Biochemical and Biophysical Research Communications 454 (2014) 581–587

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

The BIR and BIR-like domains of *Bombyx mori* nucleopolyhedrovirus IAP2 protein are required for efficient viral propagation

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ARTICLE INFO

Article history:
Received 24 October 2014
Available online 1 November 2014

Keywords:
Baculovirus
IAP
BIR
RING finger
Ubiquitin ligase
BmNPV

ABSTRACT

The baculovirus *Bombyx mori* nucleopolyhedrovirus (BmNPV) possesses two genes, *iap1* and *iap2*, which encode inhibitor of apoptosis (IAP) proteins. We previously showed that although both genes are dispensable for viral propagation, *iap2* is required for efficient viral propagation in cultured cells. BmNPV IAP2 contains three putative functional domains: a baculovirus IAP repeat (BIR), a BIR-like (BIRL) domain, and a RING finger domain. To identify the domain affecting viral growth, we generated a series of BmNPV bacmids expressing *iap2* derivatives lacking one or two domains, or possessing a single amino acid substitution to abolish IAP2 ubiquitin ligase activity. We examined their properties in both cultured cells and *B. mori* larvae. We found that either the BIR or BIRL domain of IAP2 plays an important role in BmNPV infection, and that the RING finger domain, which is required for ubiquitin ligase activity, does not greatly contribute to BmNPV propagation. This is the first study to identify functional domains of the baculovirus IAP2 protein.

1. Introduction

The family *Baculoviridae* is a large family of viruses that infect insects, particularly those of the order Lepidoptera. Baculoviruses are phylogenetically divided into four genera namely, *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* [1]. Alphabaculoviruses and betabaculoviruses are nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), respectively, both of which infect lepidopteran larvae. These viruses produce two types of virions during their infection cycle: occlusion-derived viruses (ODVs) and budded viruses (BVs). This strategy enables the viruses to replicate efficiently within infected larvae and spread their progeny among insects. ODVs are embedded in occlusion bodies (OBs) and spread from insect to insect via oral infection, whereas BVs spread infection to neighboring cells [2].

It is well known that baculoviruses have incorporated a number of genes from host insects by horizontal gene transfer [3–5]. The inhibitor of apoptosis (*iap*) gene is one of these host homologs [5]. The first *iap* genes were discovered in the genomes of *Cydia pomonella* GV and *Orgyia pseudotsugata* NPV (OpMNPV), respectively, by screening for genes that prevent the apoptosis of *Spodoptera frugiperda* cells infected with a *p35*-disrupted mutant of *Autographa californica* NPV (AcMNPV) [6,7]. The baculoviral *iap* genes are classified into five groups, *iap1*–*iap5*, according to their sequence homology [8,9]. Baculovirus genomes commonly contain multiple *iap* genes that belong to different groups. Although IAP3 proteins from most baculoviruses inhibit apoptosis [6,7,10–12], the roles of most other IAPs, including IAP2, remain unknown.

IAP proteins have 1–3 baculovirus IAP repeats (BIRs) in the N-terminal region and a RING finger domain in the C-terminal region [13]. The BIRs mediate protein–protein interactions, and an individual BIR domain is required for anti-apoptotic activity of IAP proteins [14,15], whereas the RING finger domain functions as an E3 ubiquitin ligase. The E3 ubiquitin ligase attaches ubiquitin to its substrate by interacting with E2 and the target protein. Biochemical experiments have shown that baculovirus IAPs, including *Bombyx mori* NPV (BmNPV) IAP2 and OpMNPV IAP3 have E3 ubiquitin ligase activity, but BmNPV IAP1 does not possess this activity [16,17].

The genome of BmNPV contains two *iap* genes, *iap1* and *iap2* [18]. To understand the roles of these two BmNPV *iap* genes, we previously attempted to disrupt them by inserting a *lacZ* cassette using homologous recombination in cultured cells. Isolation of an *iap1*-disrupted virus was successful, indicating that *iap1* is dispensable for BmNPV growth in cultured cells [5]. Although no phenotypic defects were observed in an *iap1*-disrupted virus in cultured cells, the mutant virus took approximately 15 h longer than the wild-type (WT) virus to kill *B. mori* larvae. This indicates that BmNPV IAP1 is a viral pathogenicity factor for *B. mori* [5].

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http://dx.doi.org/10.1016/j.bbrc.2014.10.132
0006-291X/

On the other hand, we were unable to delete *iap2* from the BmNPV genome using a conventional homologous recombination method [5]. Recently, a bacmid-based approach revealed that BmNPV *iap2* is dispensable for viral replication but is required for efficient viral propagation in cultured cells [19].

BmNPV IAP2 possesses three putative functional domains: a BIR domain, a BIR-like (BIRL) domain, and a RING finger domain. In this study, we generated a series of BmNPV bacmids, each of which expresses a mutant IAP2 protein, and showed that either the BIR or BIRL domain, but not the RING finger domain of IAP2, plays an important role in BmNPV propagation.

2. Materials and methods

2.1. Insect, cell lines, and viruses

B. mori larvae (F1 hybrid Kinshu × Showa) were reared as described previously [20]. The BmN (BmN-4) cells were cultured at 27 °C in TC-100 medium supplemented with 10% fetal bovine serum. The *polyhedrin* (*polh*)-positive BmNPV T3 strain-based bacmid, BT3Bac^{+polh} [21], was used as the wild-type (WT) virus. Viral titers were determined by the plaque assay on BmN cells [20].

2.2. Construction of recombinant bacmids possessing mutant iap2 genes

The *polh*-negative *iap2*-disrupted bacmid (Δ*iap2*) was described previously [19]. To generate a *polh*-positive bacmid lacking *iap2*, we amplified the *polh*-containing fragment by polymerase chain reaction (PCR) using the BmNPV T3 genome and primers Polh_Xho_F and Polh_Xho_R (Table S1). The PCR product was cloned into the *Xho*I site of the pFatBac1 vector (Invitrogen). The resulting plasmid (pFB-polh) was transposed into the Δ*iap2* genome and the resulting bacmid was named BmIAP2D (IAP2D) (Fig. 1A). The HA-tagged *iap2* derivatives with an endogenous *iap2* promoter (Fig. 1A) were generated by overlapping PCR [22] using the primers listed in Table S1, and cloned between the *Sph*I and *Hind*III sites of pFB-polh. DNA sequences were determined using an ABI Prism 3100 DNA sequencer (Applied Biosystems). These plasmids were transposed into the Δ*iap2* genome and the resulting bacmids were named BmIAP2DR (DR), BmIAP2CS (CS), BmIAP2BIRLD (BIRLD), BmIAP2BIRD (BIRD), BmIAP2B-BIRD (B-BLD), and BmIAP2RINGD (RINGD) (Fig. 1A). Precise transposition was verified by PCR and DNA sequencing. Detailed information about each bacmid is provided in Section 3. Recombinant viruses were produced by transfection of bacmid DNAs into BmN cells as described previously [19]. Expression of HA-tagged IAP2 was examined by Western blot analysis (Figs. 1B and 3D).

2.3. BV production and POLH expression in BmN cells

To determine the viral growth curves in BmN cells, cells were infected with BmNPVs at a multiplicity of infection (MOI) of 0.01, 0.001 or 5. Following incubation for 1 h, the virus-containing culture medium was removed and fresh medium was added [0 h post-infection (hpi)]. A small amount of the culture medium was harvested at specific time points. BV production was determined by the plaque assay. POLH expression was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously [23].

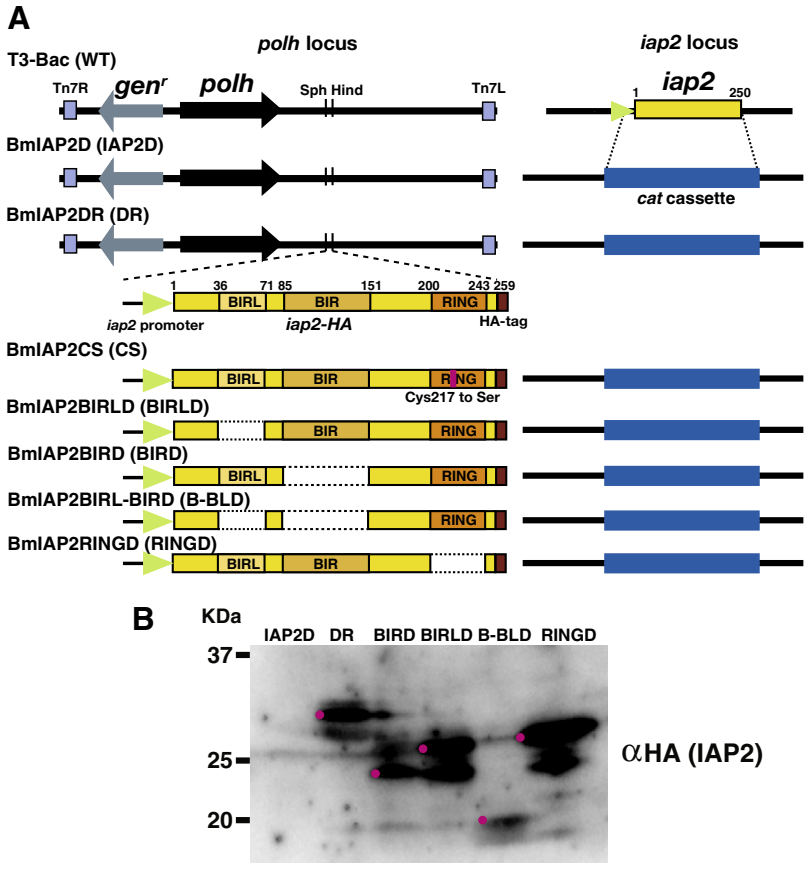

Fig. 1. Generation of BmNPV bacmids expressing *iap2* derivatives. (A) Schematic representation of the *polh* and *iap2* gene loci of WT and mutant BmNPV bacmids. (B) Expression of HA-tagged IAP2 proteins in BmNPV-infected BmN cells. BmN cells were infected with recombinant BmNPVs at an MOI of 0.001, harvested at 5 dpi, and subjected to Western blot analysis using an anti-HA antibody. Red circles indicate the signals that correspond to IAP2 proteins.

2.4. Larval bioassays

Fifth instar *B. mori* larvae were starved for several hours, injected with 50 μl of a viral suspension containing 200, 1×10^3, or 1×10^5 PFU and returned to the artificial diet at 25 °C. The hemolymph of the infected larvae was collected and the released OBs were counted using a hemocytometer [20]. The median lethal time (LT_{50}) was determined by intrahemocoelic injection of fifth instar larvae with BVs. *B. mori* larvae were intrahemocoelically inoculated with BV within 12 h after molting to the fifth instar [20]. BV titers in the hemolymph of the infected larvae were determined by the plaque assay [20].

2.5. Western blotting

BmN cells were infected with recombinant BmNPVs at an MOI of 0.001, harvested at 5 days post-infection (dpi), and subjected

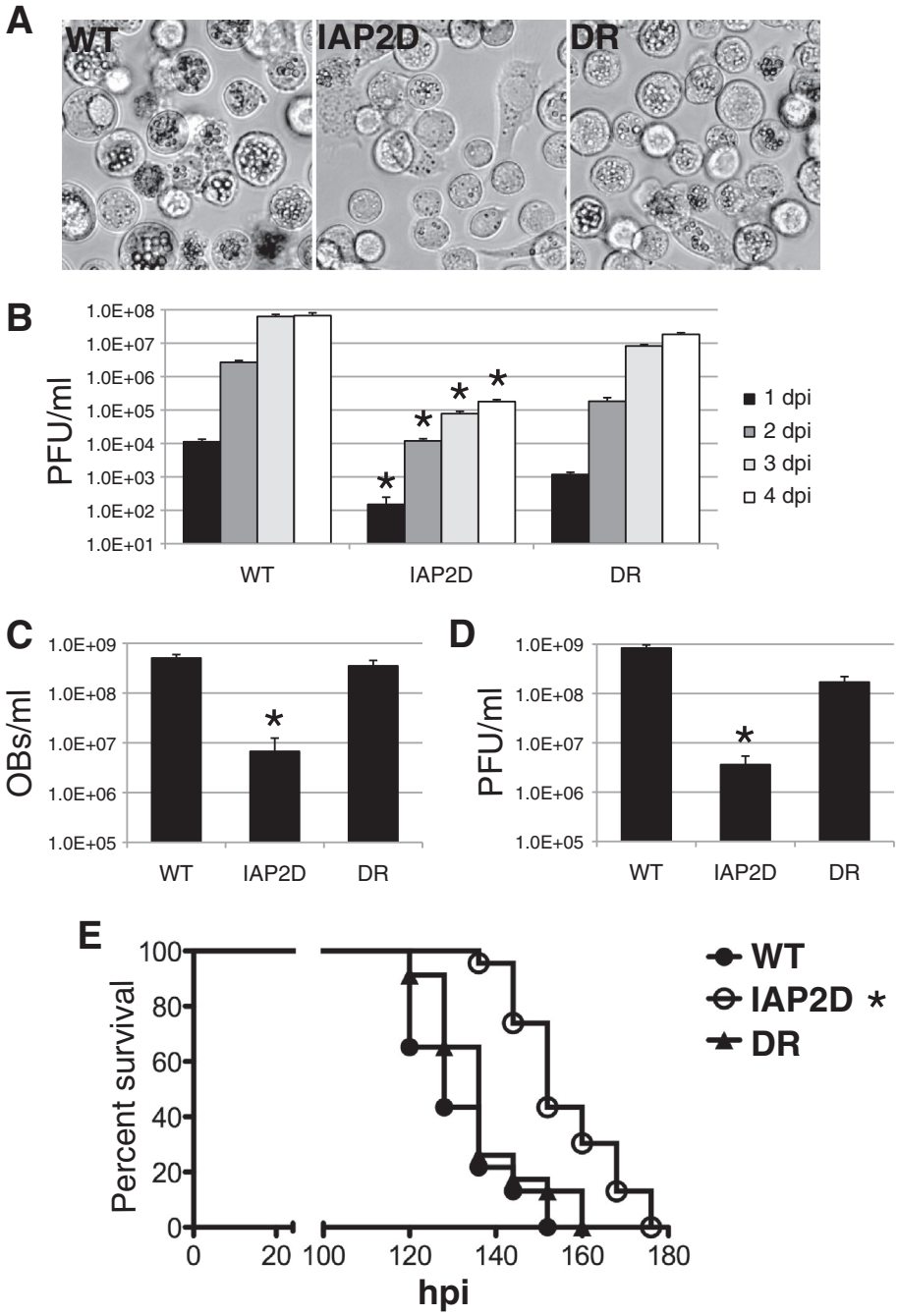

Fig. 2. Characterization of BmIAP2D in cultured cells and *B. mori* larvae. (A) Light microscopic observations of BmNPV-infected BmN cells at 4 dpi (MOI = 0.01). (B) BV production in cultured cells. BmN cells were infected with recombinant BmNPVs at an MOI of 0.01. BV titers at 1, 2, 3, and 4 dpi were determined by the plaque assay. Data are expressed as means ± SD ($n = 6$). $^*P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using WT as the comparator. (C) OB production in the hemolymph of BmNPV-infected larvae. Fifth instar *B. mori* larvae were injected with 1×10^3 PFU of BV. The hemolymph was collected at 4 dpi and the released OBs were counted using a hemocytometer. Data are expressed as means ± SD ($n = 5$). $^*P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using WT as the comparator. (D) BV production in the hemolymph of BmNPV-infected larvae. The hemolymph was collected at 4 dpi and the BV titer was determined by the plaque assay. Data are expressed as means ± SD ($n = 5$). $^*P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using WT as the comparator. (E) Survival curves. The LT_{50}s of WT, IAP2D, and DR using fifth instar larvae ($n = 20$) were 128 h, 152 h, and 136 h, respectively. $^*P < 0.05$ by the log-rank (Mantel–Cox) test with the Bonferroni correction using WT as the comparator.

to Western blot analysis using an anti-HA monoclonal antibody (Covance) as described previously [23].

2.6. Proteasome inhibition assay

BmN cells were infected with BmNPV at an MOI of 5. MG-132 (5 μM; proteasome inhibitor) or DMSO (control) was added at 1 dpi. Expression of BmNPV IAP2 and ubiquitinated proteins were examined at 2 dpi by Western blotting with anti-HA and anti-ubiquitin antibodies (Santa Cruz).

2.7. Statistical analysis

Statistical analyses were performed using the Prism 5 software (GraphPad). One-way analysis of variance (ANOVA) was performed using Tukey's post-hoc test to compare the mean of each treatment group with that of the control group. Survival curves were compared with the control using the log-rank (Mantel–Cox) test with Bonferroni correction.

3. Results and discussion

3.1. Effect of iap2 deletion on BmNPV propagation in cultured cells and B. mori larvae

We first generated a *polh*-positive *iap2*-disrupted virus, BmIAP2D (IAP2D), and a repair virus, BmIAP2DR (DR) (Fig. 1A). Western blotting showed that HA-tagged IAP2 protein was expressed in DR-infected BmN cells with a molecular mass of

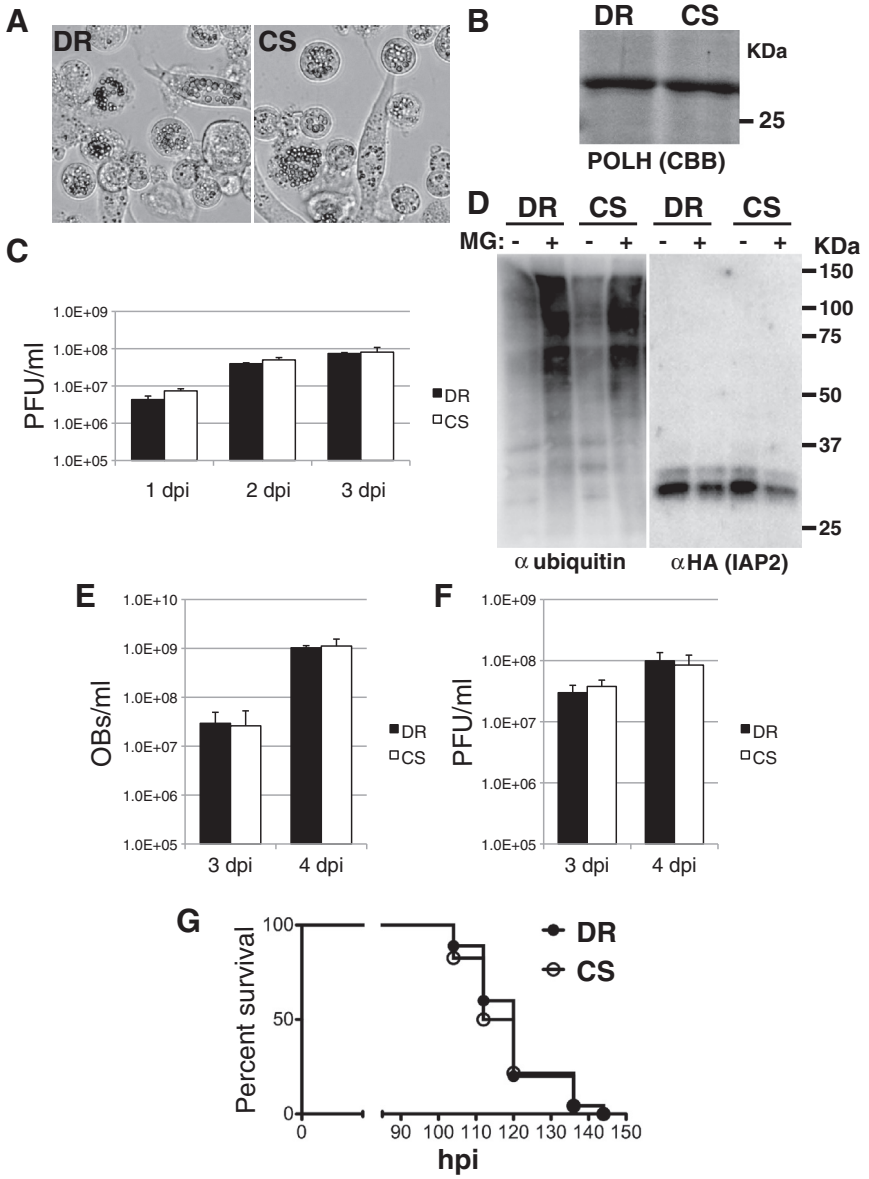

Fig. 3. Characterization of BmIAP2CS in cultured cells and *B. mori* larvae. (A) Light microscopic observations of BmNPV-infected BmN cells at 3 dpi (MOI = 5). (B) POLH expression. A gel stained with Coomassie brilliant blue (CBB) is shown. The molecular masses of protein standards are indicated to the right. (C) BV production in cultured cells. BmN cells were infected with recombinant BmNPVs at an MOI of 5. BV titers at 1, 2, and 3 dpi were determined by the plaque assay. Data are expressed as means ± SD (n = 3). (D) Effect of proteasome inhibition on IAP2 expression. BmN cells were infected with DR or CS at an MOI of 5. At 1 dpi, cells were treated with DMSO (−) or MG-132 (+). Cells were lysed at 2 dpi, and then immunoblotted with anti-ubiquitin and anti-HA antibodies. (E) OB production in the hemolymph of BmNPV-infected larvae. Fifth instar *B. mori* larvae were injected with 1×10^5 PFU of BV. The hemolymph was collected at 3 and 4 dpi and the released OBs were counted using a hemocytometer. Data are expressed as means ± SD (n = 5). (F) BV production in the hemolymph of BmNPV-infected larvae. The hemolymph was collected at 3 and 4 dpi and the BV titer was determined by the plaque assay. Data are expressed as means ± SD (n = 5). (G) Survival curves. The LT_{50}s of DR and CS using fifth instar larvae (n = 45) were 120 h and 116 h, respectively.

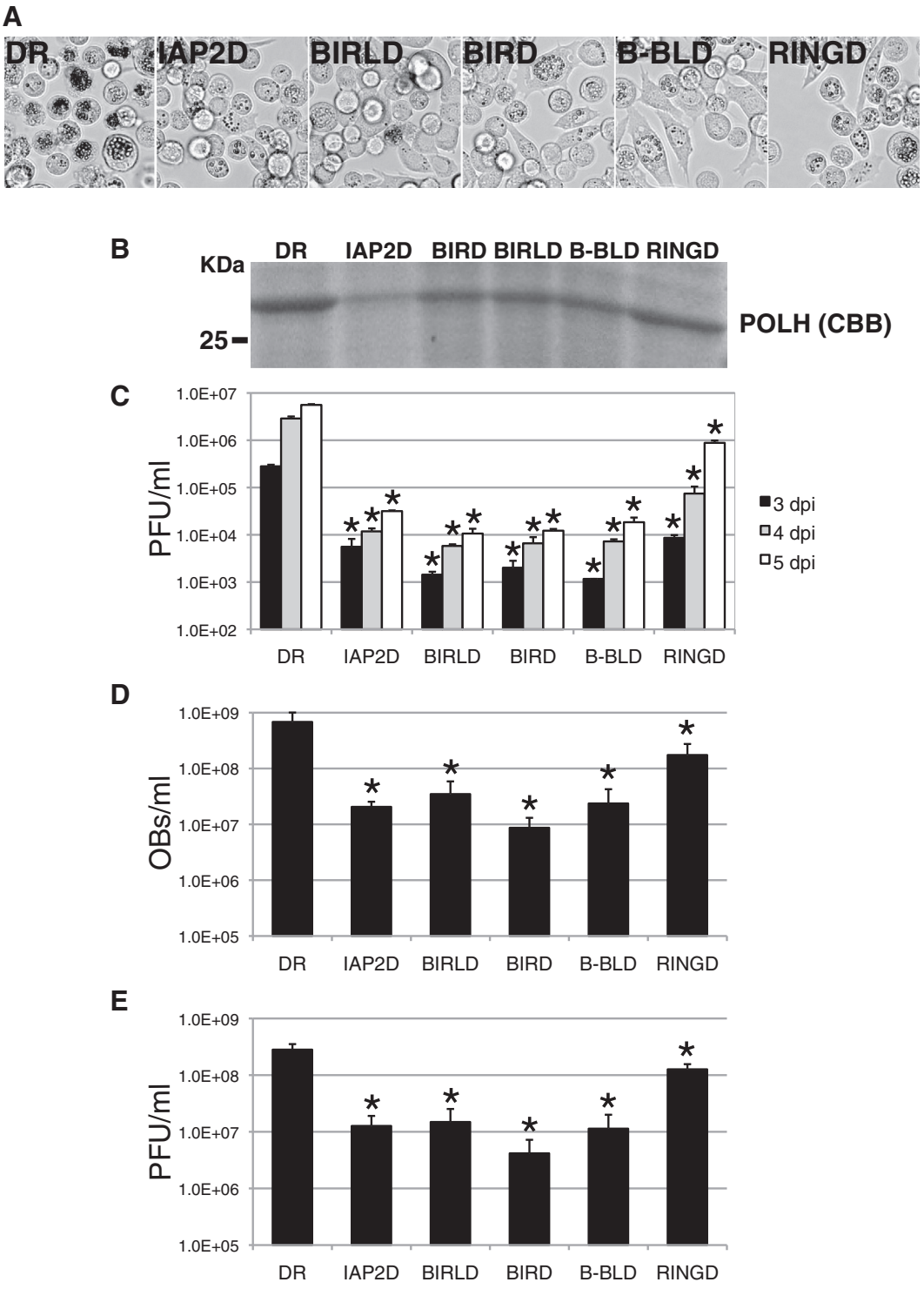

Fig. 4. Characterization of BmNPVs lacking one or two functional domains of IAP2 in cultured cells and *B. mori* larvae. (A) Light microscopic observations of BmNPV-infected BmN cells at 5 dpi (MOI = 0.001). (B) POLH expression. A gel stained with CBB is shown. The molecular masses of protein standards are indicated to the right. (C) BV production in cultured cells. BmN cells were infected with recombinant BmNPVs at an MOI of 0.001. BV titers at 3, 4 and 5 dpi were determined by the plaque assay. Data are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using DR as the comparator. (D) OB production in the hemolymph of BmNPV-infected larvae. Fifth instar *B. mori* larvae were injected with 200 PFU of BV. The hemolymph was collected at 5 dpi and the released OBs were counted using a hemocytometer. Data are expressed as means ± SD ($n = 5$). $^{*}P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using DR as the comparator. (E) BV production in the hemolymph of BmNPV-infected larvae. The hemolymph was collected at 5 dpi and the BV titer was determined by the plaque assay. Data are expressed as means ± SD ($n = 5$). $^{*}P < 0.05$ by one-way ANOVA and Dunnett's post-hoc tests using DR as the comparator.

approximately 30 kDa, whereas its expression was not detected in IAP2D-infected cells (Fig. 1B). Light microscopic observations revealed that IAP2D-infected cells did not exhibit a typical apoptotic phenotype (Fig. 2A), indicating that the IAP2 protein is not likely to be involved in the anti-apoptotic pathway during BmNPV infection. OB production in IAP2D-infected cells was strikingly low compared with that in WT- and DR-infected cells (Fig. 2A). SDS–PAGE analysis showed a marked reduction in POLH expression

(Fig. 4B), supporting this observation. IAP2D produced significantly fewer infectious BVs than WT or DR (Fig. 2B). These results indicate that deletion of BmNPV *iap2* severely reduces OB and BV production in BmN cells. In AcMNPV and *Helicoverpa armigera* NPV, deletion of *iap2* from the genome caused no phenotypic defects in cultured cells [12,24]. These results are inconsistent with the present results using BmNPV. It is intriguing that the roles of *iap2* during viral infection are different in the two closely related NPVs, BmNPV and AcMNPV.

We next examined the effects of *iap2* deletion on BmNPV propagation in *B. mori* larvae. As shown in Fig. 2C and D, IAP2D produced fewer OBs and infectious BVs compared with WT and DR. In addition, survival curves demonstrated that the LT_{50} of IAP2D was 24 h and 16 h longer than that of WT and DR, respectively (Fig. 2E). These results demonstrate that IAP2 is crucial for efficient BmNPV growth and that it enhances viral pathogenicity in *B. mori* larvae.

3.2. Effects of loss of IAP2 ubiquitin ligase activity on BmNPV propagation in cultured cells and B. mori larvae

Imai et al. showed that BmNPV IAP2 possesses ubiquitin ligase activity, and that a single amino acid substitution in the RING finger domain (cysteine to serine at residue 217) completely abolishes this activity [17]. To assess the importance of the ubiquitin ligase activity of BmNPV IAP2 during virus propagation, we generated a BmNPV mutant [BmIAP2CS (CS)] expressing ubiquitin ligase activity-deficient IAP2 (Fig. 1A). Light microscopy (Fig. 3A) and SDS–PAGE (Fig. 3B) results showed that OB production and POLH expression in CS-infected BmN cells were comparable to those in DR-infected cells at 3 dpi. BV titer in CS-infected BmN cells at 1, 2, and 3 dpi was also similar to that in DR-infected cells (Fig. 3C). These results indicate that loss of the ubiquitin ligase activity of BmNPV IAP2 did not affect viral growth in cultured cells.

We previously reported that IE2, one of the BmNPV ubiquitin ligases, is actively degraded through the ubiquitin–proteasome pathway. This process depends mainly on its own E3 activity [17,25,26]. To investigate whether the ubiquitin ligase activity of BmNPV IAP2 is involved in its own degradation, we examined IAP2 expression in the presence or absence of the proteasome inhibitor MG-132. Ubiquitinated proteins, especially the high-molecular-mass proteins, accumulated both in DR- and CS-infected cells when MG-132 was added (Fig. 3D). Expression of IAP2 in CS-infected cells was similar to that in DR-infected cells with or without MG-132 treatment (Fig. 3D). These results suggest that the ubiquitin ligase activity of BmNPV IAP2 is not required for its own degradation. Further studies are needed to identify the substrates of BmNPV IAP2.

We next assessed the virulence of CS in *B. mori* larvae. OB and BV production in CS-infected *B. mori* larvae were comparable to those produced in DR-infected larvae (Fig. 3E and F). In addition, the LT_{50}s of DR and CS, which were 120 h and 116 h, respectively, were not statistically different. These results indicate that the IAP2 ubiquitin ligase activity is not important for BmNPV propagation in *B. mori* larvae.

3.3. Effects of deletion of putative IAP2 functional domains on BmNPV propagation in cultured cells and B. mori larvae

BmNPV IAP2 has three putative functional domains: a BIRL domain, a BIR domain, and a RING finger domain (Fig. 1A). To understand the role of each domain in BmNPV propagation, we generated four bacmids expressing *iap2* derivatives lacking one or two domains. Light microscopic observation (Fig. 4A) and SDS–PAGE experiments (Fig. 4B) showed that OB production and POLH expression in BmN cells infected with mutant viruses were significantly reduced compared to those in DR-infected cells. The degree of OB production decreased in the following order: (1) DR, a virus expressing an intact IAP2; (2) RINGD, a virus expressing IAP2 protein lacking the RING finger domain; (3) BIRD, BIRLD, and B-BLD, viruses expressing IAP2 proteins lacking BIR, BIRL, or both; and (4) IAP2D, a virus that does not express the IAP2 protein (Fig. 1A). BV production in mutant virus-infected cells was also statistically lower than that in DR-infected cells (Fig. 4C). These results indicate that the BIR and BIRL domains are required for full activity for IAP2 during BmNPV infection. Deletion of the RING domain of IAP2 had a slight effect on viral growth in BmN cells, which is consistent with the result that loss of the ubiquitin ligase activity of IAP2 did not cause any phenotypic defects (Fig. 3).

We next performed larval assays using the IAP2 domain mutants. We observed a severe reduction in OB and BV production in larvae infected with BIRD, BIRLD, and B-BLD, especially with BIRD (Fig. 4D and E). Larvae infected with RINGD also exhibited lower OB and BV production, but the extent was smaller compared to that in other domain mutants (Fig. 4D and E), supporting the result that ubiquitin ligase activity is not crucial for BmNPV propagation (Fig. 3). Taken together, either the BIR or BIRL domain is important for BmNPV IAP2 functions in cultured cells and *B. mori* larvae.

4. Conclusion

Functional analyses of baculoviral IAP proteins have been primarily performed using transient expression assays. Accumulating data suggest that baculoviral IAP2 proteins have no anti-apoptotic activity, except for *Epiphyas postvittana* NPV IAP2, whose expression in *S. frugiperda* Sf-21 cells prevented apoptosis induced by various reagents [12]. In addition, till date, the role of the IAP2 protein in baculovirus infection remains elusive. In this study, we generated and characterized BmNPV *iap2* mutants using a bacmid-based strategy, and showed that the BIR and BIRL domains of IAP2 are both important for BmNPV propagation. To the best of our knowledge, this is the first study to provide experimental evidence of the role of the baculovirus IAP2 functional domains. We are now addressing the question of how the BIR or BIRL domain of IAP2 interacts with host and viral proteins to accomplish its function during BmNPV infection.

Acknowledgments

We thank Genki Ishihara and Ryuhei Kokusho for technical assistance, and Munetaka Kawamoto for clerical assistance. This work was supported by the JSPS KAKENHI Grant to S.K (Grant numbers 19688004, 22380033, and 25292196).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.132.

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