Role of \textit{BmDredd} during Apoptosis of Silk Gland in Silkworm, \textit{Bombyx mori}

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Abstract

Silk glands (SGs) undergo massive apoptosis driven degeneration during the larval-pupal transformation. To better understand this event on molecular level, we investigated the expression of apoptosis-related genes across the developmental transition period that spans day 4 in the fifth instar \textit{Bombyx mori} larvae to day 2 pupae. Increases in the expression of \textit{BmDredd} (an initiator caspase homolog) closely followed the highest \textit{BmEcR} expression and resembled the expression trend of \textit{BmIcE}. Simultaneously, we found that \textit{BmDredd} expression was significantly higher in SG compared to other tissues at 18 h post-spinning, but reduced following injection of the apoptosis inhibitor (Z-DEVD-fmk). Furthermore, \textit{BmDredd} expression correlated with changes of caspase3-like activities in SG and RNAi-mediated knockdown of \textit{BmDredd} delayed SG apoptosis. Moreover, caspase3-like activity was increased in SG by overexpression of \textit{BmDredd}. Taken together, the results suggest that \textit{BmDredd} plays a critical role in SG apoptosis.

Introduction

Programmed cell death (PCD) is a vital biological event accompanied by characteristic events including chromatin condensation, DNA fragmentation, vacuolization, and apoptotic body formation [1]. Competence of PCD is achieved by the activation of the cysteine protease (caspase) cascade. Therefore, caspases are indispensable essential during cell death [2, 3]. In insects, larval specific tissues are eliminated through PCD [4], which is triggered by endocrine hormones that lead to activation or repression of a succession of intracellular factors [5–7].

Silkworm \textit{Bombyx mori} is an important economic insect and model organism which has been used effectively in various biological researches [8]. The economic importance of silkworms is dependent on the functionality of the silk gland (SG), a specialized organ that synthesizes silk proteins in the silkworm. The SG undergoes rapid development during the fifth instar larvae with the genomic DNA content in SG cells increasing 200–400000 times through the process of endomitosis [9, 10]. Consequently, the SG becomes the largest organ in silkworm abdominal cavity until the wandering stage. Then the SG degenerates rapidly after the spinning stage in response to endocrine hormones (including juvenile hormone and ecdysone) [4, 11, 12]. This process includes 5 characteristics (nuclear condensation, DNA fragmentation, nuclear fragmentation, cells shrinkage and apoptotic body formation) and involves in PCD.
Among which, ecdysone (20E, 20-Hydroxyecdysone) plays a leading role by acting through binding to ecdysone receptor (EcR) / ultraspiracle (USP) complex. This 20E-induced PCD is similar with the mechanism found in Drosophila, where 20E/EcR/USP complex regulates primary-response genes including a number of transcription factors such as E74, Board complex (BR-C) and E93, which directly regulate secondary-response target genes to transmit and amplify the hormonal signal causing apoptosis [13]. In addition, 20E-induced SG degeneration (mainly nuclear condensation and DNA fragmentation) could be inhibited following addition of a caspase3 inhibitor to the culture medium [7]. Consequently, it is clear that 20E induced cell death involves different temporal stages in both Drosophila and silkworms [7, 13, 14], and the regulation of diverse genes [15–17]. Apart from this, we could know little details about the molecular mechanism of SG apoptosis.

Apoptosis proceeds via an intrinsic pathway that involves mitochondrial cytochrome C (Cyt C) release and an extrinsic pathway commonly [18]. Many studies have proven that the Cyt C might play little role in regulating apoptosis in Drosophila. In contrast to that reported in Drosophila, Cyt C release importantly participates in silkworm apoptosis, suggesting that silkworms may be better model systems for apoptosis research [17, 19, 20]. So far, several studies have elucidated that some homologous genes in silkworms also function similarly as in mammals. BmIce-2, Bmp53 [20] and BmDronc [21] have been reported to enhance the rate of apoptosis in BmN cells, while BmiAP [22] and BmBuffy [23] function in inhibition of apoptosis. Bombyx mori death related ced-3/Nedd2-like protein (BmDredd) has relatively high homology with the mammalian initiator caspases which include an N-terminal death effector domain (DED) or caspase recruitment domain (CARD) and a C-terminal CASc domain. The difference is that BmDredd comprises a long prodomain which replaces the mammalian N-terminal DED or CARD. Initiator caspases are gathered in a close distance through DED or CARD and finally activated by autoproteolysis in its CASc domain [24]. Recently, some evidence has showed that BmDredd functions importantly in apoptosis. Its expression level was elevated in actinmycin D induced BmE cells [17] and in actinmycin D or ultraviolet induced BmN cells [25]. In addition, overexpression of BmDredd caused the increase of caspase3/8-like protease activity in BmN-SWU1 cells [26]. However, a more advanced understanding of its role during SG degeneration has eluded researchers due to the instability of animal individual experiments. In this study, we addressed this deficiency by examining the role of BmDredd in apoptosis of the SG.

Materials and Methods

Animals

Silkworm larvae (P50 strain) were reared on fresh mulberry leaves. Silkworms were maintained at 25 ± 1°C, 70–85% humidity, and a 14 h light/10 h dark photoperiod.

RNA extraction & cDNA synthesis

Tissues (SG, gut, fat, gonad and epidermis) were dissected in PBS buffer and ground under liquid nitrogen. Total RNA was isolated using RNAiso Plus according to the manufacturer’s instructions (TaKaRa, Japan) and measured on a Bio Spec-nano (Shimadzu Biotech, Japan). For each sample, 500 ng of total RNA was used to synthesize the first cDNA strand using a Prime Script RT Master Mix (TaKaRa) according to the manufacturer’s instructions.

qRT-PCR

The BmDredd nucleotide sequences (accession number: NM_001114865) were searched against the NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) database and primers were
designed by primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Sangon Biotech, Shanghai, China (Table A in S1 File). qRT-PCR was performed in a total volume of 20 μl on an ABI7300 System (Applied Biosystems, Foster City, CA, USA) and SYBR® Premix Ex Taq™ (TaKaRa) using cycling parameters consisting of an initial denaturation at 95˚C/30 sec followed by 40 cycles of 95˚C/5 sec and 60˚C/31 sec. A melt curve analysis was used to confirm the absence of spurious products. Ct values were employed to calculate the relative expression levels [27]. Transcription levels of the target genes were normalized with Actin3 transcription levels from the same samples. All of the experiments were performed in triplicate.

Caspase inhibitor and ecdysone treatment

The caspase inhibitor, Z-DEVD-fmk (Selleck Chemicals, Houston, TX, USA), was diluted in PBS according to the manufacturer instructions and then injected a corresponding quantity into day 7 of the fifth instar (L5D7) silkworms based on its weight (22.4 μg per gram) and SGs were dissected at 18 h-post the spinning (sp18h). Similarly, ecdysone (Huzhou Silkworm Pharmaceutical Factory, China) was diluted with ddH₂O and sprayed evenly onto mulberry leaves according to the instruction (~5 μg each silkworm). Feed the leaves to experimental silkworms.

Protein extraction and caspase3 like activity assay

Dissected SGs were ground under liquid nitrogen and homogenized in100 μl tissue lysis buffer (Beyotime, Shanghai, China) per 5~10 mg tissue. Lysates were then placed on an ice bath for 5 min and centrifuged at 16000–20000 ×g at 4˚C for 15 min. The supernatant was transferred to ice cooled 1.5 ml tubes for assaying the caspase3 activity, and the total protein concentration was determined using a Bradford Protein Assay Kit (TaKaRa). Samples were maintained at -80˚C.

According to the instruction of Caspase3 Activity Assay Kit (Beyotime), 50 μl reaction buffer, 40 μl sample and 10 μl Ac-DEVD-pNA (2 mM) were mixed. The resulting mixture was incubated at 37˚C for 16 h and the absorbance measured at 405 nm. Caspase3 activities were determined based on absorbance of produced pNA in unit sample proteins.

Synthesis of dsRNA and knockdown of BmDredd

Double-stranded RNAs (dsRNA) for BmDredd were synthesized using a T7 RibofMAX™ Express RNAi System (Promega, Madison, WI, USA). PCR product templates were obtained using the following primers: 5’ ATGTTTCGACCTGACGC TTAA 3’ and 5’ TCATGGCTTAAG TAAAGTAAAG 3’. Thermocycler conditions were 98˚C for 10 sec, 55˚C for 15 sec, and 72˚C for 50 sec for 35 cycles using Prime STAR DNA Polymerase (TaKaRa). T7 RNA polymerase promoter sequence 5’ GGATCCTAATACGACTCACTATAGG3’ was added to the 5’ end of the above primers. DsRNA-BmDredd was synthesized according to the instructions. For RNA interference, each silkworm was injected with ~15 μg dsRNA-BmDredd. Control silkworm was injected with 10 μl dsRNA-EGFP. SGs were dissected 30 h post-injection for qRT-PCR and caspase3 activity detection.

Overexpression of BmDredd in Bombyx mori

Primers were designed (Table B in S1 File). To generate the recombinant plasmid PXL-BA-C-U6-Dredd-GFP, sequences for BmU6, BmDredd, and IE-GFP-SV40 were amplified by PCR and cloned into PXL-BACII according to the protocol provided with a CloExpressMutis kit (Vazyme, Nanjing, China). The plasmid PXL-BACII was linearized using XhoI and XhoI restriction sites. The reaction system contained 4 μl 5×CE Multis Buffer, 2 μl Exnase™ Multis, the above PCR products and the linearized vector. The recombinant vector (7.5 μg) was diluted
into 12 μl sterile 0.4% trehalose solution and then mixed with 1 μl of TurboFect \textit{in vivo} transfection Reagent (Thermo, Wilmington, NC, USA) immediately by pipetting. The mixture was incubated at room temperature for 20 min and injected into day 4 of the fifth larval instar silkworm. Control silkworms were injected with the same amount of 0.4% trehalose solution. After two days, the gene expression level was monitored and SGs were photographed under a fluorescence microscope (OLYMPUS BX53, Japan).

**Transmission electron microscopy**

SGs were dissected from silkworms during the middle of the spinning stage and fixed in 2.5% glutaraldehyde overnight at 4°C. Specimens were rinsed with phosphate buffer solution (0.1 M, pH 7.0) three times for 15 min each time, then fixed in 1% osmium tetroxide for 1 h, rinsed again, dehydrated in an ethanol series, and then treated with mixture of eponresin and acetone (V/V = 1:1 for 1 h, V/V = 3:1 for 3 h). Samples were finally embedded in an Epon-Araldite mixture overnight. The samples were cut into slices (70 nm) and observed by transmission electron microscopy (TEM, Hitachi HT7700, Japan).

**Immunofluorescence**

SGs were isolated and fixed in 4% formaldehyde overnight at 4°C. The tissues were dehydrated in an ethanol series and embedded in paraffin. Sections were sliced on a microtome (4 μm) and placed on slides. BmDredd rabbit polyclonal antibody was prepared by Huabio Ltd., Hangzhou, China. The samples were de-waxed and immersed in an ethanol series (95%, 85% and 75%) for 5 min each, washed with ddH2O 3 times for 5 min. Trisodium citrate repair solution was added to the sections for 4 min under high pressure. The samples were washed twice for 5 min in ddH2O and 3 times for 5 min with immunol staining washing buffer (Sangon) after cooling to room temperature. A 3% H2O2-methanol solution (50 μl) was added to each sample for 15 min. The samples were then rinsed three times for 5 min, blocked with 5% BSA for 45 min, incubated with BmDredd antibody (1:500) overnight at 4°C. Samples were rinsed four times for 5 min before incubation with Rhodamine-conjugated AffiniPure Goat Anti-Rabbit IgG (1:200; Huabio) without light for 1 h, followed by rinsing three time times with immunol staining washing buffer. DAPI (3.5 μg/ml) was added for 15 min at room temperature and washed three times same as above. Images were captured using a fluorescence microscope (OLYMPUS BX53) using the same light intensity.

**Results**

Comparison of expression levels of \textit{BmDredd} with apoptosis-related genes \textit{BmEcR} and \textit{BmIce} in the middle and posterior silk gland during metamorphosis

\textit{BmEcR} is the receptor of ecdysone, and \textit{BmIcE} is an important effector caspase. The expression level of \textit{BmEcR} peaked twice after the wandering stage in the middle silk gland (MSG), whereas the expression levels of \textit{BmIce} and \textit{BmDredd} increased rapidly starting from 24 h post-spinning (sp24h) and reached the maximum expression levels at 48 h post-spinning (sp48h). The change in \textit{BmDredd} expression was the most dramatic with an increase of over 400 times (Fig 1A). In the posterior silk gland (PSG), \textit{BmEcR}, \textit{BmIcE} and \textit{BmDredd} were elevated during the larval to pupal metamorphosis stage, which is the period when SG apoptosis begins. Additionally, \textit{BmEcR} expression was highest at the wandering stage and it peaked earlier than \textit{BmDredd} and \textit{BmIcE}. The expression of \textit{BmDredd} began to rise at sp12h in the PSG and reached the highest at sp24h, whereas \textit{BmEcR} expression began at day 7 of the fifth larval
instar (L5D7) (Fig 1A). Additionally, the expression levels of BmDredd increased both in the MSG and PSG during early (L5D3) or middle (L5D5 to L5D6) fifth instar 24 hours after feeding ecdysone, but feeding ecdysone to later (L5D7 to wandering) fifth instar impeded the normal expression of BmDredd in MSG (Fig 1B).

**BmDredd** Expression levels in different silkworm tissues and the effects of a caspase inhibitor

Silkworms present drastic changes during the transition from the spinning to the pupal stage. During this period, most larval tissues undergo quick degeneration forming fat. This

![Fig 1. Expression level interactions between BmDredd and other two apoptosis-related genes in MSG and PSG during metamorphosis.](image)

(A) Expression levels in MSG and in PSG. L5D3-L5D7: day 3 to day 7 of the fifth larval instar. W: wandering stage. sp12h: 12 h of spinning. PD1: 1-day-old pupa. PD2: 2-day-old pupa. (B) The increase of BmDredd expression induced by ecdysone in MSG and PSG, P<0.5. Early, middle and late means the time of feeding ecdysone at L5D3, L5D5 to L5D6 and L5D7 to wandering respectively.

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degeneration involves apoptosis and an increase in caspase3-like activity. We found that caspase3-like activity peaked during the wandering stage in the MSG and at sp12h in the PSG (Figure C in S1 File). At 36 h after spinning, caspase3-like activity dropped to pre-wandering stage levels, but then underwent a second period of upregulation (Figure C in S1 File). In addition, we found that BmDredd expression in the PSG was slightly higher than the MSG (Fig 2A), but expression in both tissues was significantly higher (P<0.05) than the other five tissues (fat, gut, gonad and epidermis) (Fig 2A). Moreover, addition of the caspase inhibitor Z-DEVD-fmk to silkworm significantly decreased the expression of BmDredd by ~71% and 49% in the MSG and PSG respectively (Fig 2B).
RNAi of *BmDredd* delayed the SG degeneration process

SG nuclei are initially rod-shaped in first instar larvae, but then begin to gradually branch out in the later instar stages. By the fifth instar, SG nuclei are highly branched. Nuclei in these stages had both condensed and dispersed chromatin. Typically, the more dispersed chromatin, the higher the DNA synthesis activity. We found that the amount of dispersed chromatin was significantly reduced in late fifth instars.

RNAi-mediated knockdown decreased the *BmDredd* expression in the SG (Fig 3D), in particular in the PSG (~54% reduction). The TEM samples were taken from PSG during the middle of the spinning stage. We were able to identify fibroin proteins in Fig 3A which had completed secretion. As a result, the SG cavity was empty (Fig 3A:a). In addition, condensed chromatin increased and the nucleus (NC) was disordered (Fig 3A:b). Mitochondria (Fig 3A:c,
d: red arrow) decreased in size, the structure of the ER was almost invisible, and apoptosis bodies appeared. In Fig 3B and 3C, we could see fibroin protein secretion and there was less condensed chromatin in Fig 3B than in Fig 3A and 3C. The ER was more developed in Fig 3B and 3C. More Golgi apparatus (Fig 3Bc2: red circles) and normal-functioning mitochondria were visible in Fig 3C. The caspase3 activity also showed a decrease after dsRNA interference (Fig 3E). Thus the dsRNA interference inhibited the apoptosis of the SG.

**Transient expression of BmDredd caused apoptosis of silk gland**

Green fluorescence was detected in SG 48 h-post transient transfection (Fig 4A:a2), whereas no fluorescence was detected in controls (Fig 4A:a1). Results of immunofluorescence showed

![Fluorescence microscopy images](image)

**Fig 4. Overexpression of BmDredd in silkworm.** (A) Fluorescence microscopy, a1 and a2: green fluorescence indicative of successful transfection and overexpression of the PXL-BAC-U6-Dredd-GFP. a3 and a4: DAPI and Rhodamine staining, DAPI (blue fluorescence): nuclei, Rhodamine (red fluorescence): BmDredd. a1, a3: control, a2, a4: overexpression of BmDredd. (B) BmDredd expression levels in the SG after 48h overexpression. P<0.01. (C) caspase3-like activity of SG after 48 h overexpression.

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that $BmDredd$ red fluorescence was predominantly located in the cytoplasm of the SG interior cavity wall (Fig 4A:a4) and protein levels increased after transfection with the overexpression plasmid (Fig 4A:a3, a4). $BmDredd$ transcription levels increased by ~51 times over control levels (Fig 4B) and caspase3-like activity increased more than two-fold (Fig 4C).

**Discussion**

Prior studies have shown that $BmEcR$, $BmDredd$ and $BmIce$ are important apoptosis-related genes [17]. We investigated their expression profile in SG and found that $BmDredd$, $BmEcR$ and $BmIce$ transcription levels significantly increased in the MSG and PSG during the larval-pupal transition (Fig 1A). Expression of $BmEcR$, the receptor for ecdysone, changes with ecdysone levels [15]. Recent studies have demonstrated that $BmEcR$ responds to molting hormone (ecdysone) to activate a genetic regulated cascade that results in tissue degeneration during metamorphosis. We found that the elevation in $BmDredd$ expression closely followed (~24 h) $BmEcR$ expression (Fig 1A), and was significantly increased compare to controls after feeding ecdysone in early or middle fifth instar larvae (Fig 1B). These findings indicate that $BmDredd$ functions in SG degeneration and it is most likely a downstream gene of $BmEcR$. Since $BmDredd$ belongs to the silkworm caspase family, similar to the effector caspase $BmIce-2$, it is likely an initiator caspase homolog. We suggest that $BmDredd$ can induce apoptosis with similar functions as $BmIce-2$. Furthermore, the expression levels of $BmDredd$ were significantly elevated in SG compared to other tissues (Fig 2A) demonstrating that $BmDredd$ played a remarkable role in SG apoptosis.

Caspase3 activity is a vital index for detecting apoptosis. The results indicated that apoptosis occurred in MSG and PSG from L5D7 through the spinning stage to pupa, with the apoptosis rate in MSG slightly more advanced than PSG and peaked at the wandering stage (Figure C in S1 File). This activity profile is reminiscent of $BmDredd$ expression at sp12h (Fig 2A). Z-DEVD-fmk is a specific, irreversible caspase3 inhibitor, which also potently inhibits caspase6, caspase7, caspase8 and caspase10. Z-DEVD-fmk also inhibited caspase3-like activities in SG (Figure D in S1 File) and the expression of $BmDredd$ (Fig 2B). Therefore, we speculate that SG apoptosis is associated with $BmDredd$ expression. In *Drosophila*, caspase competence is continuous from early in day 3 larvae, but it cannot induce apoptotic execution because of high DIAP1 levels block caspase activation [6]. While the specific regulatory mechanism used in silkworms is currently unknown, our results suggest that control of caspase competence in SG apoptosis is likely similar to that used in *Drosophila*.

dsRNA- $BmDredd$ reduced $BmDredd$ expression in the SG (Fig 3D). After dsRNA treatment, the SG cavity retained fibroin secretion with more dispersed nuclei than controls (Fig 3B:b1). Normal mitochondria and endoplasmic reticulum structures (Fig 3B:d2) were found in $BmDredd$ knockdown SG cells. There were no or few apoptosis bodies in Fig 3B and 3C, however, they were abundant in the control (Fig 3A:d). These changes in microstructures illustrated that knockdown of $BmDredd$ delayed SG apoptosis. However, mitochondria and condensed chromatin were also found in Fig 3B and 3C respectively. dsRNA treatment during the middle of the fifth instar hindered nucleus condensation. Given that RNAI effects for a short period of time, the condensing of the cytoplasm was not prevented. During late fifth instars, apoptosis of nuclei has already occurred before adding dsRNA- $BmDredd$, hence a certain degree of condensed nuclei were visible. Based on these findings, we inferred that apoptosis of SG may initiate from the nucleus and $BmDredd$ may be an important gene to induce nuclear condensation. This result was consistent with the study of Masatoshi Iga in 2007 [7].

*In vivo* transfection reagent is ideal for nucleic acid delivery [28, 29]. The protocol is easy and versatile that it has been adapted to many other species including mice, rat, chicken and mosquito [30–33]. We transiently overexpressed the $BmDredd$ in vivo in silkworm SG using...
TurboFect in vivo transfection Reagent (Fig 4A and 4B). The overexpression leads to light apoptosis of the SG (Fig 4C). However, whether stable overexpression of BmDredd could induce extensive apoptosis of the SG remains unknown.

In conclusion, BmDredd regulated SG apoptosis and is probably a downstream gene of BmEcR. Knockdown of BmDredd impeded SG apoptosis and overexpressing BmDredd triggered SG apoptosis. More research, however, is required to fully reveal the molecular mechanisms underlying SG apoptosis.

Supporting Information

S1 File. Fig A. Different stage of the silk gland of the silkworm during the fifth larval. A-F: L5D3-L5D8. Bar:1 cm. The SGs were dissected from Qiu feng × Bai Yu strain.

Fig B. The transformation of silk gland of silkworm during the larval to pupal. (A) the wandering stage(W). (B) middle of the spinning day. (C) prepupal stage(PP). (D) the second day of pupal. Bar:1 cm. These were dissected from Qiu feng x Bai Yu strain.

Fig C. Caspase3-like activities of SG from L5D4 to PD1. (A) Caspase3-like activities in MSG. (B) Caspase3-like activities in PSG.

Fig D. Caspase3-like activities of MSG and PSG after Z-DEVD-fmk treatment.

Table A. Primers used in real-time PCR.
Table B. Primers used for vector construction.

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