Functional analysis of a survivin-like gene in *Bombyx mori*

Hui Tang · Yu Wu · Haixiang Wu · Yan Wu ·
Huiling Wu · Wenbing Wang

Received: 5 December 2012 / Accepted: 25 February 2013 / Published online: 26 March 2013
© Springer Science+Business Media Dordrecht 2013

Abstract The survivin (*svv*) gene is a newly discovered member of the inhibitors of apoptosis gene family. In recent years, *svv* has been confirmed to have an anti-apoptosis function and to play a critical role in cell division. We identified a survivin-like gene in the silkworm, *Bombyx mori* (*Bm-svv*). In this study, to gain insight into its function, a baculovirus expression system was used to express the *Bm-svv* gene in insect cell lines. The recombinant viruses were then used as a vector to transform insect cells, and cell activity was determined using the Cell Counting Kit-8 (CCK-8), which is usually employed for detecting mammalian cell number. The results indicated that the *Bm-svv* gene plays a role in the cell growth arrest or apoptosis

induced by viruses. Furthermore, the CCK-8 kit is effective in determining the activity of insect cells.

Keywords IAP · Cell growth · AcNPV · BmNPV · Baculovirus · CCK-8

Introduction

The survivin (*svv*) gene is a new member of the inhibitors of apoptosis (IAP) protein family, which plays a critical role in the regulation of apoptosis and cell division (Li et al. 1998; Deveraux and Reed 1999). IAPs, which were first found in baculoviruses, are classified into five types, IAP1 through IAP5, according to their sequence homology (Luque et al. 2001). In recent years, *svv* has been studied in many mammalian species, such as humans, guinea pigs and mice (Ito et al. 2000; Habtemichael et al. 2010; Stauber et al. 2006). Previous studies have shown that *svv* is abundantly expressed in human cancers but is almost absent from normal differentiated cells (Ambrosini et al. 1997). However, recent evidence has indicated that *svv* is also expressed in normal tissues, suggesting a protective role against various stresses (Johnson et al. 2005; Fukuda and Pelus 2006; Kindt et al. 2008). Furthermore, *svv* inhibits Caspase activity to block apoptosis (Sah et al. 2006). It has been demonstrated that *svv* is a member of the chromosomal passenger complex (CPC) proteins and interacts with Aurora-B kinase, Borealin and inner centromere protein

H. Tang · Y. Wu · Y. Wu · H. Wu · W. Wang (✉)
Institute of Life Sciences, Jiangsu University,
301 Xuefu Rd, Zhenjiang 212013, China
e-mail: wenbingwang@ujs.edu.cn

H. Tang
e-mail: tang11_23@126.com

Y. Wu
e-mail: wooyoo4@gmail.com

Y. Wu
e-mail: wuyan@ujs.edu.cn

H. Wu
e-mail: huiling@ujs.edu.cn

H. Wu
Biomedical Development Center of Acebright Group,
1999 Zhangheng Rd, Shanghai 201203, China
e-mail: haixiang.wu@acebright.com

(INCENP) to play a significant role in cell division (Lens et al. 2006; Ruchaud et al. 2007). In addition, *svv* contains a Crm1-dependent nuclear export signal (NES), which is essential for the anti-apoptotic and regulatory role of *svv* during cell division and its correct localization (Stauber et al. 2006; Habtemichael et al. 2010; Knauer et al. 2007a, b; Colnaghi et al. 2006). This gene is highly expressed in cancers including neuroblastoma, gastric cancer, colorectal cancer, and bladder cancer (Adida et al. 1998; Lu et al. 1998; Kawasaki et al. 1998; Swana et al. 1999). In addition, a recent finding led to the hypothesis that *svv* plays a novel role in the enucleation of erythroblasts (Keerthivasan et al. 2012). Future prospective studies should focus on *svv* as a useful prognostic marker (Sah et al. 2006).

A previous study showed that *svv* contains only a single baculovirus IAP repeat (BIR) and a C-terminal coiled-coil domain or a C-terminal RING finger domain (Sun et al. 2005). *Bombyx mori* survivin (*Bm-svv*) gene is located on chromosome 17, and has three exons, consisting of 136 amino acids (Zhang et al. 2010). However, the function of *svv* in *B. mori* is still unclear.

Baculoviruses are a large family of viruses characterized by large circular, double-stranded DNA genomes that replicate in the nucleus of infected host cells. More than 600 baculoviruses have been found to date that infect species from the insect orders Lepidoptera, Diptera and Hymenoptera (Jehle et al. 2006; Katsuma et al. 2000). Recently, a modified pFastBac1 vector was developed by replacing its polyhedrin promoter with the immediate early 1 (*i.e.-1*) promoter (Su et al. 2008). IE-1 was originally identified because of its ability to transactivate early promoters of *Autographa californica* Nucleopolyhedrovirus (AcNPV) (Guarino and Summers 1986). Early genes are transcribed by host RNA polymerase II, independent of viral infection (Fuchs et al. 1983; Glocker et al. 1993). Hence, the *i.e.-1* promoter can initiate expression of the target gene without viral infection.

AcNPV was the first baculovirus whose genome was completely sequenced (Ayres et al. 1994). Recent nucleotide sequence analyses of *B. mori* NPV (BmNPV) have found that its ORFs show approximately 93 % similarity to the amino acid sequence of AcNPV (Gomi et al. 1999). Although these genes exhibit a close relationship, they present significant differences in host range. For example, AcNPV is infectious toward *Spodoptera frugiperda* 21 (Sf 21) cells, but it does not infect *B. mori* N (BmN) cells

(Kondo and Maeda 1991). In contrast, BmNPV infects BmN cells, but in Sf 21 cells, it is either not infectious or infects cells only at very low levels (Kondo and Maeda 1991; Martin and Croizier 1997). Moreover, AcNPV can infect *Trichoplusia ni* 5 (Tn 5) cells, and induces *Spodoptera littoralis* (Spli) cell apoptosis (Hink 1970; Chejanovsky and Gershburg 1995).

We constructed the recombinant viruses Bmie1-*svv* and Aciel-*svv* in which the *Bm-svv* gene was controlled by the *i.e.-1* promoter. *Bm-svv* was then expressed in non-permissive or permissive cells to determine its effect on cell activity using the Cell Counting Kit-8 (CCK-8), which has been frequently employed in mammalian cells (Weldon et al. 2009; Ding et al. 2010; Kunz et al. 2009; Tamura et al. 2010), and found that this method is also useful for the determination of insect cell activity.

Materials and methods

BmN, Sf9 and Tn5 cells were a gift from Professor X.P. Wu (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai 200031, China) and are kept in our laboratory, in Jiangsu University (Zhenjiang, China), and Spli cells were a gift from Professor J. Zhu from Soochow University (Suzhou, China). The bacterium *E. coli* DH10Bac/BmNPV was a gift from Professor E.Y. Park (Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, and Shizuoka, Japan).

Cell culture

Sf 9, Spli and BmN cells were cultured at 27 °C with TC100 insect cell medium (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (50 µg/mL). Tn 5 cells were cultured with TNM-FH medium (HyClone, Logan, UT, USA).

Cloning of the *Bm-svv* gene

The mRNA extraction and cDNA synthesis kits were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The *Bm-svv* gene [GenBank: GQ426299.1] was initially amplified using the following primers: Forward, 5'-ATG GAG AAC GAG AGC TCT TTA CT-3', and Reverse, 5'-TTA TTT CCT TGC

TAA GCA TTT TCT T-3', and an amplification program consisted of an initial step at 94 °C for 4 min, followed by 35 cycles at 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 40 s, and a final extension at 72 °C for 10 min. The amplified fragments were cloned into PMD18-T vector (TaKaRa) and sequenced.

Construction of recombinant transfer vectors

A cDNA fragment of the *Bm-svv* open reading frame (ORF) was amplified and subcloned into the *Xho* I and *Hind* III sites of the pFastBac1-ie1p transfer vector (the foreign gene was controlled by the *i.e.-1* promoter), which was constructed in our laboratory (Su et al. 2008), to generate the recombinant transfer vector pFastBac1-ie1p-svv. The *Bm-svv* fragment was amplified using the following primers: Forward, 5'-TAC CTC GAG ATG GAG AAC GAG AGC TCT TTA CT-3', and Reverse, 5'-TGC AAG CTT TTA TTT CCT TGC TAA GCA TTT TCT T-3' (the *Xho* I and *Hind* III sites are underlined). The amplification program consisted of an initial step of 94 °C for 4 min, followed by 35 cycles of 94 °C for 40 s, 56 °C for 40 s and 72 °C for 40 s. The amplified PCR product was digested with *Xho* I and *Hind* III, and ligated with the pFastBac1-ie1p vector, which had been digested with the same restriction enzymes.

Recombinant bacmid DNA construction

The recombinant vector pFastBac1-ie1p-svv was transformed into *E. coli* DH10Bac/BmNPV or AcNPV, where transposition occurred. After a 4 h incubation at 37 °C in SOC medium, the cells were transferred to a plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal, and 40 µg/mL isopropyl-β-D-thiogalactopyranoside (IPTG).

Isolation of recombinant bacmid DNA

The gene of *svv* was cloned into a pFastBac1-ie1p donor plasmid, and the recombinant plasmid was transformed into *E. coli* DH10Bac competent cells which contained the bacmid with a mini-attTn7 target site and the helper plasmid. The bacmid was propagated in DH10Bac, containing mini-F replicon, a kanamycin resistance marker, and a segment of DNA encoding the lac Zα peptide, which confers resistance

to kanamycin, and can complement a lac Z deletion present on the chromosome to form colonies that are blue (Lac⁺) in the presence of a chromogenic substrate X-gal and the inducer IPTG. The mini-Tn7 element on the pFastBac1-ie1p donor plasmid can transpose to the mini-attTn7 target site on the bacmid, and disrupts expression of the lac Zα peptide, so colonies containing the recombinant bacmid are white in a background of blue colonies harboring the unaltered bacmid. White colonies were selected and streaked onto fresh plates to verify the phenotype, and then confirmed via PCR with M13 primers (Forward, 5'- GTT TTC CCA GTC ACG AC -3', and Reverse, 5'-CAG GAA ACA GCT ATG AC -3'). The PCR amplification program included an initial step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 5 min, and a final extension at 72 °C for 10 min.

Transfection of recombinant bacmid DNA

The recombinant bacmid DNA, Bm-Bacmid-ie1-svv and Ac-Bacmid-ie1-svv, confirmed via PCR, was transfected into BmN and Sf9 cells, respectively, using a transfection reagent (Cellfection II Reagent, Invitrogen, Carlsbad, CA, USA). BmN and Sf9 cells at approximately 80 % confluence were seeded into 24-well plates and cultured for 24 h. The following solutions were prepared in micro tubes: Solution A—for each transfection, 3 µL of lipidosome (Invitrogen) was diluted into 47 µL of serum-free TC-100; and Solution B—for each transfection, 10 µL of bacmid DNA was diluted into 40 µL of serum-free TC-100. The two solutions were then mixed gently together, followed by incubation for 15 min at room temperature. The cells were washed with serum-free TC-100 medium three times, and 250 µL of a solution of serum-free TC-100 with the lipid-DNA complexes was added to each well, followed by gentle mixing. The cells were then held for 3 h in an incubator at 27 °C. After transfection, the mixtures were removed, and 500 µL of TC-100 medium containing 10 % FBS were added to the cells, followed by incubation for 72 h. Viruses (Bmie1-svv and Acie1-svv) were harvested from the culture medium at 72 h post-transfection, and held at 4 °C without light.

Cell activity assay

The CCK-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). It allows very convenient assays

to detect living cells (Wang et al. 2005). WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] is reduced by dehydrogenases in cells, resulting in an orange-colored product (Formazan). The amount of formazan dye in cells is directly proportional to the number of living cells.

According to CCK-8 kit manual, the absorbance value of the cells is from 0.1 to 2.0, preferably is 1.0. Since this method was not used in insect cells, a suitable cell concentration should be detected. BmN and Sf 9 cells were initially cultured at three cell densities, 2×10^3 , 2×10^4 and 5×10^4 per well; and, Tn 5 and Spli cells at 2×10^4 , and 5×10^4 per well.

After that, the cells were cultured in 96-well plates, and were treated with viruses, including Bmie1-*svv* and Acie1-*svv*, at multiplicities of infection (MOI) (Licari and Bailey 1991; Emery 1992) of 1, 10, or 50, for 6 h, 12 h, 24 h, 48 h, or 72 h, respectively, normal cells as a control. 10 μ L of CCK-8 was added to each well. Sf 9 and TN 5 cells were incubated at 27 °C for 2 h, BmN and Spli cells for 4 h. Using a microplate reader (BIO-RAD), the absorbance (OD) at 450 nm was read to determine the cell activity in each well, using 655 nm as the reference wavelength. The results are presented as the percentage of cell growth inhibition (%). The cell growth inhibition rate (%) of cells treated with viruses was calculated using the following equation: [(OD of normal wells—treated wells)/OD of normal wells $\times$ 100 %]. Additionally, the cell proliferation rate (%) of cells treated with viruses was calculated using the following equation: [(OD of treated wells—normal wells)/OD of normal wells $\times$ 100 %]. The experiments were performed in three different samples.

Statistical analysis

The data are presented as the mean $\pm$ SD. The statistical significance of the inhibition rate at various times was determined by two-way ANOVA with GraphPad Prism 5 software and $P < 0.05$ was considered statistically significant.

Results

Identification of recombinant bacmids

It is difficult to identify recombinant viruses (Bmie1-*svv*/Acie1-*svv*) through restriction analysis, as they

exhibit a large genome of more than 80 kb. The bacmid contained M13 forward and reverse priming sites flanking the mini-*att* Tn7 site, facilitating PCR analysis, hence, PCR is a convenient method for identification. The PCR products from the recombinant bacmids (Bm-Bacmid-ie1-*svv* and Ac-Bacmid-ie1-*svv*) were approximately 2,800 bp plus the size of the insert (*Bm-svv*, 411 bp). A fragment of 3,200 bp was amplified from the recombinant bacmid DNAs (Bm-Bacmid-ie1-*svv* and Ac-Bacmid-ie1-*svv*) (Fig. 1, lanes 2, 3). Using the specific *Bm-svv* primers, the *svv* fragment was also amplified, exhibiting a length of 411 bp (Fig. 1, lanes 4, 5).

Detection of insect cell activity using CCK-8

Four cell lines were used to detect the available concentrations in cells treated with CCK-8. The results indicated that suitable monolayer cultures of BmN and Spli cells containing 5×10^4 cells, and Sf 9 and Tn 5 cells were 2×10^4 cells (Fig. 2).

Promotion of cell proliferation by expression of *Bm-svv*

To explore the potential effect of Bm-*svv* in Sf 9, Spli, and Tn 5 cells, the Bmie1-*svv* virus was used to infect the above mentioned cells, respectively, at various MOIs, wild type BmNPV was used as a control. In Sf 9 cells, the data showed that Bmie1-*svv* could

Fig. 1 Identification of recombinant bacmids via PCR. Recombinant bacmids (Bm-Bacmid-ie1-*svv*/Ac-Bacmid-ie1-*svv*) were analyzed via PCR with the M13 forward and M13 reverse primers. The *svv* fragment was also analyzed by PCR in recombinant bacmids. Lane 1, molecular marker (DL-10,000). Lanes 2–3, PCR products from recombinant bacmids (Bm-Bacmid-ie1-*svv*/Ac-Bacmid-ie1-*svv*, indicated by the arrow). Lanes 4–5, PCR products for *svv* from recombinant bacmids (Bm-Bacmid-ie1-*svv*/Ac-Bacmid-ie1-*svv*, indicated by the arrow)

Fig. 2 Identification of the optimal cell density in CCK-8 assay. According to CCK-8 kit, the optimal cell density was identified when the absorbance (OD) reached approximately 1.0. To determine the optimal cell density, Sf 9 and BmN cells were initially cultured at three densities (2×10^3 , 2×10^4 and 5×10^4 per well), Tn 5 and Spli cells were cultured at densities of 2×10^4 and 5×10^4 cells per well

significantly promote cell proliferation during 24 and 48 h incubations compared to normal cells (Fig. 3a, b). At 72 h, cell proliferation was inhibited by viral replication, and the difference between the two types of viruses was highly significant ($***P < 0.001$, Fig. 3c). However, BmNPV inhibited cell growth under all experiment conditions. These results implied that BmNPV inhibited the growth of Sf 9 cells, even if it was not infectious for the cells. Cell growth inhibition by BmNPV was decreased when the *Bm-svv* was expressed. Similar results were found in Tn 5 cells. BmNPV inhibited Tn 5 cell growth at all examined MOIs, but Bmie1-svv virus increased Tn 5 cell growth at 24 h. It showed a highly significant difference from BmNPV ($***P < 0.001$, Fig. 4b).

In Spli cells, the results showed that the two viruses, Bmie1-svv and BmNPV increased Spli cell growth at MOIs of 1 and 10 (Fig. 4a). Cell growth was decreased when cells were incubated with BmNPV at MOI of 50 (Fig. 4a). In contrast, cell growth was increased when incubated with Bmie1-svv at MOI of 50. This evidence implied that BmNPV can promote Spli cell growth in low MOIs (1 and 10). At a very high concentration of virus (MOI 50), cell growth was decreased, but the Bmie1-svv virus could prevent this decrease.

Reducing BmN cell growth inhibition by AcNPV

To test *Bm-svv* activation in BmN cells, AcNPV and Acie1-svv viruses were added into the cell cultures, AcNPV was used as control. Both viruses inhibited

Fig. 3 The virus Bmie1-svv promotes Sf 9 cell proliferation. The BmNPV and Bmie1-svv viruses were used to infect Sf 9 cells at various MOIs (1–50) for 24 h (a), 48 h (b), and 72 h (c). BmNPV was used as a control. The error bars indicate the SD of means from three experiments (mean $\pm$ S.D., $n = 3$), * indicate significant differences at $P < 0.05$

cell growth at 24, 48, and 72 h (Fig. 5c–e). At 24 h, a significant difference was found between the viruses at MOIs of 10, and 50 ($*P < 0.05$). Inhibition activity of AcNPV is higher than that of Acie1-svv at both 48 and 72 h. At 72 h, the two viruses have a highly significant difference at MOIs of 10, and 50 ($***P < 0.001$, Fig. 5e).

To determine whether AcNPV inhibits BmN cell growth at early time points, the incubation time was decreased to 6 or 12 h. We found that both viruses also

Fig. 4 The virus Bmie1-svv promotes the proliferation of Spli and Tn 5 cells. The BmNPV and Bmie1-svv viruses were incubated with Spli (a) and Tn 5(b) cells for 24 h at various MOIs (1–50), and BmNPV served as a control. The error bars indicate the SD of means from three experiments (mean $\pm$ S.D., $n = 3$), * indicate significant differences at $P < 0.05$

inhibited cell growth (Fig. 5a, b), but compared to AcNPV, Acie1-svv showed lower inhibition activity. In addition, the both viruses exhibited a significant difference after 6 h of incubation (MOI 10), the inhibition of Acie1-svv virus was lower than AcNPV at MOIs of 1, and 50.

Resistance to viral infection

In Tn 5 cells infected with AcNPV, we found that the Acie1-svv virus (MOIs of 1, and 10) increased cell growth at 24 h, while AcNPV inhibited cell growth (Fig. 6a). When the experiment lasted to 48 h, both the viruses inhibited cell growth, but the cell growth inhibition of Acie1-svv virus was lower than that caused by AcNPV at MOIs of 1, and 10 (Fig. 6b). However, at MOI of 50, the inhibitory effect of the Acie1-svv virus was slightly higher than that of AcNPV, though this difference was not significant. Interestingly, the same results were observed in Spli cells (MOI 50, Fig. 7a, b), these data indicated that

expression of the *Bm-svv* gene decreased the growth inhibition induced by viral damage, but at a very high concentration, this effect was not apparent (Figs. 6b and 7).

Decreased apoptosis of Spli cells induced by AcNPV

In Spli cells, both BmNPV and Bmie1-svv viruses inhibited cell growth at 24 h. At MOI of 10, there was a highly significant difference in inhibition activity between the two viruses ($***P < 0.001$, Fig. 7a). Furthermore, apoptotic bodies of Spli cells were apparent under AcNPV infection at MOIs of 10, and 50. However, apoptotic bodies only occurred under Acie1-svv infection at MOI of 50. It suggests that expression of the *Bm-svv* gene delayed Spli cell apoptosis induced by AcNPV (Fig. 8a). To detect occurrence of cell apoptotic bodies at 48 h, the cells were observed under microscope (OLYMPUS CKX41, Japan). The apoptotic bodies of Spli cells were apparent under AcNPV infection at all MOIs. In Acie1-svv infection, the apoptotic bodies were found at MOIs of 10, and 50, but not at MOI of 1 (Fig. 8b).

Discussion

In previous studies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) has frequently been used in mammalian cells, tumor cells and insect cells (Wang et al. 2007; Zhou et al. 2005; Zhang et al. 2012). Recently, CCK-8 has been widely used in tumor cells, because it is easier to use and more sensitive than MTT. To confirm that CCK-8 is effective in insect cells, we firstly employed CCK-8 to determine insect cells activity. The results indicated that CCK-8 is effective in the four insect cell lines, BmN, Sf 9, Tn 5, and Spli. However, the concentration of cells and detection time are different, when an absorbance value of cell reaches approximately 1.0 (Fig. 2).

The *svv* gene is a new member of the IAP protein family (Li et al. 1998; Deveraux and Reed 1999). A homologous gene in *B. mori* was found, but its functions are unknown. Compared with other mammalian *svv*, *Bm-svv* shares 41 % amino acid identity (59 % similarity) with human *svv*, and 44 % identity (58 % similarity) with mice *svv*. To test the function of *Bm-svv* gene in apoptosis stimulated by a reagent of

Fig. 5 The virus Acie1-svv reduces BmN cell growth inhibition. The AcNPV and Acie1-svv viruses were added to BmN cells at various MOIs (1–50) for 6 h (a), 12 h (b), 24 h (c), 48 h (d), and

72 h (e), AcNPV was used as a control. The error bars indicate the SD of means from three experiments (mean $\pm$ S.D., n = 3), * indicate significant differences at $P < 0.05$

hydrogen peroxide (H_2O_2), Sf 9 cells were incubated with the Bmie1-svv and BmNPV for 24 h, respectively, and then stimulated with H_2O_2 at an IC50 concentration of 55 $\mu\text{mol/L}$ for 24 h, no difference of cell activity was found (data not shown). This implied that the *Bm-svv* products could not significantly reduce the apoptosis induced by the reagent H_2O_2 .

Normally two main methods are used to introduce genes into cells to study gene functions, by virus

infection or co-transfection with lipidosome. In insect cells, the effect of lipidosome is not quite stabilized, instead, virus infection is a convenient way to carry genes into the cells. Moreover, the *i.e.-1* promoter of baculovirus was used to express genes in most of insect cells, in this paper, this promoter was used to express *Bm-svv* gene. To avoid the influence of viral infection, we designed a cross way to study the function of *Bm-svv*, using incubation of baculovirus

Fig. 6 The virus Acie1-svv reduces Tn 5 cell growth inhibition. The AcNPV and Acie1-svv viruses were used to infect with Tn 5 cells at various MOIs (1–50) for 24 h (a), and 48 h (b). AcNPV was used as a control. The error bars indicate the SD of means from three experiments (mean $\pm$ S.D., $n = 3$), * indicate significant differences at $P < 0.05$

with non-permissive cell lines. Although BmNPV and AcNPV have approximately 93 % similarity to the amino acid sequence with each other (Gomi et al. 1999), AcNPV infects Sf 9 and Tn 5 cells, but not BmN cells and it induces Spli cell apoptosis (Hink 1970; Chejanovsky and Gershburg 1995). However, BmNPV only can infect BmN cells, but not Sf 9, Tn 5, and Spli cells. In our experiments, BmNPV inhibited the growth of Sf 9 and Tn 5 cells, but the Bmie1-svv virus promoted cell growth at early time of incubation (Figs. 3a, b and 4b). In Spli cells, BmNPV and Bmie1-svv promote cell growth at MOIs of 1, and 10 (Fig. 4a), comparing to MOIs of 1, and 10, the effect at MOI of 50 was reduced in BmNPV, but not in Bmie1-svv. This implied that high viral titer affected cell growth, and *Bm-svv* might neutralize this effect. Hence, *Bm-svv* has a role in insect cell growth.

In a cross test, a recombinant virus Acie1-svv was constructed, and used to detect *Bm-svv* function in BmN cells. The results indicated that AcNPV also inhibited BmN cell growth, this effect was decreased

Fig. 7 The virus Acie1-svv reduces AcNPV-induced apoptosis in Spli cells. The AcNPV and Acie1-svv viruses were used to infect with Spli cells at various MOIs (1–50) for 24 h (a), and 48 h (b). AcNPV was used as a control. The error bars indicate the SD of means from three experiments (mean $\pm$ S.D., $n = 3$), * indicate significant differences at $P < 0.05$

by *Bm-svv* expression (Fig. 5). In Spli cells, expression of *Bm-svv* can also reduce cell apoptosis induced by AcNPV (Fig. 7).

Viral titer may affect gene expression in insect cells. Low MOI may be an appropriate value for detection of cell growth in permissive cells. For example, significant differences were observed in Tn 5 cells between AcNPV and Acie1-svv at MOIs of 1, and 10, but not at MOI of 50 (Fig. 6b). At high MOI, the virus infection was very fast, and the infection cycle rapidly enters late stage, this resulted in the inactivation of the viral early promoters. The expression level of the genes driven by *i.e.-1* was low, so that the function of expression products might be not effective. Moreover, time also plays a critical role in these experiments. When Sf 9 cells were incubated with BmNPV or Bmie1-svv viruses for 72 h, both viruses inhibited cell growth, whereas at 24, and 48 h, only BmNPV inhibited cell growth (Fig. 3a–c). Acie1-svv

Fig. 8 The virus *Acic1-svv* reduces apoptotic bodies of Spli cells induced by viral infection Spli cells were incubated with AcNPV and *Acic1-svv* at various MOIs (1–50) for 24 h (a), and

48 h (b). The number of apoptotic bodies ($\times 400$) in Spli cells increased accompanying with an increasing virus MOI and extension of time (indicated by the arrow)

increased Tn 5 cell growth after incubation for 24 h (MOIs of 1, and 10), but when the incubation extended for 48 h, both viruses inhibited growth (Fig. 6a, b). The results indicated that *Acic1-svv* can reduce cell apoptosis in early after infection with low MOI. However, accompanying with the high MOI and extension of the time, replication of AcNPV was so rapid that *Bm-svv* expression level could not block cell apoptosis effectively. A similar result was found in Spli cells at 48 h, apoptotic bodies were observed in both incubations with AcNPV, and *Acic1-svv*, respectively.

The baculovirus system is frequently used to expression foreign protein (Smith et al. 1983; Kost et al. 2005; Summers 2006), however, an obvious shortage cannot be avoided in this system, which is that the virus kills the cells in a short time, leading to a limited foreign gene expression. To increase expression, the cells would keep activity for a long time. In this study, we found that *Bm-svv* can reduce apoptosis of the cells induced by virus infection and promote cell growth somehow. When a foreign gene is co-expressed with *Bm-svv*, the cells may grow for a longer time to produce more gene products.

In conclusion, we demonstrated that *Bm-svv* not only promotes cell proliferation in insect cells but can also reduce Spli cell apoptosis induced by AcNPV. Moreover, CCK-8 reagent can be used in insect cells to detect the cell activity conveniently.

Acknowledgments We thank Professor Jiang Zhu from Soochow University for providing the Spli cells.

Conflict of interest There is no conflict of interest in this paper.

References

- Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC (1998) Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 351:882–883
- Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 3:917–921
- Ayres MD, Howard SC, Kuzio J, Lopez-Ferber M, Possee RD (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology* 202:586–605
- Chejanovsky N, Gershburg E (1995) The wild-type *Autographa californica* nuclear polyhedrosis virus induces apoptosis of *Spodoptera littoralis* cells. *Virology* 209:519–525
- Colnaghi R, Connell CM, Barrett RM, Wheatley SP (2006) Separating the anti-apoptotic and mitotic roles of survivin. *J Biol Chem* 281:33450–33456
- Deveraux QL, Reed JC (1999) IAP family proteins—suppressors of apoptosis. *Genes Dev* 13:239–252
- Ding J, Huang S, Wu S, Zhao Y, Liang L, Yan M, Ge C, Yao J, Chen T, Wan D, Wang H, Gu J, Yao M, Li J, Tu H, He X (2010) Gain of miR-151 on chromosome 8q24.3 facilitates tumour cell migration and spreading through downregulating RhoGDI α . *Nat Cell Biol* 12:390–399
- Emery VC (1992) Baculovirus expression vectors. *Methods Mol Biol* 8:309–318
- Fuchs LY, Woods MS, Weaver RF (1983) Viral transcription during *Autographa californica* nuclear polyhedrosis virus

- infection: a novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J Virol* 48:641–646
- Fukuda S, Pelus LM (2006) Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther* 5:1087–1098
- Glockner B, Hoopes RR Jr, Hodges L, Rohrmann GF (1993) In vitro transcription from baculovirus late gene promoters: accurate mRNA initiation by nuclear extracts prepared from infected *Spodoptera frugiperda* cells. *J Virol* 67:3771–3776
- Gomi S, Majima K, Maeda S (1999) Sequence analysis of the genome of *Bombyx mori* nucleopolyhedrovirus. *J Gen Virol* 80:1323–1337
- Guarino LA, Summers MD (1986) Functional mapping of a trans-activating gene required for expression of a baculovirus delayed-early gene. *J Virol* 57:563–571
- Habtemichael N, Wunsch D, Bier C, Tillmann S, Unruhe B, Frauenknecht K, Heinrich UR, Mann WJ, Stauber RH, Knauer SK (2010) Cloning and functional characterization of the guinea pig apoptosis inhibitor protein Survivin. *Gene* 469:9–17
- Hink WF (1970) Established insect cell line from the cabbage looper *Trichoplusia ni*. *Nature* 226:466–467
- Ito T, Shiraki K, Sugimoto K, Yamanaka T, Fujikawa K, Ito M, Takase K, Moriyama M, Kawano H, Hayashida M, Nakano T, Suzuki A (2000) Survivin promotes cell proliferation in human hepatocellular carcinoma. *Hepatology* 31:1080–1085
- Jehle JA, Lange M, Wang H, Hu Z, Wang Y, Hauschild R (2006) Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. *Virology* 346:180–193
- Johnson EA, Svetlov SI, Wang KK, Hayes RL, Pineda JA (2005) Cell-specific DNA fragmentation may be attenuated by a survivin-dependent mechanism after traumatic brain injury in rats. *Exp Brain Res* 167:17–26
- Katsuma S, Deng DX, Zhou CL, Iwanaga M, Noguchi Y, Kobayashi M, Maeda S (2000) Identification of novel residues involved in nuclear localization of a baculovirus polyhedrin protein. *Virus Genes* 21:233–240
- Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N (1998) Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 58:5071–5074
- Keerthivasan G, Liu H, Gump JM, Dowdy SF, Wickrema A, Crispino JD (2012) A novel role for survivin in erythroblast enucleation. *Haematologica* 97:1471–1479
- Kindt N, Menzebach A, Van de Wouwer M, Betz I, De Vriese A, Conway EM (2008) Protective role of the inhibitor of apoptosis protein, survivin, in toxin-induced acute renal failure. *FASEB J* 22:510–521
- Knauer SK, Bier C, Schlag P, Fritzmann J, Dietmaier W, Rodel F, Klein-Hitpass L, Kovacs AF, Doring C, Hansmann ML, Hofmann WK, Kunkel M, Brochhausen C, Engels K, Lippert BM, Mann W, Stauber RH (2007a) The survivin isoform survivin-3B is cytoprotective and can function as a chromosomal passenger complex protein. *Cell Cycle* 6:1502–1509
- Knauer SK, Kramer OH, Knosel T, Engels K, Rodel F, Kovacs AF, Dietmaier W, Klein-Hitpass L, Habtemichael N, Schweitzer A, Brieger J, Rodel C, Mann W, Petersen I, Heinzl T, Stauber RH (2007b) Nuclear export is essential for the tumor-promoting activity of survivin. *FASEB J* 21:207–216
- Kondo A, Maeda S (1991) Host range expansion by recombination of the baculoviruses *Bombyx mori* nuclear polyhedrosis virus and *Autographa californica* nuclear polyhedrosis virus. *J Virol* 65:3625–3632
- Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* 23:567–575
- Kunz C, Focke F, Saito Y, Schuermann D, Lettieri T, Selfridge J, Schar P (2009) Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. *PLoS Biol* 7:e91
- Lens SM, Vader G, Medema RH (2006) The case for Survivin as mitotic regulator. *Curr Opin Cell Biol* 18:616–622
- Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 396:580–584
- Licari P, Bailey JE (1991) Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. *Biotechnol Bioeng* 37:238–246
- Lu CD, Altieri DC, Tanigawa N (1998) Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res* 58:1808–1812
- Luque T, Finch R, Crook N, O'Reilly DR, Winstanley D (2001) The complete sequence of the *Cydia pomonella* granulovirus genome. *J Gen Virol* 82:2531–2547
- Martin O, Croizier G (1997) Infection of a *Spodoptera frugiperda* cell line with *Bombyx mori* nucleopolyhedrovirus. *Virus Res* 47:179–185
- Ruchaud S, Carmena M, Earnshaw WC (2007) Chromosomal passengers: conducting cell division. *Nat Rev Mol Cell Biol* 8:798–812
- Sah NK, Khan Z, Khan GJ, Bisen PS (2006) Structural, functional and therapeutic biology of survivin. *Cancer Lett* 244:164–171
- Smith GE, Summers MD, Fraser MJ (1983) Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol Cell Biol* 3:2156–2165
- Stauber RH, Rabenhorst U, Reik A, Engels K, Bier C, Knauer SK (2006) Nucleocytoplasmic shuttling and the biological activity of mouse survivin are regulated by an active nuclear export signal. *Traffic* 7:1461–1472
- Su WJ, Wu Y, Wu HL, Zhu SY, Wang WB (2008) Expression of *Bombyx mori* nucleopolyhedrovirus ORF76 in permissive and non-permissive cell lines by a novel Bac-to-Bac/BmNPV baculovirus expression system. *Pol J Microbiol* 57:271–274
- Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. *Adv Virus Res* 68:3–73
- Sun C, Nettesheim D, Liu Z, Olejniczak ET (2005) Solution structure of human survivin and its binding interface with Smac/Diablo. *Biochemistry* 44:11–17
- Swana HS, Grossman D, Anthony JN, Weiss RM, Altieri DC (1999) Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. *N Engl J Med* 341:452–453

- Tamura K, Mawaribuchi S, Yoshimoto S, Shiba T, Takamatsu N, Ito M (2010) Tumor necrosis factor-related apoptosis-inducing ligand 1 (TRAIL1) enhances the transition of red blood cells from the larval to adult type during metamorphosis in *Xenopus*. *Blood* 115:850–859
- Wang YY, Zhou GB, Yin T, Chen B, Shi JY, Liang WX, Jin XL, You JH, Yang G, Shen ZX, Chen J, Xiong SM, Chen GQ, Xu F, Liu YW, Chen Z, Chen SJ (2005) AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. *Proc Natl Acad Sci U S A* 102:1104–1109
- Wang XJ, Chen J, Lv ZB, Nie ZM, Wang D, Shen HD, Wang XD, Wu XF, Zhang YZ (2007) Expression and functional analysis of the cellular retinoic acid binding protein from silkworm pupae (*Bombyx mori*). *J Cell Biochem* 102:970–979
- Weldon JE, Xiang L, Chertov O, Margulies I, Kreitman RJ, FitzGerald DJ, Pastan I (2009) A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity. *Blood* 113:3792–3800
- Zhang JY, Pan MH, Sun ZY, Huang SJ, Yu ZS, Liu D, Zhao DH, Lu C (2010) The genomic underpinnings of apoptosis in the silkworm *Bombyx mori*. *BMC Genomics* 11:611
- Zhang LJ, Xue YQ, Yang C, Yang WH, Chen L, Zhang QJ, Qu TY, Huang S, Zhao LR, Wang XM, Duan WM (2012) Human albumin prevents 6-hydroxydopamine-induced loss of tyrosine hydroxylase in in vitro and in vivo. *PLoS One* 7:e41226
- Zhou X, Dai LL, Jia LP, Zheng YY, Wang WB (2005) Study on the inhibitive effect of Astragalus Injection solution on hepatic fibrosis in rats. *Zhonghua Gan Zang Bing Za Zhi* 13:575–578