously described [25].

2.5. Immunoblotting

SDS–PAGE and immunoblot analysis (Western blotting) were performed as previously described [25]. Briefly, Polypeptides were separated by 12% or 15% SDS–PAGE and electrophoretically transferred to PVDF membranes. The membranes were incubated for 2 h at room temperature with the respective antibodies and the protein bands were visualized using enhanced chemiluminescence ECL reagents (Roche) and analyzed by ImageQuant LAS4000 imaging system (GE healthcare). Blue PlusTM III protein marker and EasySeeTM western marker (TransGene Biotech, China) were used as protein markers.

2.6. Treatment of BmN-SWU1 cells with actinomycin D (Act D)

The BmN-SWU1 cells were treated with actinomycin D (Act D) (Sigma–Aldrich) at a final concentration of 150 ng/ml. The cells were harvested at 12 h, 24 h or 36 h after treatment, stained with Hoechst 33258 and their proteins were visualized by immunoblotting. The caspase inhibitor Z-Val-Ala-Asp-(OMe)-Fluorome thylketone (Z-VAD-FMK) (G7231; Promega) was added to selected cells to a final concentration of 20 μM in TC-100 insect medium containing 10% FBS.

2.7. Caspase activity assay

BmN-SWU1 cells were cultured in 96-well plates (3610; Corning) at a density of 3×10^4 cells/well. The caspase activities in cell lysates collected from the BmN-SWU1 cells and *E. coli* cells were assayed fluorometrically using a GloMaxR-Multi Dection System (Promega), according to the manufacturer's protocol. The enzyme

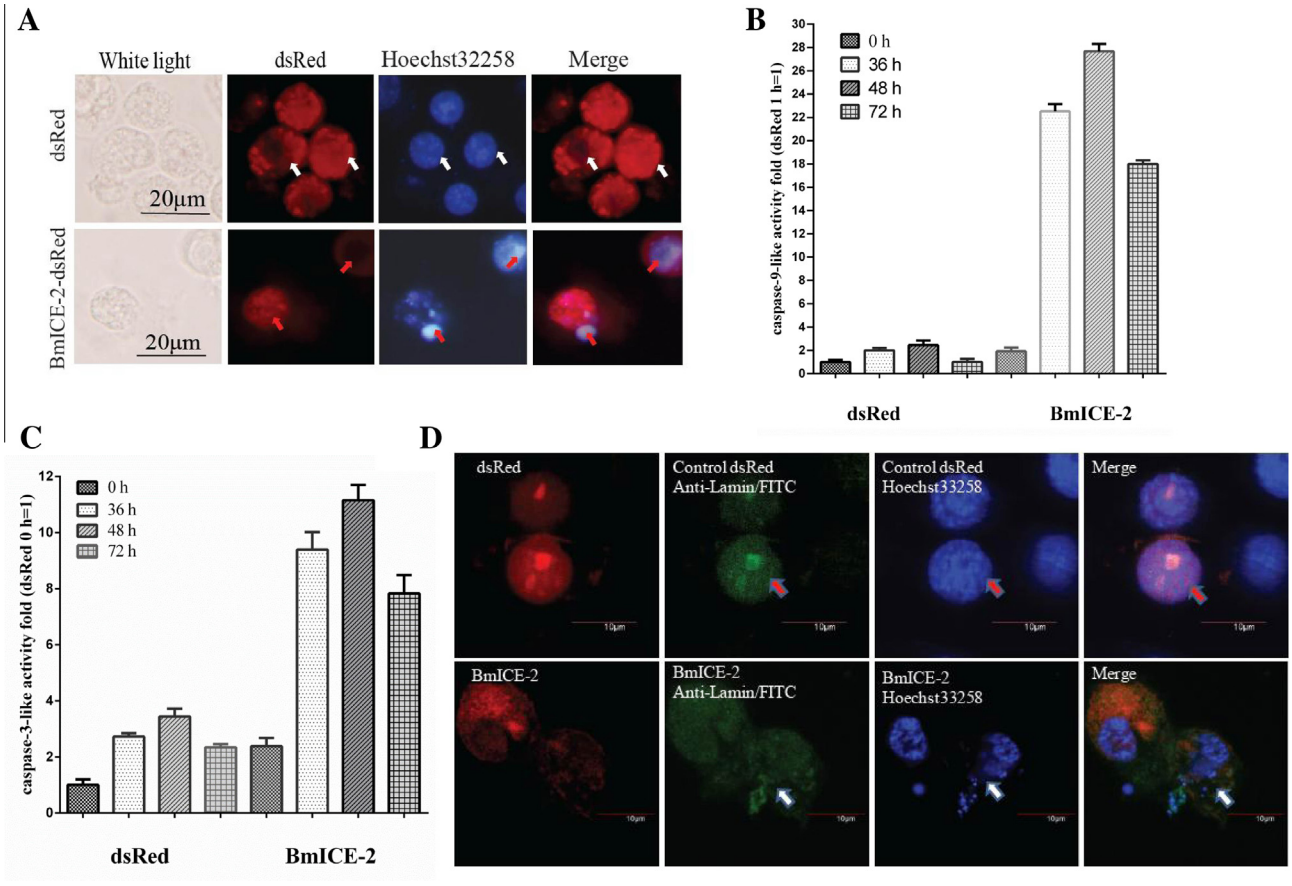

Fig. 2. Transiently expressed BmICE-2 undergoes proteolytic processing and triggers apoptosis in BmN-SWU1 cells. BmN-SWU1 cells were transfected with plasmids containing Bm-ICE-2 with an N-terminal HA-tag (BmICE-2) or with fluorescent red protein dsRed (dsRed) and examined at 0 h, 48 h and 72 h post-transfection. (A) Fluorescent microscopy reveals apoptotic cells showing characteristic irregular Hoechst 33258 staining (blue). Apoptotic nuclei are indicated by red arrows, control cells are indicated by white arrows. Scale bar represents 20 μm. (B and C) Caspase-3 and caspase-9-like protease activity were determined using Ac-DEVD-pNA and Ac-LEHD-pNA substrates, respectively. The results are normalized to the activity in dsRed cells (0 h post-transfection). The error bars represent standard deviations of the mean from three independent experiments. (D) Changes in nuclear lamins are associated with increased levels of active caspases. The fluorescent micrographs show BmN-SWU1 cells stained with antibodies against nuclear lamins (Green). The nuclei are labeled with Hoechst 33258 (blue). Cleaved nuclear lamins are indicated by white arrows and controls are indicated by red arrows. Scale bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reaction was performed for 60 min at 37 °C in a reaction mixture containing 100 μl cell lysates and an equal volume of reagent. The synthetic pNA tetrapeptides substrates, including Ac-DEVD-pNA, Ac-LETD-pNA, Ac-LEHD-pNA were all purchased from Promega.

2.8. Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from BmN-SWU1 cells which had been treated with Act D or from untreated cells (mock). Expression of *BmICE-2* (DQ360829) and *BmIAP* (NM_001043559) were evaluated by real-time quantitative reverse transcription PCR (qRT-PCR), as previously described [26]. The primers are listed in Table S1. *B. mori Actin A3* (NM_001126254) was used as an internal control. Relative gene expression ($2^{-\Delta\Delta CtA,B}$) was calculated according to the equation of $2^{-\Delta Ct}$, where ΔCt is the difference between the CT (cycle threshold) values of the target genes and BmActinA3 genes. Each experiment was performed in triplicate.

3. Results

3.1. Cloning and substrate specificity of BmICE-2 expressed in E. coli

The full length *BmICE-2* cDNA contains a continuous ORF encoding a protein of 284 amino acids residues with a predicted molecular mass of 32,720 Da. As shown in Fig. 1A, a motif search with InterProScan v. 4.8 revealed that the deduced *B. mori* BmICE-2 polypeptide has a short prodomain (amino acid residues 1–51) with a characteristic QACRG sequence in the large subunit (P20; amino acid residues 52–173,) and a small subunit (P10; amino acid residues 190–284). The QACRG sequence matches the sequence surrounding the catalytic cysteine (C171), which is consistent with the consensus sequence QAC(R/Q/G)(G/E) found in most caspases (Fig. 1A).

Examination of the polypeptides in transformed *E. coli* cells expressing BmICE-2 with C- and N-terminal His-tag, demonstrated that IPTG treatment induced a major polypeptide of approximately 32 kDa and two minor polypeptides of approximately 28 kDa and 13 kDa (Fig. 1B). Based on the knowledge that caspases were primarily cleaved, the 32, 28 and 13 kDa polypeptides were considered to represent full-length BmICE-2, cleaved BmICE-2 and the small subunit containing His-tags at the C-terminus, respectively (Fig. 1B).

To determine whether the recombinant BmICE-2 expressed in *E. coli* possessed caspase activity, cell lysates from *E. coli* cells expressing BmICE-2 were examined using different synthetic human caspases substrates. The results showed that BmICE-2 had high proteolytic activity for the caspase-9 Ac-LEHD-pNA substrate (Fig. 1C). However, less caspase activity was observed for caspase-3 Ac-DEVD-pNA and caspase-8 Ac-LETD-pNA substrates.

3.2. Transient expression of BmICE-2 induces apoptosis in BmN-SWU1 cells

BmN-SWU1 cells transfected with PIZ/V5-HA-BmICE-2 or PIZ/V5-dsRed plasmids were examined by fluorescent microscope to determine the level of apoptosis. The nuclei of cells transiently expressing BmICE-2 exhibited irregular Hoechst 33258 staining, which is characteristic of apoptotic cells (Fig. 2A). In order to determine whether recombinant BmICE-2 expressed in BmN-SWU1 cells possessed caspase activity, various synthetic human caspases substrates were employed. The results demonstrated that BmN-SWU1 cells transfected with BmICE-2 had high proteolytic activity for the caspase-9 Ac-LEHD-pNA substrate (Fig. 2B), which triggered the proteolytic activity towards the caspase-3 Ac-DEVD-pNA substrate (Fig. 2C). Nuclear lamins have been considered as caspase substrates and have been implicated with increased caspase-3 activity leading to cell apoptosis [27]. Therefore, we investigated whether changes in the levels and localization of nuclear lamins

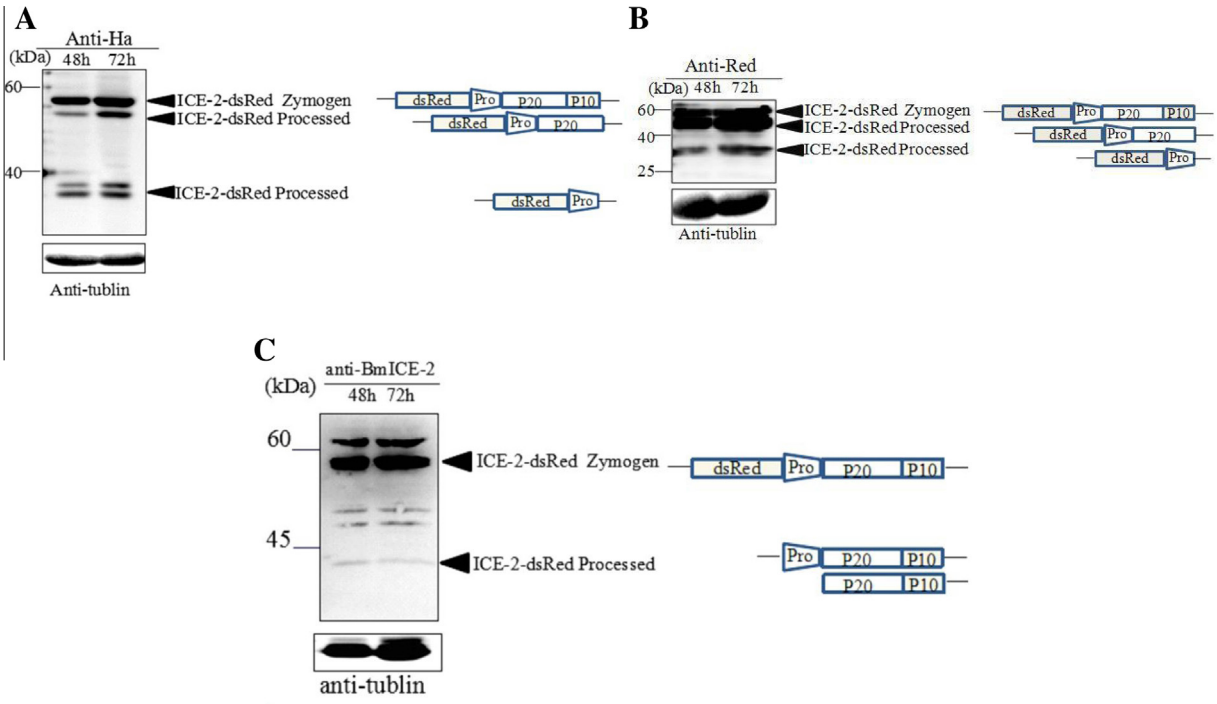

Fig. 3. Transiently expressed BmICE-2 undergoes proteolytic processing in BmN-SWU1 cells. (A–C) Expression of Bm-ICE-2 analyzed by immunoblotting using anti-Ha mAb, dsRed mAb and anti-Bm-ICE-2, respectively. The arrowheads indicate full-length and cleaved BmICE-2 proteins. The diagrams on the right indicate probable proteolytic protein sites based on the predicted domain structure. Anti-tubulin was used as a loading control.

in BmN-SWU1 cells were associated with increased levels of active caspase. Fluorescent staining showed that 72 h post-transfection, cells transfected with BmICE-2 had increased levels of cytoplasmic nuclear lamins compared to control cells transfected with dsRed. These results indicated that cleaved lamins and active caspase-3 were abundant in the cytoplasm of BmICE-2-transfected BmN-SWU1 cells (Fig. 2D). These findings indicated that transient expression of BmICE-2 could promote the degradation and localization of nuclear lamins in BmN-SWU1 cells and trigger caspase-3 proteolytic activity.

3.3. Enzymatic activity of BmICE-2 transiently expressed in BmN-SWU1 cells

To further examined whether transient expressed BmICE-2 underwent proteolytic processing in BmN-SWU1 cells, we transfected the cells with PIZ/V5-HA-BmICE-2-dsRed (BmICE-2) or PIZ/V5-dsRed (dsRed) plasmids. Immunoblot analysis of the cell lysates collected 48 h and 72 h post-transfection revealed one major polypeptides at approximately 58 kDa and the two minor polypeptides at approximately 52 kDa and 32 kDa (Fig. 3A and B). Based on the predicted molecular masses 32 kDa for BmICE-2 and 25 kDa for dsRed, these observations indicated that the BmN-SWU1 cells expressed a fused BmICE-2-dsRed protein, representing the full-length BmICE-2-dsRed protein and the large subunit containing the N-terminus Ha-tags. In addition, the fused BmICE-2-dsRed protein, the cleaved BmICE-2 protein and the full-length protein cells could also be recognized in the BmICE-2 antibody (Fig. 3C). The results indicated that transiently expressed BmICE-2 underwent proteolytic processing in BmN-SWU1 cells.

3.4. Apoptosis in BmN-SWU1 cells induced by Act D accompanies BmICE-2 cleavage

To determine whether endogenous BmICE-2 underwent proteolytic processing in BmN-SWU1 cells during Act D-induced apoptosis, untreated BmN-SWU1 cells (mock) and BmN-SWU1 cells exposed to Act D were examined for endogenous BmICE-2. Changes in Hotechest32258 staining patterns demonstrated that apoptosis was significant 12 h, 24 h, and 36 h after exposure to Act D (Fig. S2). QRT-PCR at the same time points showed that BmICE-2 was significantly upregulated >16-folds compared to mock controls (Fig. 4A). In contrast, *BmIAP* expression was downreguated >10-folds (Fig. 4B). Immunoblot analysis was performed to test whether BmICE-2 was involved in Act D-induced apoptosis. The results showed that the BmN-SWU1 cells exposed to Act D

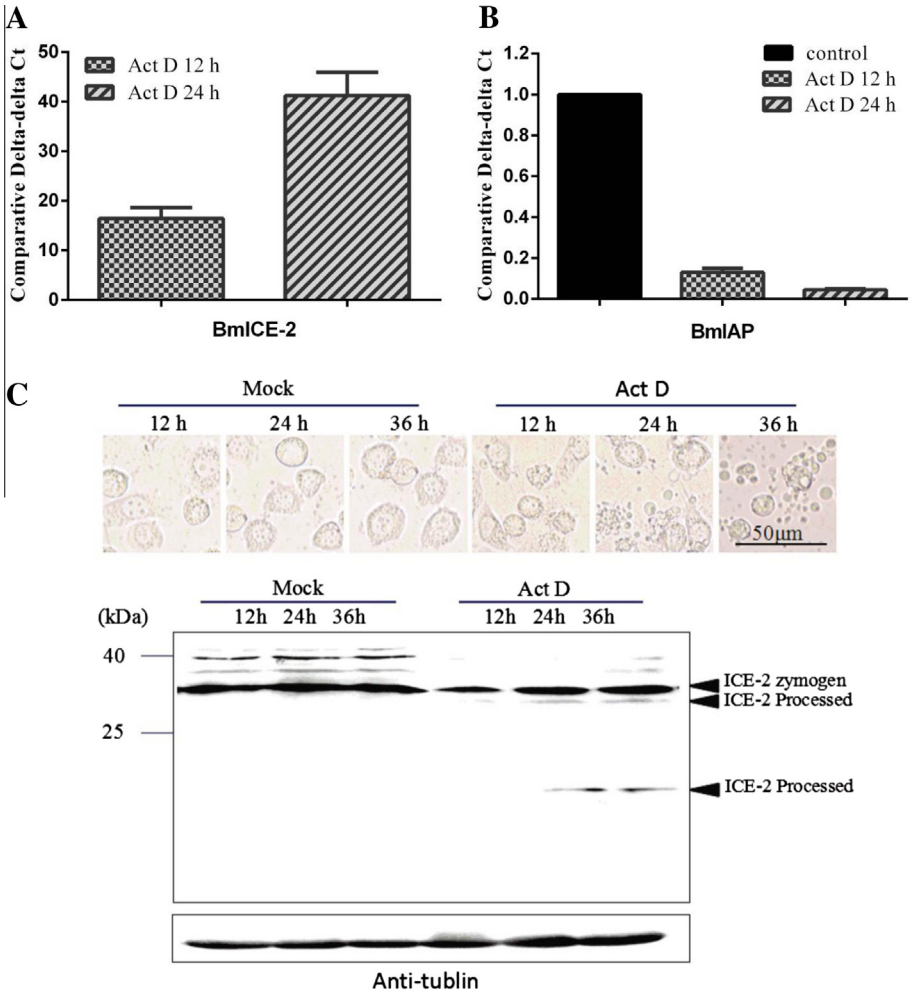

Fig. 4. BmICE-2 undergoes cleavage in BmN-SWU1 cells exposed to Act D. (A and B) qRT-PCR analysis at 12 h, 24 h and 36 h post-exposure to Act D shows the induction of apoptosis, and the upregulation of BmICE-2 and downregulation of BmIAP, respectively. Statistical differences were determined by Student's *t*-test based on three independent experiments. (C) The micrographs and immunoblot analysis compare BmICE-2 expression at 12 h, 24 h and 36 h post-exposure to Act D. The micrographs show the un-exposed (mock) cells and exposed cells. Scale bars = 50 μm. The immunoblots in the lower panel show the full-length and cleaved BmICE-2 proteins, indicated by the arrowheads. Anti-tubulin was used as a loading control.

contained a small, but significant, amount of cleaved BmICE-2 24 h after exposure (Fig. 4C). These results indicated that apoptosis induced by Act D was accompanied by expression of BmICE-2.

3.5. Transient expression of BmICE-2 does not induces apoptosis in Sf9 cells

To determine whether transiently expressed BmICE-2 induced apoptosis in other *Lepidoptera* cell line, PIZ/V5-HA-BmICE-2-dsRed was transfected into *S. frugiperda* Sf9 cells. Although BmICE-2 was successfully expressed and underwent proteolytic processing in Sf9 cells (Fig. S3A), no apoptosis and caspase-3-like protease activity occurred (Fig. S3B and C). These results demonstrated that the expression of BmICE-2 was not involved in the stimulation of effector caspases or the induction of apoptosis in Sf9 *S. frugiperda* cells.

4. Discussion

In this study, we demonstrated that *BmICE-2* was involved in apoptotic processes in BmN-SWU1 cells derived from the silkworm, *B. mori*. BmICE-2 expressed in *E. coli* cells exhibited high proteolytic caspase-9-like activity. In BmN-SWU1 cells, transiently expressed underwent spontaneous cleavage which triggered caspase-3 activities and induces apoptosis. Further investigations in BmN-SWU1 cells transiently expressing BmICE-2 confirmed that BmICE-2 underwent proteolytic processing in these cells: the transiently expressing cells exhibited high proteolytic caspase-9 and caspase-3 activity, and the structure nuclear lamins were found to be damaged and translocate to the cytoplasm. These findings suggested that BmICE-2 might be an active caspase or trigger other proteins involved in the cleavage of nuclear lamins in BmN-SWU1 cells.

Our results also indicated that BmICE-2 underwent spontaneous cleavage. Only full-length BmICE-2 was detected in mock cells; whereas both full-length BmICE-2 and its cleaved form were detected in BmN-SWU1 cells undergoing apoptosis. These findings suggested that BmICE-2 is present in BmN-SWU1 cell as inactive zymogens and is activated by proteolytic processing in response to apoptotic stimuli. BmICE-2 displays a high level of proteolytic cleavage activity for the LEHD substrate of human caspases-9. This substrate-specificity of BmICE-2 is similar to that of the initiator caspase BmDronc [13]. As BmCaspase-1 clusters into the effector caspase family with DRICE and DCP-1 of *Drosophila melanogaster*, therefore the evolutionary relationship between *B. mori* and *D. melanogaster* has resulted in additional caspase family members, BmICE and BmICE-2 and BmICE-5, being found in *B. mori* [11]. Our results indicated that BmICE-2 may act as an initiator caspase and is involved in apoptosis in BmN-SWU1 cells, suggesting that alterations in some of these genes occurred after *B. mori* diverged from the common ancestor. This may have led to a divergence in apoptosis regulators between *B. mori* and *D. melanogaster*. Consequently, the apoptotic mechanism in *drosophlia* and mammal cannot accurately reflect the regulatory mechanism in *B. mori*, therefore further investigation in *B. mori* is required.

BmIAP has been implicated in the inhibition of an upstream initiator caspase in the conserved apoptotic mitochondria pathway [28]. Our data showed that expression of *BmIAP* was downregulated >10-folds in BmN-SWU1 cells after exposure to Act D; in contrast, *BmICE-2* was significantly upregulated >16-folds compared to mock cells. The molecular mechanism of regulator apoptosis induced by Act D in BmN-SWU1 is not yet clear, but our results indicated that Act D triggered the activation of *BmICE-2* at the mRNA level in BmN-SWU1 cells. Whether BmIAP inhibited the cleavage of BmICE-2 in BmN-SWU1 cells, and the precise mechanism underlying the interaction between BmICE-2 and BmIAP, remain to be investigated. *BmDronc* plays an important role in silkworm apoptosis as an initiator caspase [13]. Our results suggested that *BmICE-2* played a similar role in the apoptotic process in BmN-SWU1 cells. However, this effect may have been induced by other initiator or effector caspases that are involved in Act D apoptosis. In addition, our results show that the endogenous BmICE-2 existed as an uncleaved full-length molecule in BmN-SWU1 cells and underwent cleavage during apoptosis exposure to Act D. This further demonstrate that BmICE-2 acted as a pro-apoptotic caspase and was involved in Act D-induced apoptosis in BmN-SWU1 cells.

Conversely, our results demonstrated that BmICE-2 did not trigger caspase-3-like protease activity and apoptosis in Sf9 cells, indicating that BmICE-2 was unable to activate the effector caspase in *S. frugiperda*. This may provide important insights into differences between the structure, funcation and divergence of BmICE-2 in *B. mori* and *S. frugiperda*, and suggests that each insect family may possess a unique mechanism of apoptotic regulation. Initiator and effector caspase in *Drosophila* and mammalians play important roles in the development of their respective organisms [3]. By analogy, and based on our findings in this study, we propose that *BmICE-2* plays a critical role in silkworm development. However, further studies will be necessary to elucidate the biological significance of the *BmICE-2* gene.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.139.

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