INTRODUCTION

Certain endoparasitic wasps carry polydnaviruses (PDVs), a family of insect viruses characterized by a multipartite or segmented double-stranded DNA genome (Fleming, 1992; Webb, 1998). Two genera are recognized in this virus family: the bracoviruses, which are associated with braconid wasps, and the ichnoviruses, which are associated with the ichneumonid wasps (Stoltz et al., 1995). Because virus particles classified in each genus are quite different in morphology, it is thought that their lineages are separate. However, both bracoviruses and ichnoviruses are of crucial importance for the survival of the braconid wasps and ichneumonid wasps, respectively. During oviposition, female wasps inject PDV virions with egg(s) into their host insect, mostly lepidopteran larvae. They do not replicate in the parasitized hosts but they induce a variety of physiological changes in development and immunity through expression of their genome genes. Such an alteration of the host physiology contributes to survival of the eggs and larvae of the wasps. Hence, PDVs are unique in terms of the obligate mutualistic association with their parasitoid wasps.

PDV genomes exist in two states, as integrated proviral DNA in the wasp chromosomal DNA and as extrachromosomal DNA segments within the virions (Fleming and Summers, 1991). Both forms of viral DNAs are essential for vertical transmission as an endogenous provirus. It has been reported that the replication of PDVs is observed in the nucleus of specialized cells (calyx cells) of the female wasp reproductive tract from where they are secreted into the oviduct. Most of this basic knowledge of PDVs stem from investigations with the ichnovirus of *Campoletis sonorensis*, although some of the common characters of PDVs in bracoviruses have also been demonstrated (Stoltz, 1993; Albrecht et al., 1994).

*Cotesia kariyai* polydnavirus (CkPDV) is a bracovirus which has been demonstrated to be an essential factor for the successful parasitization of *C. kariyai* wasps against the host insect *Mythimna separata* larvae (Hayakawa et al., 1994). Larval weight gain was significantly reduced and pupation was delayed in CkPDV-injected larvae relative to sham injected larvae (Hayakawa and Yasuhara, 1993). Further, one of the CkPDV envelope proteins functions as an immunoevasive mediator for the cellular defence reaction of the host insect.

**Analysis in the course of polydnavirus replication in ovarian calyx cells of the parasitoid wasp, Cotesia kariyai** (Hymenoptera: Braconidae)

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Abstract

The parasitoid wasp *Cotesia kariyai* oviposits into larvae of the armyworm *Mythimna (=Pseudaletia) separata*. At this time, the coinjection of a polydnavirus and venom is prerequisite for successful parasitoid development. Polydnaviruses are responsible for developmental arrest and interference with the host’s immune system; thereby they are called symbiont viruses of the parasitoid wasps. In this study, we demonstrated that *C. kariyai* polydnaviruses (CkPDVs) replicate in ovarian calyx cells of the host female wasps after pupation. In the course of the replication, DNA synthesis commences by one day after pupation, being followed by capsid protein synthesis. Almost one day after the initiation of the capsid protein synthesis, envelope proteins become visible in the lateral oviducts. Therefore, it is reasonable to assume that the complete virion of CkPDV is not composed simultaneously but requires gradual steps.

**Key words:** Parasitoid wasp, polydnavirus, calyx cells
(Hayakawa and Yazaki, 1997). However, not only the replication mechanism of CkPDV but also the replication site has not been investigated. For any species of PDVs the mechanism by which the multiple viral genome DNAs are encapsidated in nucleocapsids and enveloped has not been elucidated. Therefore, it is certainly important to identify the replication site of CkPDV and analyze the process of replication.

MATERIALS AND METHODS

Animals. Armyworms M. separata, were reared on an artificial diet at 25±1°C with a photoperiod of 16 h light:8 h dark. Parasitization by C. kariyai was carried out by exposing prospective hosts (day 0 last instar larvae) to female wasps. Endoparasitoid wasp C. kariyai was reared on the host M. separata under the above conditions. Adult wasps were maintained with honey. Penultimate instar larvae undergoing ecdysis between 2 and 2.5 h after lights on were designated as day 0 last instar larvae (Hayakawa, 1990).

Preparation of anti-C. kariyai polydnavirus (CkPDV) capsid protein antibodies. C. kariyai polydnavirus (CkPDV) particles were purified by using sucrose density gradient centrifugation as described in Hayakawa et al. (1994). Purified CkPDV particles were washed five times in NaCl/Pi (8 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl and 2.7 mM KCl, pH 7.2) by sedimentation and resuspension. After washing, the particles were incubated in NaCl/Pi containing 1% Nonidet P-40 on ice for 15 min, centrifuged at 11,000 × g for 5 min at 4°C and the pellet was washed three times with 1% Nonidet P-40 in NaCl/Pi (Webb and Luckhart, 1994). The washed pellet was subcutaneously injected into mice for preparation of anti-capsid protein serum. IgG in the serum was purified by ammonium sulfate fractionation and an affinity column of protein A agarose (Harlow and Lane, 1988).

Preparation of anti-CkPDV envelope protein antibodies. We previously identified two components of the CkPDV envelope proteins and named them immunoevasive protein-1 (IEP-1) and -2 (IEP-2), because they function as an immunoevasive mediator in the cellular defence reaction of the host armyworm larvae (Hayakawa and Yazaki, 1997). IEP-1 produced by a bacterial expression system was used as antigens for immunizing rabbits. Anti-IEP-1 antibody was used as CkPDV envelope protein IgGs. Previous experiments demonstrated that this antibody cross-reacts with IEP-2 at the same extent as IEP-1.

Identification and quantification of CkPDV capsid and envelope proteins. To identify CkPDV capsid and envelope proteins, the reproductive tracts were collected from female wasps into liquid nitrogen and homogenized in NaCl/Pi containing 1% SDS. The homogenates were centrifuged at 17,400 × g for 10 min to remove cell debris and the supernatants used as samples for SDS/PAGE (10%) after incubating with 80 mM Tris/HCl buffer (pH 8.8) containing 1% SDS and 2.5% 2-mercaptoethanol in boiling water for 5 min. The PAGE gels were developed and stained with Coomassie Brilliant Blue R-250 (Laemmli, 1970). Protein bands on SDS-PAGE gel were electrically transferred to PVDF membrane filters essentially according to the method of Burnette (1981). Immunostaining with the anti-IEP-1 IgG was performed using peroxidase conjugated secondary antibodies according to the method of Hiraoka et al. (1995).

Immunoreactive bands were photographed using a cooled CCD camera and quantified using the AIS image analysis system (Imaging Research Co., St. Catharines, Ontario, Canada). For each experiment, a standard curve was calculated using linear regression and the equivalence point was determined (Noguchi and Hayakawa, 2001).

Immunocytochemical detection of DNA synthesis. Female reproductive tracts were dissected three days after pupation and incubated in TC-100 medium (Sigma Chemical Co., U.S.A.). Cells in S phase were identified by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) using a Cell Proliferation Kit (Amersham). Tissues were fixed in 4% paraformaldehyde in NaCl/Pi, embedded in paraffin and 5 μm-thick sections were prepared for immunocytochemical detection as described in the manufacturer’s instructions.

Measurement of DNA synthesis. The lateral oviduct regions containing the calyx cells were excised from the reproductive tracts of three female wasps and incubated in 50 μl of TC-100 medium containing 1 μCi 3H-thymidine at 25°C for 6 h. The incubated tissues were washed three times in NaCl/Pi by sedimentation and resuspension. Tis-
sues were then lysed with 100 μl 0.3 N NaOH and the incorporated radio activity counted with a liquid scintillation counter (Aloka LSC-5100).

**Electron microscopy.** Adult female reproductive tracts were dissected in cold HEPES buffered saline (HBS) (50 mM HEPES, 37 mM NaCl and 27 mM KCl, pH 7.5) and fixed with 4% glutaraldehyde in HBS at 4°C. Postfixation and staining was performed in 2% aqueous OsO_4 and 2% aqueous uranyl acetate, respectively. The tissue was embedded in Epon 812 (TAAB Laboratories Equipment Ltd., England) after dehydration. Thin sections were cut on an Ultracut microtome (Reichert-Jung, Germany). For electron microscopy, thin sections were briefly stained in 2% aqueous uranyl acetate and 0.1% lead citrate. Micrographs were taken with a JEM-1200EX (Jeol Ltd., Japan) electron microscope.

**RESULTS AND DISCUSSION**

*C. kariyai* polydnavirus (CkPDV) particles isolated from adult female wasps consist of nucleocapsids and envelope proteins (Hayakawa and Yazaki, 1997). Proteins extracted from the isolated nucleocapsids and immunoevasive protein-1 (IEP-1, a component of viral envelope proteins) were used to rise anti-capsid protein and envelope protein IgGs. Five CkPDV capsid proteins and two envelope proteins that cross-reacted with the respective antibodies were also detectable in the extracts of reproductive tracts isolated from adult female wasps (Fig. 1). To determine the points in development when capsid and envelope proteins are synthesized, both of the immuno-positive proteins were quantified throughout pupal-adult development. While capsid proteins were detectable from two days after pupation, envelope proteins became visible from three days after pupation; thereby, syntheses of capsid proteins preceded that of envelope proteins by one day (Fig. 2A, B). When envelope protein synthesis was initiated the lateral oviduct region of the reproductive tract began enlarging (Fig. 2C). The measurement of DNA synthesis in the calyx regions of the female reproductive tracts showed that DNA synthesis commenced one day after pupation (Fig. 2D).

To confirm that the DNA synthesis occurs in the calyx cells adjacent to the lateral oviducts, microscopic observations were performed after labelling the reproductive tracts with a 6-h pulse of 5-bromo-2'-deoxyuridine (BrdU). As we expected, only the calyx cells incorporated BrdU (Fig. 3). The central cells in the calyx region seemed to be stained weaker than the surrounding cells, however, we do not know if this difference is due to the intrinsic difference of these cells.

Finally, we tried to identify CkPDV virions in the reproductive tracts by electron microscopy. Since DNA synthesis was detected only in the calyx cells, the end region of the ovary containing the oviduct and calyx cells were extensively observed. As shown in Fig. 4, electron-dense virus-like particles were found in the ovarian calyx cells of adult female wasps. By magnifying the micrograph, the virions composed of nucleocapsids enveloped by a single unit membrane could be identified. Because it has been reported that this ultrastructure is typical of bracoviruses (Stoltz, 1993), it is reasonable to conclude that these virus-like particles are CkPDV virions.

In the present study, we investigated the process of CkPDV replication in the reproductive tracts of female wasps by measuring the concentration of major components of the virions, such as genomic DNAs, capsid proteins and envelope proteins. Genomic DNA synthesis commenced one day after pupation and synthesis of capsid proteins began two days after pupation, while synthesis of envelope proteins began three days after pupation. Further analysis of Polydnavirus Replication 325
ther, prior studies demonstrated that the envelope proteins, IEP-1 and -2, were not synthesized in the calyx cells but in the lateral oviduct cells (Tanaka et al., unpublished). Based on these observations, it is reasonable to assume that the viral genome DNAs and some proteins are synthesized to form nucleocapsids in the calyx cells and released into the oviducts, where envelope proteins cover the surface of the immature virions. It has been demonstrated that the envelope protein, IEP-1, possesses an immunoevasive activity from the cellular defence reaction of the host *M. separata*. Therefore, the wasp eggs with many virions attached could avoid being attacked by the cellular defence reaction from the *M. separata* larvae.

It has been reported that *C. sonorensis* PDV replication was induced by treatments that increased the ecdysteroid titer (Webb and Summers, 1992). Although it is plausible that CkPDV replication is also regulated by such hormones, we have not yet investigated this possibility. Because PDVs are indispensable factors for many parasitoid wasps, basic analyses about PDVs will undoubtedly advance our understanding on the evolution-
ary or physiological relationships between parasitoid wasps and their host insects.

REFERENCES


Noguchi, H. and Y. Hayakawa (2001) Dopamine is a key factor for the induction of egg diapause of the silkworm.


