Biochemical and Biophysical Research Communications 447 (2014) 237–243

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of *Bmbuffy* in hydroxycamptothecine-induced apoptosis in BmN-SWU1 cells of the silkworm, *Bombyx mori*

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ARTICLE INFO

Article history:
Received 17 March 2014
Available online 29 March 2014

Keywords:
Bmbuffy
Bcl-2 family
HCPT
Bmp53
Mitochondrial apoptotic pathway

ABSTRACT

Bcl-2 family proteins have been reported previously to play important roles in the mitochondrial apoptotic pathway. Particularly, *Bmbuffy* has been identified as a key homologue of Bcl-2 in silkworm; however, its exact function is unknown. In this study, we investigated the role of *Bmbuffy* in hydroxycamptothecine (HCPT)-induced apoptosis of BmN-SWU1 cells. By conducting confocal microscopy studies, we found that *Bmbuffy* is located on the outer membrane of mitochondria and endoplasmic reticulum (ER). Furthermore, we discovered that the hydrophobic transmembrane domain at the COOH terminus is a putative anchor for the subcellular localization of *Bmbuffy*. Overexpression of *Bmbuffy* inhibited cytochrome c release, activation of caspase-3 and cell apoptosis, while RNAi-mediated silencing of *Bmbuffy* promoted apoptosis. In the absence of a hydrophobic membrane anchor, we revealed that *Bmbuffy* is unable to block apoptosis. These results indicate that *Bmbuffy* acts as an anti-apoptotic protein, located on the mitochondrial outer membrane and is involved in the mitochondrial apoptotic pathway. Moreover, in HCPT-induced apoptosis, we showed that the translocation of endogenous *Bmp53* from the nucleus to the mitochondria is a slow and progressive process, followed by cytochrome c release. This suggests that mitochondrial *Bmp53* accumulation may contribute to membrane permeability. The co-localization of *Bmp53* and *Bmbuffy* suggests the interaction of the two proteins, which was further confirmed by Co-IP assay. In addition, overexpression of *Bmp53* increased cytochrome c release and the cell apoptotic rate, whereas *Bmbuffy* overexpression blocked these. All the data suggest that *Bmbuffy* functions as an anti-apoptotic protein and interacts with *Bmp53* in HCPT-induced apoptosis of silkworm cells.

1. Introduction

The mechanisms of apoptosis are conserved and complex in many species [1]. Two apoptotic pathways are involved in this process; the intrinsic mitochondrial and extrinsic death-receptor pathways [2]. Bcl-2 family proteins play key roles in these pathways [3]. Based on the structural and functional characteristics in mammalian cells, the Bcl-2 family can be divided into two subgroups; multi-domain (comprising three or four BH domains) and BH3-only. The multi-domain group consists of both pro- and anti-apoptotic members, whereas BH3-only proteins are typically pro-apoptotic [4]. Interestingly, in *Caenorhabditis elegans*, two members of the Bcl-2 family; EGL-1 and CED-9, have been found to regulate programmed cell death in worm cells [5]. Furthermore, in *Drosophila*, two Bcl-2 family proteins homologous to Bok (a mammalian pro-apoptotic member), have been identified successfully [6].

In the intrinsic apoptotic pathway, anti-apoptotic proteins of Bcl-2 family are typically located on the mitochondrial outer membrane (MOM) via the hydrophobic carboxyl-terminal domain. These proteins neutralize the activity of pro-apoptotic members, leading to the inhibition of MOM permeability and release of mitochondrial cytochrome c [7]. The pro-apoptotic protein, p53, is an important component of several stimuli-induced apoptotic processes, specifically the p53-dependent pathway [8]. Recent reports have indicated both a direct and indirect association of p53 with Bcl-2 family members in relation to the mitochondrial apoptotic pathway [9]. As an effective apoptosis inducer, hydroxycamptothecine (HCPT) has significant anti-tumour activity and is less toxic against many types of cancer cells. Furthermore, HCPT is known to act as an active ingredient of plant-derived pesticides [10]. However, the specific mechanisms of HCPT-induced apoptosis in insect cells are yet to be explored. To better understand the

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http://dx.doi.org/10.1016/j.bbrc.2014.03.093
0006-291X/

interrelations of cell apoptotic factors, *Lepidoptera* has been recommended as the insect model system for apoptotic process studies [11]. As the representative of *Lepidoptera*, the silkworm, *Bombyx mori*, is gradually becoming the model species for a wide range of investigations, in particular, in the field of agricultural pest control. In silkworm, *Bmbuffy* has been found and identified as a homologue to Bok [12]. However, no other proteins homologous to Bcl-2 family members have been reported in silkworm and the role of *Bmbuffy* in apoptosis is unknown. Interestingly, the BmN-SWU1 cell line separated and cultured from silkworm ovarian tissues has good growth and genetic stability and may be a suitable target for detailed apoptosis pathway studies. In this paper, we investigated the role of *Bmbuffy* in HCPT-treated BmN-SWU1 cells to reveal apoptotic protein interactions in the mitochondrial pathway in silkworm. Our findings lay the theoretical foundation for further research on the apoptotic mechanisms of insect cells.

2. Materials and methods

2.1. Cells culture and transfection

The BmN-SWU1 cell line [13] was maintained at 27 °C in TC-100 insect medium (Invitrogen, USA) supplemented with 10% (vol/vol) faetal bovine serum (FBS; PAA Laboratories), penicillin G (200 Unit/mL) and streptomycin sulphate (200 Unit/mL). Monolayer culture of BmN-SWU1 cells (1×10^6) were incubated in 6-well plates (Corning 3516) for 24 h, in TC-100 and Grace medium with 10% FBS. Subsequently, the medium was discarded and replaced with TC-100 or Grace medium lacking antibiotics and serum. According to the manufacturer's instructions, the cells were transiently transfected using 2 μg of each plasmid and 8 μL of liposome (Roche, CH). After 12 h of incubation, transfection mixtures were replaced with either TC-100 or Grace medium containing 10% FBS.

2.2. Cloning of genes and construction of plasmids

Total RNA was extracted from BmN-SWU1 cells using an RNA-easy Mini kit (Qiagen, China) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by AMV Reverse Transcriptase (Promega, USA), according to the protocol provided by the manufacturer. The cDNA of *Bmbuffy* was amplified by PCR in a 50 μL reaction mixture with primers F1 and R1 (Table 1). Similarly, *Bmbuffy-ΔTM* cDNA was amplified with F2 and R2 primers (Table 1). The PCR products were purified using an DNA gel extraction kit (Omega, USA) according to the manufacturer's protocol and cloned into *PIZ-dsRed* vectors (Invitrogen, USA) to generate *PIZ-Bmbuffy-dsRed* and *PIZ-Bmbuffy-ΔTM-dsRed*, *Bmbuffy* and *Bmbuffy-ΔTM* were cloned into the *BamH*I/*Eco*RI sites of *PIZ-dsRed* with primers F3 and R3, F4 and R4 (Table 1). *Bmp53* was cloned into the *BamH*I/*Eco*RI sites of *PIZ-dsRed* with primers F5 and R5. The miRNA sequences of *Bmbuffy* and *Bmp53* were designed with miTarget software. Subsequently, the miRNA fragments of *Bmbuffy* and *Bmp53* were synthesized and cloned into the *Asc*I and *Sac*II sites of *PIZ/V5-dsRed*. Using the same approach, *PIZ-HA-Bmbuffy-dsRed* and *PIZ-Flag-Bmp53-dsRed* encoding transient expression of HA and FLAG tags at the respective C-terminals were constructed for Co-IP assays.

2.3. Observation of Bmbuffy localization in BmN-SWU1 cells

Cells were grown overnight on coverslips (20 × 20 mm) in 6-well plates. At 72 h after transfection with either *PIZ-Bmbuffy-dsRed* or *PIZ-Bmbuffy-ΔTM-dsRed*, the cells were incubated with 300 nM Mito-Tracker Green/ER-Tracker Green (Beyotime, China) for 15 min at 37 °C. The stained cells were then washed twice with PBS and visualized using confocal microscopy (Olympus, Japan), using an emission wavelength of 488 nm.

2.4. Immunofluorescence staining

Cells were grown on coverslips (10 × 10 mm) in 24-well plates. After HCPT-induced apoptosis, the cells were washed 3 times with PBS and fixed with 4% cold paraformaldehyde for 10 min at room temperature. The washed cells were permeabilized with 0.1% Triton X-100 for 15 min, rinsed again with PBS and then blocked with 1% bovine serum albumin (BSA) and 10% Lowlenthal serum overnight at 4 °C. Cells were incubated with anti-cytochrome c mouse monoclonal antibody, anti-*Bmp53* rabbit monoclonal antibody and anti-*Bmbuffy* rabbit monoclonal antibody (cytochrome c diluted at 1:50; *Bmp53* diluted at 1:100; *Bmbuffy* diluted at 1:100, Beyotime, China) for 1 h, at 37 °C. After washed repeatedly (6 times) with PBS, the cells were stained with DAPI (Beyotime, China) for 15 min at room temperature, and then cells were washed with PBS for 6 times followed by incubation with FITC or Cy3-conjugated secondary antibody (Invitrogen, USA) for 40 min at 37 °C, and then thoroughly washed with PBS and incubated with 200 nM Mito-Tracker Green (Beyotime, China) in complete medium (at 37 °C, for 30 min). The modified coverslips were mounted onto glass slides and observed with confocal laser scanning microscopy.

2.5. Flow cytometric analysis of cell apoptosis

The Annexin V-fluorescein isothiocyanate (V-FITC) apoptosis detection kit (Roche, CH) was used according to the manufacturer's instructions to quantify the apoptotic cells. Cell surface expression of phosphatidylserine (PS) was detected with Annexin V during the progression of apoptosis. BmN-SWU1 cells (5×10^5 cells/mL) were cultured in the presence of 100 ng/mL HCPT for 0, 24, 48, and 72 h. Attached and suspension cells were harvested with a pipette, washed twice with PBS, and resuspended in a 500 μL binding buffer. Annexin V-FITC and propidium iodide (PI) (5 μL of each) were added to the cells and incubated at room temperature for 15 min before flow cytometric analysis. Data analysis was performed using the CellQuest software (Becton Dickinson).

Table 1
List of primers used in this study.

Genes/vectors	Primers
Bmbuffy	F1 5′-CGCGAATTCATGCGTCGAAAGCTCA-3′ R1 5′-CCGGGATCCCCACTATATATGAGTCGCCGT-3′
PIZ-Bmbuffy-dsRed	F2 5′-CCATGCGTCGAAAGCTCAGCAC-3′ R2 5′-CGGCGCGCAGACGATCTATTA-3′
Bmbuffy-ΔTM	F3 5′-CCGGGATCCATGCGTCGAAAGCTCA-3′ R3 5′-CGCGAATTCCCGACTATATATGAGTCGCCGT-3′
PIZ-Bmbuffy-ΔTM-dsRed	F4 5′-CCGGGATCCATGCGTCGAAAGCTCAGCAC-3′ R4 5′-CGCGAATTCCCGGCGCGCAGACGATCTATTA-3′
PIZ-Bmp53-dsRed	F5 5′-CGGGATCCATGGAAACACGAAATCATGACATCTCTT-3′ R5 5′-CGGAATTCGTCTTCACCGTTTTGGCTCCGATAA-3′
PIZ-Bmbuffy-miRNA-dsRed	F6 5′-CGGCGTAGTAATCAGCGGAG-3′ R6 5′-GGTTCGTCGCCGAGACCGCGGC-3′
PIZ-Bmp53-miRNA-dsRed	F7 5′-AGGCGCGCCAATAATGAATTAATACGACTCACTA-3′ R7 5′-TCCCCGCGGTCTCGGCGACGAACCTCGCAACTTC-3′

2.6. Measurement of cytochrome c release and mitochondrial Bmp53

The subcellular fraction was independently isolated using a mitochondria isolation kit (GENMED Scientifics Inc., USA), according to the manufacturer's instructions. BmN-SWU1 cells (2×10^7) were collected by centrifugation at 400×*g* for 10 min (at 4 °C). The cells were washed twice with ice-cold PBS (pH 6.9), followed by centrifugation at 400×*g* for 5 min. The cell pellet was then resuspended in ice-cold cell extraction buffer supplemented with PMSF for 2 min. The cells were then homogenized with a glass dounce B-type pestle (30 strokes), transferred to Eppendorf centrifuge tubes and centrifuged at 700×*g* for 10 min at 4 °C to eliminate nuclei and unbroken cells. The supernatant was further centrifuged at 12,000×*g* for 15 min at 4 °C to obtain the cytosolic and mitochondrial fractions. The resulting solutions were stored at −80 °C and used for Western blot analysis. Mitochondrial fractions were dissolved with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% NonidetP-40) and homogenized. The protein content was measured using a standard colorimetric assay kit (Bradford kit). Proteins (20 μg per lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, CH). Subsequently, the membranes were probed with primary antibodies (cytochrome c diluted at 1:200; *Bmp53* diluted at 1:1000) overnight at 4 °C, and incubated with a horseradish peroxidase (HRP) coupled secondary antibody (HRP; 1:5000, Beyotime, China). Immunolabeling was detected using an electrogenerated chemiluminescence (ECL) Western blotting system (Roche, CH), with either tubulin or COXIV (1:1000, Beyotime, China) as the loading control. The results were quantified with a scanning densitometer (Bio-Rad, USA).

2.7. Assay of caspase-3-like activity

The Caspase-Glo® 3 reagent (Promega, USA) was prepared freshly by mixing and equilibrating at room temperature, prior to assay measurements. Concurrently, 96-well plates containing cells from the incubator were also removed and equilibrated to room temperature. Subsequently, 100 μL of the prepared Caspase-Glo® 3 reagent was added to each well, containing 100 μL of blank, negative control and treated cells in culture medium. The contents of the wells were gently mixed for 30 s, using a plate shaker at 300–500 rpm and incubated at room temperature for 1 h. The luminescence of each sample was measured in a plate-reading luminometer (Promega, USA) according to the manufacturer's instructions.

2.8. Co-IP assay

BmN-SWU1 cells (1×10^6) were transfected with either 1 μg of *PIZ-HA-Bmbuffy-dsRed* or *PIZ-Flag-Bmp53-dsRed*, along with 8 μL of X-tremeGENE HP DNA transfection reagent (Roche, CH). At 72 h after transfection, the cells were further treated with 100 ng/mL HCPT (untreated cells were assigned as the control group) for 72 h, at 27 °C, and then washed with PBS (2 times). The cells were then resuspended in 1 mL of immunoprecipitation (IP) buffer (25 mM Tris, 150 mM NaCl, pH 7.2) containing 10 μL PMSF. The protein suspension was lysed for 30 min and centrifuged at 13,000×*g* for 30 min at 4 °C. Subsequently, 500 μL of supernatant was incubated with the anti-FLAG reagent at 4 °C overnight. The following day, the protein A+G agarose was introduced in the protein mixture and further incubated at 4 °C for 6–9 h. The protein suspension was centrifuged at 3000×*g* for 5 min at 4 °C, and the captured proteins were eluted in IP buffer. Following the addition of 10 μL SDS sample buffer and boiled for 10 min, the samples were centrifuged for a further 5 min at 13,000×*g* (room temperature). The resulting suspension samples were separated by 12% SDS–PAGE as described previously. The antibodies used here were: (i) mouse monoclonal anti-FLAG, 1:5000; (ii) mouse monoclonal anti-HA 1:5000.

2.9. Statistical analysis

The experiments were repeated a minimum of three times. All data were expressed as means ± SD. Statistical differences between control and treated groups were evaluated using Student's *t*-test. Differences between groups were considered statistically significant at $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. The localization of Bmbuffy in BmN-SWU1 cells

By analyzing the hydrophobic transmembrane domain of *Bmbuffy* online (http://www.cbs.dtu.dk/services/TMHMM/), we found the COOH terminus contained a putative hydrophobic membrane anchor (Fig. 1A). To identify the localization of *Bmbuffy* in silkworm cells, the BmN-SWU1 cells were transfected with *PIZ-Bmbuffy-dsRed* and *PIZ-Bmbuffy-ΔTM-dsRed*. Using confocal microscopy, we found that *Bmbuffy* was located on both the outer mitochondrial membrane (MOM) and endoplasmic reticulum (ER) membrane (Fig. 1B), whereas, *Bmbuffy-ΔTM* lacking of transmembrane domain (261–280aa) was unable to anchor to the membranes. These observations indicate that the hydrophobic transmembrane domain of *Bmbuffy* is essential for localization.

3.2. Bmbuffy inhibits apoptosis in HCPT-treated cells

To investigate the role of *Bmbuffy* in apoptosis of silkworm cells, BmN-SWU1 cells were transfected with one of the following plasmids, respectively: *PIZ-Bmbuffy-dsRed*, *PIZ-Bmbuffy-ΔTM-dsRed* and *PIZ/V5-Bmbuffy-miRNA-dsRed*. Confocal microscopy revealed an apparent decrease in cytochrome c release by cells that were transfected with *Bmbuffy* (Fig. 2A). Additionally, the activity of caspase-3 was lowest in *Bmbuffy*-transfected cells (Fig. 2B). Flow cytometric analysis showed a decrease in the percentage of apoptosis in *Bmbuffy*-transfected cells, while the rate remained unchanged in *Bmbuffy-ΔTM* transfected cells. Furthermore when the *Bmbuffy* was silenced by RNAi, we observed an increase in the apoptotic rate (Fig. 2C). These results indicate an anti-apoptotic role for *Bmbuffy* in the mitochondrial apoptotic pathway of HCPT-treated cells.

3.3. Cytochrome c release is associated with Bmp53 translocation

In HCPT-untreated cells, *Bmp53* was almost located in the nucleus; however, it was able to gradually translocate to the mitochondria in HCPT-treated cells (Fig. 3A). After treatment of BmN-SWU1 cells with HCPT for 72 h, the majority of *Bmp53* was found to accumulate within mitochondria (Fig. 3B). Moreover, the release of mitochondrial cytochrome c increased gradually with this translocation (Fig. 3A), suggesting that cytochrome c release is linked to *Bmp53* translocation.

3.4. Bmp53 interacts with Bmbuffy in HCPT-treated cells: the pro-apoptotic function of exogenous Bmp53 is weakened by co-expression with Bmbuffy

Co-immunoprecipitation and immunofluorescence staining analyses showed that *Bmp53* interacts with *Bmbuffy* in HCPT-treated cells (Fig. 4A and B). The release of cytochrome c increased in

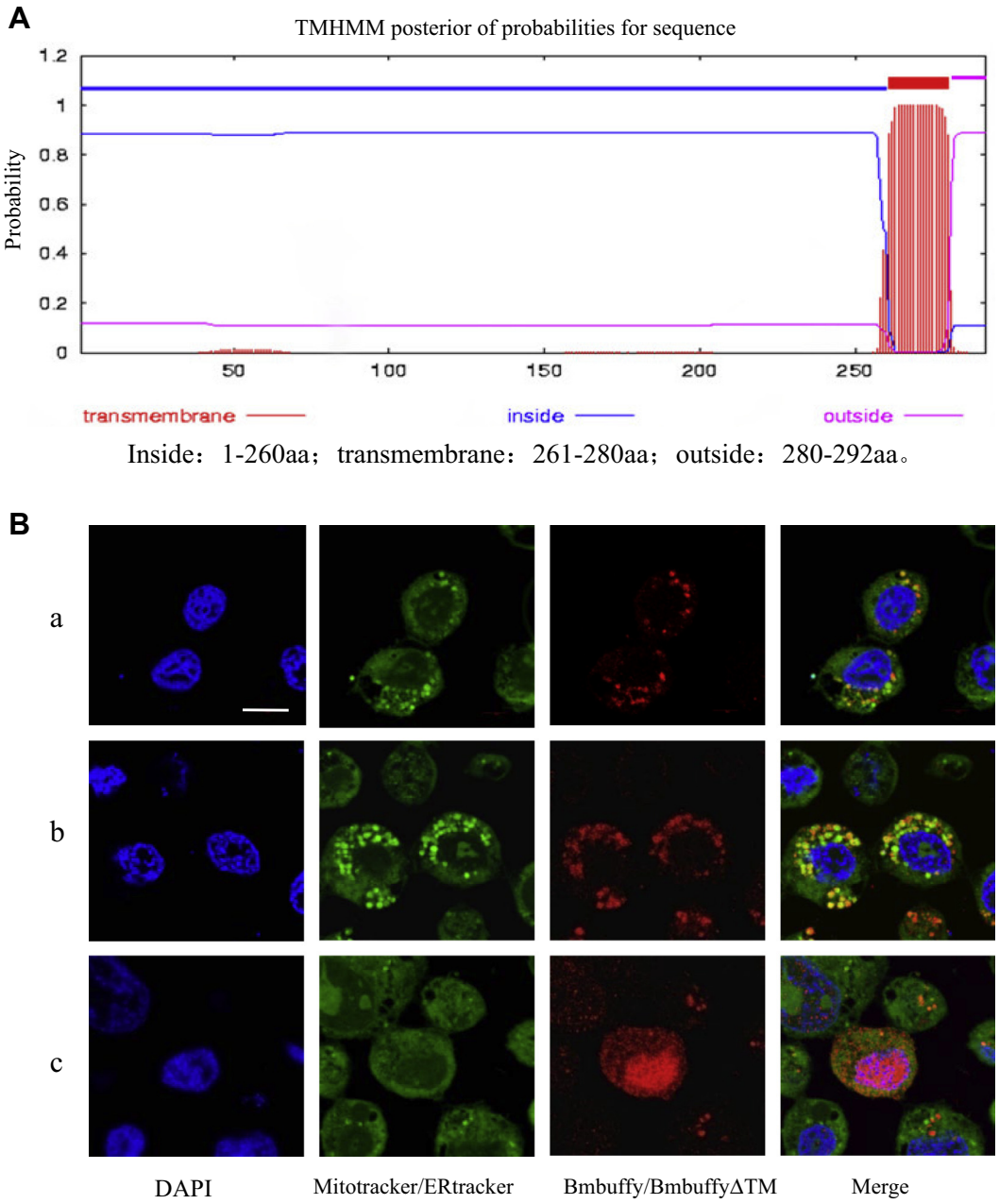

Fig. 1. *Bmbuffy* is located on both the outer membrane of mitochondria and ER. (A) Schematic diagram of the transmembrane region of *Bmbuffy*. The COOH terminus contains a putative hydrophobic membrane anchor (261–280aa). (B) The localization of *Bmbuffy* in BmN-SWU1 cells. (a) *Bmbuffy* (red) is co-located with the mitochondria (green). (b) *Bmbuffy* (red) is co-located with the endoplasmic reticulum (green). (c) *Bmbuffy-ΔTM* is unable to anchor to the membrane and is located in both the nucleus and cytosol. Scale bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Bmp53 transfected cells; however, following co-expression of *Bmp53* and *Bmbuffy*, cytochrome c release decreased (Fig. 4C). The apoptotic rate increased after *Bmp53* overexpression in HCPT-treated cells, whereas the rate decreased following co-expression of *Bmp53* and *Bmbuffy* (Fig. 4D). These results demonstrate that *Bmbuffy* and *Bmp53* formed a complex in HCPT-treated BmN-SWU1 cells, and that their co-expression inhibits apoptosis, which is typically promoted by exogenous *Bmp53*.

4. Discussion

In mammals, several studies have concluded that anti-apoptotic and pro-apoptotic Bcl-2 family proteins are the molecular determinants for the release of cytochrome c and activation of caspase-3 [14,15]; thus, playing key roles in the mitochondrial pathway [16]. *Bmbuffy* has a similar structure to Bok, and is commonly found and identified in silkworms [12], although its precise function is unknown.

In this study, we investigated the role of *Bmbuffy* in the intrinsic mitochondrial pathway of BmN-SWU1 cells. We found the COOH terminus of *Bmbuffy* contains a putative hydrophobic membrane anchor (261–280aa), which is capable of anchoring *Bmbuffy* to the MOM and ER. We observed that *Bmbuffy* is randomly distributed within the cytoplasm and nucleus in the absence of the hydrophobic transmembrane domain. Moreover, overexpression of *Bmbuffy* in HCPT-treated cells inhibits; (i) the release of cytochrome c from the mitochondria, (ii) the activation of caspase-3, and (iii) cell apoptotic rate. In contrast, the reverse effects were observed following RNAi-mediated silencing of *Bmbuffy*, indicating an anti-apoptotic role for *Bmbuffy* in the mitochondrial apoptotic

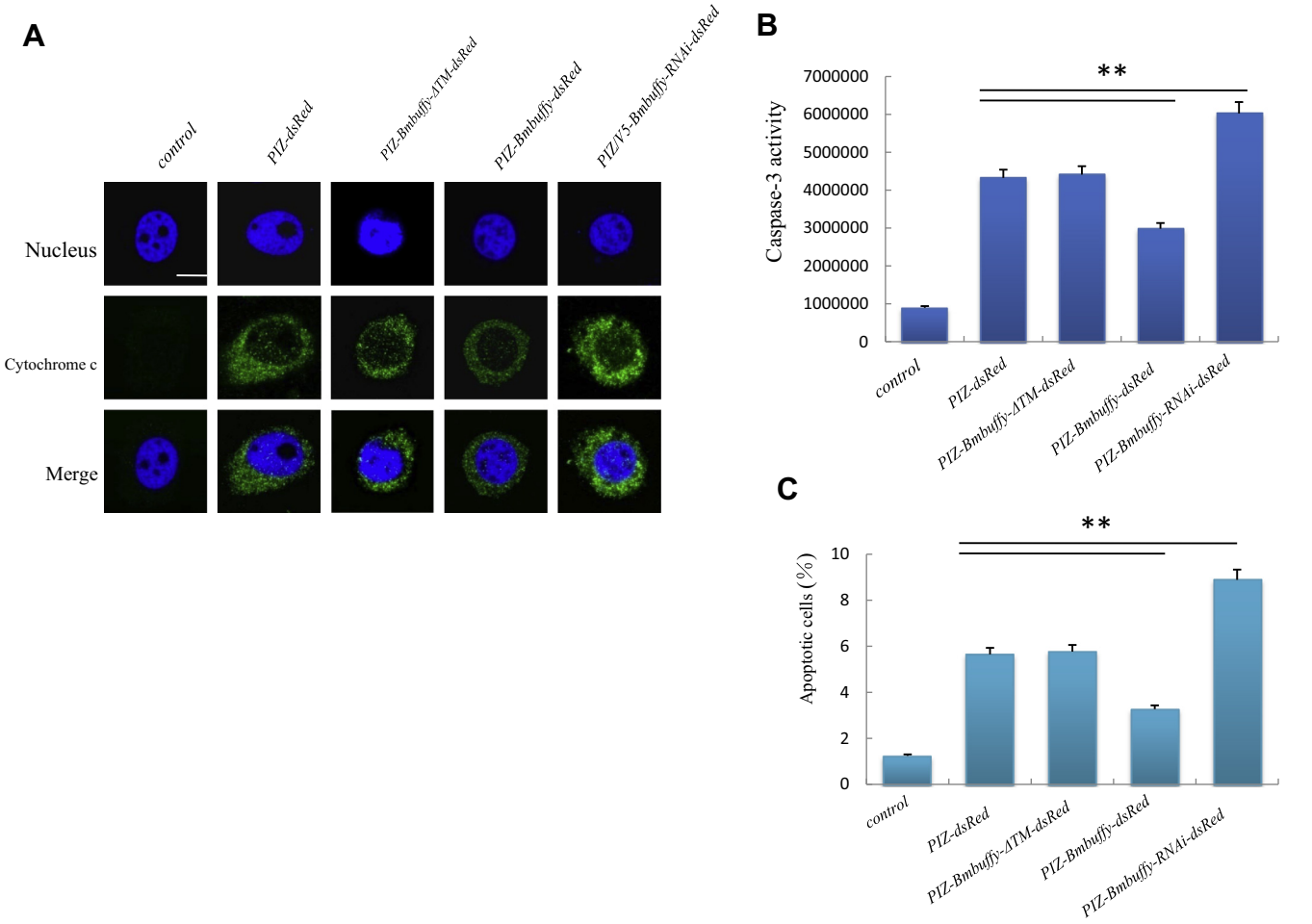

Fig. 2. *Bmbuffy* inhibits apoptosis in HCPT-treated BmN-SWU1 cells. Cells untreated with the HCPT were assigned as the control group; the others were treated with 100 ng/mL HCPT for 48 h after vector transfection for 72 h. (A) The release of cytochrome c decreased in *Bmbuffy* overexpressing cells. Scale bar, 10 μm. (B) The activity of caspase-3 is lowest in cells transfected with *Bmbuffy*. Bars represent means ± SD, $^{**}P < 0.01$. (C) The apoptotic rate decreased in *Bmbuffy* overexpressing cells, whereas the rate remained unchanged in cells transfected with *Bmbuffy-ΔTM*. Bars represent means ± SD, $^{**}P < 0.01$.

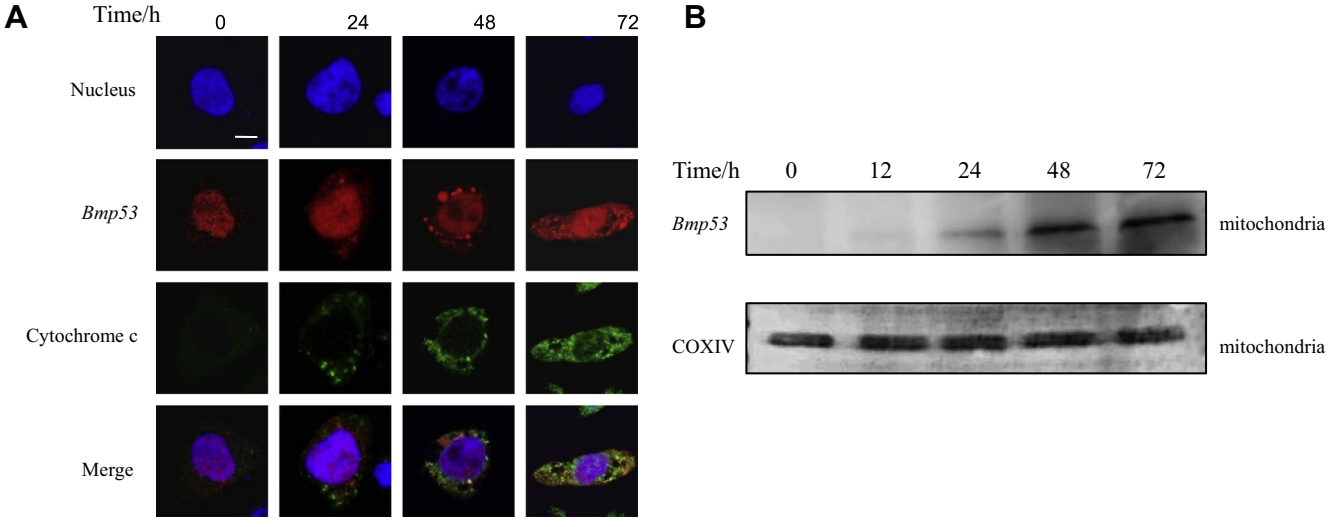

Fig. 3. *Bmp53* translocation from the nucleus to mitochondria in HCPT-treated BmN-SWU1 cells. Cells were treated with 100 ng/mL HCPT for 0, 24, 48, and 72 h. (A) The release of cytochrome c was associated with *Bmp53* translocation. After treatment with HCPT for 72 h in cells, a majority of *Bmp53* translocated from nucleus to mitochondria, along with high level of cytochrome c release. Scale bar, 10 μm. (B) *Bmp53* gradually translocates to the mitochondria over time in HCPT-treated cells.

pathway. In the absence of the hydrophobic membrane anchor, *Bmbuffy* has no effect on HCPT-induced apoptosis, showing that mitochondrial localization is required. ER localization indicates the involvement of *Bmbuffy* in various other physiological and biochemical processes in silkworm cells. Although *Bmbuffy* is a structural homologue of Bok, it acts as a Bcl-2 family pro-survival

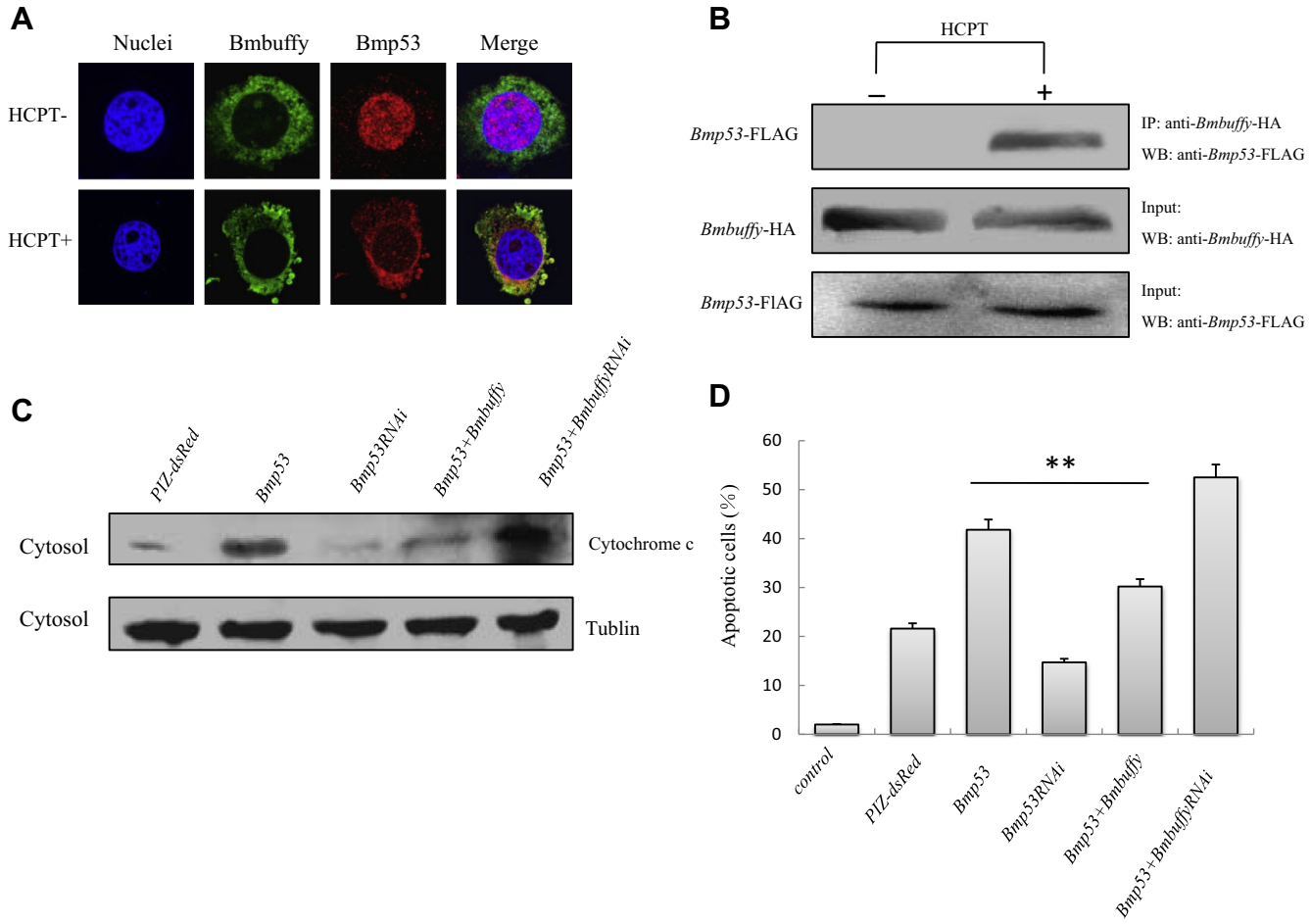

Fig. 4. *Bmbuffy* interacts with *Bmp53* in HCPT-treated BmN-SWU1 cells. Overexpression of *Bmbuffy* inhibits the pro-apoptotic function of *Bmp53*. (A) Co-localization of *Bmbuffy* and *Bmp53* in HCPT-treated cells. Scale bar, 10 μm. (B) *Bmbuffy* interacts with *Bmp53* in HCPT-treated cells. (C) Co-expression of *Bmbuffy* and *Bmp53* inhibits cytochrome c release which is promoted by *Bmp53* overexpression. (D) Co-expression of *Bmbuffy* and *Bmp53* inhibits cell apoptosis which is promoted by *Bmp53* overexpression.

protein, and the hydrophobic transmembrane domain is crucial for the localization and anti-apoptotic function.

It is well-known that mitochondrial p53 localization is required for p53-dependent apoptosis and does not occur during p53-independent apoptosis, nor does it occur during the arrest of p53-mediated cell cycle progression [17]. In mammalian cells, p53 accumulation in the mitochondria is rapid (starting after 1 h) and precedes early dysfunctional changes in cytochrome c release, and procaspase-3 activation [18]. In our research, endogenous *Bmp53* accumulation in the mitochondria was a slow and progressive process, followed by cytochrome c release. Importantly, the level of cytochrome c release was closely related to mitochondrial *Bmp53* localization. This relationship suggests that *Bmp53* localization contributes to mitochondrial membrane permeability, leading to the release of cytochrome c.

The observation of the co-localization of endogenous *Bmp53* and *Bmbuffy* during the apoptotic process confirms this interaction, which was further validated in Co-IP assays. *Bmp53* overexpression promoted HCPT-induced apoptosis, while RNAi-mediated *Bmp53* silencing blocked apoptosis. We showed that mitochondrial *Bmp53* interacts with *Bmbuffy* to block its anti-apoptotic ability and promotes apoptosis progression. Importantly, the rate of cell apoptosis promoted by *Bmp53* overexpression appeared to be reduced by *Bmp53* and *Bmbuffy* co-expression in HCPT-treated cells. These findings indicate an antagonistic relationship between *Bmbuffy* and *Bmp53*. Interestingly, unlike anti-apoptotic Bcl-2 proteins, *Bmbuffy* lacks the BH4 domain at the NH_4-terminus (data not shown), but it functions in a similar manner to pro-survival Bcl-2 proteins. The results of this study are instructive for further studies on the molecular mechanisms of Bcl-2 family proteins in *Lepidoptera*.

In conclusion, the revelation of the anti-apoptotic function of *Bmbuffy* may contribute to studies on apoptotic mechanisms in insect cells and potentially indicate directions for the identification of other Bcl-2 family members in silkworm.

Acknowledgments

This work was supported by Grants from the National High-tech R&D Program of China (No. 2013AA102507), and the National Natural Science Foundation of China (Nos. 31172269 and 31272505).

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