The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 469–477
DOI 10.1007/s12275-012-2010-z

Identification and Characterization of Host Factors Interacting with *Bombyx mori* Nucleopolyhedrovirus ORF8[§]

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(Received January 6, 2012 / Accepted March 5, 2012)

The *orf8* gene (*Bm8*) in *Bombyx mori* nucleopolyhedrovirus (BmNPV) is one of 17 genes unique to group I NPVs and is expressed as an early gene. We have reported that *Bm8* may play an important role during viral infection and that Bm8 protein co-localized with IE1 to specific nuclear foci throughout infection. It was also demonstrated that both IE1 and BmNPV *hr* facilitate this localization of Bm8. To investigate further, host proteins interacting with Bm8 were screened using a yeast two-hybrid system. We identified 6 host clones as Bm8-interacting partners from three cDNA libraries derived from BmN cells or *B. mori* larvae. Further assays showed that the N-terminal region of Bm8 is important for the interaction with most host clones and that two of the clones can associate with IE1. Cloning and sequencing of full-length cDNAs revealed that most of the clones potentially encode either membrane-bound proteins or secreted proteins. Quantitative RT-PCR analysis revealed that some of these host genes were slightly induced during the early stage of infection in BmN cells, and that the expression of all genes was markedly reduced during the late stage of infection. Generation of mutant BmNPVs over-expressing these host genes also identified a gene that potentially functions as a negative factor during BmNPV infection. These features of Bm8-interacting host proteins strongly support that Bm8 is a multifunctional protein involved in multiple signaling pathways in host cells.

Keywords: BmNPV, Bm8, BV/ODV-E26, Ac16, baculovirus

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[§]Supplemental material for this article may be found at http://www.springer.com/content/120956

Introduction

Baculoviridae is a large family of viruses that infect insects of the Lepidopteran, Dipteran, and Hymenopteran orders. Baculoviruses contain circular double-stranded DNA genomes of 80 to 180 kb that are contained within an enveloped, rod-shaped virion. The viruses are divided phylogenetically into four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* (Jehle *et al*., 2006). Alphabaculoviruses and betabaculoviruses infect lepidopteran larvae whereas gammabaculoviruses and deltabaculoviruses infect larvae from Hymenoptera and Diptera, respectively. Alphabaculovirus can be further subdivided into groups I and II based on phylogenetic studies (Herniou *et al*., 2001). The *Bombyx mori* NPV (BmNPV) and *Autographa californica* multiple NPV (AcMNPV) are group I alphabaculoviruses.

In this study, we focused on host proteins interacting with Bm8 of BmNPV. The *Bm8* gene and its homologues are found only in group I alphabaculovirus genomes (Herniou *et al*., 2003). Our laboratory has demonstrated that the Bm8 protein, which is expressed as an early gene, may play an important role during viral infection and co-localizes with IE1 to specific nuclear sites throughout the infection (Imai *et al*., 2004). Furthermore, we showed that IE1 and *hr* facilitate the localization of Bm8 to specific nuclear sites where viral DNA replication occurs (Kang *et al*., 2005). These data suggest that Bm8 may be involved in viral DNA replication and/or transcription. The *Ac16* gene, a *Bm8* homologue of AcMNPV, is also expressed during the early stage of infection (O'Reilly *et al*., 1990). Ac16 is an envelope protein (BV/ODV-E26) of both budded virus (BV) and occlusion-derived virus (ODV) (Beniya *et al*., 1998). In addition to its role as a structural protein, interaction with AcMNPV IE0 and IE1 suggested that Ac16 might play a role in viral transcription or replication (Nie *et al*., 2009). Recently, Nie and Theilmann (2010) showed that Ac16 and Ac17 are required for rapid viral gene expression in AcMNPV-infected cultured cells.

It has been shown that Bm8/Ac16 interacts with baculoviral and cellular proteins and that these interactions with other proteins are important for their functions. As described above, Bm8/Ac16 interacts with IE1 and/or IE0. Using a yeast two-hybrid assay, it was demonstrated that the N-terminal region containing the coiled-coil domain of Bm8 is required for IE1 interaction. Furthermore, this region is sufficient for the localization to specific nuclear sites in the presence of IE1 and *hr3*, suggesting that the N-terminal region containing the coiled-coil domain is important for Bm8 function (Kang *et al*., 2005). Consistent with this, the Ac16 binding domain of IE0 or IE1 also contains a coiled-

coil domain, suggesting that coiled-coil domains facilitate the interaction between Bm8/Ac16 and IE0 or IE1 (Nie *et al.*, 2009). In addition to IE0/IE1, Ac16 also forms a complex with viral FP25K and cellular actin and associates with membranes via palmitoylation (Beniya *et al.*, 1998; Burks *et al.*, 2007). It has also been suggested that Ac16 is involved in the trafficking of ODV-E66 as well as FP25K (Braunagel *et al.*, 2004, 2009). These accumulating data suggest that this gene family encodes a multifunctional protein that accomplishes specific needs of group I alphabaculoviruses. To further identify cellular proteins interacting with Bm8, we used a yeast two-hybrid system. This study describes the identification and characterization of host factors interacting with Bm8.

Materials and Methods

Virus, cell line, infection, and larval assay

The BmN-4 (BmN) cell line was maintained in TC-100 with 10% fetal bovine serum as described previously (Maeda, 1989a, 1989b). The BmNPV and recombinant viruses were propagated on BmN cells as described (Maeda, 1995). Mabb-derivatives expressing Bm8-interacting host clones under the control of the *polyhedrin* promoter were constructed by co-transfection of plasmids described below (pBm31-derivatives) with *Bsu*36 I-digested *Bm*NPV-abb (Mabb) genomic DNA (Ko *et al.*, 2000) into BmN cells, using Lipofectin (GibcoBRL), and isolated by identification of plaque formation. Insertion of each host factor into the *polyhedrin* locus was confirmed by PCR using primers (pEPS1: 5′-CAATCCTATTTGTAA-3′ and poly21: 5′-ACGCACAGAATCTAACGCT-3′) and DNA sequencing. Two *Bm8*-disrupted mutants, Mabb/8D and Mabb-2-11/8D, were generated by *LacZ* cassette insertion as described previously (Imai *et al.*, 2004). For viral growth curves, BmN cells were infected with either BmNPV-Mabb or recombinant BmNPVs carrying each of the Bm8-interacting host factors at a multiplicity of infection (MOI) of 5. BV production was determined by plaque assay. The median lethal time (LT_{50}; time in hours required to kill half of the population) was determined by hemocoelic injection of BV (50 μl of a viral suspension containing 1×10^5 plaque forming units). *B. mori* larvae were injected with BV within 8 h of molting to the fifth instar. Statistical analyses were performed with Prism 5 (Graphpad).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the ProQuest™ two-hybrid system (Gibco BRL) as described (Kang *et al.*, 2003) using pDB-orf8 (Kang *et al.*, 2005). In addition to the BmN cell cDNA libraries described (Kang *et al.*, 2003), a cDNA library constructed using mRNA purified from the epidermis of BmNPV-infected larvae [2 days post infection (d p.i.)] was also used. Primary transformants (5 to 7×10^5) were selected for growth on histidine dropout plates containing 25 mM 3-aminotriazole (AT). His^+ colonies were subsequently analyzed for β-galactosidase activity by filter-lift experiments and checked for growth on uracil dropout plates. Plasmids from positive clones were rescued by transformation of *Escherichia coli* with total yeast DNA. Each plasmid DNA was amplified in *E. coli* and retransformed into yeast together with pDB-orf8. Transformants were checked for growth on histidine dropout plates containing 50, 75, and 100 mM 3-AT and assayed for β-galactosidase activity in order to confirm the interaction.

The interaction between Bm8-interacting host factors and IE1 was investigated using the yeast two-hybrid system. A pDBLeu plasmid containing each host clone described below and a pPC86 plasmid containing the *ie1* gene (Kang *et al.*, 2005) were transformed into yeast and examined. To reconfirm the interaction between Bm8 and host clones, one of these pDBLeu derived plasmids containing host clones, and a pPC86 plasmid containing the *Bm8* gene, were transformed into yeast and the reporter expression was checked.

Construction of plasmids

To investigate interactions with IE1 and confirm the interaction in a reciprocal manner, insert-DNA fragments of host clones from the pPC86 vector were re-inserted into pDBLeu using the *Sal*I and *Not*I restriction sites.

For protein expression in bacteria, insert-DNA fragments of host clones in the pPC86 vector were fused with glutathione S-transferase (GST) in the pGEX-6P-2 vector (Amersham biosciences) using the *Sal*I and *Not*I restriction sites (called pGEX-2-11, -2d2, and 2d4, respectively). For a host clone 2d-4, *Pst*I digestion and self-ligation were performed to eliminate 3′-untranslated region and then the *Sal*I/*Not*I fragment was inserted into the pGEX vector.

To construct transfer vectors for the expression of host clones, DNA fragments were inserted into the transfer vector pBm31, in which individual genes were expressed from the *polyhedrin* promoter (Ko *et al.*, 2000). For clones 2-11 and 2d-4, the DNA fragments were amplified using the full-length cDNAs fcaL27p08f or fcaL-P15_F_N13 with the primers pFull (5′-AGCTGCTCGAGTGTAA-3′) and p11-RV1a (5′-AGTTTCTTACATAGCGCA-3′) or p2d4-R1 (5′-TATTTAGTTTATTAGCACCA-3′). For clone 2d-2, the DNA fragment was amplified using the full-length cDNA fner-33c19f and primers p2d2-EcoATG (5′-GGGAATTCGATAATGAACTCAAA-3′) and p2d2-R1 (5′-GAATTTAATCAGAGAT-3′). The amplified fragments were then digested with *Eco*RI and inserted into the *Eco*RI and *Eco*RV restriction sites of pBm31 (called pBm31-2-11full, pBm31-2d4full, and pBm31-2d2full, respectively).

Full-length clones and DNA sequencing

To obtain the full-length host clone cDNAs, we screened *B. mori* cDNA libraries (unpublished data, partially available at http://www.ab.a.u-tokyo.ac.jp/Bombyx_EST/) and genome sequences (Mita *et al.*, 2004; Xia *et al.*, 2004, 2009) using the BLAST program. The full-length cDNAs for m-9 (fe100PO4_F_F17), 2-11 (fcaL27p08f), 2d-2 (fner33c19f), and 2d-4 (fcaL-P15_F_N13) were obtained.

The nucleotide sequences of clones obtained from the yeast two-hybrid screening and the full-length cDNA clones were completed by primer walking using an ABI sequencer. Sequence assembly and analysis were performed using GENETYX-MAC and 4Peaks (version 1.7.2). A domain

Table 1. List of host clones interacting with Bm8 obtained from yeast 2-hybrid screening

Clone	Library	Full length cDNA	Length of cDNA (nt)	CDS aa (MW, kDa)	Accession number
m-9	BmN (Mock)	+	3,698	1,062 aa (111)	AB559580
2-5	BmN (2 h p.i.)	-	1,477	440 aa (49.1)[a]	AB559578
2-7	BmN (2 h p.i.)	-	3,481b	1,069 aa (118.9)[a]	AB046365[b]
2-11	BmN (2 h p.i.)	+	4,464	1,410 aa (169.3)	AB559579
2d-2	Larvae (2 d p.i.)	+	1,861	193 aa (22.9)	AB559581
2d-4	Larvae (2 d p.i.)	+	1,549	452 aa (48.3)	AB559582

[a] partial clone which does not cover intact ORF, [b] not this study (Kang *et al.*, 2003)

search within the ORFs was performed using SMART MODE (http://smart.embl-heidelberg.de/).

The nucleotide sequence data presented in this paper have been submitted to the GenBank database and assigned the accession numbers shown in Table 1.

RT-PCR analysis

For quantitative RT-PCR (qRT-PCR) analyses, cDNAs used in Fig. 4 were prepared by Katsuma and Shimada (2009). The primers for PCR are shown in Supplementary data Table S1 and qRT-PCR experiments were performed as described previously (Katsuma *et al.*, 2008; Katsuma and Shimada, 2009). Briefly, BmN cells were infected with BmNPVs at an MOI of 5. Following incubation for 1 h, the virus-containing culture medium was removed and fresh medium was added after washing it two times with serum free TC-100 medium (0 hpi). Infected cells were harvested at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hpi. Total RNA was reverse-transcribed, diluted, and used for qRT-PCR experiments as described elsewhere (Katsuma *et al.*, 2008). mRNA expression of 2d-2, 2d-4, 2-11, and *vp39* in BmN cells infected with Mabb derivatives (Fig. 5A) were also examined by qRT-PCR using the primers listed in Supplementary data Table S1.

Antibody production and western blotting

Recombinant proteins of pGEX-clones containing 2-11, 2d-2, or 2d-4 were expressed and purified as previously described (Kang *et al.*, 2003). The purified GST-fused proteins were used to raise polyclonal antibodies in rabbits after cleavage of the GST tag using PreScission protease (Amersham biosciences). SDS-PAGE was performed in 10 to 15% polyacrylamide gels with Precision Plus protein standards (Bio-Rad) as size markers. The Western blotting protocol was described previously (Kang *et al.*, 2003). Subcellular fractionation was performed as described (Imai *et al.*, 2004).

Results and Discussion

Screening and identification of host proteins interacting with Bm8

We have previously reported BmNPV IE1 as a Bm8-interacting protein (Kang *et al.*, 2005). In order to further identify Bm8-interacting factor(s), we used pDB-orf8, in which the complete Bm8 gene was fused to the DNA-binding domain of GAL4 in pDBLeu, as bait and screened for interacting proteins from three cDNA libraries constructed using mRNAs purified from mock-infected BmN cells, BmNPV-infected BmN cells at 2 h p.i., and the epidermis of BmNPV-infected larvae at 2 d p.i.. We identified one, three, and two positive clones from the respective libraries. All of the clones had moderately strong interactions, as defined by comparing the growth on histidine dropout plates containing 3-AT, and the β-galactosidase activity, with that of control strains provided by the manufacturer.

To confirm the interaction between Bm8 and the identified host clones, pDB-orf8 was co-transformed with each of the six positive clones into yeast and assayed for reporter expression. The expression of β-galactosidase was then reproduced, confirming the interaction between intact Bm8 and all of the positive clones (Fig. 1). We next defined the region in Bm8 responsible for the interactions. Each of the six positive clones was co-transformed with a series of deletion mutants containing amino acids (aa) 1-110, 1-78, 110-230, or 73-230 (referred to as pDB-orf8dC1, -orf8dC2, -orf8dN1, and -orf8dN2 respectively) (Kang *et al.*, 2005). The C-terminal deletion mutant, orf8dC1, containing residues 1-110 activated reporter expression with clones m-9, 2-7, and 2d-4, suggesting that the N-terminal region of Bm8, which contains a coiled-coil domain, mediates interaction with these three clones. In addition, the N-terminal deletion mutant, orf8dN2, comprising residues 73-230 also activated reporter expression with clone 2d-2, indicating that the

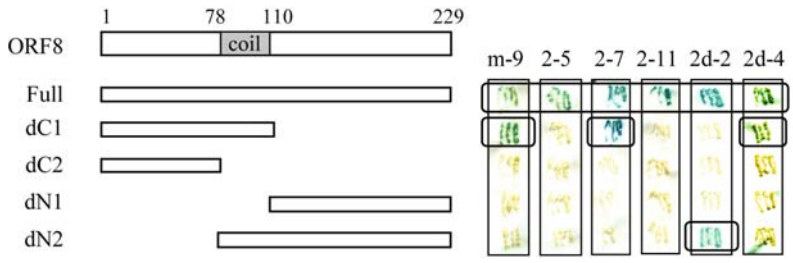

Fig. 1. Analysis of the interaction between Bm8 and host proteins using the yeast two-hybrid system. On the left are diagrams showing the structure of Bm8 and the portion of it encoded by each plasmid. The open box on the top indicates Bm8 with the predicted coiled-coil domain (residues 78–110) shown by a closed box. The open boxes below indicate the regions contained in the deletion mutants. On the right, the results of the β-galactosidase assay are shown. The highlighted results indicate positive Bm8 interactions.

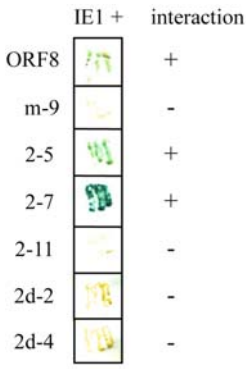

Fig. 2. Analysis of the interaction between IE1 and Bm8 interacting host factors. The pPC86 plasmid containing the *ie1* gene (Kang *et al.*, 2005) was co-ransformed into yeast with a pDBLeu plasmid containing the *Bm8* gene as a control or one of the host clones. The boxes in the middle show the results of the β-galactosidase assay. The symbols + or - indicate positive or negative results for interaction with IE1, respectively.

coiled-coil domain and the C-terminal end are responsible for the interaction with 2d-2. The entire region of Bm8 seems to be essential for interaction with clones 2-5 and 2-11, since deletions resulted in a complete loss of interaction (Fig. 1).

We have previously shown that BmNPV IE1 also interacts with Bm8 and that this interaction is important for the nuclear localization of Bm8 (Kang *et al.*, 2005). Therefore, we next examined whether Bm8-interacting host factor(s) also interact with IE1 using the yeast 2-hybrid system. Of the 6 positive clones, we found that clones 2-5 and 2-7 also interacted with IE1 (Fig. 2), suggesting that these two host proteins may be involved in Bm8 function through association with IE1.

The gene structures of Bm8-interacting host clones

Following confirmation of the interaction between Bm8 and the clones identified in the yeast two-hybrid screen, we next determined the nucleotide sequences of the positive clones. A homology search using amino acid sequences deduced from the nucleotide sequences suggested that the six clones might not include intact ORFs and the initiation codon seemed to be absent. In addition, clone 2-7 was found to be identical to the previously reported bLaminin; the insert size of clone 2-7, however, was 600 nt shorter than that of bLaminin (data not shown). The cloned bLaminin was also shown to encode a partial ORF (Kang *et al.*, 2003). In order to find clones containing an intact ORF, we screened *B. mori* cDNA libraries and found full-length cDNAs for m-9 (fe100PO4_F_F17), 2-11 (fcaL27p08f), 2d-2 (fner33c19f), and 2d-4 (fcaL-P15_F_N13). We were unable to find full-length cDNAs for either 2-5 or 2-7, which was consistent with predictions suggesting that the 2-5 and 2-7 genes likely encode very large proteins. Sequence and domain analyses were performed using sequence data derived from the yeast two-hybrid screen for clone 2-5 and the full-length cDNA clones of m-9, 2-11, 2d-2, and 2d-4 (Fig. 3 and Table 1).

Sequence analysis of m-9 revealed that this clone contained a 3,698-nt-long mRNA. The deduced amino acid sequence showed that the putative protein was composed of 1,062 amino acid residues with a molecular mass of 111 kDa. The protein encoded by m-9 contains a signal peptide sequence, an MNNL motif (represents a region of conserved sequence at the N terminus of several Notch ligand proteins) at its N-terminus, followed by a DSL motif (Delta Serrate Lag-2), multiple EGF/EGF_CA (epidermal growth factor-like domain/calcium-binding EGF-like domain) motif repeats, and a potential transmembrane domain at the C-terminus. These motifs are notable features of Notch ligand proteins. In addition, the amino acid sequence of the m-9-encoded protein showed high similarity to that of jagged-1 or serrate protein of insects that are ligands of Notch (Thomas *et al.*, 1991; data not shown). This suggests that the m-9 clone may encode a protein related to or involved in the Notch signaling pathway. The Notch pathway is a highly conserved and ubiquitous signaling system that functions in determining a diverse array of cell fates and regulates many cellular processes. Notch signaling is in-

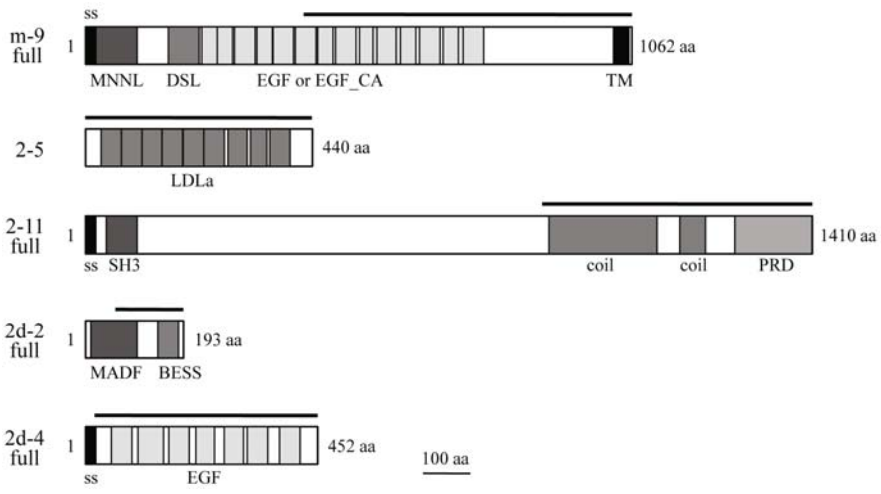

Fig. 3. Protein domain structures of Bm8-nteracting host clones. The full-length cDNAs of clones m-9, 2-11, 2d-2, and 2d-4 are 3698, 4464, 1861, and 1549 nt long mRNAs containing ORFs encoding 1062, 1410, 193, 452 amino acid residues, respectively. The cDNA of clone 2-5 obtained from the yeast two-hybrid screen is a 1477 nt-long mRNA containing a partial ORF of 440 amino acid residues. The solid bar above each ORF shows the region covered by clones obtained from the yeast two-hybrid screen. Domains detected by SMART MODE are indicated: ss, signal sequence; MNNL, a region of conserved sequence at the N terminus of several Notch ligand proteins; DSL, delta serrate ligand; EGF, Epidermal growth factor-like domain; EGF_CA, Calcium-binding EGF-like domain; TM, transmembrane; LDLa, Low-density lipoprotein receptor domain class A; SH3, Src homology 3 domain; coil, coiled-coil domain; PRD, proline-rich domain; MADF, subfamily of SANT domain (SWI3, ADA2, N-CoR and TFIIIB DNA-binding domains); BESS, named after the three proteins that originally defined the domain: BEAF (Boundary element associated factor 32), Suvar(3)7 and Stonewall. The scale bar indicates 100 amino acid residues.

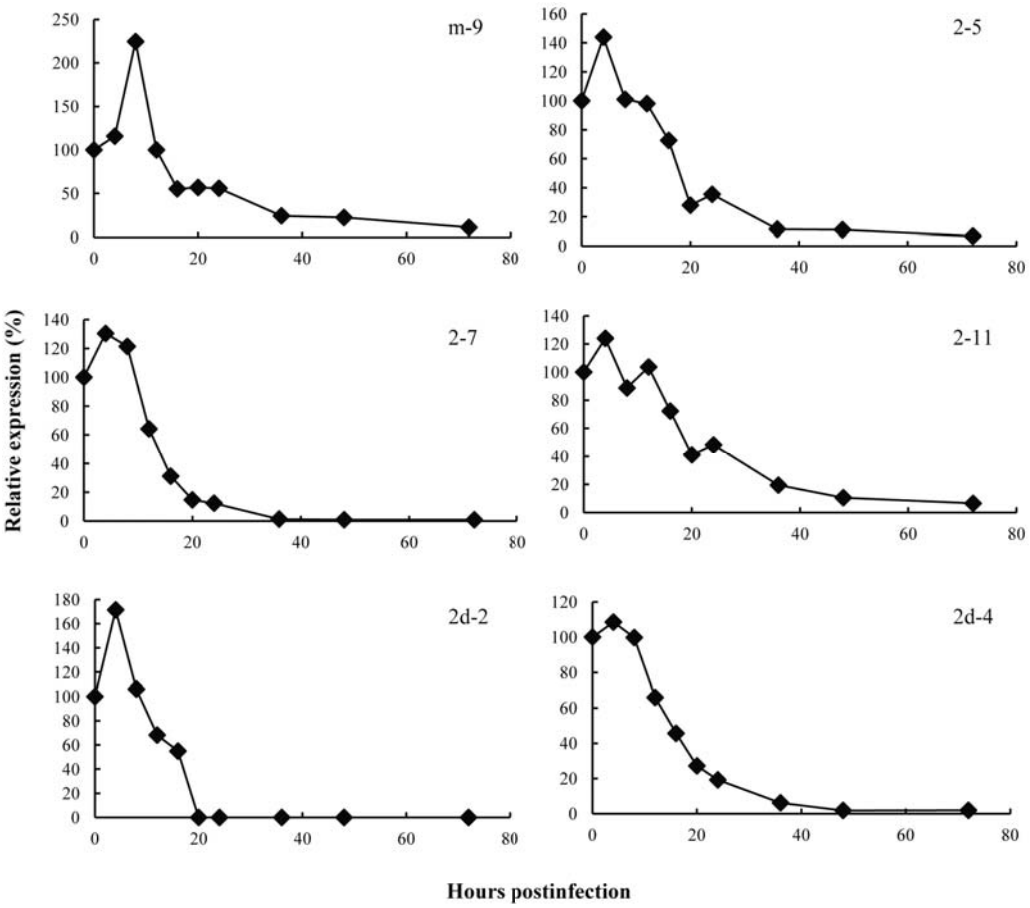

Fig. 4. Expression profiles of Bm8-interacting host clones in BmNPV-infected BmN cells. Total RNA was prepared from BmNPV T3-infected BmN cells at 0, 4, 8, 12, 16, 24, 36, 48, and 72 h p.i., and subjected to qRT-PCR. PCR primers used in this experiment are indicated in Supplementary data Table S1. Similar results were obtained in two independent experiments.

duced through direct cell-cell interactions that promote receptor activation following engagement with a membrane-bound Delta, Serrate, or Lag-2 ligand on adjacent cells. Endocytosis is critical for directing and regulating Notch activation (Nichols *et al*., 2007).

Sequence analysis of clone 2-5 identified a 1,477 nt-long mRNA containing a partial ORF of 440 amino acid residues. This partial protein contains many LDLa domains (low-density lipoprotein receptor domain class A) (Fig. 3) and showed high similarity to the low-density lipoprotein receptor of insects including *Aedes aegypti*, *Tribolium castaneum*, *Drosophila melanogaster*, and *Apis mellifera*. The LDL receptor is a prototypical endocytosis receptor that mediates the uptake of extracellular ligands to regulate cholesterol homeostasis. Other members of the LDL receptor gene family function as signaling receptors in a wide variety of cellular processes (May *et al*., 2007). The presence of LDLa domains suggests that clone 2-5 may encode a protein from the LDL receptor gene family.

The full-length cDNA of clone 2-11 was shown to contain a 4,464-nt-long mRNA. The deduced amino acid sequence showed that the putative protein was composed of 1,410 amino acid residues with a molecular mass of 160 kDa. The potential protein has a signal peptide sequence, followed by a SH3 (Src homology 3 domains) domain, coiled-coil domains and a proline-rich region. These features resemble those of the *Drosophila* TANGO1 protein (Saito *et al*., 2009). One difference is the presence/absence of a transmembrane domain. The transmembrane is present next to the coiled-coil domain in TANGO1 but is absent in clone 2-11. A BLAST search also showed that the 2-11 protein showed high similarity to TANGO1 homologs from other insects including *D. melanogaster*, *T. castaneum*, *A. mellifera*, and *A. aegypti*. Saito *et al*. (2009) showed that TANGO1 functions as a guide for loading the cargo molecule collagen VII into COPII carriers and suggested that TANGO1 exports other secretory cargo that might be involved in cell-cell attachment. Similarity to TANGO1 and well-conserved motifs suggest that the 2-11 protein may be involved in endocytosis of some secretory proteins.

Sequence analysis of clone 2d-2 identified a 1,861-nt-long mRNA containing an ORF composed of 193 amino acid residues and an extensive 3′-untranslated region (1,215-nt). The protein encoded by clone 2d-2 contains MADF and BESS domains that are often associated with each other. The MADF (myb/SANT-like domain in alcohol dehydrogenase transcription factor-1) domain directs sequence specific DNA binding. The BESS domain is often found in *Drosophila* transcription factors and directs a variety of protein-protein interactions including interactions with itself. These features suggest that the protein encoded by clone 2d-2 may play a role as a transcription factor.

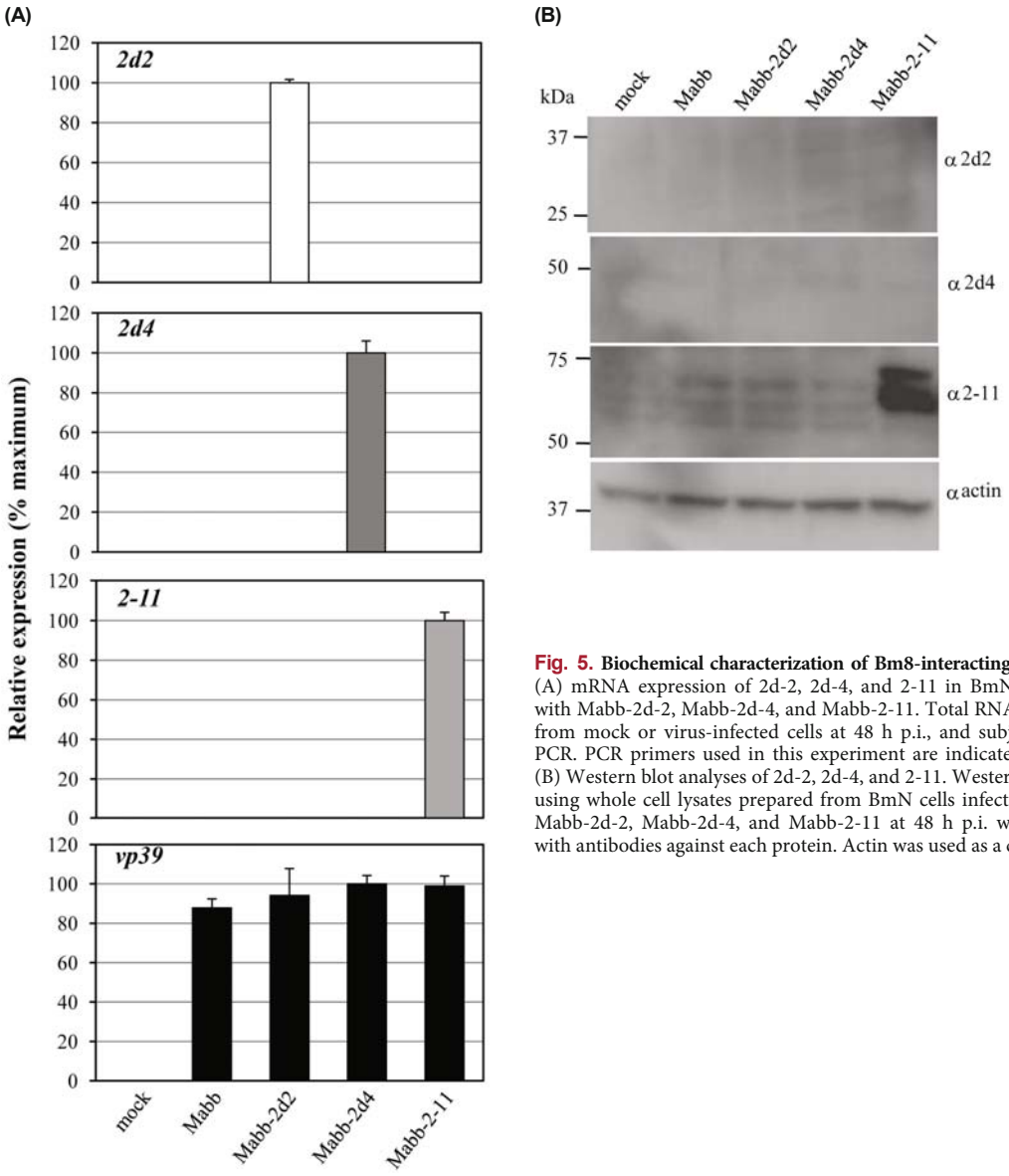

Fig. 5. Biochemical characterization of Bm8-interacting host proteins. (A) mRNA expression of 2d-2, 2d-4, and 2-11 in BmN cells infected with Mabb-2d-2, Mabb-2d-4, and Mabb-2-11. Total RNA was prepared from mock or virus-infected cells at 48 h p.i., and subjected to qRT-PCR. PCR primers used in this experiment are indicated in Table S1. (B) Western blot analyses of 2d-2, 2d-4, and 2-11. Western blot analyses using whole cell lysates prepared from BmN cells infected with Mabb, Mabb-2d-2, Mabb-2d-4, and Mabb-2-11 at 48 h p.i. were performed with antibodies against each protein. Actin was used as a control.

The full-length cDNA of clone 2d-4 contains a 1,459-nt-long mRNA encoding a putative protein composed of 452 amino acid residues with a molecular mass of 48 kDa. The potential protein has a signal peptide sequence and seven EGF domains. The molecular mass and domain structures (signal peptide and numbers of EGF domains) are conserved only in insects (eg. *Anopheles gambiae*, *A. aegypti*, *Culex quinquefasciatus*, *T. castaneum*, and *D. melanogaster*), suggesting that these proteins may have evolved in insects. Their function, however, remains undefined.

Transcriptional analysis of Bm8-interacting host clones

The expression of Bm8-interacting host clones in BmN cells during BmNPV infection was investigated by qRT-PCR analysis. The expression profiles of the Bm8-interacting host clones were similar (Fig. 4). In all cases, transcription was slightly up-regulated following infection, then decreased gradually, and finally vanished. The extent of the up-regulation and the timing of maximum expression, however, differ in each Bm8-interacting host clone.

Biochemical characterization of Bm8-interacting host proteins

To examine biochemical properties of Bm8-interacting host proteins, we raised polyclonal antibodies against bacterially expressed 2d-2, 2d-4, and 2-11 proteins. However, by Western blot analysis using these antibodies, we were not able to detect endogenous expression of the three proteins in BmN cells (data not shown). This suggested that these proteins are likely expressed in BmN cells at very low levels. Thus, we next examined the expression of these proteins in BmN cells infected with recombinant BmNPVs

(Mabb-2d-2, Mabb-2d-4, and Mabb-2-11) expressing each Bm8-interacting host gene under the control of the *polyhedrin* promoter. qRT-PCR experiments verified that cells infected with Mabb-2d-2, Mabb-2d-4, and Mabb-2-11 expressed more than 100-fold transcripts compared with those observed in mock or Mabb-infected cells (Fig. 5A). Subsequent Western blot analyses using whole cell lysates detected the expression of 2-11 protein, but not that of 2d-2 or 2d-4 (Fig. 5B).

Next, we examined the cellular localization of 2-11 protein in BmNPV-infected cells. Since Bm8 is expressed only in the nuclei of BmNPV-infected cells (Imai *et al*., 2004), 2-11 protein should be localized in the nuclei if this protein interacts with Bm8. Western blot analysis using nuclear fractions showed that 2-11 protein was expressed as 60, 85, and 120 kDa polypeptides in the nuclei of BmNPV-infected cells (data not shown), suggesting that 2-11 protein possibly interacts with Bm8 in the nuclei of BmNPV-infected cells. We further attempted to detect the interaction between Bm8 and 2-11 by immunoprecipitation experiments, but we did not succeed in this, presumably due to antibody property and/or a low expression level of Bm8.

Effects of over-expression of Bm8-interacting host clones on virus propagation and *in vivo* virulence

We also examined the effect of over-expression of the 2d-2, 2d-4, and 2-11 genes on BmNPV infection. We first examined the effect of over-expression on BV production. BmN cells were infected with Mabb as a control virus or three Mabb-derivatives (Mabb-2d-2, Mabb-2d-4, and Mabb-2-11) at an MOI of 5, and yields of BV were determined by plaque assay. The Mabb recombinants expressing 2d-2 and 2d-4 exhibited similar patterns of BV production to that of the corresponding Mabbs (Fig. 6A). In contrast, the growth of Mabb-2-11 showed a reduced rate of BV production in BmN cells after 48 h p.i. (Fig. 6A). To ask if this negative effect on

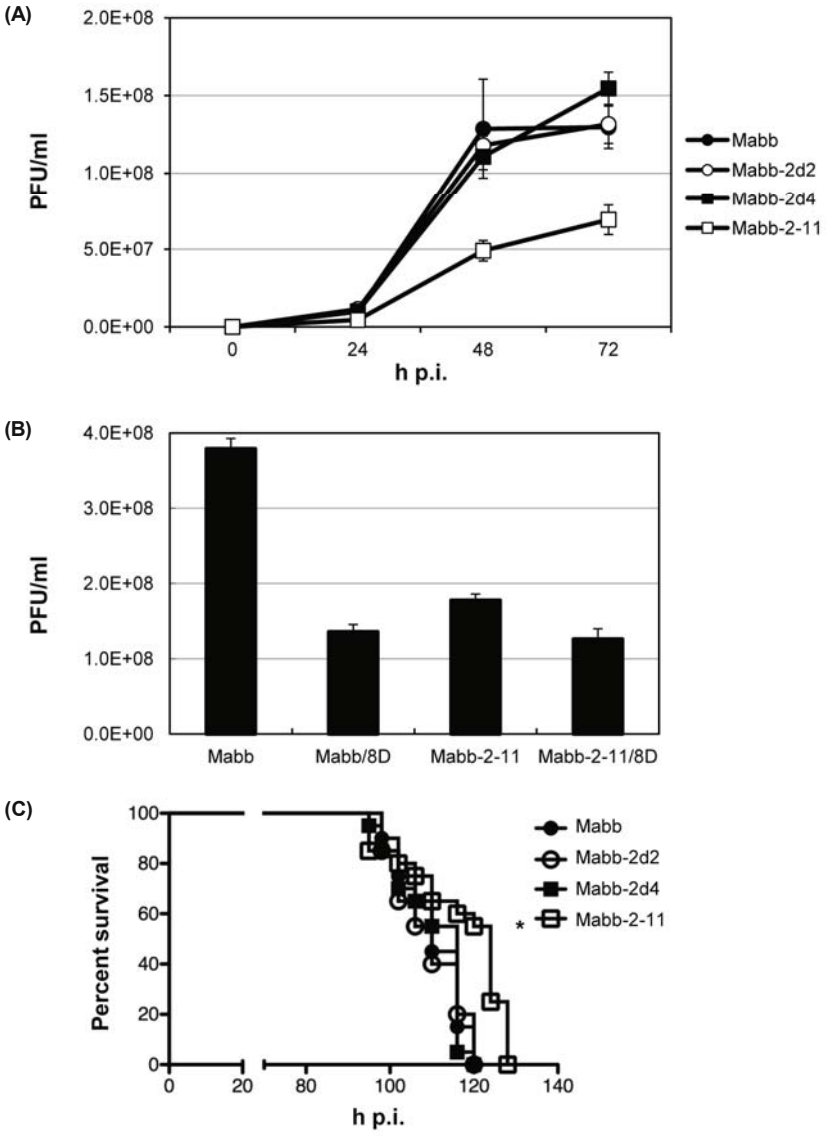

Fig. 6. Effects of over-expression of Bm8-interacting host clones on virus propagation and *in vivo* virulence. (A) BV production of recombinant viruses expressing Bm8 interacting host protein in BmN cells. BmN cells were infected with BmNPVs at an MOI of 5. BV titers were determined by plaque assay. Data shown are means±SD (N=3). (B) Effects of *Bm8* deletion on BV production. BmN cells were infected with Mabb-derivatives at an MOI of 5. A small amount of the culture medium was harvested at 72 h p.i., and then BV titers were determined by plaque assay. Data shown are means±SD (N=3). (C) Survival curves for *B. mori* larvae infected with Mabb-derivatives. The LT_{50}s of Mabb, Mabb-2d-2, Mabb-2d-4, and Mabb-2-11 were 110 h, 110 h, 116 h, and 124 h, respectively. *$p<0.05$, compared with Mabb.

BV production was due to the interaction between Bm8 and 2-11, we generated two additional mutants, Mabb/8D and Mabb-2-11/8D, in which *Bm8* was functionally disrupted by *LacZ* cassette insertion into the genomes of Mabb and Mabb-2-11. As shown in Fig. 6B, Mabb/8D produced fewer BVs than the Mabb, which was consistent with our previous finding that deletion of *Bm8* from the BmNPV genome resulted in the production of fewer BVs (Imai *et al*., 2004). Also, we observed that Mabb-2-11/8D produced similar amounts of BVs compared with those in Mabb/8D-infected cells (Fig. 6B), suggesting a functional interaction between Bm8 and 2-11 proteins. Furthermore, by comparing the survival time of *B. mori* larvae infected with Mabb and Mabb-2-11, we found that Mabb-2-11 took approximately 14 h longer than Mabb to kill *B. mori* larvae when tested by BV injection (Fig. 6C). Although we were not able to obtain biochemical evidence supporting the interaction between Bm8 and 2-11, these results suggest that 2-11 may play a role as a negative factor during BmNPV infection, possibly through interaction with Bm8.

Conclusion

In this study, we identified six host proteins interacting with Bm8 by a yeast two-hybrid system. Proteins encoded by m-9, 2-5, 2-11, and 2d-4 may be receptors/ligands for cell signaling pathways or endocytosis. Regarding the interaction of Bm8 with these proteins, two possible functions in BmNPV infection can be considered. First, Bm8 may interact with these proteins as a structural protein of the virus particle, suggesting that the interaction with these proteins may be involved in entry and budding of virus particles. Secondly, Bm8 may function in gene expression by binding these signal transducers. It is known that gamma herpes viruses use components of Notch and Wnt pathways to regulate their own viral gene expression and additionally alter host gene expression through mimicry or manipulation of downstream pathway responses (Hayward *et al*., 2006). In this study, we identified 2d-2, a putative transcription factor, as a Bm8-binding partner. Bm8 may play a role in regulation of gene expression by interaction with 2d-2. Also, we found that the 2-11 protein, the *Bombyx* TANGO1 homolog, is involved in BmNPV propagation in BmN cells and virulence in *Bombyx* larvae, suggesting that Bm8 might play a role in TANGO1-mediated trafficking of host proteins during BmNPV infection. Further detailed analyses of Bm8-interacting host proteins will explore the molecular mechanisms by which Bm8 enhances virus production and virulence in host insects.

Acknowledgements

We thank J. J. Hull for critical reading of the manuscript and R. Nakazawa and S. Tanaka (Bioarchitect Research Group, RIKEN) for DNA sequencing. The full length cDNA clones used in this study were supplied by the National Bioresource Project (Silkworm). This research was supported by the Bioarchitect program of the Science and Technology Agency of Japan, the Agrigenome Project of the Ministry of Agriculture, Forestry and Fisheries and the National Institute of Agrobiological Sciences, Japan, KAKENHI (Japan Society for the promotion of Science) Nos. 19688004 and 22380033 to S. K. and the Professional Program for Agricultural Bioinformatics, Ministry of Education, Culture, Sports, Science and Technology, Japan.

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