Full Length Research Paper

Expression and functional analysis of apoptosis-related gene *BmICAD* in silkworm, *Bombyx mori*

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The *ICAD* gene plays an important role in the growth and development processes in insects. We conducted a molecular cloning and functional analysis to study a specific silkworm gene *BmICAD* related to apoptosis. The *BmICAD* gene was obtained from the fifth instar larvae of the silkworm by RT-PCR and over-expressed in *Escherichia coli* as His-tagged fusion proteins. Subcellular localization of the protein indicated that BmICAD was found in the cytoplasm near the nucleus. RNAi assay indicated that the apoptosis rate of Bm5 cells increased markedly. A His pull-down assay was used to investigate proteins that bind to rBmICAD. Two proteins, DNA supercoiling factor (SCF) and secreted protein acidic and rich in cysteine (SPARC), were found. These results indicate that down regulation of *BmICAD* can increase the apoptosis rate of Bm5 cells, and that SCF and SPARC may have important roles by interacting with BmICAD during this process.

Key words: *Bombyx mori*, BmICAD, subcellular localization, RNAi, protein interaction.

INTRODUCTION

Apoptosis, or programmed cell death (PCD), is an essential process during normal development, cell senescence, cell damage and homeostasis of a multi-cellular organism (Van de Craen et al., 1997; Feng et al., 2004). Apoptosis is the process of cellular self-destruction, regulated by increasing the number of genes that are conserved in all metazoans and recognized by characteristic morphological and molecular changes.

DNA fragmentation is a prominent feature of apoptosis (Wyllie, 1980). DFF (DNA fragmentation factor)-caused DNA breaks is an important step in apoptosis in vertebrate cells (Eckhart et al., 2007). DFF is a protein complex, composed of the inhibitor of caspase-3-activated DNAase (ICAD) and caspase-activated DNase (CAD) (Enari et al., 1998; Liu et al., 1997). ICAD cannot only inhibit the activity of CAD DNase, but also has a chaperone-like activity specific for CAD (Sakahira et al., 1998). Caspase 3 is a protease that is activated during apoptosis and can cleave and inactivate its CAD-inhibitory effect, releasing CAD to enter the nucleus and thus cause DNA cleavage and nuclear enrichment (Sakahira et al., 1998; Liu et al., 1998). Previous studies showed that both DFF45/ICAD and DFF40/CAD contain a motif known as a CIDE (cell death-inducing DFF45-1ike effectors) domain. It has been shown previously that the homologous N-terminal domains of CAD and ICAD bind to each other and that this binding is a crucial step in formation of the complex between CAD and ICAD (Liu et al., 1998). CIDEs are pro-apoptotic proteins that induce DNA fragmentation as well as other apoptotic features such as membrane blebbing and nuclear condensation (Hoffmann et al., 2004; Inohara et al., 1998).

Bombyx mori is an important model organism for Lepidoptera, undergoing complete metamorphosis (Kuroda et al., 1989). PCD is crucial in body restructuring during metamorphosis of holometabolous insects (Tettamanti et al., 2007); it is therefore important to study the mechanism of apoptosis during the metamorphosis of silkworm. Most studies on ICAD have been limited to gene cloning, sequence analysis and expression (Wang et al., 2006); there are few reports on the apoptotic

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Abbreviations: OFR, Open reading frame; CIDE, cell death-inducing DFF45-1ike effectors; CAD, caspase-activated Dnase; ICAD, inhibitor of caspase-3-activated DNAase; DFF, DNA fragmentation factor; PCD, programmed cell death; SCF, supercoiling factor; SPARC, secreted protein acidic and rich in cysteine.

mechanisms of ICAD. In this study, we identified a silkworm ICAD complementary DNA (cDNA; *Bm-ICAD*) from the fifth instar larvae of *Bombyx mori* by RT-PCR. Using bioinformatic analysis, the open reading frame (ORF) of *BmICAD* gene, molecular weight and conserved domain of the BmICAD protein were predicted. Immunocytochemistry and Western-blot analysis were used to determine the subcellular localization and expression levels of BmICAD in *E. coli*. Furthermore, the biological function of BmICAD was studied by RNA interference analysis and possible interaction proteins with BmICAD were identified by pull-down assays. The results provide an important foundation for further studies on the functional roles of BmICAD during the metamorphosis of silkworm.

MATERIALS AND METHODS

Bioinformatic analyses

The *BmICAD* gene was obtained from the fifth instar larvae of *Bombyx mori* by RT-PCR. The similarity analysis of nucleotide and protein sequences was performed using the BLAST algorithm from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The characteristics of this gene were analyzed using DNAstar software.

Insects, strains, growth media and reagents

The *Bombyx mori* strain used in this study was the progeny of Jingsong×Haoyue. Silkworms were reared on mulberry leaves under standard conditions. The fifth instar larvae were dissected, frozen immediately in liquid nitrogen, and stored at –80°C. *Escherichia coli* strains TG1 and BL21 (DE3) were grown at 37°C in LB media, pH 7.5, containing 5 g yeast extract, 10 g tryptone peptone, and 10 g NaCl per liter. Recombinant *E. coli* strains were grown in the presence of kanamycin (50 μg/ml). Bm5 cells (a gift from Prof. Zhang ZF) were cultured in TC-100 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Gibco BRL) at 27°C. 4'-6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma. Cy3-labeled goat anti-rabbit IgG was purchased from Proteintech Group Inc.

Cloning, expression, purification and antibody preparation

Total RNA was extracted from silkworm fifth instar larvae using Trizol regent (Invitrogen) as instructed by the manufacturer. The Ready-To-Go T-Primed First-Strand Kit (Amersham) was used to synthesize first-strand cDNAs from total RNA. Using the first-strand cDNA as a template, the *BmICAD* ORF was amplified by polymerase chain reaction (PCR). Based on the *BmICAD* cDNA sequence, a pair of primers was designed. For subcloning purposes, two restriction endonuclease recognition sites, *SacI* and *XhoI*, were included at the 5′-ends of the primers. The forward primer was P1 5′-CGAGCTCATGGAAAACGACATTAAC-3′, and the reverse primer was P2 5′- CCCTCGAGTTAGCAATTCCATAGC-3′. The *BmICAD* ORF was subcloned into the prokaryotic expression vector pET-28a (+) to construct the recombinant plasmid pET-28a(+)-*BmICAD*. *Escherichia coli* BL21 (DE3) cells were transformed with the recombinant plasmid. The transformed *E. coli* were grown in culture, and recombinant protein expression was induced by incubation at 25°C for 12 h with 1 mM IPTG. The recombinant His-tagged fusion protein was extracted from the

bacteria and purified using Ni-Sephadex^{TM} G-25 Superfine (HiTrap^{TM} Desalting Column, Amersham) metal-chelating affinity chromatography. Purified 6×His-BmICAD was used to generate anti-BmICAD polyclonal antibody in a New Zealand rabbit. Antiserum from the immunized rabbit was passed through a Protein A HP chromatography column to obtain purified rabbit IgG. ELISA was used to determine the polyclonal antibody titer, and the specificity of the polyclonal antibody was determined by Western blot analysis; the purified polyclonal antibody was diluted 1:1,000 and incubated with the immunoblot at room temperature for 2 h, the goat anti-rabbit secondary antibody was diluted 1:1,000.

Subcellular localization of BmICAD

Bm5 cells were cultured overnight on glass cover slips and grown to 50-60% confluence, washed for 10 min three times in phosphate buffered saline (PBS), and fixed (3.7% poly-formaldehyde) at room temperature for 15 min. The cells were then permeabilized by incubation with 0.2% Triton X-100 at room temperature for 5 min and washed three times in PBS. The cells were blocked in 5% BSA in PBS at room temperature for 2 h, followed by three 10 min washes in PBS containing 0.05% Tween-20 (PBST). Cells were then incubated with antiserum containing anti-BmICAD polyclonal antibody (diluted 1:200 in blocking buffer) at 4°C overnight. Simultaneously, control cells were incubated with the negative serum obtained from the rabbit before immunizing with the antigen. After three 10-min washes in PBST, cells were incubated with Cy3-labeled goat anti-rabbit IgG (diluted 1:1500 in PBS; Promega) at 37°C for 2 h and then washed twice for 10 min in PBST. Cells were then incubated with 4′-6-diamidino-2-phenylindole (diluted 1:2000 in PBS; Promega) at room temperature for 15 min, and washed three times in PBST. Cells were examined using a Nikon ECLIPSE TE2000-E Confocal Microscope; images were analyzed using EZ-C1software.

RNAi and cell transfection

To determine the biological function of BmICAD, Bm5 cells were transfected with *BmICAD* dsRNA. The *BmICAD* ORF was amplified by PCR using primers (Pa, GGATCC<u>TAATACGACTCACTATAG</u>GATGGAAAACGACATTAAC, Pb, GGATCC<u>TAATACGACTCACTATAGG</u>TTAGCAATTCCATAGC P1 and P2) containing T7 promoter sequences (underlined). pET-28a$^{(+)}$-BmICAD was used as a template for the PCR, and the two single strands were amplified using two primer pairs: Pa and P2, and Pb and P1. Using the T7 RiboMAXExpress RNAi System kit (Promega), the dsRNA was synthesized and the PCR products were purified according to the manufacturer's instructions. The products were quantified spectrophotometrically at 260 nm and 280 nm, and sizes were confirmed by agarose gel electrophoresis. Silkworm Bm5 cells were seeded in 24-well plates containing complete TC-100 medium and left overnight to attach, then the cells were transfected with *BmICAD* dsRNA (0.9 μg/well) using liposomes (Invitrogen, Carlsbad) in serum-free media. Cells transfected with liposome was using as negative control. Following continuous culture for 6 hours, media was with fresh normal growth media and incubated for a further 72 h at 27°C.

Detection of BmICAD protein expression by Western blot

Both *BmICAD* dsRNA transfected cells and control group cells were harvested and lysed in PBS. The lysates were centrifuged at 12000 r/min at 4°C for 20 min to collect the supernatants. The proteins concentration was determined by Bradford methods. For each
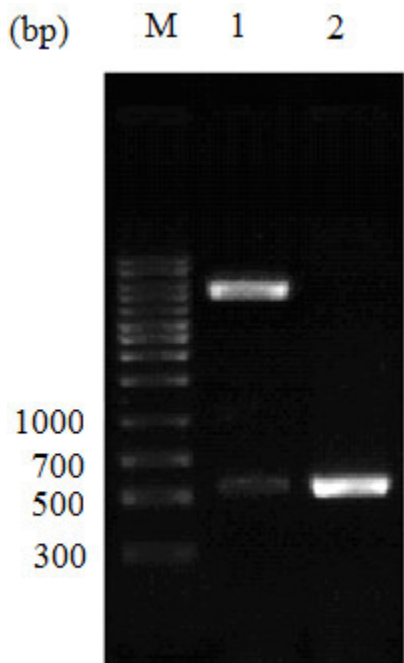
Figure 1. The identification of recombinant plasmid pET28a(+)-*BmICAD*. M, DNA ladder marker; lane 1, recombinant plasmid digested with *SacI/XhoI*; Lane 2, the amplification products of PCR using pET28a (+)-*BmICAD* as a template.

protein sample, 20 µg proteins were loaded to each well of a 10% SDS-PAGE gel and electrotransferred onto PVDF membrane. After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with the purified primary polyclonal antibody diluted by 1:1,000 at room temperature for 2 h at 37°C. After three washes with TBST for 15 min each at room temperature, the membranes were incubated with the goat anti-rabbit secondary antibody diluted by 1:1,000. Densitometric analysis was performed using an image scanner and analyzing software (NIH image Ver. 1.61).

Evaluation of Bm5 cell viability

Bm5 cells were seeded in 96-well plates containing complete TC-100 media and left overnight to attach. The complete media was then removed and the cells were treated with dsRNA for 72 h or with a UV crosslinker (model UVC2500) for 10 min. After 10 min UV radiation treatment, cells were cultured in complete media for another 72 h. The Bm5 cells viability was estimated after treatment with dsRNA, UV radiation, or dsRNA plus UV radiation; cells transfected with liposomes were used as a negative control and those treated with UV radiation as a positive control. The effectiveness of the RNAi or UV radiation experiment for 72 h was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide; Sigma) assay. Briefly, 1 mg/ml MTT (final concentration) was added to the well and incubated for 4 h at 27°C. The medium was removed and cells were lysed with 2-isopropanol containing 0.04 mol/l HCl. The absorbance measured at 490 nm was used to calculate the relative cell viability ratio (Dimmeler et al., 1997).

Evaluation of Bm5 cells apoptosis

The effects of RNAi on cell growth and death were determined by examining chromosomal DNA fragmentation. For quantitative determination, Bm5 cell apoptosis was measured as DNA fragmentation. DNA fragmentation was evaluated by measuring histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions. Briefly, attached cells were harvested and the cell suspension was pelleted by centrifugation. Floating and attached cells were lysed. After centrifugation, the supernatant was measured by ELISA (Yu et al., 2006).

In vitro binding assays

An *in vitro* His pull-down assay was used to identify the interactions between BmICAD and total extracted protein from Bm5 cells. The Bm5 cells were collected and lysed in PBS, the total protein were extracted for binding assays. The total protein (10 µg) and purified 6×His-BmICAD (10 µg) were loaded onto a 0.1 ml HiTrap™ desalting column (Amersham) overnight at 4°C. The mixture was transferred to a 2 ml tube and washed three times with 1.5 ml of wash buffer (20 mM Tris–HCl (pH 7.4), 0.5 M NaCl and 20 mM imidazole), centrifuged for 10 s at 12000 g at 4°C. The pellet was washed three times with 1.5 ml of elution buffer (20 mM Tris-HCl (pH 7.4), 0.5 M NaCl and 200 mM imidazole), centrifuged for 10 s at 12000 g at 4°C. Samples without purified 6×His-BmICAD or Bm5 cell total protein were used as negative controls. Samples were separated by SDS-PAGE and digested with trypsin; peptide sequences were determined using an ABI4700MALDI-TOF /TOF MS. The obtained peptide mass fingerprinting (PMF) was searched in the NCBI protein data bank.

RESULTS

Bioinformatic analyses

The BmICAD cDNA was 844 bp in length and contained a 522 bp ORF (269–791 bp) which encoding a protein of 174 amino acids with a predicated molecular weight of 19.6 kDa. Using NCBI BLAST, we found that this protein contains an N-terminal CIDE-N domain that was conserved in other known pro-apoptotic CIDE-N proteins, a novel family of cell death activators. These results reveal that BmICAD is a member of this family and its biological activity is possibly similar to the ICAD in mammals. In normal cells it might also act as the inhibitor of CAD by its binding to CAD.

Prokaryotic expression of BmICAD and preparation of polyclonal antibody

The *BmICAD* ORF was subcloned into the pET-28a (+) prokaryotic expression vector (Figure 1). A BmICAD fusion protein was expressed successfully in *E. coli*. The molecular weight of the recombinant fusion protein, as determined by electrophoresis, was 25 kDa, which is consistent with the predicted value (Figure 2A). After Ni^{2+} affinity chromatography, recombinant BmICAD was highly purified as detected by electrophoresis (Figure2B).
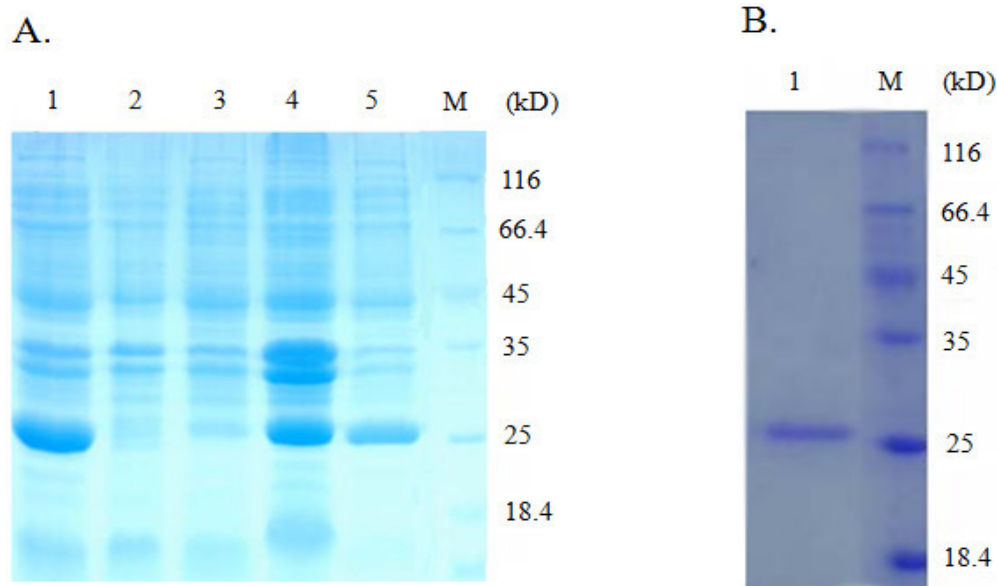
Figure 2. Expression and purification of rBmICAD. A. lane M, protein molecular-weight marker (values are in kDa); lane 1, total extract of BL21-pET-28a-*BmICAD* bacteria after induction with IPTG; lane 2, total extract of BL21-pET-28a(+) bacteria after induction with IPTG; lane 3, total extract of BL21-pET-28a-*BmICD* bacteria without induction; lane 4, the precipitate of sonicated lysate; lane 5, the supernatant of sonicated lysate. B. lane M, protein molecular-weight marker (values are in kDa); lane 1, purified His-tagged BmICAD.

The anti-BmICAD polyclonal antibody titer was determined by ELISA, positive serum extinction value/negative serum extinction value (P/N) ≥ 2.1 was positive; 1.5 ≤ P/N < 2.1 was suspicious expression; P/N < 1.5 was negative. Finally, the titer of the antibodies was determined to be 1:25,600, which could meet the requirements for all next experiments. Western blot was used to analyze the specificity of BmICAD polyclonal antibodies; the antibody was incubated with the prokaryotic expression product. The results indicate that the antibody could react specifically with rBmICAD.

Subcellular Localization of BmICAD

The cells that had been treated with Cy3-labeled goat anti-rabbit IgG and DAPI were examined using a Nikon ECLIPSE TE2000-E Confocal Microscope, and images were analyzed using EZ-C1software. Cy3-labeled goat anti-rabbit IgG emits red fluorescence when stimulated with light of wavelength 550 nm, and DAPI-stained nuclei emit blue fluorescence when stimulated with light of wavelength 353 nm. Our results reveal that BmICAD is mainly located in the cytoplasm near the nucleus (Figure 3).

Effect of BmICAD dsRNA transfection on BmICAD expression

To test the role of BmICAD in the process of Bm5 cell

apoptosis, dsRNA against *BmICAD* were synthesized. 1% agarose gel electrophoresis showed that the size of synthetic dsRNA was consistent with the predicted value. The absorbance of a diluted dsRNA (40×) sample was measured at 260 and 280 nm; the A260/A280 ratio was between 1.8 and 2.0 and the concentration of the BmICAD dsRNA was 1.87 µg/µl. Synthesized dsRNA was transfected into the Bm5 cell, normal cells transfected with liposome only were used as a negative control. Western blot demonstrated that synthesized dsRNA could inhibit the expression of BmICAD protein effectively (Figure 4) and it was therefore used for further studies.

Effect of RNAi on Bm5 cell viability

To determine the role of BmICAD in Bm5 cell viability, MTT assay was used to test the cell viability after RNAi and UV radiation treatment. MTT experiments demonstrated that the optimal concentration of the BmICAD dsRNA for use in RNAi experiments was 0.9 µg/well and that the optimal duration of the interference was 72 h (data not shown). The MTT results showed that the activity of Bm5 cells decreased significantly following RNAi or UV radiation treatment, indicating that silencing the BmICAD gene resulted in increased cell death (Figure 5).

Effect of RNAi on Bm5 cell apoptosis

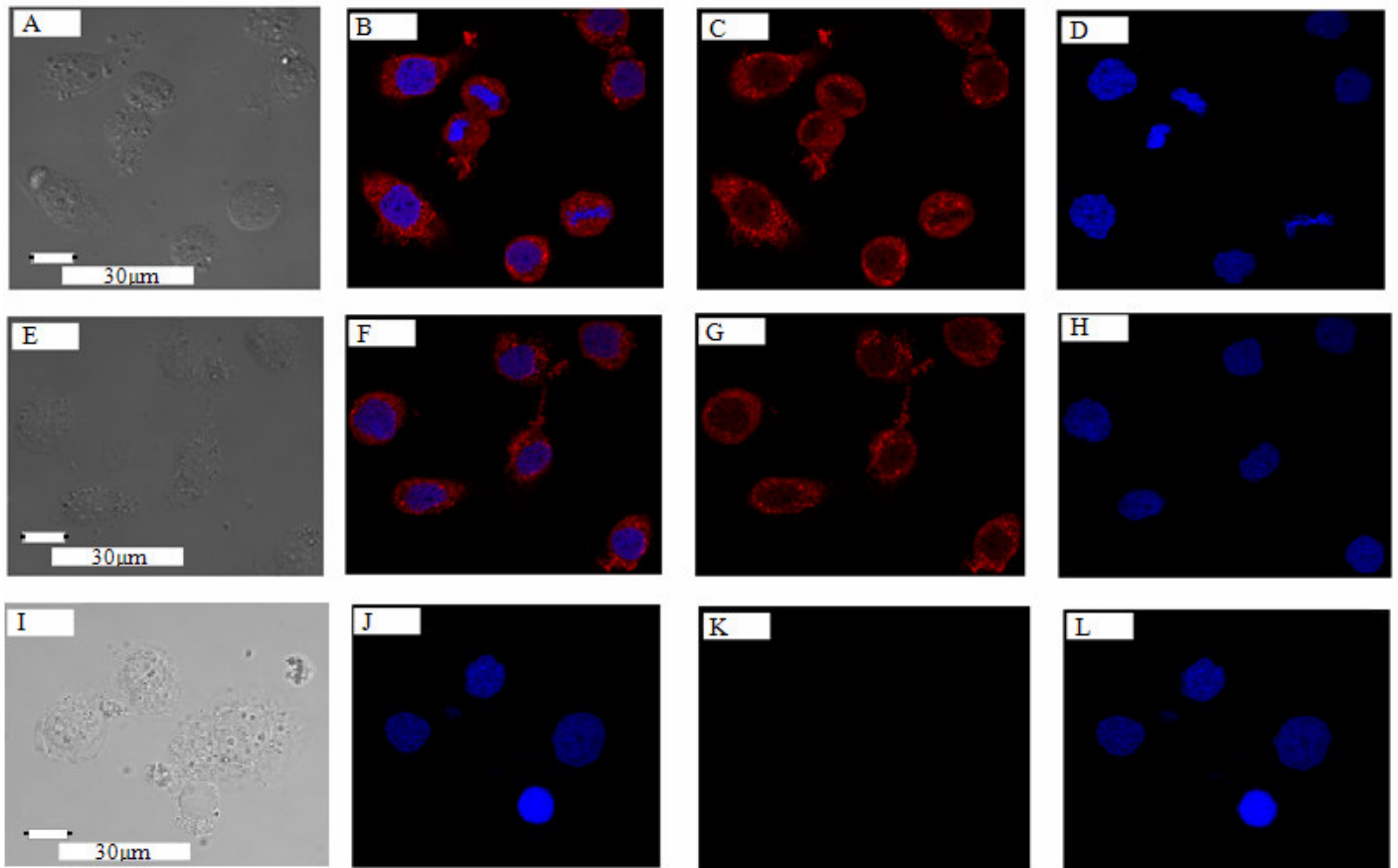To determine whether *BmICAD* gene plays an important

Figure 3. The subcellular localization of BmICAD. A, E, I. Cells in the light transmission. B, F, J. Merged image. C, G, K. Intracellular BmICAD stained with Cy3. D, H, L. Nucleolus stained with DAPI. I-L.Negative control. Scale bars represent 30μm.
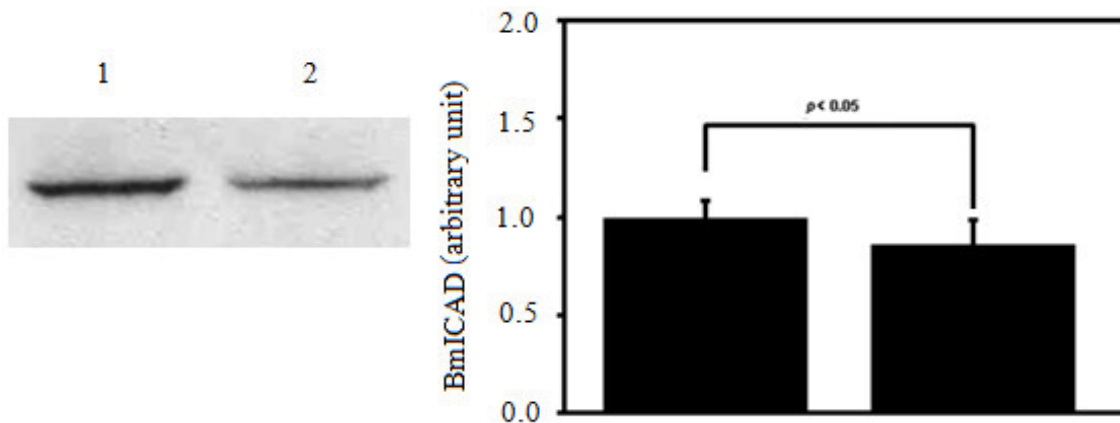

Figure 4. Western blot analysis of *BmICAD* dsRNA interference. Lane 1, control group: cells were transfected with liposome; lane 2, RNAi group: cells were transfected with *BmICAD* dsRNA. Right panels show the results of densitometric analyses of immunoblotting (mean ± SEM, n=3).

role in the process of Bm5 cells apoptosis, RNAi and UV radiation treatment were carried out to test the rate of cells apoptosis. As measured by DNA fragmentation,

Bm5 cells apoptosis was significantly increased by RNAi or UV radiation treatment and was particularly increased after treatment with dsRNA and UV radiation, compared
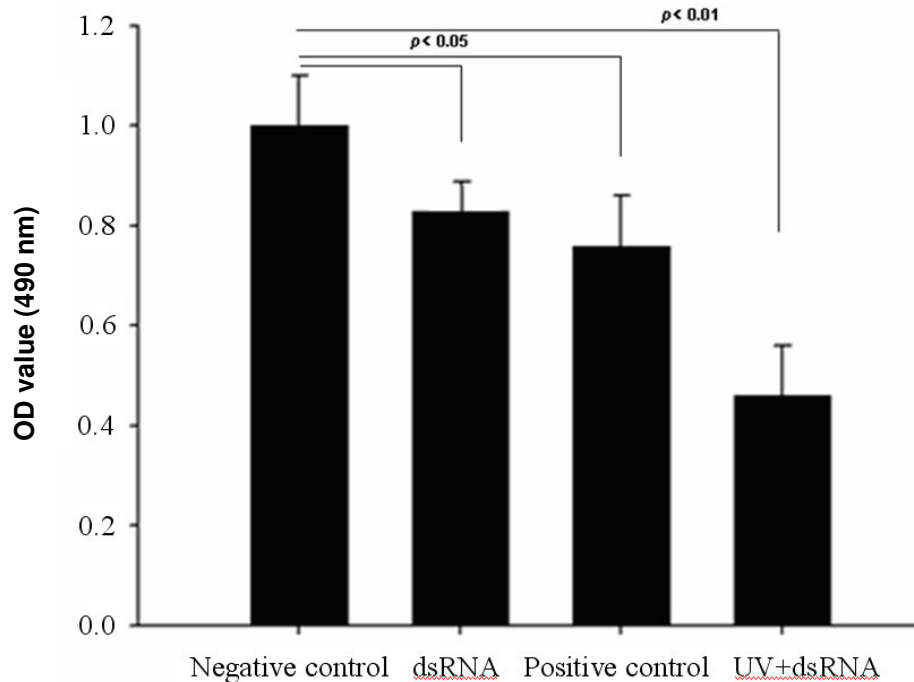
Figure 5. Effect of RNAi or UV radiation treatment on Bm5 cell viability. Negative control, cells were transfected with liposome; dsRNA, cells were treated with *BmICAD* dsRNA for 72 h; positive control, cells were treated with UV radiation for 10 min; UV+dsRNA, cells were treated with UV radiation for 10 min and *BmICAD* dsRNA for 72 h. Cell viability was evaluated at 72 h after dsRNA treatment or UV radiation by measuring the optical density at 490 nm (mean ± SEM, n =3).

with the control group (Figure 6). Consistent with this effect, changes in cell viability was also decreased by RNAi or UV radiation treatment (Figure 5).

Analysis of BmICAD-binding proteins

To analyze proteins that interact with BmICAD, samples eluted from HiTrap^{TM} Desalting columns were separated by SDS-PAGE (Figure 7). Compared with the negative control groups (without purified 6×His-BmICAD), five different protein points were selected and peptide sequences were further determined by ABI4700MALDI-TOF/TOF MS after trypsin digests. After screening the protein sequence databases, two possible interacting proteins, BmSCF (DNA supercoiling factor) and BmSPARC (secreted protein acidic and rich in cysteine), were identified (Table 1).

DISCUSSION

Apoptosis has a very important role during larval tissue destruction in the silkworm. Previous studies revealed that ICAD is involved in this process because it is the substrate of activated caspase 3 during the execution

phase of apoptosis (Yokoyama et al., 2000). However, it was unknown whether BmICAD is also involved in the execution of cell death as ICAD in other species and its role during the process of cell death. In this study, we cloned and expressed the *BmICAD* gene in *E. coli* and analyzed the possible relationship between BmICAD and apoptosis by RNAi and DNA fragmentation. The results show that silencing the *BmICAD* gene resulted in increased cell death and downregulation of cell activity. These results are in agreement with previous results in HeLa cells (Tsuruta et al., 2007). They suggested that RNAi could decrease the level of CAD and further inhibited internucleosomal DNA degradation in HeLa cell. In normal cells, CAD is produced as a complex with ICAD; ICAD specifically inhibits CAD-induced degradation of nuclear DNA and DNase activity (Enari et al., 1998; Liu et al., 1997). So the effect of *CAD* RNAi and the binding of CAD/ICAD complex are similar, both of them can decrease cell apoptosis by downregulating the activity of CAD. Our results suggest that *BmICAD* dsRNA might promote cell apoptosis by downregulating the expression of *BmICAD* and therefore releases CAD, allowing CAD to enter the nucleus and degrade chromosomal DNA.

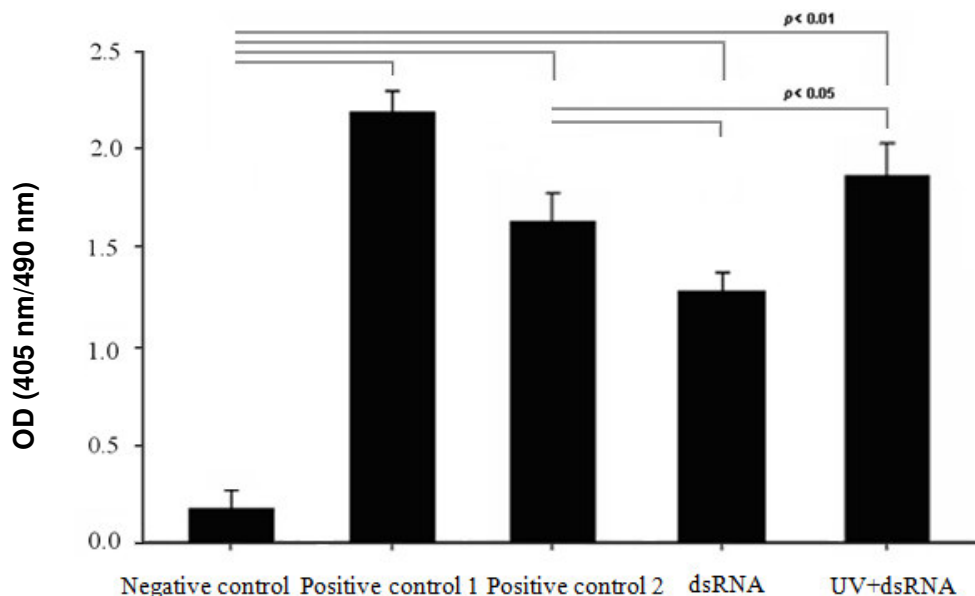Immunostaining of endogenous ICAD showed it to be in the nucleus of human epithelial and HeLa cells (Liu et

Figure 6. Detecting the effect of *BmICAD* RNAi or UV radiation treatment on Bm5 cell death by ELISA. Negative control, cells were transfected with liposome; positive control 1, DNA-histone complex (provided with kit); positive control 2, cells were treated with UV radiation for 10 min; dsRNA, cells were treated with dsRNA of *BmICAD* for 72 h; UV+dsRNA, cells were treated with UV radiation for 10 min and dsRNA of *BmICAD* for 72 h. Cell apoptosis was evaluated by measuring the optical density at 405 nm/490 nm (mean ± SEM, n =3).
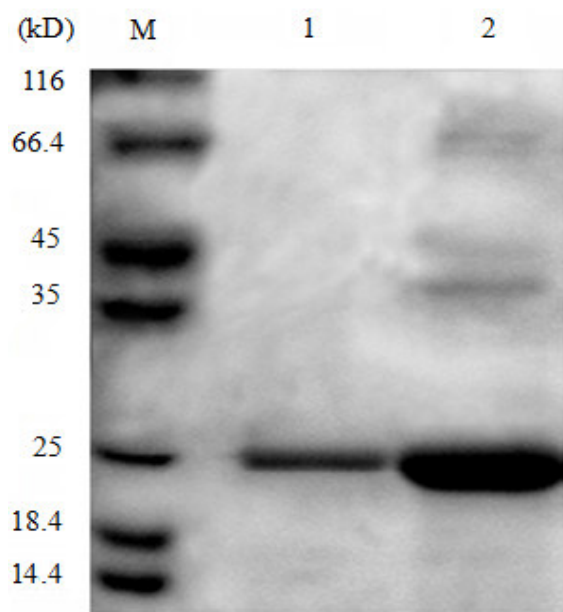

Figure 7. His pull-down assay. The purified 6×His-BmICAD and total extracted protein from Bm5 cells were loaded onto a HiTrap™ Desalting Column overnight. After washing, the bound proteins were separated by SDS-PAGE. Lane M, protein molecular-weight marker (values are in kDa); lane 1, the control group, eluted proteins after the incubation of Bm5 cell total extracted protein with HiTrap™ Desalting Column; lane 2, experimental group, eluted proteins after the incubation of total extracted protein from Bm5 cells with column purified 6×His-BmICAD.

al., 1998; Lechardeur et al., 2000), but in the cytoplasm of activated thymocytes and Hodgkin's lymphoma cells (Nagata et al., 2002; Xerri et al., 2000). These contradictory results may indicate that ICAD is localized in the nucleus or cytoplasm in a cell-specific manner. Our subcellular localization results showed that BmICAD was mainly found in cytoplasm near the nucleus. This result is in agreement with previous results in *Drosophila*; *Drosophila* ICAD does not have a nuclear localization signal (NLS) at the position corresponding to the NLS of mammalian ICAD proteins (Yokoyama et al., 2000; Mukae et al., 2000). Therefore, insect ICAD may be also localized to the cytoplasm due to the lack of a NLS. The localization of BmICAD in cytoplasm is near to nucleus, it might facilitate the released CAD to enter the nucleus and thus, cause DNA cleavage and nuclear enrichment (Sakahira et al., 1998; Liu et al., 1998).

In the protein interaction studies, several specific bands were found, five different protein points were selected and peptide sequences were further determined by MS. According to the obtained PMF, we searched the NCBI protein data bank, only two possible interacting proteins, BmSCF and BmSPARC, were found. We couldn't find more information about the other three samples; they might be new proteins which are not included in the protein data bank. We will perform extensive studies to address this in our next work.

As well known, the primary role of BmSCF is as a DNA helicase (Ohta et al., 1995); it is necessary to uncoil the super-helix structure of DNA to facilitate the cleavage of

Table 1. The identification result of possible BmICAD-binding proteins.

Spot ID	Mr	pI	Protein name
gil 112983659	37870.9	4.49	DNA supercoiling factor (*Bombyx mori*)
gil 114051654	37359.2	4.86	secreted protein acidic and rich in cysteine (*Bombyx mori*)

Spot ID is the accession number from NCBInr; Mr: molecular mass (kD); pI: isoelectric point.

DNA. BmSCF was thought to localize to the nucleus, but our results show that BmICAD was localized to the cytoplasm (Figure 3) raising the question of how the two proteins can interact. BmSPARC can inhibit cell proliferation and induce apoptosis in tumor cells (Tai and Tang, 2008). However, the mechanism by which BmICAD interacts with BmSCF to inhibit cell apoptosis and the effects of BmSPARC on BmICAD during silkworm cell apoptosis remains unknown. The answers to these questions will be important to understand the specific apoptotic mechanism during silkworm metamorphosis and the developmental phase.

ACKNOWLEDGMENTS

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