A viral histone H4 encoded by *Cotesia plutellae* bracovirus inhibits haemocyte-spreading behaviour of the diamondback moth, *Plutella xylostella*

Wael Gad and Yonggyun Kim

Histone H4 is highly conserved and forms a central-core nucleosome with H3 in eukaryotic chromatin. Its covalent modification at the protruding N-terminal region from the nucleosomal core can change the chromatin conformation in order to regulate gene expression. A viral H4 was found in the genome of *Cotesia plutellae* bracovirus (CpBV). The obligate host of the virus is an endoparasitoid wasp, *C. plutellae*, which parasitizes the diamondback moth, *Plutella xylostella*, and interrupts host development and immune reactions. CpBV has been regarded as a major source for interrupting the physiological processes during parasitization. CpBV H4 shows high sequence identity with the amino acid sequence of *P. xylostella* H4 except for an extended N-terminal region (38 aa). This extended N-terminal CpBV H4 contains nine lysine residues. CpBV H4 was expressed in *P. xylostella* parasitized by *C. plutellae*. Western blot analysis using a wide-spectrum H4 antibody showed two H4s in parasitized *P. xylostella*. In parasitized haemocytes, CpBV H4 was detected predominantly in the nucleus and was highly acetylated. The effect of CpBV H4 on haemocytes was analysed by transient expression using a eukaryotic expression vector, which was injected into non-parasitized *P. xylostella*. Expression of CpBV H4 was confirmed in the transfected *P. xylostella* by RT-PCR and immunofluorescence assays. Haemocytes of the transfected larvae lost their spreading ability on an extracellular matrix. Inhibition of the cellular immune response by transient expression was reversed by RNA interference using dsRNA of CpBV H4. These results suggest that CpBV H4 plays a critical role in suppressing host immune responses during parasitization.

**INTRODUCTION**

The chromosome is a complex structure of DNA and proteins, and efficiently transmits the genetic entity of an organism from one generation to the next due to its compact structure. To achieve this compactness in eukaryotic cells, naked DNA is linked to histone proteins and formed into nucleosomes. The nucleosome core particle consists of 146 bp of DNA wrapped around an octameric complex of core histones (two each of H2A, H2B, H3 and H4) and is the highly conserved, basic structural unit of chromatin in all eukaryotic cells (Wolffe, 1992). However, this association between DNA and histones is a physical hindrance to gene expression activity so that there is a conflicting need between compacting and accessing the DNA structure. The histone N termini (15–38 aa) extend from the core and form the histone ‘tails’, where they can be subjected to enzyme-catalysed, post-translational modifications (Wu et al., 1986) that affect their charge and function. In particular, specific lysine residues can be acetylated at ε-amino groups by a family of enzymes, the histone acetyltransferases (HATs) (Kuo & Allis, 1998). In fact, acetylation of lysine-16 of histone H4 modulates both higher-order chromatin structure and functional interactions between a non-histone protein and the chromatin fibre (Shogren-Knaak et al., 2006). Histone acetylation is reversed by a second enzyme family, the histone deacetylases (HDACs), which can also mediate gene inactivation as components of large complexes in a variety of eukaryotes (Pazin & Kadonaga, 1997). Several transcriptional activators or repressors form complexes with HATs or HDACs in particular regions of chromatin and regulate gene activity (Turner, 1993; Grunstein, 1997).

A previous report described a novel viral H4 encoded by *Cotesia plutellae* bracovirus (CpBV) (Ibrahim et al., 2005). *C. plutellae* is an endoparasitoid of the diamondback moth, *Plutella xylostella*, in which the symbiotic polydnavirus CpBV plays a critical role in altering physiological status favourable for wasp survival and development (Kim et al., 2007). CpBV, which is present as a provirus on the host wasp chromosome, replicates only in the ovarian calyx during the wasp pupal stage (Kim & Ryu, 2007). The replicated CpBV genome consists of at least 27 DNA segments in which more than 470 kb of non-overlapping nucleotide sequences have been determined, and is known...
to encode several putative gene families (Choi et al., 2005; Kim et al., 2007). CpBV protein tyrosine phosphatases (PTPs) are the largest gene family in CpBV and play a significant role in immunosuppression, probably by altering the phosphorylation status of the infected haemocytes (Ibrahim & Kim, 2007; Ibrahim et al., 2007). CpBV15β is a 15 kDa protein-encoding CpBV gene and also inhibits haemocyte-spreading of P. xylostella (Nalini & Kim, 2007).

The CpBV genome also contains one H4 protein, which has been shown to be expressed in parasitized P. xylostella (Ibrahim et al., 2005). Its putative open reading frame indicates lysine-rich residues in an unusually extended N-terminal region of CpBV H4, suggesting induction of nucleosomal aberration, which may in turn alter host physiological status. This study focused on analysing the effect of CpBV H4 on the cellular immune response of P. xylostella. To this end, we analysed its expression and localization in haemocytes of parasitized P. xylostella using an immunofluorescence assay (IFA). Using a transient expression technique (Ibrahim & Kim, 2007), viral gene expression was then induced in non-parasitized P. xylostella and assessed in an immune capacity by estimating haemocyte-spreading behaviour. The observed adverse effect of CpBV H4 on haemocytes was further confirmed by RNA interference (RNAi).

METHODS

Insect rearing and parasitization. P. xylostella larvae were reared at 25 ± 1 °C under a 16:8 h (light:dark) photoperiod with cabbage leaves. Late second-instar larvae (4 days after oviposition at 25 °C) were parasitized by about half the host number of C. plutellae females for 12 h. The parasitized larvae were reared on cabbage leaves in the environment described above. Adults emerging from the cocoons (11 days after parasitization at 25 °C) were collected and allowed to mate with each other for 24 h before use for parasitization.

Haemolymph collection and haemocyte-spreading assay. Fifteen larvae were surface-sterilized and their haemolymph was collected in 150 μl anticoagulant buffer, which was freshly prepared by dissolving 4 mg L-cysteine hydrochloride (Sigma) in 5 ml Tris-buffered saline [50 mM Tris/HCl (pH 7.5), 100 mM glucose, 5 mM KCl, 2.5 mM MgCl2, and 50 mM NaCl]. Haemocyte monolayers were made by using 50 μl of this suspension for each monolayer and were left in a moist chamber for 60 min. Spread haemocytes were observed at 400× magnification under a phase-contrast microscope (BX41; Olympus). Haemocyte spreading was determined by cytoplasmic expansion (Nalini & Kim, 2007). The percentage of spread haemocytes was scored by counting 100 haemocytes from ten randomly selected microscopic fields.

cDNA construction and RT-PCR. Total RNA was extracted using Trizol reagent (MRC) and followed by reverse transcription using RT PreMix (Bioneer) with an oligo(dT) primer (5'-CCAGTGAGCACGATGGTCCGAGGAGGAGCAGCTGCTCAAGCTTGTGGTTTGTTTTT-3') and subsequent RNase H (1 unit μl⁻¹) treatment. The synthesized cDNA was used as a template for PCR amplification. A control PCR was performed to check for DNA contamination using RNA extract as template.

Cloning of P. xylostella H4. Based on conserved H4 amino acid sequences from Drosophila hydei (GenBank accession no. X52576), Tribolium castaneum (GenBank no. XM_962775) and Aphis mellifera (GenBank no. NM_001011609), the following degenerative primers were designed to clone the entire open reading frame: 5'-ATGACTGGCGGGAAGGTGTAARGG-3' (forward) and 5'-TTAAGCCGAAA-KCCRTANAGRTNGC-3' (reverse). The cDNA was amplified with these primers using the following conditions: 94 °C for 1 min, followed by 35 cycles of 50 °C for 1 min and 72 °C for 2 min. The amplified PCR product was cloned into pCR2.1 (Invitrogen) and submitted for sequencing (Macrogen) using M13 forward and reverse primers.

Bacterial expression of CpBV H4 and production of polyclonal antibody. To produce specific antibody against CpBV H4, the extended N-terminal region (38 aa), which differed from the host H4, was cloned into expression vector pBAD-TOPO (Invitrogen) according to the manufacturer's instructions and transformed into Escherichia coli TOP10. This vector contains a poly(His) tail and a V5 epitope in the recombinant protein for purification using an affinity column and Western blotting using anti-V5 antibody (Invitrogen). The recombinant bacteria were cultured and induced to overexpress the target protein by addition of the inducer L-arabinose to a final concentration of 0.002%. Recombinant protein was purified using a Ni-NTA column (Qiagen) and confirmed by Western blotting using anti-V5 antibody as described below. Antibody was produced using rabbit (Peptron).

Extraction of nuclear proteins and Western blotting of various histone H4 subunits. Parasitized and non-parasitized larvae were ground with a glass/glass homogenizer in 50 mM PBS (pH 7.0) containing 0.7% NaCl and supplemented with 5 mM sodium butyrate to retain the level of histone acetylation. After centrifugation at 200 g for 5 min, the supernatant extract was quantified using the Bradford (1976) method and mixed with the same volume of denaturing buffer [62.5 mM Tris/HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol]. After heat treatment for 5 min at 95 °C, the samples were analysed by 15% SDS-PAGE. Electrophoresis was performed under denaturing conditions (Laemmli, 1970) until the tracking dye migrated to the end of the gel. Gels were stained with 0.125% Coomassie Blue R-250 in 50% methanol/10% acetic acid. The separated proteins on the gel were transferred onto a nitrocellulose membrane using the method of Towbin et al. (1979). Non-specific sites were blocked with 5% skimmed milk for 1 h at room temperature. After three washes with PBS, the membrane was incubated for 1.5 h at room temperature with a primary antibody raised against CpBV H4, a general antibody against H4 (Millipore) or antibody against acetylated H4 (Millipore). After three washes with PBS, the membrane was incubated for 1 h at room temperature with a secondary antibody against rabbit IgG (1:2000 dilution) conjugated with alkaline phosphatase (Sigma). After three washes with PBS, the membrane was stained with alkaline phosphatase substrate solution containing nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 10 mM phosphate buffer (pH 9.5).

IFA. An IFA was performed according to the method of Nalini & Kim (2007) using the three antibodies against CpBV H4, pan-H4 and acetylated H4 as described above. Briefly, monolayers were prepared as described above, washed and fixed in 3.7% formaldehyde for 20 min. Cells were permeabilized using PBS containing 0.2% Triton X-100 for 5 min and blocked using non-immunized goat serum for 15 min at room temperature. Each of the three antibodies was diluted 1:500 with PBS and incubated with haemocytes for 45 min at room temperature. After three washes with PBS, the cells were incubated with a 1:80-diluted fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit secondary antibody for 45 min. The resulting haemocytes were washed three times in PBS to remove unbound or non-specific
binding dye and observed under a confocal microscope (IX70; Olympus) in FITC mode after adding one drop of 50 % glycerol on a coverslip.

**Transient expression in non-parasitized *P. xylostella*.** A full-length CpBV H4 ORF was produced using two gene-specific primers: 5'-GGATCCATGGCAGTCAGCTC-3' (forward) and 5'-GAATTCTCAACTTTACATACAG-3' (reverse; BamHI and EcoRI sites are underlined). After restriction digestion with the indicated enzymes, the PCR product was cloned into a eukaryotic expression vector, pBacPAK9 (Clontech Laboratories) using T4 DNA ligase (Bioneer). For in vivo transfection of the vector into *P. xylostella* larvae, we used Metafectene PRO transfection reagent (Biontex). Briefly, 0.5 μg pBacPAK9 or pBacPAK9 recombinant vector was mixed with 3 μl transfection reagent and incubated for 20 min at room temperature to allow DNA–lipid complexes to be formed before injection into the haemocoel of second-instar *P. xylostella*. The success of transfection was analysed by RT-PCR using gene-specific primers, and protein expression was analysed by Western blotting or IFA using anti-CpBV H4 antibody.

**RNAi.** RNAi used dsRNA prepared with a Megascript RNAi kit (Ambion) according to the manufacturer’s instruction. The N-terminal protruding region was amplified with two gene-specific primers: 5’-GGATCCATGGCAGTCAGCTC-3’ (forward) and 5’-GAATTCTCAACTTTACATACAG-3’ (reverse). The PCR product (190 bp) was cloned into pcR2.1 and its orientation confirmed to get two oppositely inserted clones. After digestion at the 3’ end with BamHI, the two directional linearized DNA templates were used for in vitro transcription reactions. Sense and antisense strands were synthesized using T7 RNA polymerase. Annealing of both strands to form dsRNA was performed by heating the reaction to 75 °C for 5 min followed by mixing and cooling to room temperature. DNA and ssRNA were digested using DNase I and RNase, respectively. The resulting dsRNA was purified and eluted in elution buffer [10 mM Tris/HCl (pH 7.0), 1 mM EDTA]. For transfection, 1 μg dsRNA was mixed with 0.5 μg pBacPAK9 vector or its recombinant, into which 3 μl Metafectene PRO transfection reagent was added, and injected into the haemocoel of second-instar *P. xylostella* larvae. The efficiency of RNAi was evaluated by RT-PCR in terms of the knockdown of CpBV H4 gene expression. As a reference, *P. xylostella* H4 gene expression was determined.

**Statistical analysis.** Treatment means and variances were analysed in a one-way ANOVA using PROC GLM of the sas program (SAS Institute, 1989). All means were compared by least-squared difference (LSD) test using type I error=0.05.

**RESULTS**

**Comparison of host and viral H4 genes**

Compared with a typical H4, CpBV H4 has an extended N-terminal region with several lysine residues (Ibrahim *et al.*, 2005). This structure led us to suggest that CpBV H4 may interfere with target insect nucleosome structure. To begin with, we needed to clone the H4 gene of *P. xylostella*, which could be used as a target molecule to compete with CpBV H4. Based on known insect H4 amino acid sequences, degenerate primers were designed and used to produce a single PCR product, which was sequenced (Fig. 1a). The predicted amino acid sequence strongly matched those of known insect H4s, with 97–100 % identity (Fig. 1b).

CpBV H4 was longer by 38 aa in the N-terminal region than H4 of *P. xylostella* (Fig. 1c). When the H4 amino acid sequence of *P. xylostella* was compared with that of CpBV H4, they shared 82.5 % identity except for the protruding N-terminal region. Lysine residues in the N-terminal region are usually target sites for H4 acetylation (Peterson & Laniel, 2004). The protruding N-terminal region of CpBV H4 contained nine lysine residues, which were 6, 12, 16, 19, 23, 26, 30, 33 and 37 from the N terminus.

**Developmental changes of the host and viral H4 proteins**

Protein levels of CpBV H4 in the parasitized *P. xylostella* were monitored using an antibody raised against the protruding N-terminal as an epitope (Fig. 2). A pan-H4 antibody reacted with the nuclear extracts of both parasitized and non-parasitized *P. xylostella*. As expected, parasitized *P. xylostella* showed two different H4 bands. The antibody specific for CpBV H4 reacted only with the nuclear extract of parasitized *P. xylostella*. Interestingly, an antibody raised against acetylated H4 reacted significantly only with the nuclear extract of parasitized *P. xylostella*, in which the response increased with developmental stage of the parasite.

**Presence of CpBV H4 in haemocytes**

To determine the localization of CpBV H4 in the target cells, haemocytes of parasitized and non-parasitized *P. xylostella* were used and compared (Fig. 3). The pan-H4 antibody reacted against both parasitized and non-parasitized haemocytes and showed that most H4 was located in the nucleus. In contrast, the CpBV H4-specific antibody reacted only with parasitized haemocytes, showing localization in the nucleus. As expected from the Western blot assay, the antibody raised against acetylated H4 reacted only with parasitized haemocytes.

**Effect of transient expression of CpBV H4 on haemocyte-spreading behaviour**

The observations that CpBV H4 is expressed and mostly localized to the nucleus of haemocytes suggested that it might interrupt the functional defence response of parasitized haemocytes. A eukaryotic vector, pBacPAK9, was recombined with CpBV H4 to induce transient expression in non-parasitized *P. xylostella*. *P. xylostella* transfected with the recombinant vector produced CpBV H4 for at least 3 days, as shown by RT-PCR (Fig. 4a) and Western blotting (Fig. 4b). Haemocytes collected from transfected *P. xylostella* also showed CpBV H4 expression by IFA as shown in Fig. 3. The transfected haemocytes were analysed based on their spreading ability (Fig. 4c), in which the vector together with CpBV H4 significantly interfered with the haemocyte-spreading efficacy of the transfected larvae compared with larvae transfected with vector alone.
Rescue effect of RNAi on the inhibitory activity of CpBV H4

The inhibitory effect of transiently expressed CpBV H4 on haemocyte-spreading behaviour was further proven by specific knockdown of the gene through RNAi (Fig. 5). Injection of dsRNA prepared from the N-terminal region of CpBV H4 resulted in complete knockdown of CpBV H4 expression, but did not affect the expression of the target host (P. xylostella) H4 (Fig. 4a). The larvae transfected with dsRNA were significantly rescued from the inhibitory action on haemocyte spreading induced by CpBV H4 (Fig. 4b).

DISCUSSION

H4 is required for the chromatin structure of eukaryotes and exhibits a highly conserved amino acid sequence among biological taxa (DeLange & Smith, 1971; Thatcher & Gorovsky, 1994; Pineau et al., 2004). In this study, we cloned the H4 gene of P. xylostella, the predicted amino acid sequence of which reflects this highly conserved nature. In comparison, CpBV H4 is a novel viral type of histone H4. Similar polydnaviral H4s have been found in the genomes of Cotesia congregata bracovirus (CcBV) (Espagne et al., 2004) and Cotesia glomerata bracovirus
Fig. 3. Indirect IFA of host and viral H4 in haemocytes of P. xylostella non-parasitized (NP) or parasitized (P) by C. plutellae. Haemocytes were collected from 5-day-old larvae after parasitization. The haemocytes were observed under a confocal microscope in differential interference contrast (DIC) or FITC mode using a 60× oil-immersion lens. H4, Ac-H4 and CpBV H4 represent pan-H4 antibody, antibody against acetyl-lysine H4 and antibody against CpBV-expressed H4, respectively.

Fig. 4. Transient expression of CpBV H4 in non-parasitized P. xylostella larvae by microinjection of pBacPAK9 recombined with CpBV H4, or vector alone. Results are shown for RT-PCR (a) and Western blotting (b) of CpBV H4 after microinjection. In (b), vector alone did not show a band (data not shown). (c) Haemocyte-spreading assay of injected larvae (2 days after microinjection). Each treatment was replicated ten times. Different letters (x–z) above SD bars indicate a significant difference among their means at type I error $\alpha = 0.05$ (LSD test).

Fig. 5. Recovery of immune responsiveness by RNAi using dsRNA of CpBV H4 in P. xylostella transfected with a transient recombinant expression vector, pBacPAK9-CpBV-H4. (a) RTPCR of CpBV H4 with or without dsRNA treatment. H4 expression of P. xylostella (Px H4) served as a positive control to confirm the specificity of the dsRNA treatment. (b) Effect of dsRNA on the haemocyte-spreading behaviour of P. xylostella transfected with pBACPAK9-CpBV-H4. Each treatment was replicated ten times. Different letters (x–z) above SD bars indicate a significant difference among their means at type I error $\alpha = 0.05$ (LSD test).
(CgBV) (Kim et al., 2006b). All three H4s have their own extended N-terminal region compared with the typical host H4, and CpBV H4 shows high amino acid sequence identities with CcBV-H4 (73.8 %) and CgBV-H4 (97.2 %), respectively. These H4 genes, as well as viral lectin genes, are found only in Cotesia-associated bracoviruses, whilst some gene families such as the ankyrin repeat motif or protein tyrosine phosphatase families are found in relatively broad polydnaviral groups (Kroemer & Webb, 2004; Kim et al., 2006a, 2007). These specific gene families according to viral groups appear to support the hypothesis that the polydnavirus comes from the wasp chromosome and can be regarded as a delivering package derived from the host wasp (Whitfield, 1990).

CpBV H4 is 38 aa longer than the typical eukaryotic histone H4s including that of P. xylostella. The extra residues are in the N-terminal extended region, which contains nine lysine residues. Relative activities of HAT and HDAC may control acetylation status at these lysines. The histone code hypothesis explains that a single acetylation at lysine-16 is a prevalent and specific post-translational modification in eukaryotes and that acetylation of H4 (especially at the N-terminal region of genes (Dhalluin et al., 1999). The other comes from destabilization of the chromatin fibre by histone acetylation, because acetylation neutralizes the positive charge of the lysine residue, which would bind a negatively charged component in an adjacent nucleosome, resulting in increased local accessibility to the transcriptional machinery (Tse et al., 1998; Wolffe & Hayes, 1999). For example, acetylation of lysine-16 is strongly associated with a specific regulatory function in yeast, where its acetylation state regulates the extent of silent heterochromatin (Kimura et al., 2002). Considering the overall inhibitory action of parasitism, CpBV H4 may compete with target host H4 in terms of lysine acetylation at the histone tail. Thus, trapping of different sequence-specific acetyl-lysine-binding complexes to the viral H4 may adversely affect gene expression of P. xylostella to maintain its normal developmental physiology.

The current observation of CpBV H4 in the haemocytes of parasitized larvae suggests that the gene product may interrupt haemocyte function. To test this hypothesis, we used a transient expression technique to specifically deliver this viral gene to non-parasitized P. xylostella. Transient expression has been used as a powerful method of studying polydnavirus gene function: transient transfection of Glc1.8 of Microplitis demolitor bracovirus into High Five and S2 insect cell lines revealed its role in inducing loss of adhesion (Beck & Strand, 2005). The recombinant pBacPAK9 containing CpBV H4 under a polyhedrin promoter expressed the viral gene effectively in transfected P. xylostella. Generally, in a baculovirus, the polyhedrin gene can be optimally expressed by the trans-activating action of specific transcriptional factors (Lu & Miller, 1995; Lin & Blissard, 2002). Expression of CpBV H4 in pBacPAK9 suggested that its level may be basal or that transcriptional factor(s) originating from P. xylostella may trans-activate the polyhedrin promoter to express CpBV H4. Similar transient expression has been performed for expression of CpBV-PTP genes under the polyhedrin promoter in non-parasitized P. xylostella, which also showed significant gene expression (Ibrahim & Kim, 2007). After transient expression of CpBV H4, haemocytes significantly lost their spreading activity. The inhibitory effect of CpBV H4 on the haemocyte population was further demonstrated by an RNAi-mediated decrease in CpBV H4 expression. dsRNA together with CpBV H4 completely reduced the corresponding mRNA levels and resulted in the recovery of haemocyte-spreading activity.

There have been several immunosuppressive genes identified so far in CpBV. CpBV PTPs, which comprise more than 30 genes encoded by CpBV, inhibit plasmacytoid spreading and encapsulation of P. xylostella (Ibrahim et al., 2007; Kim et al., 2007). CpBV154 inhibits haemocyte spreading by interfering with protein synthesis in the haemocytes (Nalini & Kim, 2007). In addition, CpBV EP1-like protein and CpBV IkB (a viral ankyrin gene) have been suspected to play immunosuppressive roles in parasitized P. xylostella (Kim et al., 2006a; Lee et al., 2005). Moreover, teratocytes and calyx fluid also showed an additive effect on the immunosuppressive actions of CpBV (Andrew et al., 2006). Thus, we may ask why C. plutellae needs several factors to induce host immunosuppression. They may act
at different levels of immune reactions such as immune-associated molecular targets or different stages of parasitism. Alternatively, an estimation of polydnavirus gene expression in the parasitized host may address this question as even the most highly expressed *Chelonus inanitus* bracovirus genes were shown to reach maximal levels that were still 250 times lower than actin (Weber et al., 2007). This suggests that several CpBV immunosuppressive genes may have certain limitations in their contribution because of the low levels of their transcripts and so should act together in a concerted manner, in which teratocyte and calyx fluid can give additive effects on inducing host immunosuppression as well as developmental alteration.

This study showed an immunosuppressive effect of CpBV H4 on haemocyte spreading of *P. xylostella* by transient expression and RNAi assays. However, its persistent expression pattern and its increase in acetylation status in the late developmental stage of parasitism suggest that CpBV H4 may influence the expression of various genes in parasitized *P. xylostella*. This hypothesis may be further explored by proteomic analysis.

**ACKNOWLEDGEMENTS**

This study was funded by Biogreen 21 programme of the Rural Development Administration. W. G. was financially supported by the second stage of BK21. We appreciate Dr Jae Young Choi and Dr Yeon Ho Je of Seoul National University, Seoul, Korea, for sharing genome sequence information for CpBV. We also appreciate Dr Nalini Madanagopal for her critical comments on this manuscript.

**REFERENCES**


