Microplitis bicoloratus bracovirus regulates cyclophilin A-apoptosis-inducing factor interaction to induce cell apoptosis in the insect immunosuppressive process

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Abstract
Microplitis bicoloratus bracovirus (MbBV) induces apoptosis in hemocytes of the host (Spodoptera litura) via the cyclophilin A (CypA)-mediated signaling pathway. However, the mechanisms underlying CypA-mediated signaling during apoptosis remain largely unknown. Therefore, in this study, we investigated how CypA and apoptosis-inducing factor (AIF) interact during MbBV-mediated apoptosis. Our findings showed that MbBV induces apoptosis through the CypA-AIF axis of insect immune suppression. In MbBV-infected Spli221 cells, both the expression of the cypa gene and the release of AIF from the mitochondria increased the number of apoptotic cells. CypA and AIF underwent concurrent cytoplasm-nuclear translocation. Conversely, blocking of AIF release from mitochondria not only inhibited the CypA-AIF interaction but also inhibited the cytoplasmic-nuclear translocation of AIF and CypA. Importantly, the survival of the apoptotic phenotype was significantly rescued in MbBV-infected Spli221 cells. In addition,
we found that the cyclosporine A-mediated inhibition of CypA did not prevent the formation of the CypA and AIF complex; rather, this only suppressed genomic DNA fragmentation. In vitro experiments revealed direct molecular interactions between recombinant CypA and AIF. Taken together, our results demonstrate that the CypA-AIF interaction plays an important role in MbBV-induced innate immune suppression. This study will help to clarify aspects of insect immunological mechanisms and will be relevant to biological pest control.

**KEYWORDS**
apoptosis, apoptosis inducing factor (AIF), cyclophilin A (CypA), immunosuppression, hemocytes, *Microplitis bicoloratus* bracovirus (MbBV)

## INTRODUCTION

*S. litura* is an agricultural insect pest that is parasitized by the wasp *Microplitis bicoloratus*. In this parasitic relationship, the wasp completes its development by injecting eggs into *S. litura* larvae and suppressing the host’s immune system (K. Luo & Pang, 2006; K. J. Luo et al., 2007; Strand & Pech, 1995). The eggs of *M. bicoloratus* carry the *M. bicoloratus* bracovirus (MbBV), a polydnavirus (PDV), and thus MbBV is transferred to the host during parasitism. The viral genes are involved in the inhibition of the host immune response to ensure the proper development of the wasp larvae (Falabella et al., 2007; Strand & Burke, 2012; Yu et al., 2016). In the immunosuppressive process, MbBV can cause the apoptosis of host hemocytes and thereby lower cellular immunity (Dong et al., 2017; Kou et al., 2017; M. Li et al., 2014; K. Luo & Pang, 2006). However, the mechanisms by which the host hemocyte apoptosis is initiated by MbBV have not yet been determined.

Cyclophilin A (CypA) is an 18 kDa cytosolic protein, and *S. litura* CypA, encoded by the *ppia* gene, consists of 165 amino acids. It is a member of the immunophilins and an intracellular receptor of the immunosuppressive drug cyclosporine A (CsA) (Davis et al., 2010; Handschumacher et al., 1984; Tian et al., 2019). In addition to participating in the immunosuppressive process, CypA is also involved in various other cellular activities, including inflammation (L. Liu et al., 2010; Seizer et al., 2012; Takapoo et al., 2011), apoptosis (Candé et al., 2004; Piao et al., 2012; Tanaka et al., 2011; Zhu et al., 2007), viral infection and replication (He et al., 2012; Jie et al., 2014; Zhou et al., 2012), and cancer proliferation and metastasis (Grigoryeva et al., 2014; Guo et al., 2017; Mingjun et al., 2011; Obchoei et al., 2011). Our previous study demonstrated that CypA is required for MbBV-induced apoptosis during the insect cellular immune response, as CypA undergoes nuclear translocation in MbBV-infected cells (Tian et al., 2019). However, the molecular mechanism of this translocation remains unclear.

Apoptosis-inducing factor (AIF), a flavoprotein normally distributed in the mitochondrial inter-membranous space, can translocate from the mitochondria to the nucleus through the cytoplasm and participate in apoptosis-associated chromatinolysis under apoptotic stimuli (Daugas et al., 2000; Joza et al., 2001a; Susin et al., 1999). Additionally, AIF and CypA co-translocate to the nucleus in neurocytes and participate in apoptosis-associated chromatinolysis (Candé et al., 2004). Therefore, we wondered whether AIF is also involved in the MbBV-induced translocation of CypA and host cell apoptosis in the insect immunosuppressive process.

To explore this conjecture, we attempted to clarify the mechanism of CypA-induced apoptosis in MbBV-infected cells and the MbBV-regulated CypA-AIF interaction for induction of cell apoptosis under host immune suppression. In our
study, the immunoprecipitation technique and pull-down assays were applied to examine the interaction between CypA and AIF. We found that CypA and AIF interacted directly and underwent cytoplasmic-nuclear translocation, resulting in the fragmentation of genomic DNA and the increase of apoptosis in the MbBV-infected cells. The blocking of AIF released from mitochondria inhibited the formation of the CypA-AIF complex, suppressed the cytoplasmic-nuclear translocation of AIF and CypA, and rescued the MbBV-induced apoptosis during MbBV infection. Moreover, the results of an electrophoretic mobility shift assay (EMSA) showed that the AIF-His fusion protein could directly bind but not degrade DNA, implying that CypA may be responsible for the DNA fragmentation. These results suggest that the MbBV-CypA-AIF pathway plays an important role in host immune suppression.

2 MATERIALS AND METHODS

2.1 Insect rearing and experimental animals

A colony of *S. litura* was reared on an artificial diet as described by G. Li et al. (1998). Rearing conditions were 27°C ± 1°C, relative humidity (RH) 60%-80%, and a 12:12 h (L:D) photoperiod. The parasitoid *M. bicoloratus* was maintained on laboratory-reared *S. litura* larvae, and the adults were supplied with honey as a dietary supplement. The parasitoid colony was passaged as previously described (K. J. Luo et al., 2007).

2.2 Isolation of hemocytes from *S. litura* larvae

Hemocyte samples were isolated from parasitized 2nd instars of *S. litura* larvae at 6 days post-parasitization (p.p.) (K. J. Luo et al., 2007). The samples were centrifuged for 5 min at 1000 × g, and the resulting pellets were collected as parasitized (P) hemocytes. We also collected hemocytes from nonparasitized 4th instar *S. litura* larvae at 6 days to serve as nonparasitized (NP) controls.

2.3 Cell culture

Spli221 (TUAT-Spli221) adherent cells (Thermo Fisher Scientific) were derived from the pupal ovaries of *S. litura* (Yanase et al., 1998). The cells were cultured in the TNM-FH insect culture medium with 10% fetal bovine serum (FBS) (HyClone, GE Life Sciences). The cultures were maintained at 27°C and passaged in 25-cm² tissue culture flasks (Corning).

2.4 Western blot analysis

Western blot analysis was performed as previously described (T. Liu et al., 2013). Briefly, 50 µg of protein was loaded per sample. Total protein was isolated and analyzed by blotting using appropriate antibodies: anti-CypA antibody, which was a rabbit polyclonal antibody generated for CypA, cloned from *M. bicoloratus* hemocytes and expressed in *Escherichia coli* using the pET-32a-CypA plasmid (1:2000; Bioworld Technology, Inc.), anti-rabbit AIF polyclonal antibody (1:2000; cat. no. bs-0037R; Bioss Technology, Inc.), mouse anti-GAPDH antibody (1:2000; cat. no. T0004; Affinity, USA), mouse anti-VDAC1 monoclonal antibody (1:2000; cat. no. AF1027; Beyotime), anti-mouse V5 Tag monoclonal antibody (1:2000; cat. no. R960-25; Invitrogen), anti-α tubulin monoclonal antibody (1:2000; Cat. no. K006154P; Solarbio), anti-mouse GST tag monoclonal antibody (1:2000; Cat. no. AG768; Beyotime), and a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000; Cat. no. 0216; Beyotime). ImageJ (National Institutes of Health) was used to measure protein band density.
2.5 | Isolation and purification of MbBV particles and infection of Spli221 cells

Viral particles were purified using a published protocol (K. Luo & Pang, 2006). In detail, 50 fresh adult *M. bicoloratus* were frozen at −20°C for 10 min and then put on ice. The reproductive tracts of female wasps were excised under a binocular stereomicroscope, and the separated ovaries were collected into 1.5-ml Eppendorf tubes on ice. The calyces were punctured using forceps. The exuded calyx contents were collected in 1 × phosphate buffered saline (PBS, pH 7.4) using a 2.5-ml syringe. The mixture was centrifuged for 3 min at 960 × g, 4°C to remove eggs and cellular debris. A 0.45-μm syringe filter was used to purify the viral particles. Spli221 cells were seeded in a six-well culture plate at a density of 2.5 × 10⁶ cells per well and cultured for 2 h. The experiments were expressed in wasp equivalents. One wasp equivalent in 20 μl of cell culture medium was inoculated per 1 × 10⁵ Spli221 cells.

2.6 | Plasmid construction and expression

The plasmid pLZT/V5-His-CypA (CypA-V5) was provided by our laboratory and was successfully expressed in Spli221 cells (Tian et al., 2019). To track CypA transfer in MbBV-infected cells, we constructed the recombinant plasmid pLZT-CypA-GFP (CypA-GFP) for expressing the fusion CypA-GFP protein (Figure 3g). Briefly, CypA was PCR amplified using a L4440-CypA plasmid (provided by our group) as a template. The primers containing Sac1 and Xba1 sites (underlined) were as follows: CypA-GFP-F (5′-GAGCTCATGGCTTTACCCCGAGT-3′) and CypA-GFP-R (5′-TCTAGAGGAGAGTTGACCGCAGT-3′). The gene was inserted in-frame into a pLZT-AIF3-GFP plasmid (derived from a pLZT/V5-His plasmid, Invitrogen).

Protein for expression in the *E. coli* strain BL21-CodonPlus (DE3), CypA was PCR amplified using the L4440-CypA plasmid as a template and the primers pGEX-CypA-F (5′-GAATTCATGGCTTTACCCCG-3′) and pGEX-CypA-R (5′-CTCGAGGGAGAGTTGACCGCAGT-3′) containing EcoRI and XhoI restriction sites (underlined). The gene was directionally cloned in-frame into pGEX-6p-1 (Amersham, Pharmacia Biotech) (Figure 6a). Similar to CypA, AIF was PCR amplified using cDNA as a template and the primers PET32a-AIF-F (5′-CCATGGATGCGGGAGGTCG-3′) and PET32a-AIF-R (5′-AAGCTTCTTAGTAAGACAAGGGCCC-3′) containing NcoI and HindIII sites (underlined in the figure). The gene aif was inserted in-frame into a pET32a(+) plasmid (Figure 6a). The pET32a (+)-AIF plasmid was used to express the AIF-His fusion protein (Figure 6b,c), and the pGEX-6p-1-CypA plasmid was used to express the fusion protein GST-CypA (Figure 6d,e) in *E. coli*.

2.7 | Preparation of recombinant proteins

The recombinant GST or His tagged proteins were expressed in *E. coli* strain BL21-CodonPlus (DE3) with 1 mM IPTG for 4 h at 37°C. The cells were collected and lysed in PBS (pH 7.4) supplemented with 0.1% Triton-100, 1 mM PMSF and 1 mg/ml lysozyme. After sonication on ice for 30 min, the samples were centrifuged, and the supernatants were purified using a His-tag protein purification kit (Cat. no. P2226; Beyotime) and a GST protein purification kit (Cat. no. P2262; Beyotime).

2.8 | Immunofluorescence

Immunofluorescence was performed as previously described (T. Liu et al., 2013) with minor modifications. Briefly, Spli221 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well for transfection. The cells were washed with PBS and fixed for 15 min in 3.7% formaldehyde. CypA was identified using the CypA-specific antibody (1:2000; Bioworld Technology, Inc.) and AlexaFluor 568 goat anti-mouse IgG (H + L) secondary antibody (1:2000;
Invitrogen). AIF was identified using anti-rabbit AIF polyclonal antibody (1:2000; bs-0037R; Bioss) and AlexaFluorR 568 goat anti-mouse IgG (H + L) secondary antibody (1:2000; Invitrogen). Before imaging, the labeled cells were incubated with phalloidin (1:40; Sigma) diluted in PBS for 1 h at 37°C. Cells were washed with 1 × PBS and incubated with 4', 6-diamidino-2-phenylindole (DAPI) (1: 1000; Roche) for 5 min. A 5-μl drop of fluoroshield with N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) buffer (EMS Technical Data Sheets Cat.17985-30) was applied to the center of the well. Cells were imaged using an Olympus 71 inverted-fluorescence microscope.

2.9 | Analysis of apoptotic cells

Analysis of apoptotic cells was performed using an annexin V–FITC/PI apoptosis detection kit (Cat. no. A211-02; Vazyme) according to the manufacturer’s instructions. Briefly, Spli221 cells at 24 h posttransfection with 20 μl MbBV particles or hemocytes at 6 days p.p. were harvested and incubated with 400 μl FITC-binding buffer for 5 min at room temperature. Then, 5 μl FITC-conjugated antibody and 5 μl PI were added and incubated for 20 min on ice in darkness. An Olympus 71 inverted fluorescence microscope was used to detect apoptotic cells. Annexin V–FITC was used to identify the early apoptotic hemocytes that showed green fluorescence; PI was used to identify the late apoptotic hemocytes that showed red fluorescence.

2.10 | Mitochondrial membrane potential detection

Analysis of mitochondrial membrane potential (∆Ψm) was performed using a Mitochondrial Membrane Potential Assay Kit with JC-1 (JC-1, Beyotime, cat. no. C2006) according to the manufacturers’ instructions. Spli221 cells or hemocytes were incubated in the diluted JC-1 solution for 20 min at 37°C and then washed with 1 × JC-1 staining buffer. The stained cells were observed under an Olympus 71 inverted-fluorescence microscope, and images were captured using an Olympus CCD camera. The images were grouped under different treatment conditions, and five dots on individual images were sampled to measure the overall fluorescence intensity by using NIS software (Nikon, Japan). Experiments were conducted with three individual replicates.

2.11 | Blocking of AIF release

Spli221 cells were seeded in a six-well culture plate at a density of 5 × 10^5 cells per well. After cell adhesion, the cells were treated with 50 μM of 4-amino-1, 8-naphthalimide (4-AN, an inhibitor blocking the release of AIF from mitochondria, Cat. no. sc-200125; Santa Cruz Biotechnology) and cultured at 27°C for 24 h. The cultures were infected with MbBV for an additional 24 h. The experiments were expressed in wasp equivalents. One wasp equivalent in 20 μl of cell culture medium was inoculated per 5 × 10^5 Spli221 cells, and Western blot analysis was performed as described in Section 2.4.

2.12 | Cell nuclear protein extraction

Cell nuclear proteins were extracted using a Nuclear Protein Extraction Kit (Cat. no. BB-3102, BestBlo) according to the manufacturer’s instructions. Briefly, Spli221 cells were pelleted at 500 × g for 5 min. The resulting pellet was washed with ice-cold PBS buffer several times and gently resuspended in the same buffer. Then, the cells were incubated on ice in lysis buffer A and oscillated at 2°C–8°C for 20 min. After the cellular lysate was centrifuged at
2000 × g for 5 min, the supernatant was carefully separated from the pellet and collected as cytoplasmic protein. The precipitate was again oscillated and lysed using lysis buffer B at 2°C–8°C for 20 min, followed by centrifugation at 12,000 × g for 10 min. The resulting pellet was collected as nuclear protein. The cytoplasmic protein and nuclear protein were analyzed by Western blot analysis using anti-CypA antibody and anti-rabbit AIF polyclonal antibody, respectively. Experiments were conducted with three individual replicates.

2.13 | Isolation of mitochondrial protein

Isolation of mitochondrial proteins was performed using a Cell Mitochondria Isolation Kit (Cat. no. C3601, Beyotime) according to the manufacturers’ instructions. Briefly, Spli221 cells were collected at 4°C by centrifugation at 600 × g for 5 min. The pellet was suspended and incubated in Mitochondrial Separation Reagent with PMSF at 0–4°C for 15 min. Then, the cell suspension was transferred to a glass homogenizer and homogenized 30 times. After homogenates were centrifuged at 600 × g for 5 min, the precipitate was discarded, and the supernatant fluid was centrifuged at 3500 × g and 4°C for 10 min. The resulting pellet was lysed in mitochondrial lysate with PMSF, and the lysate was centrifuged at 12,000 × g and 4°C for 5 min. The supernatant was used as mitochondrial protein for Western blot analysis using anti-CypA antibody or anti-rabbit AIF polyclonal antibody.

2.14 | Time-lapse imaging

Time-lapse images were acquired at 6 h intervals for 24 h on a Living Cell Observation System (DMI8LASX, Leica, Wetzlar, Germany) immediately after each treatment. During imaging, cells were held in an incubation chamber at 27°C. Pedigrees for each cell were established for a period of 24 h after the start of observation. The CypA-GFP fusion in Spli221 cells was excited using a 488 nm excitation light source and was rendered as green.

2.15 | Inhibition of CypA

Spli221 cells were seeded in a six-well culture plate at a density of 5 × 10^5 cells per well. After cell adhesion, the cells were treated with 1 μM of cyclosporine A, an inhibitor of CypA (Cat. no. 12088; Cayman Chemical) and cultured at 27°C for 24 h. The cells were infected with MbBV for an additional 24 h. The experiments were expressed in wasp equivalents. One wasp equivalent in 20 μl of cell culture medium was inoculated per 5 × 10^5 Spli221 cells, and Western blot analysis was performed as described in Section 2.4.

2.16 | Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using a protein A+G agarose kit (Cat. no. P2012; Beyotime) according to the manufacturer’s instructions. Briefly, cells were lysed with RadioImmunoPrecipitation Assay (RIPA) buffer (Cat. no. P0013B; Beyotime). The prepared lysates were immunoprecipitated with anti-CypA antibodies (Bioworld Technology, Inc.) or anti-mouse V5 Tag monoclonal antibodies (cat. no. R960-25; Invitrogen, USA) at 4°C for 12 h. Each experiment using this assay was performed at least three times independently. Immunoblotting was performed with the following primary antibodies: anti-CypA, anti-rabbit AIF polyclonal, or anti-mouse V5 Tag monoclonal antibodies. A goat anti-mouse horseradish peroxidase-conjugated antibody was used as the secondary antibody.
2.17 | In vitro pull-down assays

The expression of GST-CypA fusion protein or AIF-His fusion protein in E. coli BL21 was induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C. The bacteria expressing the fusion protein were lysed by sonication in nondenatured lysis buffer with lysozyme (P2226-5, Beyotime). GST fusion proteins were purified and eluted using the BeyoGold™ GST-tag Purification Resin Kit (P2253, Beyotime) according to the manufacturer’s instructions. The His fusion proteins were purified and eluted using the His-tag Protein Purification Kit (P2226, Beyotime) according to the instructions. The purified fusion proteins were quantified using a BCA protein concentration assay kit (BCA02, DingGuo Prosperous).

After purification, purified GST-CypA fusion protein (5 μg) or GST protein (5 μg) was incubated with GST-tag Purification Resin for 2 h at 4°C in the binding buffer (50 mM Tris-cl, 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 10% glycerol, 0.5% NP40). After washing to remove unbound protein, the GST-CypA-bound resin or the GST-bound resin alone was incubated with His-AIF fusion protein (5 μg) for 12 h at 4°C in the binding buffer. The resin was then washed four times with 200 μl binding buffer. The proteins were eluted with SDS sample buffer, separated via 8% SDS-PAGE and transferred onto a PDVF membrane. The membrane was probed with anti-CypA, anti-AIF, or anti-GST tag monoclonal antibody (AG758, Beyotime).

2.18 | Electrophoretic mobility shift assay (EMSA)

The DNA-binding properties of AIF were measured by EMSA. In detail, different amounts of purified His-AIF protein (0 μg, 30 μg, 60 μg, or 90 μg, Figure 6j) were mixed with 1 μg DNA fragment (non-loading buffer; Cat. no. G451A; Promega, USA) and 10 × binding buffer (50 mM Tris-HCl, 200 mM NaCl, 1 mM DTT, 1 mM PMSF, 10% glycerin, 0.5% NP40, pH 7.4) and incubated for 30 min at room temperature. The mixture was then subjected to agarose gel electrophoresis and observed for retardation of the DNA shift.

2.19 | DNA ladder

DNA fragmentation in nuclei of MbBV-infected cells was assessed using a DNA Ladder Extraction kit (Cat. no. c0008; Beyotime) according to the manufacturer’s instructions. Briefly, the spli221 cells were collected, washed with PBS buffer (pH 7.2) with 0.1% DTT and centrifuged for 1 min at 1000 × g. The pellet was then incubated in a lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% NP-40, with 5 mg/ml RNaseA and protease K) at 70°C for 5 min, and the supernatant was collected after being centrifuged at 1600 × g for 5 min. The DNA was precipitated with ethanol, dissolved in TE buffer, and separated by electrophoresis on 1% agarose gels.

2.20 | Data analysis

Data were analyzed using GraphPad Prism ver. 5 (GraphPad Inc.). Statistical significance in experiments with two treatments was examined using an unpaired t-test, and the differences of three group data were analyzed by one-way ANOVA. Semi-quantification of the bands was performed using ImageJ software ver. 1.49v (National Institutes of Health), with the intensity values normalized to the corresponding band of the internal reference; *p < 0.05, **p < 0.01, and ***p < 0.001 were considered statistically significant. All data were represented as the mean ± SEM of at least three independent experiments.
3 | RESULTS

3.1 MbBV upregulates the expression of CypA and promotes apoptosis

To determine if CypA was involved in MbBV-mediated apoptosis, the protein levels of CypA were quantified by Western blot analysis following M. bicoloratus parasitization or MbBV infection. CypA was significantly upregulated in the hemocytes at 6 days p.p. (*p < 0.05, Figure 1a) and in the MbBV-infected Spli221 cells after a 24 h viral induction period (*p < 0.05, Figure 1d). The apoptosis of both the hemocytes and the MbBV-infected cells was measured by detection of annexin V-conjugated FITC (Figure 1b,c). In both cases, the proportion of apoptotic cells increased significantly (Figure 1c,f, *p < 0.05; Figure 1f, *p < 0.01). These data were consistent with previous results (Tian et al., 2019) and suggested that MbBV upregulates CypA expression and promotes insect cell apoptosis that may be involved in the effect of CypA.

3.2 MbBV promotes the decrease of mitochondrial membrane potential and induces the release of AIF from mitochondria

Mitochondria play a key role in innate immunity and apoptosis metabolism in mammals (Friedman & Nunnari, 2014; Yang et al., 2007). Oxidative stress can affect mitochondrial function and decrease the mitochondrial membrane potential (ΔΨm). This can lead to increased mitochondria outer membrane permeability and the outflow of mitochondrial matrix contents such as AIF, cytochrome c (Cyt c), and Endo G (Garrido et al., 2006; Takumi et al., 1997). MbBV infection may induce AIF release from mitochondria to the cytoplasm. We used JC-1 fluorescent probe to examine the ΔΨm and found that parasitism significantly decreased the ΔΨm of the parasitized hemocytes compared with the non-parasitized hemocytes (Figure 2a and B, **p < 0.01). Similar results were obtained in the MbBV-infected studies (Figure 2c,d, **p < 0.01). To verify that AIF was released from the mitochondria to cytoplasm during MbBV infection, we tested the AIF protein in MbBV-infected Spli221 cells and found that the total AIF protein in the cytoplasm of virus-infected cells was similar to that of the uninfected cells (Figure 2e,f); however, the AIF protein of mitochondria was significantly decreased (Figure 2g,h, *p < 0.05). These results demonstrated that the AIF protein was transferred from the mitochondria to other parts of the cells under MbBV infection. This may have resulted from the decreased ΔΨm of the mitochondria.

3.3 MbBV promotes the CypA-AIF interaction and the cytoplasmic-nuclear translocation of CypA and AIF

To determine if AIF released from mitochondria forms a complex with CypA, we used an Immunoprecipitation (IP) assay to investigate the interaction of AIF and CypA in MbBV-infected cells. Compared to the untreated cells, MbBV infection promoted the formation of the CypA-AIF complex (Figure 3a). To confirm this interaction, we expressed recombinant CypA-V5 (pIZT/V5-His-CypA, CypA-V5) in Spli221 cells (Figure 3b). After 24 h MbBV infection of the Spli221 cells post-transfected with CypA-V5, the interaction complex of CypA-V5 and AIF was detected by an IP assay in the MbBV-infected cells (Figure 3c). These data indicated that MbBV promoted the formation of the interaction complex of CypA and AIF during the MbBV-induced immunosuppressive process. Both CypA and AIF can promote apoptosis (Tian et al., 2019; Joza et al., 2001b; Xie et al., 2019), and it is possible that both proteins enter the nucleus of MbBV-infected cells during the immunosuppressive process. To examine this possibility, we isolated the MbBV-infected nuclear protein and analyzed CypA and AIF by Western blot analysis. Compared to the untreated cells, the CypA and AIF proteins in the nuclei of the MbBV-infected cells were increased (Figure 3d,e). Using an immunofluorescence assay, we determined the localization of CypA and AIF. The
**Figure 1**  *Microplitis bicoloratus* parasitism (MbBV) infection upregulates CypA expression and promotes apoptosis both in hemocytes and in Spli221 cells. (a) The results of semi-quantitative analysis of Western blot signals showed a significant increase of CypA in hemocytes at 6-d.p.p. (b) Apoptotic hemocytes induced by *M. bicoloratus* parasitism stained with Annexin V-FITC/PI and observed under the inverted fluorescence microscope. The samples were analyzed for green fluorescence (FITC) and red fluorescence (PI). Different labeling patterns of the apoptotic hemocytes: early apoptotic cells, annexin V-FITC positive and PI negative; late apoptotic cells, both annexin V-FITC and PI positive. The squares in both NP and P show that the representative cells were in the late stages of apoptosis. Scale bar, 10 μm. (c) Comparison of the percentages of apoptotic cells between NP and P hemocytes. (d) CypA protein expression was significantly upregulated in the MbBV-infected Spli221 cells. (e) The squares in MbBV-infected or noninfected Spli221 cells show that the representative cells were in the late stages of apoptosis. Scale bar, 20 μm. (f) Statistical analysis of apoptotic cell death in Spli221 cells infected with MbBV. The Spli221 cells infected by MbBV had significantly higher levels of apoptosis at 24 h postinfection. NP, nonparasitized hemocytes; P, parasitized hemocytes. Data are presented as mean ± SEM of three independent experiments. Significant differences are indicated by asterisks (*p* < 0.05, **p** < 0.01, two-tailed Student's *t*-test)
FIGURE 2  (See caption on next page)
immunofluorescence results revealed that CypA and AIF were normally distributed in the cytoplasm of untreated cells. However, MbBV infection resulted in a transfer of CypA and AIF to the nucleus, indicating co-localization (Figure 3f). We also constructed an expression plasmid of the CypA-GFP fusion protein (Figure 3g) and transfected it into the High Five cells. A Time-lapse Fluorescence Microscope was employed to show that the CypA-GFP protein gradually accumulated in the nucleus following the MbBV-induced cell apoptosis (Figure 3h). Next, we used a DNA ladder assay to measure the degradation of the nuclear genome in the CypA-GFP plasmid-transfected cells induced by MbBV. At 12 and 24 h postinfection, the nuclear genome of infected cells had become fragmented. At 48 h after infection, the DNA ladder disappeared (Figure 3i), suggesting further degradation of the DNA fragments. These results indicated that MbBV infection promoted the interaction and nuclear transfer of CypA and AIF. This may cause apoptotic cell death in the infected cells by genomic DNA fragmentation.

3.4 | AIF blocking inhibits the formation of the CypA-AIF complex and the cytoplasmic-nuclear translocation of AIF or CypA, thereby rescuing the MbBV-induced apoptosis

MbBV induced the release of AIF from the mitochondria and promoted the CypA-AIF interaction (Figure 3). It was unknown whether the formation of the CypA-AIF complex could be inhibited by blocking the release of AIF from mitochondria. To clarify this, we used 4-amino-1, 8-naphthalimide (4-AN), an inhibitor that blocks the release of AIF from mitochondria (Bae et al., 2010; Schlicker et al., 1999; Zhang et al., 2005) to block the release of AIF from mitochondria. The AIF protein in the 4-AN-treated cell mitochondria was not reduced compared to those in the control groups, indicating that AIF was not released from the mitochondria (Figure 4a), and the CypA-AIF complex had disappeared (Figure 4b). The nuclear distribution of AIF and CypA in 4-AN-treated cells was decreased (Figure 4c,d), and the fragmentation of genomic DNA was also reduced (Figure 4e). Apoptosis analysis indicated that inhibiting the AIF release could reduce apoptosis of the MbBV-infected cells (Figure 4f,g). Taken together, these results suggested that the blocking of AIF release from mitochondria inhibited the formation of the CypA-AIF complex, suppressed the cytoplasmic-nuclear translocation of AIF and CypA, and rescued the MbBV-induced apoptosis during MbBV infection.

3.5 | Inhibition of CypA does not affect the binding of CypA and AIF and weakens the DNA fragmentation induced by MbBV

CsA is an immune inhibitor of CypA that can form a CypA-CsA complex via binding to CypA (Handschohmacher et al., 1984). To determine the effect of CypA inhibition on the formation of the CypA-AIF complex...
**FIGURE 3** *Microplitis bicoloratus* bracovirus (MbBV) promotes the interaction and the cytoplasmic-nuclear translocation of CypA and AIF. (a) IP analyses of the interaction between cyclophilin A (CypA) and apoptosis-inducing factor (AIF) in Spli221 cells infected with MbBV for 24 h. Whole cell lysates (WCLs) of Spli221 cells were immunoprecipitated (IP) with CypA antibodies and subjected to immunoblotting (IB) analyses. (b) The 18-kDa CypA-V5 fusion protein was detected in Spli221 cell lysates at 48 h posttransfection. (c) IB analyses of IP with V5 antibodies in WCLs of MbBV-infected Spli221 cells transfected with CypA-V5 for 48 h. (d) Western blot analysis showing an increase of nucleus CypA in MbBV-infected Spli221 cells. (e) Western blot analysis showing increased nuclear AIF in MbBV-infected Spli221 cells. (f) Representative immunofluorescence images showing that CypA and AIF were co-transferred into the nucleus in MbBV-infected Hi5 cells. CypA is labeled in red, and AIF is labeled in green. (g) Schematic diagrams of pIZT-CypA-GFP and pIZT/V5-His-CypA-GFP plasmid construction. The recombination pIZT-CypA-GFP plasmid containing the OpIE1 promoter was used for expression of Zeocin protein, and the OpIE2 promoter for expression of CypA-GFP fusion proteins using the pIZT/V5 vector. (h) Time-lapse fluorescence images of MbBV-infected Hi5 cells expressing CypA-GFP fusion proteins. (i) DNA ladder detection shows the fragmentation of genomic DNA in the pIZT-AIF-GFP plasmid transfected Hi5 cells infected by MbBV. Scale bar = 20 μm. WB: Western blot analysis
complex, we used CsA to suppress the immunological properties of CypA. Surprisingly, the binding of CypA and AIF was unaffected by the inhibition of CypA (Figure 5a), but the inhibition weakened the DNA fragmentation induced by MbBV (Figure 5b). These data showed that the binding site of CypA-CsA was different from that of CypA-AIF, and the binding of CsA to CypA affected the biological activity of CypA, consistent with the results of Tian et al. (2019).
3.6 | The combination of CypA and AIF occurs through direct interaction

It was unclear whether the interaction of CypA and AIF in the immunosuppressive process was direct or indirect. To study this, we constructed a pET32a(+)-AIF and a pGEX-6p-1-CypA (GST-CypA) plasmid (Figure 6a) and expressed the AIF-His protein (Figure 6b,c) and the GST-CypA protein (Figure 6d,e) in vitro, respectively. With the help of His-tag protein purification and a GST protein purification kit, we purified the GST protein (Figure 6f), the GST-CypA protein (Figure 6g) and the AIF-His protein (Figure 6h). In a pull-down assay, we found that in vitro the AIF-His protein could bind to GST-CypA but not to GST (Figure 6i). Furthermore, the AIF-His protein was able to prevent DNA from moving in the gel electrophoresis. Moreover, ladders of DNA fragments were not observed (Figure 6j). These observations indicated that AIF directly interacts with CypA to form the CypA-AIF complex and non-specifically bind DNA.

4 | DISCUSSION

Previous studies have demonstrated that CypA is required for MbBV-mediated apoptosis and that it undergoes nuclear translocation in MbBV-infected cells (Tian et al., 2019); however, little is known concerning how CypA is transported into the nucleus. Therefore, we examined the molecular mechanisms by which the CypA-AIF interaction is promoted under the immunosuppression induced by MbBV. As expected, the expression of CypA was upregulated by natural parasitism or by MbBV infection, resulting in immunosuppressive phenotypes. These included increases in apoptosis and genomic DNA fragmentation. Furthermore, MbBV infection facilitated the cytoplasmic-nuclear translocation of CypA and AIF in virus-infected cells. The blocking of AIF release from mitochondria inhibited the CypA-AIF interaction mediated by MnBV infection and reduced apoptosis and genomic DNA fragmentation induced by MbBV. AIF can interact with CypA directly to form a CypA-AIF complex and non-specifically bind DNA. These findings indicated that the CypA-AIF complex induced by MbBV promotes cell apoptosis in response to immunosuppression.

In this study, we found that CypA and AIF could enter the nucleus simultaneously in MbBV-infected cells (Figure 3d to H). To clarify the mechanisms of CypA nuclear translocation, we blocked the release of AIF using 4-AN, which can suppress the release of AIF from mitochondria (Bae et al., 2010; Schlicker et al., 1999; Zhang et al., 2005), and inhibited the biological activity of CypA using CsA. The results showed that the binding of CypA and AIF was blocked by 4-AN (Figure 4b) and the nuclear translocation of CypA was inhibited (Figure 4d), indicating that the
Figure 6 (See caption on next page)
cytoplasmic-nuclear translocation of CypA requires the assistance of AIF, perhaps in the form of a CypA-AIF complex. Meanwhile, we observed the differences between inhibition of CyPA and blocking of AIF release on the formation of CypA-AIF complex. When 4-AN was used to block AIF release from the mitochondria, MbBV infection could not induce the formation of the CypA-AIF complex (Figure 4b). However, under the same conditions of MbBV induction, the inhibition of CyPA by CsA did not prevent the formation of the complex (Figure 5a). The most plausible explanation for this difference is that AIF and CsA have different binding sites with CypA that may lead to the formation of a CypA-AIF-CsA complex under the conditions of CsA treatment. Further study is needed to examine this possibility.

During the binding of CypA and AIF in the cytoplasm, the key step is the release of AIF from the mitochondria to the cytoplasm after MbBV infection. We observed that decreases of ΔΨm and AIF in the mitochondria were induced by parasitism or MbBV (Figure 2). However, the total amount of AIF protein was unchanged in the MbBV-infected cells compared to untreated cells (Figure 2e,f), and an increase of AIF in the cytoplasm was not detected by Western blot analysis after virus infection. It is possible that the AIF concentration in the cytoplasm was too low to be detected or that AIF remained in the cytoplasm for too brief a time. AIF increased in the nucleus but decreased in the mitochondria under the same virus infection (Figures 2g, 3e,f). Therefore, these data indicate the release of AIF into the cytoplasm from mitochondria followed by plasma-nuclear translocation.

CypA and AIF co-translocated to the nucleus and caused chromatinolysis during MbBV infection (Figures 3h,i); however, it was unclear as to which factor caused the DNA fragmentation. When CsA was used to inhibit the biological activity of CypA, the genomic DNA ladders induced by MbBV disappeared in the CsA-treated cells (Figure 5b), indicating that the inhibition could rescue the chromatinolysis induced by MbBV. Next, an EMSA assay was used to test the binding of AIF and DNA. The results showed that the AIF-His protein was able to block the mobility shift of DNA and did not result in DNA fragmentation (Figure 6i). For the CypA-AIF complex in the nucleus, these data suggest that AIF may bind with genomic DNA, and then CypA degrades the DNA during MbBV-induced immunosuppression. Our findings are consistent with previous research demonstrating that AIF had no significant endonuclease activity and could cut DNA directly by recruiting or regulating the activity of nonspecific endonucleases (Bano & Prehn, 2018), while CypA does have endonuclease activity (Montague et al., 1997). Based on these data, we concluded that CypA is responsible for the cleavage of genomic DNA and that AIF is responsible for transporting CyPA and binding DNA in the apoptosis-associated chromatinolysis mediated by MbBV.

Although CypA can bind to AIF in the MbBV-infected cells (Figure 3a,c), it was unclear whether the interaction between CypA and AIF was direct or indirect. To address this question, AIF-His, GST-CypA and GST were obtained from E. coli BL21 bacteria by expression and purification in an in vitro experiment (Figure 6a–h). AIF-His could form a complex with GST-CypA but not with GST (Figure 6i). Because the interference of other cytokines was excluded in the in vitro system, these results show that AIF can directly combine with CypA in the immunosuppressive

**FIGURE 6** Apoptosis-inducing factor (AIF) can directly interact with CypA and bind nonspecifically to DNA. (a) Schematic diagrams of pET-32(a+)-AIF and pGEX-6P-1-CypA plasmid construction. The recombinant pET-32(a+)-AIF plasmid containing a six-histidine (His) tag was driven by a T7 promoter, and the recombinant pGEX-6P-1-CypA plasmid was driven by the Tαc promoter. (b,c) The AIF-His fusion protein (84 kDa) was detected in E. coli strain BL21-CodonPlus (DE3) lysates induced by IPTG for 4 h at 37°C. (b: western blot analysis; c: Coomassie brilliant blue staining). (d,e) The 46-kDa GST-CypA fusion protein was detected in DE3 lysates induced by IPTG for 4 h at 37°C. (d: Western blot analysis; e: Coomassie brilliant blue staining). (f,g) The GST protein and the GST-CypA protein were purified from IPTG-induced DE3 lysates using a GST protein purification kit. (h) The AIF-His protein was purified from IPTG-induced DE3 lysates using a His-tag protein purification kit. (i) The interaction of GST-CypA and AIF-His was measured using in vitro pull-down assays. (j) The binding of AIF-His and DNA was tested using an electrophoretic mobility shift assay (EMSA); the shift of genomic DNA was retarded by AIF-His proteins in gel electrophoresis.
response. Similarly, in mice AIF must interact with CypA to form a complex and co-translocate to the nuclei (Candé et al., 2004; Tanaka et al., 2011; Zhu et al., 2007).

In our study, we used two different cell lineages to verify the molecular mechanism: Spil221 cells and High Five cells (Hi5 cells). The Spil221 cells were derived from the pupal ovaries of S. litura (Yanase et al., 1998). The Hi5 cells were derived from Trichoplusia ni embryos (Granados et al., 1994). Considering that the cells arising from the same species could better reflect the changes of MbBV-infected Spodoptera litura, most of our experiments were carried out using the Spil221 cells. However, recent studies from our laboratory have shown that Hi5 cells can be infected by MbBV, and this is also an ideal model for exploring the mechanism of MbBV-induced insect cell apoptosis (Tian et al., 2019; Yan et al., 2018). In our research, we observed that Hi5 cells had better cell morphology than Spil221 cells and that the timescale for apoptosis was slower after MbBV infection. Thus, in the experiments with immunofluorescence and time-lapse imaging, we used Hi5 cells as host cells to better illustrate the translocation of CypA in MbBV-infected cells.

In inflammatory disease, CypA expression is induced by reactive oxygen species (ROS) (Nigro et al., 2013; Satoh et al., 2008). However, we do not know which viral gene regulates CyPA expression in the MbBV-mediated insect immunosuppression. In our previously published study, we performed whole-genome sequencing of MbBV and identified 116 genes, 28 of which belonged to the IkB-like viral ankyrin (vank) family, one of the largest viral families. Among the 116 genes, 13 MbBV genes were expressed in hemocytes undergoing MbBV-induced apoptosis. In further analysis, only three viral IkB-like genes (vank86, vank92, vank101) were expressed in hemocytes collected from S. litura larvae parasitized by M. bicoloratus (Yu et al., 2016). NF-κB/IκB signaling pathways are known to regulate cell survival and apoptosis. It is tempting to speculate that the three viral IkB-like genes may be involved in the regulation of CypA expression during MbBV-induced apoptosis, although further study must be carried out to confirm this.

In summary, our analyses have established a causal link between innate immunosuppression and the CypA-AIF complex. This linkage is regulated by MbBV. We demonstrated that MbBV promotes the interaction of CypA and AIF to form a proapoptotic DNA degradation complex and induce insect cell apoptosis. These findings suggest that the MbBV-CypA-AIF axis plays an important role in the insect immunosuppressive process, and the results may aid in clarifying insect immunological mechanisms.

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AUTHOR CONTRIBUTIONS

Yan Hu: Methodology, investigation, validation, data collection, formal analysis, writing—original draft. Ya-Ping Liang: Methodology, validation, investigation, formal analysis, writing—original draft. Han-Yu Tian, Cui-Xian Xu, Dan Yu, and Pang Zhang: Methodology, investigation, validation. Hui Ye: Supervision and funding acquisition. Ming Li: Conceptualization, methodology, formal analysis, writing—original draft, writing—review and editing, supervision, project administration, funding acquisition.

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