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Functional characterization of *Bombyx mori* nucleopolyhedrovirus CG30 protein

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ABSTRACT

The baculovirus *cg30* gene is present in the genomes of most alphabaculoviruses. The gene product, CG30, contains two putative functional domains, a RING finger motif and a leucine zipper motif. A gene-knockout study in *Autographa californica* nucleopolyhedrovirus (AcMNPV) revealed that a *cg30*-disrupted virus did not show any striking differences compared with wild-type virus. To determine the roles of *cg30* in another alphabaculovirus, we constructed two *Bombyx mori* NPV (BmNPV) mutants lacking a functional *cg30* by *lacZ* cassette insertion and characterized its infectivity in BmN cells and *B. mori* larvae. The mutants produced fewer budded viruses (BVs) in BmN cells and *B. mori* larvae compared with wild-type BmNPV. We also observed a decrease in the release of occlusion bodies (OBs) in the hemolymph of the larvae infected with the *cg30* mutants. To investigate the role of a RING finger domain of CG30 during virus growth, we further constructed two mutants; one expressed a mutant CG30 where a RING finger motif is disrupted by a single amino acid substitution, whereas the other possessed a CG30 derivative that completely lacked the RING finger domain. Both produced fewer OBs in the hemolymph of *B. mori* larvae, demonstrating that a RING finger domain of CG30 is involved in maximum OB production in the hemolymph of *B. mori* larvae. We also revealed that CG30 is expressed as a nuclear protein with a molecular mass of 30 kDa in BmNPV-infected cells.

1. Introduction

Baculoviridae is a large family of pathogens that infect insects, particularly the order Lepidoptera. Baculoviruses have a large circular, supercoiled, and double-stranded DNA genome packaged into rod-shaped virions. Baculoviruses are phylogenetically divided into four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus* (King et al., 2011). Alphabaculoviruses can be further subdivided into group I and II nucleopolyhedroviruses (NPVs) (Herniou et al., 2003). NPVs produce two types of virions during their infection cycle, which enable the virus to replicate efficiently within infected larvae and spread their progeny among insects. Occlusion-derived viruses (ODVs), which are occluded in occlusion bodies (OBs), spread from insect to insect through oral infection, whereas budded viruses (BVs) spread infection to neighboring cells (Keddie et al., 1989). At the late stage of infection, a markedly enhanced locomotion behavior of infected larvae is observed (Goulson, 1997), followed by dramatic degradation of the host cadaver (Federici, 1997).

The baculovirus *cg30* gene is not a core set of baculovirus genes; however, it is present in most (about 80%) alphabaculoviruses. The *cg30* genes of *Autographa californica* NPV (AcMNPV) and *Bombyx mori* NPV (BmNPV) are expressed as delayed early genes (Thiem and Miller, 1989; Lu and Iatrou, 1996) and encode a 30-kDa protein with two putative functional domains, a RING finger motif and a leucine zipper motif (Thiem and Miller, 1989). To investigate the roles of *cg30* during NPV infection, gene-knockout studies have been performed in AcMNPV and BmNPV. Recombinant AcMNPVs lacking a functional *cg30* gene produced wild-type levels of BV and OB in *Spodoptera frugiperda*- and *Trichoplusia ni*-derived cells. Larval bioassays also revealed that the oral infectivity and survival time of the *cg30* mutants did not differ from those of the wild-type virus (Passarelli and Miller, 1994). These results indicated that *cg30* deletion from the AcMNPV genome had little effect on in vitro and in vivo virus growth. A *cg30*-disrupted BmNPV bacmid was recently generated and characterized in cultured cells (Bm5 cells) and *B. mori* larvae. In contrast to AcMNPV, *cg30* deletion reduced BV production in cultured cells and slightly prolonged the survival of infected larvae (Zhang et al., 2012). In both cases, however, the precise function of this protein has not been clarified.

Several lines of evidence have suggested that RING finger proteins have E3 ubiquitin ligase activity, which is dependent on the RING finger domain (Deshaies and Joazeiro, 2009). The BmNPV genome possesses six genes that potentially encode a RING finger protein. Among these, four proteins, namely IAP2, IE2, PE38, and CG30, were shown to be polyubiquitinated in the presence of zinc ions using MBP-fused recombinant proteins and rabbit reticulocyte lysates (Imai et al., 2003), suggesting that these proteins

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might have E3 ubiquitin ligase activity. Subsequently, recent studies have revealed that IE2 is rapidly degraded by the proteasome during BmNPV infection and that this degradation depends mainly on its own E3 ligase activity (Imai et al., 2005; Katsuma et al., 2011a). However, the functions of the RING finger domains of IAP2, PE38, and CG30 remain unknown.

In this study, we investigated the roles of *cg30* in BmNPV infection using *lacZ* insertional mutants and RING finger domain mutants. We found that *cg30* disruption reduced BV and OB production in both cultured cells and *B. mori* larvae. We also showed that the RING finger motif of BmNPV CG30 is involved in OB production in the hemolymph of *B. mori* larvae. These results, combined with our observation that CG30 is localized mainly in the nucleus, suggest that this protein may transcriptionally regulate very late genes directly or indirectly in insect larval tissues.

2. Materials and methods

2.1. Insect, cell lines, and viruses

B. mori larvae (F1 hybrid Kinshu × Showa) were reared as described previously (Katsuma et al., 2012a). The BmN (BmN-4) cells were cultured at 27 °C in TC-100 medium supplemented with 10% fetal bovine serum. BmNPV T3 was used as the wild-type virus. Virus titers of T3 and recombinant BmNPVs were determined by plaque assay on BmN cells (Katsuma et al., 2012a).

2.2. Construction of recombinant BmNPVs possessing the mutated cg30 genes

A 3.5-kb fragment (nt 65,313–68,842; GenBank Acc. no. L33180) was purified from the BmNPV T3 genomic clone (Maeda and Majima, 1990) and inserted into pTZ19R to construct a plasmid for the deletion of BmNPV *cg30*. The plasmid was then digested with *Pst*I and *Bal*I and ligated to a β-galactosidase gene cassette containing a *Drosophila melanogaster* heat shock protein promoter (*hsp70-lacZ* cassette; Kamita et al., 1993). The resultant plasmid (lacZ-cg30) was cotransfected with wild-type (T3) BmNPV DNA into BmN cells using Cellfectin (Invitrogen). Two *cg30* deletion mutants (BmCG30D-1 and BmCG30D-2; Fig. 1A) were isolated by identifying plaques expressing β-galactosidase (Katsuma et al., 2012a). Deletion of *cg30* was confirmed by polymerase chain reaction (PCR) using the primers shown in Supplementary Table 1.

To construct the *cg30* revertant virus BmCG30DR, a 3.9-kb PCR fragment (nt 65,413–69,274) containing full-length *cg30* was cloned into pcDNA3.1(−)(Invitrogen) and designated pcDNA-cg30. The pcDNA-cg30 plasmid was cotransfected with *Bsu*36I-digested BmCG30D-1 genomic DNA into BmN cells as described previously (Katsuma et al., 2012a). Recombinants identified as white plaques were isolated by plaque assay with agarose overlays containing X-Gal. To confirm whether the replacement of *lacZ* with intact *cg30* was performed correctly, the *cg30* gene region of isolates with the white plaque phenotype was amplified by PCR and DNA sequences were determined using an ABI Prism 3100 DNA sequencer (Applied Biosystems) as described previously (Katsuma et al., 2012a).

pcDNA-cg30 was used as a template to generate mutations in the *cg30* gene for constructing recombinant BmNPVs expressing CG30 where a RING finger motif is disrupted by a single amino acid substitution (BmCG30C39S) or that completely lacks its RING finger domain (BmCG30RingD). Mutagenesis was performed using the KOD-plus-Mutagenesis Kit (TOYOBO) according to the manufacturer's protocols with the primers shown in Supplementary Table 1. The resultant plasmids were cotransfected with *Bsu*36I-digested BmCG30D-1 genomic DNA into BmN cells, and recombinant viruses were isolated by the identifying plaques that did not express β-galactosidase, as described above. The presence of the mutated *cg30* genes in these viruses was confirmed by PCR and DNA sequencing, as described above.

To examine the expression and localization of CG30 protein in BmNPV-infected cells, two repair mutants of BmCG30D, BmCG30D-ResFLAG and BmCG30D-Res, expressing FLAG-tagged or non-tagged CG30, respectively, under an authentic *cg30* gene promoter (inserted immediately upstream of the *polh* gene) were generated by two step process as described previously (Katsuma et al., 2012b) with modifications. Firstly, the lacZ-cg30 plasmid was cotransfected with the genomic DNA of an OB-negative virus BmNPV-abb (Kang et al., 1998) into BmN cells. A recombinant BmNPV (mabb-CG30D), which is an OB-negative virus lacking an endogenous *cg30* gene, was plaque-purified by identifying blue plaques. In the second step, a FLAG-tagged or non-tagged *cg30* gene under the authentic *ptp* gene promoter was cloned into the transfer vector pBmEPS1 (a *polh*-positive transfer vector; Kang et al., 1998), and the recombinant transfer plasmids were cotransfected with mabb-CG30D genomic DNA into BmN cells. Recombinant viruses were identified by the formation of OB-positive plaques expressing β-galactosidase.

2.3. Assays for BV production

To determine the virus growth curves, BmN cells were infected with T3 or recombinant BmNPVs at an MOI of 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 plaque assay.

2.4. OB production in B. mori larvae and BmN cells

Fifth instar *B. mori* larvae were starved for several hours, injected with 50 μl of a viral suspension containing 1×10^5 PFU, and returned to the artificial diet at 25 °C. At 96 hpi, the hemolymph of the infected larvae was collected and the released OBs were counted using a hemocytometer as described previously (Katsuma and Shimada, 2009). BmN cells infected with T3 or recombinant BmNPVs at 96 hpi were gently scraped using a rubber policeman, and the total OB production was measured as described previously (Katsuma and Shimada, 2009).

2.5. Larval bioassays

The median lethal time (LT_{50}) was determined either by intrahemocoelic injection of 5th instar larvae with BVs (1×10^5 PFU per larva). *B. mori* larvae were intrahemocoelically inoculated with BV within 12 h after molting to the 5th instar. To determine the median lethal concentration (LC_{50}) of OBs, 1st instar larvae were orally inoculated within 8 h after hatching. OBs were purified from the hemolymph of BmNPV-infected 5th instar *B. mori* larvae. OBs were washed twice with distilled water, once with 1% sodium dodecyl sulfate (SDS), and twice with distilled water. Purified OBs were diluted in distilled H_2O and quantified using a hemocytometer. First instar larvae were orally inoculated by exposing them to a 960-mm^2 area of artificial diet that was surface-contaminated with different concentrations of OBs (Nakanishi et al., 2010). Approximately 20 larvae per dose were used in each of the experiments. Virus titers in the hemolymph of the infected larvae were determined by plaque assay.

2.6. Quantitative reverse transcription-PCR (qRT-PCR)

BmN cells were infected with BmNPVs at an MOI of 5. Following incubation for 1 h, the virus-containing culture medium was