Role of the ubiquitin–proteasome system in
*Bombyx mori* nucleopolyhedrovirus infection

Susumu Katsuma,¹ Ayumi Tsuchida,¹ Noriko Matsuda-Imai,²
WonKyung Kang² and Toru Shimada¹

¹Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
²Molecular Entomology Laboratory, RIKEN ASI, 2-1 Hirosawa, Wako 351-0198, Japan

The ubiquitin–proteasome system plays a central role in the degradation of intracellular proteins and is often required for efficient virus infection. Homologues of ubiquitin are found in all group I nucleopolyhedroviruses (NPVs), but their roles in NPV infection are still unclear. This study found that the specific proteasome inhibitor MG-132 markedly reduced budded virus (BV) production and polyhedrin expression in *Bombyx mori* NPV (BmNPV)-infected BmN-4 cells. Western blot analysis revealed that treatment of cells with MG-132 resulted in delayed and/or dysregulated viral gene product expression. Application of MG-132 significantly reduced BV production when applied up to 12 h post-infection (p.i.), whereas suppression of polyhedrin expression was almost abolished when applied after 6 h p.i. These results suggested that proteosomal degradation of viral and/or host proteins is required at an early stage of infection for efficient polyhedrin expression. To examine further the possible roles of ubiquitin signalling in BmNPV infection, the baculoviral ubiquitin gene (*v-ubi*) was deleted from the BmNPV genome. Deletion of *v-ubi* affected neither BV production nor polyhedrin expression. Furthermore, Western blots also showed that *v-UBI* was not required for degradation of IE2, which is known as a target viral protein of the ubiquitin–proteasome system.

INTRODUCTION

Ubiquitin is a small, highly conserved protein that is involved in a number of cellular processes, including the targeting of proteins for degradation (Rechsteiner, 1991). In eukaryotic cells, the ubiquitin-dependent proteolytic system is one of the major routes by which intracellular proteins are selectively destroyed. Misfolded or damaged proteins are specifically marked for destruction by the covalent attachment of ubiquitin. Ubiquitylation of a target protein involves multi-step enzymic reactions catalysed by a cascade of enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). After ubiquitin is linked to the protein substrate, a polyubiquitin chain is usually formed in which the C terminus of each ubiquitin unit is linked to a specific lysine residue of the previous ubiquitin. The polyubiquitylated substrates are rapidly recognized and degraded by the 26S proteasome (Ciechanover et al., 2000).

The family *Baculoviridae* is a large family of pathogens that infect arthropods, particularly insects of the order Lepidoptera. Members of the genus *Nucleopolyhedrovirus* within this family have a large circular, supercoiled, dsDNA genome packaged into rod-shaped virions. Nucleopolyhedroviruses (NPVs) produce two types of virion, occlusion-derived virions (ODVs) and budded virions (BVs). These two types differ in the origin and composition of their envelopes, as well as their roles in the virus life cycle. ODVs, contained in occlusion bodies (OBs), transmit viruses from insect to insect via oral infection, whereas BVs spread infection to neighbouring cells (Keddie et al., 1989).

Homologues of ubiquitin are found in most lepidopteran baculoviruses (Katsuma et al., 2008). Baculoviral ubiquitin protein (*v-UBI*) is 75% identical to eukaryotic ubiquitin and appears to be present on the inner surface of viral envelopes (Guarino et al., 1995). *v-UBI* may be involved in the formation of viral particles, because a *v-ubi* mutant of *Autographa californica* multiple NPV (AcMNPV) showed a five- to tenfold reduction in BV production (Reilly & Guarino, 1996). Furthermore, biochemical experiments have suggested that *v-UBI* may function in baculovirus replication to block the destruction of short-lived proteins by the host degradative pathway (Haas et al., 1996).

We have shown previously that three *Bombyx mori* NPV (BmNPV) proteins, IAP2, PE38 and IE2, have E3 ligase activity using bacterially expressed recombinant proteins (Imai et al., 2003). In addition, transient expression studies have suggested that IE2 is actively degraded by the proteasome, which depends mainly on its own E3 activity.
Effects of MG-132 on BmNPV infection

To examine whether the ubiquitin–proteasome system is involved in BmNPV propagation, we examined the effects of the specific proteasome inhibitor MG-132 on BV and OB production. We first assessed the cell viability of MG-132-treated BmN-4 (BmN) cells to test whether the effects of MG-132 were caused by cytotoxicity. Mock- and BmNPV-infected BmN cells were treated with 5 μM MG-132, and cellular metabolic activity was measured by cleavage of the tetrazolium salt WST-1. As shown in Supplementary Fig. S1(a) (available in JGV Online), MG-132 treatment did not exhibit a negative effect on cell survival of BmN cells. Also, no decrease in cell viability compared with DMSO-treated cells was observed during BmNPV infection (Supplementary Fig. S1b). These results indicated that MG-132 treatment at a concentration of 5 μM was not associated with cytotoxicity of BmN cells.

Next, we assessed the effects of MG-132 on BV production. As shown in Fig. 1(a), treatment with MG-132 (5 μM) significantly reduced BV production from 24 h post-infection (p.i.), and a tenfold reduction was observed at 72 h p.i. in MG-132-treated cells compared with in DMSO-treated cells. We also observed that treatment with MG-132 (5 μM) completely inhibited OB production at 72 h p.i. in BmNPV-infected BmN cells, whereas a large number of OBs were observed in cells treated with DMSO (control treatment) (Fig. 1b). Western blot analysis indicated that MG-132 markedly inhibited polyhedrin protein expression, suggesting that the reduced OB production by MG-132 was due to the inhibition of polyhedrin synthesis (Fig. 1c). Taken together, these results suggested that the ubiquitin–proteasome system is required for efficient production of BVs and OBs during BmNPV infection.

Effects of MG-132 on expression of viral gene products and accumulation of ubiquitylated proteins in BmNPV-infected cells

We next examined the effects of MG-132 on the expression of BmNPV gene products. Western blot analysis showed that expression of an early gene product, DNA-binding protein (DBP), a late gene product, viral chitinase (v-CHIA), and a very late gene product, polyhedrin, was delayed and/or reduced in MG-132-treated cells compared with in DMSO-treated cells (Fig. 1c). These results indicated that inhibition of the ubiquitin–proteasome system results in reduced virus production, coinciding with delayed and/or dysregulated expression of viral gene products.

Next, we examined ubiquitylation of proteins expressed in BmNPV-infected cells by Western blotting with anti-ubiquitin mAb. As shown in Fig. 1(c), the ubiquitylated proteins, especially high-molecular-mass proteins, disappeared abruptly from 48 h p.i. in BmNPV-infected cells, whereas treatment with MG-132 resulted in a gradual increase in the accumulation of ubiquitylated proteins throughout the infection. We also observed that expression of the housekeeping protein actin disappeared almost completely from 48 h p.i. in BmNPV-infected DMSO-treated cells, whereas its expression was still observed in MG-132-treated cells, even at 72 h p.i. This suggested that MG-132 treatment delays a global shut-off of host protein synthesis by BmNPV infection. Collectively, these results strongly suggested that the ubiquitin–proteasome system is required for efficient BmNPV infection.

Effects of v-ubi deletion on virus production in BmN cells

To determine further the role of ubiquitin signalling in BmNPV infection, we deleted the v-ubi gene from the BmNPV genome by homologous recombination and successfully isolated a v-ubi-deficient virus, BmUbiD (see Supplementary Fig. S2a, available in JGV Online). Western blot analysis showed that v-ubi deletion did not affect polyhedrin and actin expression (Fig. 2a). Furthermore, we examined the effect of v-ubi deletion on BV production. BmN cells were infected with the wild-type BmNPV T3 isolate or BmUbiD, with or without MG-132 treatment (1 or 5 μM), and yields of BV were determined by plaque assay. The titre of BmUbiD at 72 h p.i. was comparable to that of T3 regardless of treatment or not with MG-132 (Fig. 2b). These results suggested that deletion of v-ubi did not affect BV and OB production in BmNPV-infected BmN cells.

To identify more specifically the stage of BmNPV infection targeted by MG-132, we examined BV production or polyhedrin expression after applying the inhibitor at different time points p.i. (0–12 h p.i.). As shown in Fig. 3(a), MG-132 significantly reduced BV production in BmN cells infected with T3 or BmUbiD at all time points tested. In contrast, we observed that suppression of polyhedrin expression by MG-132 was significantly reduced when applied after 6 h p.i. (Fig. 3b). These results suggested that degradation of viral and/or host proteins by the ubiquitin–proteasome system at an early stage of BmNPV infection (0–6 h p.i.) is essential for efficient polyhedrin expression.

v-UBI is not required for the ubiquitin–proteasome-dependent degradation of IE2

In a previous study using confocal microscopy, we observed that MG-132 treatment resulted in IE2 protein accumulation (Imai et al., 2005), suggesting that IE2 is
actively degraded by the ubiquitin–proteasome system. Also, transient expression experiments have shown that the degradation process is seemingly regulated by its own E3 ligase activity (Imai et al., 2005). These results indicate that IE2 is being auto-ubiquitylated and degraded during BmNPV infection. To verify this, we generated a mutant virus, BmIE2CS, expressing an E3 ligase activity-deficient IE2 derivative (IE2C243S; Imai et al., 2003, 2005) (Supplementary Fig. S2b) and examined IE2 expression during BmNPV infection. As reported previously (Imai et al., 2005), we observed that IE2 formed several foci in the nucleus at 2 h p.i. and then gradually diminished after 4 h p.i. in T3-infected BmN cells (see Supplementary Fig. S3, available in JGV Online). As expected, IE2 foci in BmIE2CS-infected cells were brighter compared with those in T3-infected cells. In addition, foci in BmIE2CS-infected cells were clearly detected as late as 12 h p.i., although those in T3-infected cells were rarely detected at this stage (Supplementary Fig. S3). However, it should be noted that the foci in BmIE2CS-infected cells also diminished gradually after 4 h p.i., suggesting that IE2 could be degraded in the absence of its E3 ligase activity. Western blot analysis clearly showed that treatment with MG-132 did not inhibit degradation of IE2CS, indicating that an alternative degradation mechanism is apparently responsible for IE2CS degradation (Fig. 4a).

Next, we examined whether v-UBI is involved in IE2 degradation in BmNPV-infected BmN cells. BmN cells were infected with T3, BmUbiD, BmIE2CS or BmIE2D and harvested at 4 or 12 h p.i. As shown in Fig. 4(a, b), we observed that IE2 accumulation was indistinguishable between T3- and BmUbiD-infected cells with or without MG-132 treatment. In BmIE2CS-infected cells, IE2 accumulated strongly at 4 h p.i. and then decreased at 12 h p.i. (Fig. 4b). This was consistent with confocal microscopic
observations (Supplementary Fig. S2). On the basis of these results, we concluded that v-UBI is not required for IE2 degradation in BmNPV-infected cells.

**DISCUSSION**

The ubiquitin–proteasome system has a crucial role in the degradation of intracellular proteins and regulates a variety of cellular pathways. Using proteasome inhibitors such as MG-132, viruses belonging to several different families have been shown to utilize or modulate this system to their advantage during their infection cycles (Raaben et al., 2010; Satheshkumar et al., 2009; Teale et al., 2009). In this study, we examined whether the ubiquitin–proteasome system is required for efficient BmNPV infection. First, we investigated the effects of MG-132 on OB and BV production and
expression of BmNPV gene products. As shown in Fig. 1(a, b), OB and BV production was significantly reduced by MG-132 treatment. It should be noted that the effect of MG-132 on OB production was more prominent than that on BV production. Western blot analysis also showed that treatment of BmNPV-infected cells with MG-132 markedly delayed or reduced expression of early, late and very late gene products of BmNPV (Fig. 1c). These results strongly suggested that the ubiquitin–proteasome system plays a role during BmNPV infection.

We observed that the suppression of polyhedrin expression was significantly reduced when applied at or after 6 h p.i.; however, decreased BV production was evident even when applied at 12 h p.i. (Fig. 3). This suggested that ubiquitin-mediated protein degradation during the early stage of infection has different roles in expression of late and very late gene products. In addition, we observed that ubiquitylated proteins disappeared abruptly from 48 h p.i. in BmNPV-infected cells (Fig. 1c). As these conjugates accumulated in the presence of MG-132 at these time points, this suggested that the ubiquitin–proteasome system is responsible for their degradation after 48 h p.i.

Surprisingly, we did not observe any phenotypic defects when v-ubi was deleted from the BmNPV genome. This result is inconsistent with that from a study of a closely related AcMNPV (Reilly & Guarino, 1996). The AcMNPV mutant Vubi-FS, with a frameshift mutation in the coding region of v-ubi, produced five- to tenfold fewer BVs than the wild-type, suggesting that v-UBI plays an important role in BV production during AcMNPV infection. This group also showed that AcMNPV v-UBI may function in baculovirus replication to block the destruction of short-lived proteins by the host degradative pathway (Haas et al., 1996). Amino acid differences in some proteins have been shown to result in the phenotypic discrepancies between AcMNPV and BmNPV (Katsuma et al., 1997; Katou et al., 2010; Katsuma et al., 2006b; Morishima et al., 1998; Nakanishi et al., 2010). Despite the fact that the amino acid sequences of v-UBI are completely identical between these two viruses, the roles of v-UBI may be different in the infection cycle of each NPV.

Host ubiquitin (H-UBI) and v-UBI are known to be structural proteins of BVs and are anchored to the inner face of the BV envelope by covalently linked phospholipids (Guarino et al., 1995). This has led to the hypothesis that BV-associated ubiquitin may be utilized for proteasome-dependent degradation of viral and/or host proteins immediately after virus entry. Because IE2 is the only known baculoviral protein degraded via the ubiquitin–proteasome system (Imai et al., 2005), we examined IE2 expression in BmUbiD-infected BmN cells. Western blot analysis clearly showed that IE2 accumulation was indistinguishable between T3- and BmUbiD-infected cells, suggesting that H-UBI is utilized for IE2 degradation.

v-UBIs are more closely related to each other than to animal ubiquitin, and most of the substitutions occur at the same amino acid residues, suggesting that v-UBI has evolved for a specific role in the baculovirus life cycle (Reilly & Guarino, 1996). Although no phenotypic abnormality of BmUbiD was observed in BmN cells, the modern lepidopteran baculovirus has seemingly acquired a v-ubi gene from an ancestral host insect to obtain evolutionary advantages. Further studies using insect larvae instead of cultured cells may help us understand the significance of v-UBI in the BmNPV life cycle. Furthermore, to comprehend the direct contribution of the viral ubiquitin system to BmNPV infection, we are currently generating a mutant BmNPV in which all of the viral ubiquitin system-related genes (e.g. v-ubi, iap2, ie2 and pe38) are inactivated.

**METHODS**

**Materials.** The proteasome inhibitor MG-132 was purchased from Calbiochem and dissolved in DMSO. The final concentration of DMSO in the cell culture medium was 0.1 % (v/v). The ubiquitin mAb was obtained from Santa Cruz Biotechnology. Antibodies against BmNPV DBP, BmChi-h (v-CHA) and IE2 were as reported previously (Daimon et al., 2007; Imai et al., 2005; Okano et al., 1999; Shimada et al., 1994).

**Cell line and virus.** The BmN-4 (BmN) cell line was maintained at 26 °C in TC-100 medium with 10 % FBS. The BmNPV T3 isolate (Maeda et al., 1985) was propagated in BmN cells. The ie2 deletion mutant BmIE2D has been reported previously (Gomi et al., 1997). Viruses were propagated in BmN cells, and BV titres were determined by plaque assay.

**Proteasome inhibition assay.** BmN cells were infected with BmNPV at an m.o.i. of 5. After 1 h of incubation, virus-containing culture medium was removed, the cells were washed twice with TC-100 medium, and fresh medium with MG-132 (5 μM) or DMSO (control) was added (0 h p.i.). A small amount of culture medium was collected at various time points, and BV production was determined by plaque assay. Accumulation of ubiquitylated proteins and expression of viral gene products were examined by SDS-PAGE and Western blotting.

**Cell viability.** BmN cells were infected with BmNPV at an m.o.i. of 5. After 1 h of incubation, virus-containing culture medium was removed and fresh medium with or without MG-132 was added (0 h p.i.). We used the WST-1 assay kit (Roche Applied Science) to assess viable cell numbers as described previously (Katsuma et al., 2005).

**SDS-PAGE and Western blotting.** SDS-PAGE and Western blotting were performed as described previously (Katsuma et al., 2007). Western blot analysis of the ubiquitylated proteins DBP and v-CHA was carried out using anti-ubiquitin, anti-DBP and anti-BmChi-h antibodies, respectively. Polyhedrin expression was examined by SDS-PAGE as described previously (Katsuma et al., 2007).

**Construction of the v-ubi deletion mutant by insertion of a β-galactosidase gene cassette.** To construct a plasmid for the deletion of v-ubi, a 5.4 kb fragment (nt 21548–27087; GenBank accession no. L33180) was purified from the BmNPV T3 genomic clone (Maeda & Majima, 1990) and inserted into pTZ19R. The plasmid was then digested with BstXI and ligated to a β-galactosidase gene cassette containing the *Drosophila melanogaster* heat-shock protein promoter (hsp70-laZ cassette). The resultant plasmid was co-transfected with T3 genomic DNA into BmN cells using *Caenorhabditis*
(Invitrogen). The v-ubi-deleted BmNPV (BmUsbID) was isolated by identification of plaques expressing β-galactosidase (Katsuma et al., 2006a). Deletion of v-ubi was confirmed by PCR (see Supplementary Fig. S2a) using the primer set v-ubiF (5'-AACGGAACCAGC-GAGACG-3') and v-ubiR (5'-CTCTCTGTAATCGTAAACCC-3').

Construction of BmIE2CS expressing an E3 ligase activity-deficient IE2. A PCR fragment encoding an E3 ligase activity-deficient IE2 (IE2C243S; Imai et al., 2003) was cloned into pBluescript II SK(−) (Stratagene), and designated pBS-IE2Cs. The pBS-IE2cs plasmid was co-transfected with the Bsal-digested BmIE2D genomic DNA into BmN cells as described previously (Katsuma et al., 2006a). Recombinants identified as white plaques were isolated by plaque assay with agarose overlays containing 400 μg X-Gal per 60 mm dish. To confirm whether the replacement of lacZ with the mutated ie2 was performed correctly, the ie2 gene region of isolates with a white plaque phenotype was amplified by PCR (see Supplementary Fig. S2a) using the primer set ie2F (5'-ATTCT-TCGTTGTGACTTCG-3') and ie2R (5'-AACCTGCAATATGGA-TGACTCTGTAACC-3'), and DNA sequences were determined using an ABI Prism 3100 DNA sequencer (Applied Biosystems).

Immunohistochemistry and confocal microscopy. Infected cells were harvested at designated times and subjected to immunohistochemistry. Immunohistochemistry and confocal microscopy were performed as described by Okano et al. (1999). For immunohistochemistry, anti-IE2 antibody (1:200 dilution) and Cy5-conjugated goat anti-rat IgG (1:200 dilution; Jackson Immuno Research) or FITC-conjugated anti-IE2 antibody (1:200 dilution) and Cy5-conjugated goat anti-rat IgG (1:500 dilution; Cappel) were used.

ACKNOWLEDGEMENTS

This work was supported by the Program for Agricultural Bioinformatics from MEXT, Japan grant nos 19688004 and 22380033 to S. K. and grant no. 17018007 to T. S.

REFERENCES


