Article

BMDREDD REGULATES THE APOPTOSIS COORDINATING WITH *BMDAXX*, *BMCIDE-B*, *BMFADD*, AND *BMCREB* IN *BM*N CELLS

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The apoptosis mechanisms in mammals were investigated relatively clearly. However, little is known about how apoptosis is achieved at a molecular level in silkworm cells. We cloned a caspase homologous gene named BmDredd *(where* Bm *is* Bombyx mori *and Dredd is death-related ced-3/Nedd2-like caspase) in* Bm*N cells from the ovary of* Bm *and analyzed its biological information. We constructed the N-terminal, C-terminal, and overexpression vector of* BmDredd, *respectively. Our results showed that the transcriptional expression level of* BmDredd *was increased in the apoptotic* Bm*N cells. Furthermore, overexpression of* BmDredd *increased the caspase-3/7 activity. Simultaneously, RNAi of* BmDredd *could save* Bm*N cells from apoptosis. The immunofluorescence study showed that* Bm*Dredd located at the cytoplasm in normal cell otherwise is found at the nucleus when cells undergo apoptosis. Moreover, we quantified the transcriptional expressions of apoptosis-related genes including* BmDredd, BmDaxx *(where Daxx is death-domain associated protein),* BmCide-b *(where Cide-b is cell death inducing DFF45-like effector),* BmFadd *(Fadd is fas-associated via death domain), and* BmCreb *(where Creb is cAMP-response element binding protein) in* Bm*N cells with dsRNA interferences to detect the molecular mechanism of*

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apoptosis. In conclusion, BmDredd *may function for promoting apoptosis and there are various regulatory interactions among these apoptosis-related genes.* © *2016 Wiley Periodicals, Inc.*

Keywords: silkworm *Bombyx mori* (*Bm*); apoptosis; *Bm*Dredd; caspase

INTRODUCTION

Apoptosis is a cellular suicide process to eliminate unwanted, damaged, and potentially dangerous cells from multicellular organisms, playing an important role in the development and homeostasis of metazoans (Thompson, 1995; Vaux and Korsmeyer, 1999). It is associated with distinct morphological changes and biochemical events such as phosphatidylserine extrusion, DNA fragmentation, chromatin condensation, membrane blebbing, vacuolization, and apoptotic body formation (Lockshin and Zakeri, 2004). Physical factors such as UV (Aragane et al., 1998) or chemical substances, such as ecdysone (Fujiwara and Ogai, 2001; Tsuzuki et al., 2001; Tian et al., 2012) and actinomycin D (Act D; Kleeff et al., 2000; Yi et al., 2014), can induce cell apoptosis.

Generally, there exist two basic apoptosis signaling pathways in mammals, an "extrinsic" pathway triggered by receptor systems (Fas/ tumor necrosis factor [TNF]) or cytotoxic stress (Raff, 1998; Schmitz et al., 2000; Movassagh and Foo, 2008) and an "intrinsic"" pathway, that is, the mitochondrial pathway (Reed, 1997; Green and Reed, 1998). Interestingly, researchers have found that some vital elements also exist in these two apoptosis pathway in silkworm (Terashima et al., 2000; Chen et al., 2015). In both pathways, apoptotic stimulus results in the activation of the cysteine protease (caspase) cascade (Thornberry, 1998). Therefore, caspases are essential factors involved in apoptosis. Caspases were divided into effector caspases (such as caspase 3, caspase7) and initiator caspases (such as caspase 8; Thornberry, 1998; Earnshaw et al., 1999).

In mammals, initiator caspases contain specific protein–protein interaction motifs, such as caspase recruitment domain (CARD) or death effector domain (DED), which could activate downstream effectors (Yang, 2015). Activated effector caspases cleave intracellular structure proteins such as lamins and cytokeratins, causing nuclear decomposition and inducing genomic lysis (Nicholson, 1999). Gene sequence analysis showed high identity between *Dm*Dredd (*Drosophila melanogaster* death-related ced-3/Nedd2-like caspase) and mammalian caspase-8. Simultaneously, the activated *Dm*Dredd participates in the apoptosis process and Imd pathway (Po Chen and Tien Thach, 1998; Myllymaki et al., 2014). An ortholog of *Dm*Dredd was also found in the silkworm, namely *Bm*Dredd (where *Bm* is *Bombyx mori*). Though *Bm*Dredd has P20 and P10 domains, unlike mammalian caspase, the N-terminal sequence in *Bm*Dredd lacks the DED and caspase recruitment domain (Zhang et al., 2010).

Additionally, many other factors are involved in apoptosis pathway in silkworm, such as EcR (ecdysone receptor), TNF superfamily, Daxx (death domain associated protein), Fadd (fas-associated via death domain), cell death inducing DFF45-like effector (Cide-b), cAMP-response element binding protein (Creb), and so on. In mammals, Daxx and Fadd are two important Fas-binding proteins that activate a series of downstream genes and proteins to induce apoptosis (Yang et al., 1997). Cide-b is a proapoptotic mitochondrial protein inducing apoptosis (Chen et al., 2000). It was identified by its high homology with both of the N-terminal domains of DFF40 and DFF45. Creb is a kind of protein modulating DNA transcription, which induces the expression of Bcl-xl and Bcl-2 to prevent

cyt C release (Mori et al., 2001). However, at present, little is known about how apoptosis is achieved at a molecular level in silkworm cells. Therefore, studies on the silkworm cell apoptosis are certainly needed. Here, we cloned *BmDredd*, characterized its role in *Bm*N cell apoptosis, and inferred the possible apoptosis signaling pathway among above important apoptosis-related genes.

MATERIALS AND METHODS

Cells

*Bm*N cells (originated from the ovary of *Bm*) or sf9 cells were maintained at 27°C and cultivated in TC-100 insect medium (Applichem, Germany) supplemented with 12% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 0.35 g/L $NaHCO_3$.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the collected *Bm*N cells using RNAiso Plus according to the reagent instruction (TaKaRa BioTechnology, Dalian, China). First-strand cDNA was synthesized using the Prime Script RT Master Mix (TaKaRa). About 500 ng RNA was mixed with 0.5 μl of the Prime Script RT Master Mix followed by incubating immediately in 37°C for 30 min and then the enzymes were inactivated by incubating at 85°C for 5 sec. The product was diluted 20-fold with ddH_2O and stored at –20°C.

Quantitative Real-Time PCR

The primers for qRT-PCR were designed by primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA), and synthesized by Sangon Biotech, Shanghai, China (Supplementary Table 1). qRT-PCR was performed using an ABI7300 System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex TaqII (TaKaRa). The two-step amplification consisted of a 95°C for 30 sec followed by target amplification of 40 cycles at 95°C for 5 sec and 60°C for 31 sec. The transcript levels of the targeted genes were normalized with *Actin3* gene. The comparative C_t method (Livak and Schmittgen, 2001) was used for analysis, where ΔC_t is the given C_t value for target gene normalized to the *Actin3*.

Construction of Overexpression Plasmids and Transfection

Templates of *BmU6* and *IE-GFP-SV40* were preserved in our laboratory. According to the instructions of Vazyme CloExpress Mutis kit, primers were designed (Supplementary Table 2) and sequences of *BmU6*, *BmDredd*, *IE-GFP-SV40* were amplified by PCR and purified using DNA gel extraction kit (Sangon). The plasmid PXL-BACII was cut in *Xba* I and *Xho* I sites to form a linear vector. The reaction system contained 4 μl 5× CE Multis Buffer, 2 μl Exnase Multis, the three above PCR products, and the linearized vector. The mixture was kept at 37°C for 30 min, and then placed on ice for 5 min according to the instruction, yielding the recombinant plasmid PXL-BAC-U6-Dredd-GFP. For N-terminal (1–885 bp) and C-terminal (883–1,629 bp) sequences, the PCR products were connected into the 18T-Simple vector (TaKaRa) and then digested with *Eco*RI and *Xho* I and cloned into pIZ/v5-his vector (Invitrogen, Carlsbad, CA, USA) to generate pIZ/v5-N-Dredd-his and pIZ/v5-C-Dredd-his.

About 1.2 μg plasmid DNA and 5 μl of Lipofectamin 2000 Reagent (Invitrogen Life Technologies) were mixed into 100 μl serum-free medium followed with incubating at room temperature for 5 min, and then the transfection complex was dropped into 12-well cell culture plate. The samples were exposed under fluorescence microscope (Olympus IX71, Japan) after 48 h.

Assay of Caspase-3/7 Activity

The Caspase-Glo 3/7 buffer and lyophilized Caspase-Glo 3/7 substrate (Promega, Madison, WI, USA) were equilibrated to room temperature. The buffer solution was mixed with the substrate. The cells were suspended by pipetting and added 80 μl into white 96-well plate per well. The mixed reagent was added into the corresponding wells with 1:1 ratio (i.e., 80 μl). The plate was shaken at 300–500 rpm for 30 sec and incubated at room temperature for 2 h. Finally, the caspase activities were detected by the GloMax-96 Luminometer (Promega).

RNA Interference of BmDredd, BmDaxx, and Bmcide-b genes in BmN cells

Primers for dsRNA synthesis of *BmDredd*, *Bmcide-b*, and *BmDaxx* were designed by primer 5.0 software (Supplementary Table 3). The dsRNAs were synthesized based on the instruction of T7 RiboMAXTM Express RNAi System kit (Promega), then named as dsRNA-*Bmcide-b*, dsRNA-*BmDaxx*, and dsRNA-*BmDredd*, respectively.

*Bm*N cells were seeded onto 6-well plates for 24 h, then above dsRNAs and a negative control dsRNA were added into cell culture medium, respectively. qRT-PCR was performed with ABI7300 (Applied Biosystems) to detect the transcriptional levels of apoptosis-related genes.

UVC Irradiation and Ecdysone/Actinomycin D Treatment

The medium was gently removed when *Bm*N cells covered 70–80% of the dish area. The cells were irradiation with UVC (power 8 W, wavelength 245 nm, distance 40 cm) for 1, 2, and 3 min, respectively. Then, fresh medium was added into each culture dish for successive incubation for 24 h at 27°C. The cells were pictured under microscope (Nikon Eclipse Ti-s, Japan). In addition, when *Bm*N cells covered the dish at 70–80% confluence, ecdysone (Huzhou silkworm pharmaceutical factory, Zhejiang, China) was added into the medium at corresponding concentration of 200 and 250 ng/μl or Act D at 65 ng/mL, respectively.

Flow Cytometric Analysis

*Bm*N cells were treated with dsRNA-*Bm*Dredd, UVC, or ecdysone, respectively. After 36 h, the cells were collected at 800 × *g*, washed with phosphate buffer saline (PBS) twice, and stained with FITC and PI according to the instruction of Annexin V-FITC Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China). After treatment, cells were detected by flow cytometry (Beckman coulter FC-500, Kraemer Boulevard Brea, CA, USA).

Antibody Preparation

Specifically expressed sequence (231–346 bp) was selected and acquired using primer pET-P231-F (CCGGAATTCGAAACAAATCCAAATAAT) and pET-P346-R (CCGCTCGA-

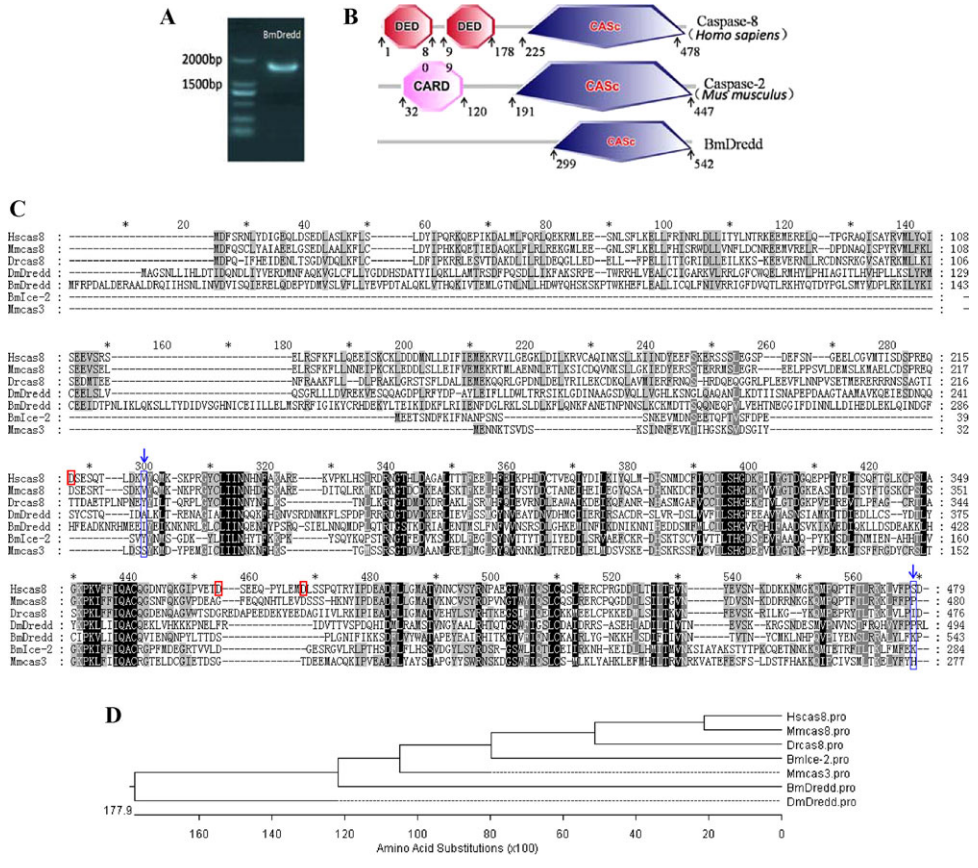

Figure 1. Biological information about *BmDredd*. (A) PCR products of *BmDredd*. (B) Protein sequences analysis of mammalian caspase-2, caspase-8, and *BmDredd*. (C) Conserved amino acids in *BmDredd*. The red frame is three autocleavage sites. Between the two blue boxes are the CASc domains. (D) Phylogenetic tree. The GenBank accession numbers of sequences used for the alignment are: *Homo sapiens* Caspase 8 (*Hs*Cas-8) AAD24962.1, *Mus musculus* Caspase 8 (*Mm*Cas-8) AAH49955.1, *Danio rerio* Caspase 8 (*Dr*Cas-8) NP_571585.2, *Mus musculus* Caspase 3 (*Mm*Cas-3) AAH38825.2, *BmDredd* BAF98475.1, *Bm*ICE-2 ABC94941.1, *Drosophila melanogaster* Dredd (*Dm*Dredd) AAC33117.1, *Mus musculus* Caspase 2 AAH34262.1.

GAATTCTGTCTTTGGTT). The sequence was digested with *Eco*RI and *Xho* I, and cloned into the pET-30a, yielding the recombinant plasmid named pET-P-*Bm*Dredd. Above plasmid was transformed into *E. coli* BL21 competent cells and fusion protein (Fig. S1) was purified and injected into New Zealand white rabbit for polyclonal antibody production. The serum was collected and purified by Huabio, China.

Western Blotting

After SDS-PAGE, the proteins were transferred onto a nitrocellulose (NC) membrane and subsequently washed with TBST (50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and blocked in 5% dried skimmed milk/TBST for 1 h at room temperature followed by incubating in *Bm*Dredd polycolonal antibody (1:100) or 6× His-tag horseradish peroxidase (HRP, 1:2,000) at room temperature for 1 h. The membrane was washed 3 times/5 min and incubated with 1:2,000 diluted second antibody ImmunoPure Goat Anti-Rabbit

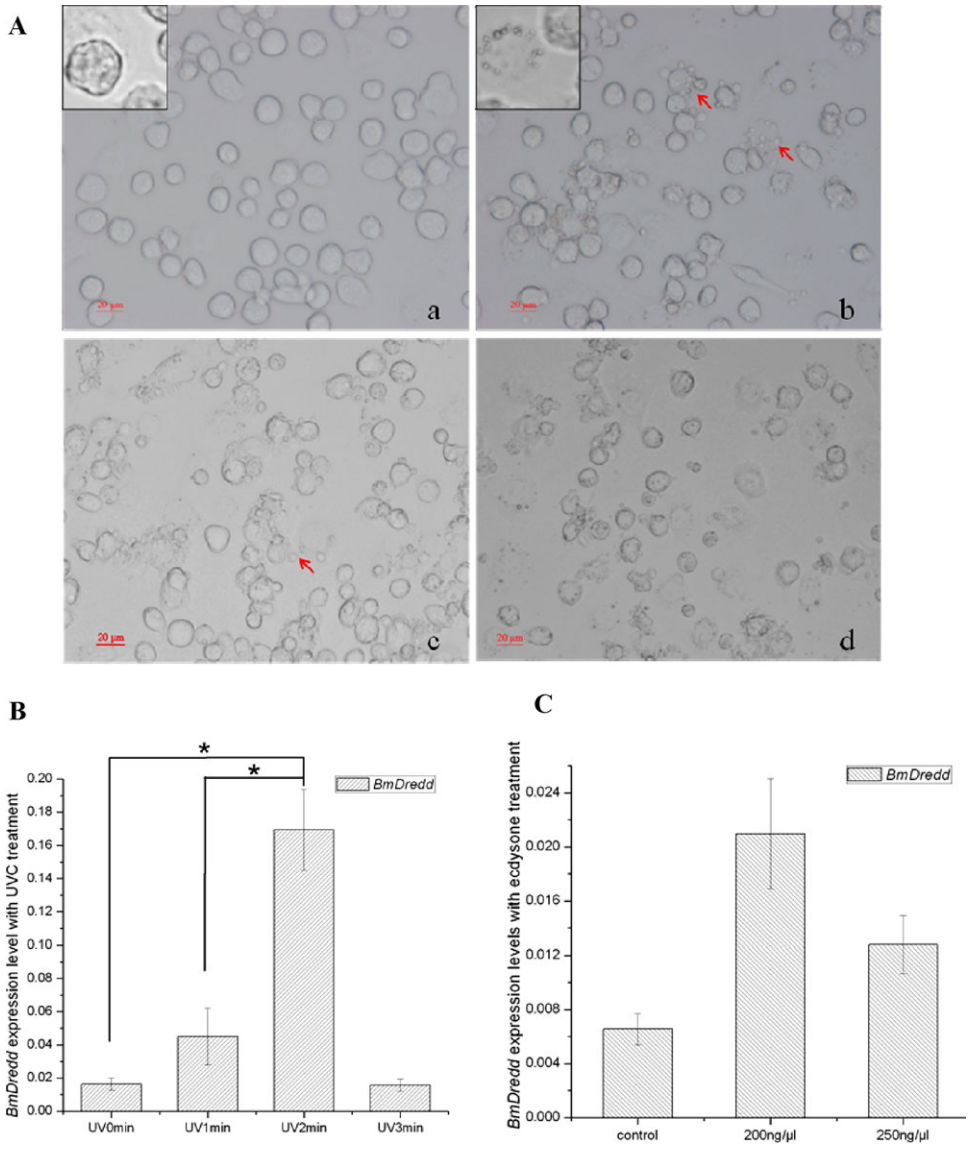

Figure 2. (A) UVC induced *B. mori* ovary cell apoptosis. a: Control *Bm*N cells; b: *Bm*N cells with UVC irradiation for 1 min; c: *Bm*N cells with UVC irradiation for 2 min; d: *Bm*N cells with UVC irradiation for 3 min. Arrows: apoptotic bodies. (B) Expression levels of *BmDredd* with UVC irradiation. (C) Expression levels of *BmDredd* with ecdysone treatment. $^{*}P < 0.05$. Error bar means standard deviation.

IgG, (H + L) for 1 h at room temperature. The specific protein band was visible using FDbio-Dura ECL Western Blotting Detection Reagents (Fdbio science, Hangzhou, China).

Immunofluorescence

*Bm*N cells were grown on coverslips (10 × 10 mm) in 24-well plate. At 48 h post corresponding treatments, the cells was washed 2 times/2 min with PBS, and fixed with 80%

cold acetone for 15 min following with 3% Triton X-100 for 10 min. The cells were rinsed 3 times/5 min with PBS and incubated in 1:100 diluted *Bm*Dredd polyclonal antibody or 1:1500 diluted 6× His-tag HRP (Huabio) as first antibody for 1 h at 37°C. After washed 3 times/5 min with PBST, the cells were incubated in Rhodamine-conjugated AffiniPure Go at Anti-Rabbit IgG (Huabio) keeping out of light for 1 h followed by rinse three times with PBST again. 4',6-diamidino-2-phenylindole (DAPI, 3.5 μg/ml) was added into the wells for 15 min at room temperature and washed three times same as above processing. The modified coverslips were mounted in glycerol on glass slides and sealed, and finally observed with confocal laser scanning microscopy (Olympus FV1000).

Statistical Analysis

All experiments were performed in three independent biological replication and reactions of each sample were carried out in triplicate. All data were analyzed by Excel 2007 and SPSS Statistics 19. Paragraphs were exported by Origin 8.

RESULTS

Cloning and Analysis of BmDredd

We could know the open reading frame (ORF) of *BmDredd* contains 1632 bp (Fig. 1A) and codes 543 amino acids. It includes a long prodomain and a CASc domain that starts at 299 amino acids and ends at 542 amino acids (Fig. 1B) after bioinformatic analysis (http://smart.embl-heidelberg.de/). There exists 1–3 amino acids slide in domain location when using NCBI online prediction (http://www.ncbi.nlm.nih.gov/pubmed/, Fig. S2). *BmDredd* shares 22.7% identity with *Homo sapiens* caspase 8, 21.8% identity with *Mus musculus* caspase 8, 25.1% identity with *Danio rerio* caspase 8, 23.4% identity with *Dm*Dredd, 19.9% identity with *Bm*Ice-2, and 33.1% identity with *Mus musculus* caspase 3 (Fig. 1C and D). *BmDredd* lacks the DED domains compared to *Homo sapiens* caspase 8 and CARD domain compared to *Mus musculus* caspase 2 (Fig. 1B). All these suggested a close relation between *Bm*Dredd and caspases.

UVC and Ecdysone Induced B. mori Cell Apoptosis and Expression of BmDredd Increased

Ultraviolet and ecdysone have the competence of inducing apoptosis. Apoptotic bodies were visible when *Bm*N cells were exposed to UVC for 1, 2, and 3 min, respectively, or added with ecdysone. The normal *Bm*N cells were oval, uniform cytoplasm (Fig. 2A: a). After irradiation with UVC for 1 min, *Bm*N cells presented membrane blebbing, that is, apoptotic bodies (Fig. 2A: b, red arrow). This phenomenon was more obvious 2 min after UVC irradiation (Fig. 2A: c, red arrow). After the cells were irradiated for 3 min, most of the cell structures would be incomplete and appeared to dissolve (Fig. 2A:d).

Apoptosis occurred on *Bm*N cells after UVC irradiation. At this time, we found the amount of expressed *BmDredd* increased by 938% after 24 h with 2-min treatment compared with the control (Fig. 2B). Furthermore, expression of *BmDredd* increased by 220% at 24 h treated with 200 ng/μl ecdysone in *Bm*N cells. After UVC irradiation for 3 min or 250 ng/μl ecdysone treatment for 24 h, the expression of *BmDredd* decreased (Fig. 2C), which may be resulted from the decline of cell number and the deepening of apoptosis degree.

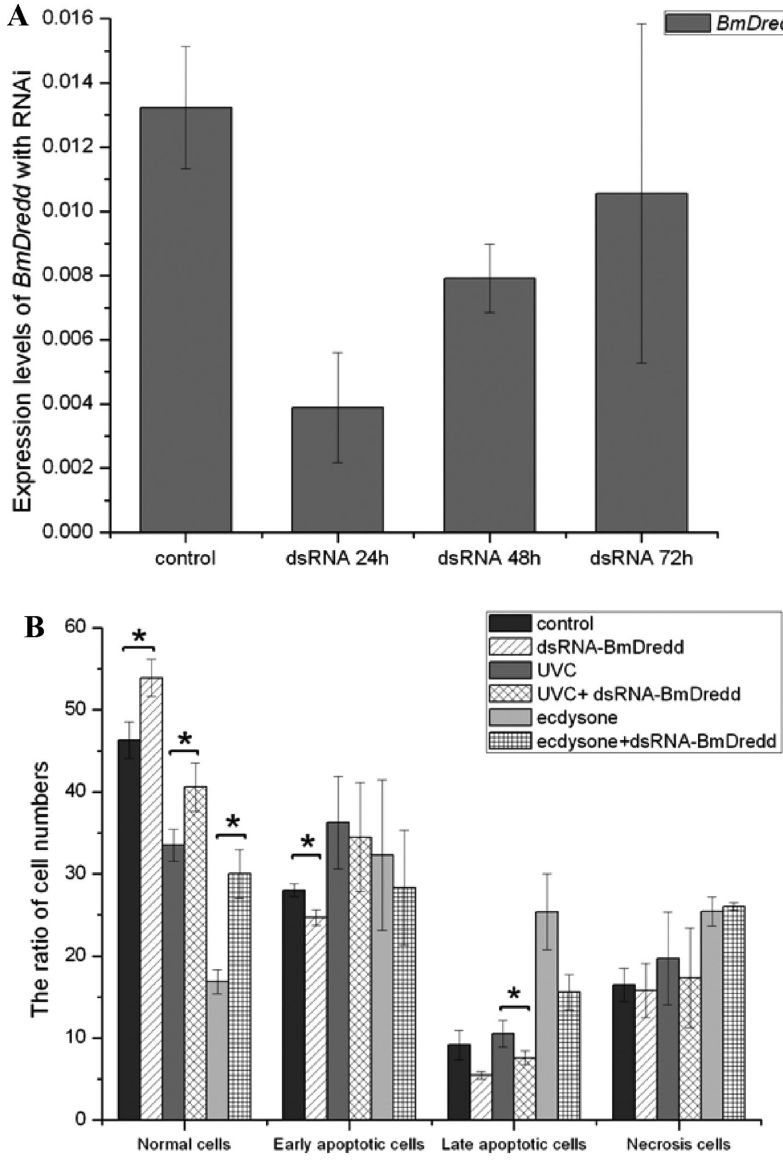

Figure 3. RNAi of *BmDredd*. (A)The expression levels of *BmDredd* after adding dsRNA-*Bm*Dredd in *Bm*N cells. (B) Cell apoptosis with different treatment (%). UVC, 30 sec, ecdysone, 200 ng/μl. $^{*}P < 0.05$. Error bar means standard error.

dsRNA Interference of BmDredd Reduced Cell Apoptosis Rate

To further investigate the role of *BmDredd*, we employed RNAi and the flow cytometry to detect the apoptosis rate (Fig. 3). The expression of *BmDredd* was decreased by 70.61% at 24 h and 40.23% at 48 h after adding dsRNA-*BmDredd* (Fig. 3A). Compared to the control dsRNA, adding dsRNA-BmDredd improved the normal cell (FITC–/PI–) number and reduced the early apoptotic cell (FITC+/PI–) number significantly. The late apoptotic cells (FITC+/PI+) also showed a slight descent (Fig. 3B). After UVC irradiation, normal cells

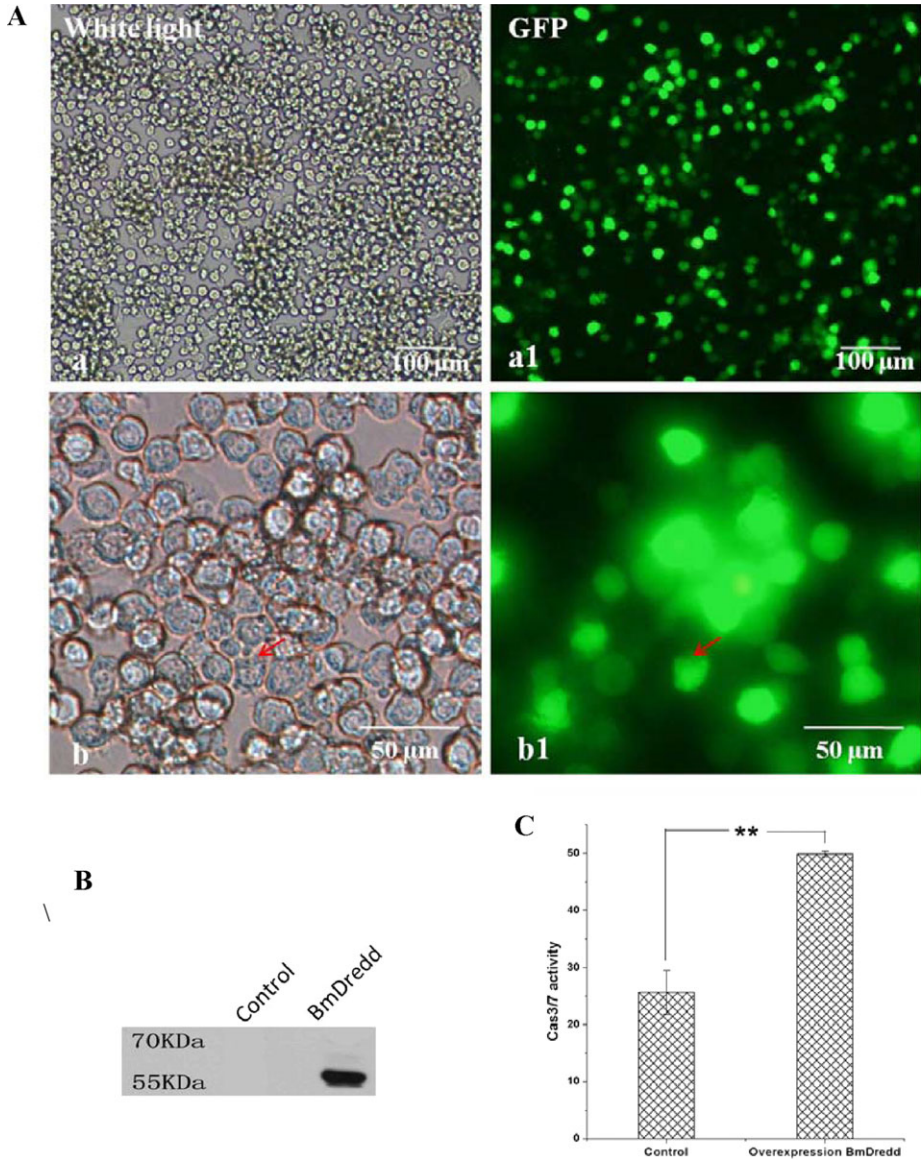

Figure 4. Overexpression of *BmDredd* in *Bm*N cells. (A) *Bm*N cells under fluorescent microscopy. a, a1 bar: 100 μm; b, b1 bar: 50 μm. The red arrows in b and b1 pointed a membrane blebbing cell undergo apoptosis. (B) Western blotting of overexpressed *Bm*Dredd. (C) The cas3/7-like activity of *Bm*N cells at 48 h after transfection of PXL-BACII (control) and PXL-BAC-U6-Dredd-GFP (overexpression *Bm*Dredd), respectively. $^{**}P < 0.01$. Error bar means standard deviation.

accounted for 33.51%, early apoptotic cells reached 36.25%, and late apoptotic cells' rate was 10.56%. We added dsRNA-*BmDredd* after UVC irradiation; the number of normal cells had a significant increase by 7.06%; early apoptotic cells and late apoptotic cells decreased 1.75 and 2.98%, respectively (Fig. 3B). The normal cells in ecdysone-treated group accounted for 16.88% and it rose to 30.02% after the addition of dsRNA-*Bm*Dredd. Its early

apoptotic cells and late apoptotic cells were dropped by 3.95 and 9.8%, respectively (Fig. 3B).

BmDredd Activated Caspase-3/7 and Induced Cell Apoptosis

The *Bm*N cells were screened with green fluorescence at 48 h posttransfection (Fig. 4A:a1 and b1). The protein band was consistent with the predicted molecular weight of 63 kDa (Fig. 4B) after transient overexpression. As the important effector caspases, caspase-3 and caspase-7 activities are vital index in detecting the apoptosis degree. The cas3/7-like activity in *BmDredd*-overexpressed cells was increased by 94.56% than control (Fig. 4C).

Location of BmDredd in BmN Cells

The role of *BmDredd* prodomain was unclear. Thereby, we employed immunofluorescence to analyze the conceivable function, simultaneously gave a clear view of the functional location of *BmDredd* in *Bm*N cells. In normal *Bm*N cells, *BmDredd* was expressed in cytoplasm and not found in the nuclei (Fig. 5A: Control). Act D was inserted into the DNA double helix in the minor groove, impeded the function of RNA polymerase, and inhibited the RNA synthesis, especially mRNA synthesis, which causes cell stress and induce cell apoptosis (Fig. S4). After adding Act D into the cell culture medium 36 h, *BmDredd* was present in the nuclei (Fig. 5A: Act D), which was identical to overexpression of *BmDredd* (Fig. 5A: *BmDredd*). However, after overexpression of its N- or C-terminal sequences (Fig. 5C), the cleaved proteins N-Dredd and C-Dredd did not appear in the nuclei (Fig. 5B: b, b1) even when cells were induced to apoptosis with Act D (Fig. 5B:b2, b3).

Interactions Among Bombyx Apoptosis-Related Genes BmDredd, BmDaxx, Bmcide-b, BmCreb, and Bmfadd

The dsRNA and the negative control dsRNA were added into the *Bm*N cells. The cells were collected after 24 and 48 h, respectively. Expression levels of *BmDredd*, *BmDaxx*, *Bmcide-b*, *BmCreb*, and *BmFadd* were measured by qRT-PCR.

BmDredd had a decrease of approximately 66% compared with the control at 24 h postinterference after dsRNA-*Bm*Dredd interference. While the expression of *BmDaxx* and *Bmcide-b* both showed an increase at this time and fell back to normal levels intentionally at 48 h postinterference. The expression level of *BmCreb* decreased at 24 h after dsRNA-*Bm*Dredd interference and increased at 48 h postinterference, which was opposite to trends of *BmDaxx* and *Bmcide-b* (Fig. 6A). Moreover, we found that there were no changes in expression levels of *BmDaxx*, *Bmcide-b*, and *BmFadd* after overexpression of *BmDredd* (Fig. 6D).

The expressed quantity of *Bmcide-b* dropped by approximately 64% compared with the control cells at 24 h and revealed a tiny rise at 48 h after dsRNA-*Bm*cide-b interference. The expression levels of *BmDredd*, *BmDaxx*, and *BmCreb* continued to rise from 24 to 48 h after interference (Fig 6C).

Compared to the control, the expression level of *BmDaxx* also exhibited a decrease by 50% at 24 h after dsRNA-*BmDaxx* interference and returned to normal levels at 48 h. However, the expression trends of other genes, *BmDredd*, *Bmcide-b*, *BmCreb*, and *BmFadd*, were entirely consistent with *BmDaxx*, which was different from knockdown of *BmDredd* or *Bmcide-b* (Fig. 6B).

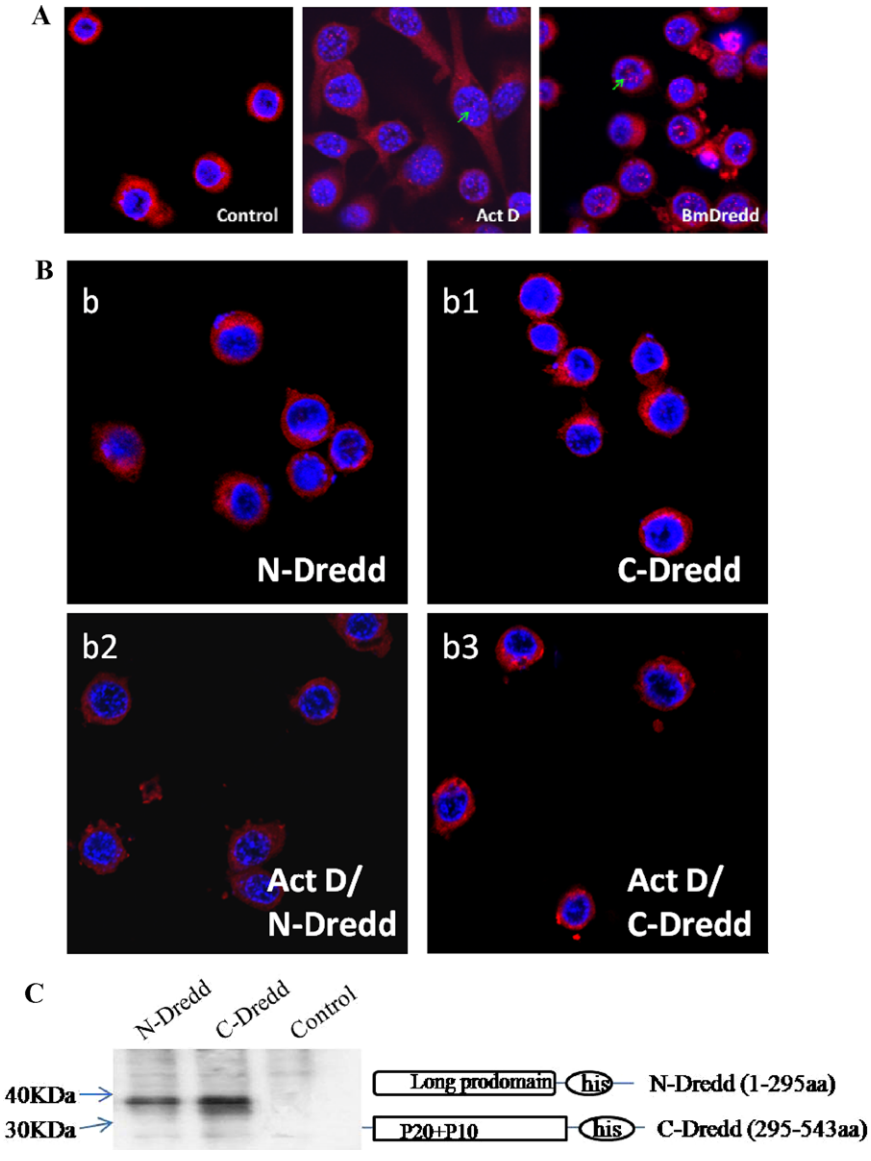

Figure 5. Immunofluorescence. (A) Location of *BmDredd* (red), nucleus was stained with DAPI (blue). Control: the endogenous *BmDredd*. *BmDredd*: overexpressed *BmDredd*. Green arrow: *BmDredd* appeared in nucleus. (B) Location of N-terminal sequence and C-terminal sequence of *BmDredd*. (C) Western blotting. Control was *Bm*N cells transfected with empty plasmid.

DISCUSSION

In mammals, there are two basic signaling pathways that lead to apoptosis. It is unclear whether mitochondria involves in apoptosis in model organism *Drosophila* (Dorstyn et al., 2004; Dorstyn and Kumar, 2006), but some studies found that these fundamental pathways may appear in the silkworm (Yi et al., 2014). By sequence blast, we have known that *Bm*Dredd unveils higher homology with mammalian caspase 8 than *DmDredd*, which

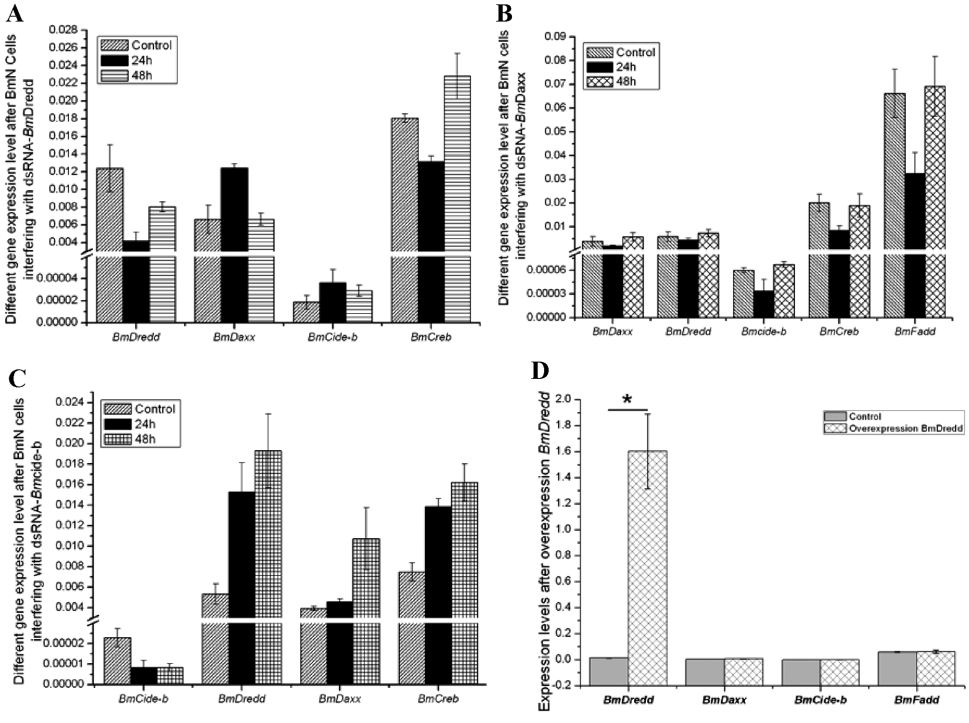

Figure 6. Relative expression levels of apoptosis-related genes in *Bm*N cells. (A) dsRNA interference. a: Treated with dsRNA-*BmDredd*; b: treated with dsRNA-*Bm*cide-b; c: treated with dsRNA-*Bm*Daxx. (B) Overexpression of *BmDredd* after 48 h. Error bar means standard deviation.

has been demonstrated regulating the apoptosis in *Drosophila*. The sequence identity is 25.1% with *Dr*cas8 and 25.8% with *Mm*cas3. We found the expression levels of *BmDredd* in *Bm*N cells increased significantly after UVC irradiation or ecdysone treatment. This finding suggested that *BmDredd* participated in regulating apoptosis of *Bm*N cells. Results of flow cytometry showed that percentage of normal cells increased and the number of apoptotic cells decreased when *Bm*N cells treated with UVC or ecdysone were added with dsRNA-*Bm*Dredd. These manifested that knockdown of *BmDredd* restrained *Bm*N cells from tending to death.

Furthermore, caspase-3/7-like activities displayed a significant rise compared to the control after overexpression of *Bm*Dredd, which indicated that affluent *Bm*Dredd within *Bm*N cells could induce cell apoptosis in a way. In addition, heterologous expression of *Bm*Dredd in sf9 cells caused the abnormal cell morphologies (Fig. S3). Sequence alignment has shown that 23.4% identity exists between *Bm*Dredd and *Dm*Dredd. Hence, we speculated that *Bm*Dredd also impacted the growth of sf9 cells. All of these data suggested that *Bm*Dredd may function as a caspase.

*Bm*Dredd was mainly expressed in the cytoplasm of normal cells. However, after cells were exposure to Act D or overexpressing *Bm*Dredd, it was detected in the nucleus. The work suggested that *Bm*Dredd caused apoptosis and would locate into nucleus when apoptosis occurred in cells. Nevertheless, overexpressed N-Dredd and C-Dredd were not found in the nucleus even by adding Act D to induce apoptosis (Fig. 5B). These findings demonstrated nuclear localization signal of *Bm*Dredd situated in the center of its protein sequence and *Bm*Dredd probably referred to DNA damage.

Prior studies have indicated that the apoptotic pathway in *Bm*N-SWU1 and *Bm*12 cells was extremely similar with that in mammals (Yi et al., 2014; Xie et al., 2016). We selected *BmDaxx*, *Bmcide-b*, *BmCreb*, *BmFadd*, and *BmDredd* to survey preliminary whether the regulation relationship in *Bm*N was the same as in mammals. Results showed that change trends of expression of other genes were entirely consistent with *Bm*Daxx after dsRNA-*Bm*Daxx interference, while the trends were completely contrary to *Bm*cide-b after dsRNA-*Bm*cide-b interference. Moreover, we found *BmDredd* transcription quantity was raised by overexpression of *Bm*Fadd and reduced by knockdown of *BmFadd* (data not shown). This proved that *BmDaxx* located in the upstream of singal pathway and *Bmcide-b* in the downstream. Meanwhile, the decline of *BmDredd* expression caused the expression escalation of *BmDaxx* and *Bmcide-b* except *BmCreb* that exhibited a descent. We concluded that there are complex interactions among these genes.

*Bm*Daxx may be located in the upstream of apoptosis pathway. When the stimulus signal of apoptosis activates *Bm*Daxx, it further activates the downstream *Bm*Dredd and *Bm*cide-b directly or indirectly. Meanwhile, activated *Bm*Dredd and *Bm*cide-b have positive feedback effect on *Bm*Daxx. This showed great consistency with mammals.

Overall, we discover that *Bm*Dredd has the function of activating apoptosis in *Bm*N cells and it coordinates with above genes. However, this could not accurately reflect the apoptosis pathway of *Bm*N cells, further investigations are required.

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