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Apparent Functional Role for a Cysteine-Rich Polydnavirus Protein in Suppression of the Insect Cellular Immune Response†

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Polydnaviruses suppress the cellular immune response and inhibit growth and development in their lepidopteran host, allowing survival of their endoparasitic hymenopteran host. Characterization of genes disrupting insect physiological systems is a major objective in the study of polydnaviruses. Recently, a cysteine-rich gene family encoding a motif composed of invariable cysteine residues flanking hypervariable intercysteine amino acids was described (S. D. Dib-Hajj, B. A. Webb, and M. D. Summers, Proc. Natl. Acad. Sci. USA 90:3765–3769, 1993). They noted similarities to the positive selection pressure for mutations within the vertebrate major histocompatibility complex (MHC) class II genes and speculated that this class of polydnavirus genes may target and disrupt the insect immune system. To study the functional activity of this family of predicted cysteine-rich proteins, the VHv1.1 gene product was produced from bacterial and baculovirus expression systems. Polyclonal antiserum produced from the bacterial fusion protein reacted with a 30-kDa protein from hemocytes, cell-free plasma, and fat body of parasitized larvae. Immunofluorescence analysis of hemocytes from parasitized insects detected the 30-kDa protein bound to granulocytes and plasmatocytes. To assay the functional activity of the 30-kDa VHv1.1 protein, a recombinant baculovirus was constructed allowing in vivo expression of the 30-kDa polydnavirus protein from infected insects. Expression of the VHv1.1 protein from the baculovirus system reduced the encapsulation response to washed wasp eggs relative to controls. The experimental evidence demonstrates that *Campoletis sonorensis* polydnavirus-infected cells secrete VHv1.1 into the hemolymph, where it binds to hemocytes and is associated with the inhibition of the cellular immune response.

Parasitization by endoparasitic wasps produces rapid alterations in host physiology that allow survival of the wasp egg and result in the eventual death of the parasitized insect (30). Polydnaviruses (1, 12, 13), venom (19, 32), teratocytes (9), and ovarian proteins (36) interact to disrupt the affected physiological systems in various host-parasite systems. Introduction of the *Campoletis sonorensis* polydnavirus (CsPDV) into *Heliothis virescens* larvae causes dramatic host physiological changes that result in retarded growth and suppression of the cellular immune response (12, 13) mimicking natural parasitization (34). Injection of inactive CsPDV, venom, and ovarian proteins in any combination does not produce the complement of characteristic symptoms associated with natural parasitization (10, 12, 13, 33).

Polydnaviruses have been found in parasitic members of the order *Hymenoptera* in the families *Braconidae* and *Ichneumonidae* and are characterized by double-stranded, superhelical DNA genomes that are heterologous in size (29). The genome of CsPDV consists of at least 28 superhelical DNA segments, ranging in size from 5 to 21 kb (4, 20). Superhelical DNA segments are integrated into wasp chromosomes and are vertically transmitted (14, 16, 28, 41). The virus replicates in the calyx epithelium of the wasp oviduct and buds into the oviduct lumen. During oviposition, CsPDV is injected with venom and ovarian proteins into the lepidopteran host, *H. virescens*. Viral replication is not detected in parasitized larvae (33), but viral transcripts appear as early as 2 to 6 h postparasitization (pp) and continue throughout the course of parasitization (9 days) (2, 4, 5, 15, 20).

Two viral genes, WHv1.0 and WHv1.6, encode 1.0- and 1.6-kb mRNAs, respectively, that are abundantly expressed in parasitized insects and map to superhelical DNA segment W (3). These two genes have regions of significant similarity in nucleotide and predicted amino acid sequences (3, 11). Two other abundantly expressed mRNAs of 1.1 and 1.4 kb, also hybridized to the same viral genomic (5) and cDNA (39) probes but did not hybridize with WHv1.0 and WHv1.6. On the basis of hybridization analyses, the 1.1- and 1.4-kb genes were thought to represent a polydnavirus gene family that was unrelated to the 1.0- and 1.6-kb genes (5). However, sequence comparison of the VHv1.1, WHv1.0, and WHv1.6 genes demonstrated that these genes have related structures that include absolutely conserved splicing sites in coding and noncoding regions (11). Comparison of predicted amino acid sequences identified conserved cysteine-rich motifs similar to those of conotoxins (23). Moreover, the conserved cysteine residues are separated by highly variable intervening residues, an organization that is similar to the cysteine motifs described from conotoxins (23). On the basis of their similar gene structure, their conserved cysteine motifs, and their expression only in parasitized insects, Dib-Hajj et al. (11) proposed that these genes constitute a cysteine-rich polydnavirus gene family. Although the abundant, species-specific expression of this gene family strongly suggests an important biological activity, functions for the cysteine-rich genes have not been described.

To elucidate the functional activities of cysteine-rich CsPDV proteins, we have expressed a polydnavirus gene in a bacterial

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system to facilitate antibody production. The antiserum detected a viral gene product which was found only in parasitized insects and was associated with host granulocytes and plasmatocytes. A baculovirus construct was then developed to express the VHv1.1 gene in infected larvae. Expression of the 30-kDa VHv1.1 gene product in larvae reduced the encapsulation response, demonstrating that this polydnavirus gene product inhibits the host's cellular immune response.

MATERIALS AND METHODS

Vector construction and gene expression. To express the VHv1.1-encoded protein in a bacterial expression system, the 3′ terminus of the VHv1.1 gene was released by restriction digestion of pcHv900 (39) with *Hae*III and *Eco*RI to produce an 879-bp fragment. This portion of the VHv1.1 gene has 567 nucleotides, encoding the C-terminal 189 amino acids of the 30-kDa protein (30-kDa/VHv1.1) and 312 bp of 3′ noncoding sequence. All of the 5′ untranslated leader sequence and 84 nucleotides encoding the N-terminal 28 amino acids, including the putative 16-amino-acid signal peptide, were removed from the VHv1.1 gene in this construct. A phosphorylated *Eco*RI linker [d(GGAATTCC)] was ligated to the 5′ end of the gene to produce an *Eco*RI fragment, which was then digested with *Eco*RI and ligated to a compatibly digested bacterial fusion vector (pMAL-cr1) (22). This construct allowed in-frame insertion of the VHv1.1 gene 3′ to the *malE* gene, to generate a maltose-binding protein (MBP)-polydnavirus fusion protein (MBP/VHv1.1). The orientation and junctions of the construct were confirmed by DNA sequence analysis from the *malE* primer (5′-GGTCGTCAGACTGTCGATGAAGCC-3′; New England BioLabs). The MBP fusion protein was induced by IPTG (isopropyl thiogalactopyranoside) and purified from bacterial lysates according to the manufacturer's specifications (New England BioLabs, Beverly, Mass.) (22). The 30-kDa/VHv1.1 protein was cleaved from MBP with factor Xa for immunization and immunoblot analyses.

To express the VHv1.1 gene in the baculovirus system, a full-length VHv1.1 cDNA was constructed in pBluescript(+) (Stratagene). A 5′ PCR fragment (PCR119) produced to complete the VHv1.1 cDNA sequence (11) was digested with *Eco*RI and *Sph*I. The 81-bp PCR fragment containing 54 bp of 5′ coding cDNA sequence was ligated to the C-terminal portion of the VHv1.1 cDNA at the *Sph*I site. The C terminus was released from pcHv900 by *Sph*I and *Pst*I digestion to allow the complete cDNA to be directionally cloned into an *Eco*RI- and *Pst*I-digested pVL1393 baculovirus transfer vector (40). The junctions of transfer vector recombinants were confirmed by DNA sequencing with pVL1393 reverse (5′-GGATTTCCT TGAAGAGAGTGAG-3′) and forward (5′-CAATATAT AGTTGCTGATATCATGGAG-3′) primers. The recombinant transfer vector was then cotransfected with the E2 strain of *Autographa californica* nuclear polyhedrosis virus (Ac*M*-NPV) into *Spodoptera frugiperda* (Sf9) cells to produce the recombinant virus (40). Isolation of recombinant baculovirus was performed by plaque assay according to standard procedures (40). Recombinant virus identity was confirmed by hybridization of pcHv900 cDNA probes to infected-cell lysates (40).

To examine the expression of the VHv1.1 gene, Sf9 cells were infected with the VHv1.1/pVL1393 recombinant baculovirus. A total of 6×10^5 cells per well were seeded in 24-well tissue culture plates and infected with VHv1.1/pVL1393 at a multiplicity of infection of 10. Cells and supernatants were collected at 6-h intervals from 24 to 72 h. Cells and media were separated by centrifugation for immunological analysis by Western blotting (immunoblotting).

Immunological analyses. Preliminary studies indicated that the cleaved 30-kDa/VHv1.1 protein was susceptible to breakdown, while the fusion protein was stable. Therefore, the initial immunization procedure was done with the fusion protein. The purified fusion protein was separated by sodium dodecyl sulfate (SDS)–8% polyacrylamide gel electrophoresis (PAGE), stained with Coomassie blue, and destained (39). The 70-kDa fusion protein was cut from the gel, soaked in 500 ml of distilled water (two times for 30 min each), frozen in liquid N_2, and ground to powder. Immunization procedures were done with Freund's complete and incomplete adjuvants as previously described (18). The instability of the 30-kDa/VHv1.1 protein was later found to be related to freeze-thaw cycles of protein samples. Because reactivity to the MBP could contribute to nonspecific binding, a second antiserum to the 27-kDa protein was similarly produced after it was cleaved from MBP by factor Xa digestion.

Antiserum binding, specificity, and reactivity were analyzed by immunoblot as previously described (37, 38). Briefly, proteins were transferred from 8% gels to Immobilon-P (Millipore; Bedford, Mass.) membrane with a Trans-blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, Calif.) at 15 V for 30 min. The membrane was blocked with BLOTTO (5% nonfat milk in PBS-T [135 mM NaCl, 2.5 mM KCl, 5 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% Tween 20, pH 7.4]) for 1 h and then incubated with the polyclonal antiserum (1:500 to 1:1,500 dilution in BLOTTO) overnight. The membrane was then washed with PBS-T three times for 30 min each at room temperature. Alkaline phosphate-conjugated anti-rabbit immunoglobulin G (IgG; 1:1,000; Sigma) was added in PBS-T to the membrane and incubated for 1 to 3 h. The membrane was washed three times as above with PBS-T to remove secondary antibody and then washed once with PBS for 5 min. Antibody binding was detected by using 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium chloride (GIBCO BRL) as chromogenic substrates.

Insect tissue preparation. *H. virescens* larvae were reared as described by Krell et al. (20). Fourth-instar larvae were parasitized by mated 3- to 5-day-old female wasps. For some experiments, parasitization was confirmed by recovery of parasitoid eggs from the larval hemocoel. Larvae were chilled on ice, and hemolymph from each insect was collected onto Parafilm and then immediately transferred to an ice-cold microcentrifuge tube. The samples were diluted in 1 volume of PBS and 2 volumes of 2× disruption buffer (1× disruption buffer is 125 mM Tris [pH 6.8], 2% SDS, 1% glycerol, 100 mM 2-mercaptoethanol, 0.001% bromophenol blue). Equal volumes (10 μl) of diluted hemolymph were loaded per lane, allowing qualitative comparisons between parasitized and nonparasitized proteins.

For tissue preparations, parasitized and unparasitized fourth instar larvae were chilled on ice and dissected in Pringle's saline (24) under low magnification. Fat body, nervous system, midgut, and Malpighian tubules were individually collected into 200 μl of ice-cold Pringle's saline and then washed four times with 500 μl of cold Pringle's saline. Tissues were resuspended in 200 μl of cold Pringle's saline and homogenized with manual micro-tissue grinders (Fisher). After homogenization, the protein concentration was determined by the Bradford assay (7). Then, an equal volume of 2× disruption buffer was added, and the samples were stored at 4°C for immediate use or at −20°C for later analyses. Equal protein concentrations (10 μg) of tissue samples were used for immunoblot analysis.

For hemocyte preparations, hemolymph was collected from parasitized fourth-instar larvae at 24 h pp. Similarly staged, unparasitized insects were used as controls. Samples were diluted in an equal volume of cold Pringle's saline and pelleted by tabletop centrifugation for 15 min at 4°C and 1,500 × *g*. The supernatant (plasma) was transferred to a fresh microcentrifuge tube, recentrifuged for 10 min at 4°C and 1,500 × *g* to remove any remaining hemocytes, and used for immunoblot analysis. Hemocytes were then washed three times with 500 μl of ice-cold Pringle's saline by resuspending and reprecipitating the hemocytes. Hemocytes and plasma protein concentrations were determined by the Bradford assay (7). Equal concentrations (10 μg) of hemocyte and plasma proteins were used for immunoblot analysis.

Indirect immunofluorescence. For each sample, 15 μl of parasitized or unparasitized insect hemolymph was collected and added to 50 μl of Grace's medium on glass slides (36). Cells were allowed to settle for 5 min, and the medium was replaced with fresh Grace's medium to remove nonadherent cells and to prevent melanization. Cells were allowed to spread in humidified chambers for 1 h, fixed in 3.7% formaldehyde, rinsed in P_i-NaCl solution (100 mM NaCl, 25 mM phosphate buffer [pH 7]), and permeabilized with 0.2% Triton X-100 for 2 min. Cells were then rinsed three times with 50 μl of P_i-NaCl and blocked with normal goat serum (diluted 1:20 with P_i-NaCl) for 15 min at room temperature. The 30-kDa/VHv1.1 antiserum was added (50 μl, 1:500 dilution in P_i-NaCl), and slides were incubated at 37°C for 45 min and then rinsed with P_i-NaCl (1 rinse, 15 min). Fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma; 100 μl, 1:100 in P_i-NaCl) was added, and the slides were incubated for 45 min at 37°C in the dark and washed as described above. To observe fluorescent antibody binding, 1 to 2 drops of 1:1 PBS-glycerol were added to each slide, and hemocytes were examined with Nikon Optiphot epifluorescent illumination at 1,000× magnification.

For overlay experiments, 15 μl of unparasitized hemolymph was added to 50 μl of Grace's medium on glass slides and incubated for 5 min to allow cells to adhere. The medium was removed and replaced with 50 μl of diluted parasitized insect plasma (15 μl of parasitized insect hemolymph, 24 h pp; 35 μl of Grace's medium). Parasitized insect plasma was prepared by collecting parasitized insect hemolymph into an Eppendorf tube and removing hemocytes by centrifugation for 10 min at 2,000 × *g* and 4°C. Cell-free plasma was examined under a microscope to confirm that hemocytes were removed from the plasma.

DNA hybridization. Hemolymph was collected from 20 parasitized fourth-instar larvae at 0 and 24 h pp. Hemocytes and plasma were separated by centrifugation (1,000 × *g*, 4°C, 15 min), and then hemocytes were resuspended and recentrifuged to remove remaining plasma. DNA was extracted from hemocytes and plasma separately by proteinase K digestion and phenol-chloroform-isoamyl alcohol extraction (25:24:1) (15). The DNA was ethanol precipitated, washed with 70% ethanol, and resuspended in 100 μl of TE buffer. Each sample was loaded on the nylon membrane by using a 24-well slot-blot. DNA samples were cross-linked to the membrane by UV treatment and hybridized with the VHv1.1 probe. Membranes were prehybridized at 42°C for 3 h in 6× SSC (1× SSC is 0.15 M sodium citrate plus 0.015 M NaCl)–5× Denhardt's reagent–0.5% SDS–100 μg of denatured, fragmented salmon sperm DNA per ml–50% formamide and then hybridized to ^{32}P-radiolabeled cDNA probe (5×10^8 to 5×10^9 dpm/μg) that was generated by random primer labeling (27) for 18 to 24 h at 42°C. After hybridization, membranes were washed in 2× SSC–0.5% SDS for 5 min, in 2× SSC–0.1% SDS for 15 min at room temperature, and twice in 0.1× SSC–0.5% SDS for 15 min each at 37 and 68°C. Membranes were exposed to X-ray film for 24 h at −70°C.

Recombinant virus injection. Washed eggs were prepared from 20 chilled *C. sonorensis* female wasps. The eggs were dissected from the ovaries of female wasps 5 days after mating. Eggs were suspended in 1 ml of Pringle's saline and collected by centrifugation (1,500 × *g*, 7 min). Eggs were then resuspended in Pringle's saline and pelleted five times to remove CsPDV and ovarian proteins. Recombinant virus (10^4 PFU) in a volume of 1 μl was injected into chilled fourth-instar *H. virescens* larvae with a 10-μl Hamilton microsyringe. The E2 strain of wild-type virus or a saline solution (Pringle's solution) was injected into control larvae (1 μl). Twenty-four hours postinjection, pretreated larvae were injected with washed wasp eggs (8 to 12 eggs per larva) with a finely drawn glass capillary. The encapsulation response to eggs was determined 24 h after egg injection. Preliminary studies indicated that in the absence of virus and ovarian proteins, a strong encapsulation response to parasite eggs was seen at 24 h. If one or more of the eggs had 100 or more adherent hemocytes covering at least one-third of the egg surface, the insect was scored as immunoresponsive. If fewer than 100 hemocytes were adherent on all the eggs recovered, the insect was scored as nonimmunoresponsive. Naturally parasitized insects at 24 h pp were used as controls and always had fewer than 100 adherent hemocytes. Data were analyzed by χ^2 analysis (21).

RESULTS

Expression of the VHv1.1 gene product in bacteria for antibody production. To express the VHv1.1 gene product, the C terminus of the VHv1.1 fragment was subcloned into bacterial fusion vector pMal-cr1 (Materials and Methods). Upon IPTG induction of transformed bacteria, the VHv1.1/pMal-cr1 construct produced VHv1.1 mRNA (data not shown) and a 70-kDa fusion protein composed of a 27-kDa VHv1.1 CsPDV protein fused to the bacterial MBP (Fig. 1A; MBP, 42 kDa). The fusion protein was purified from bacterial lysates by MBP affinity chromatography and then cleaved by factor Xa to release the 42-kDa MBP and a 27-kDa VHv1.1 CsPDV protein (Fig. 1A). A minor band (24 kDa) was also detected after cleavage (Fig. 1A) and may be due to contaminating protease activity or other instability of the protein. It was noted that the amount of the 24-kDa protein increased upon repeated freeze-thaw cycles of the sample. No other factor Xa sites are encoded by the VHv1.1 gene. Injection of the VHv1.1 protein purified from bacterial lysates into *H. virescens* larvae had no detectable effect on larval growth or immunity (data not shown).

Polyclonal antiserum raised against the VHv1.1/MBP fusion protein reacted with both the MBP and the VHv1.1 CsPDV protein (Fig. 1B and 2A). Preimmune serum did not react with VHv1.1 CsPDV protein (Fig. 1B). Parasitized and control insect tissues did not react with an antibody against MBP (data not shown).

Protein detection in parasitized insects. To determine if the VHv1.1/MBP antiserum detected a polydnavirus protein in vivo, hemolymph from naturally parasitized and unparasitized insects was collected for analysis. The 30-kDa/VHv1.1 protein was detected in the parasitized insect hemolymph beginning at 5 h pp and increased in apparent concentration up to 14 h pp. After 14 h, the 30-kDa/VHv1.1 immunoreactive signal remained constant through at least 7 days pp (Fig. 2A). The 14-h sample contains an immunoreactive band at approximately 28 kDa. This sample was subjected to repeated freeze-thaw cycles and is included to illustrate the breakdown of the 30-kDa/

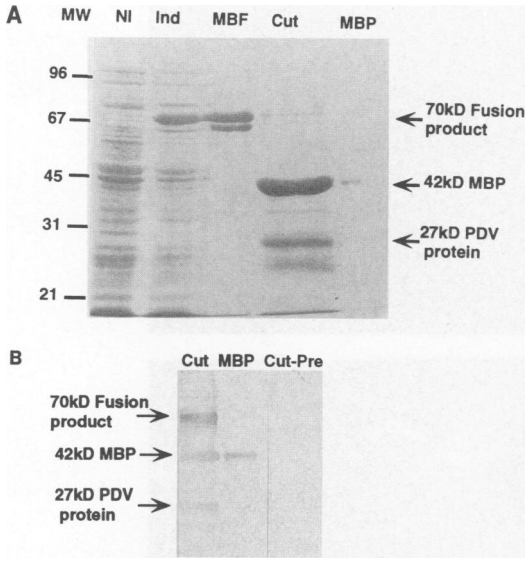

FIG. 1. Expression and detection of the VHv1.1/MBP fusion protein and Western blot. The MBP fusion protein was induced by IPTG and purified from bacterial lysates according to the manufacturer's specifications (New England BioLabs). (A) Recombinant CsPDV fusion protein was visualized on a Coomassie blue-stained SDS–8% PAGE gel. MW, molecular size markers; NI, uninduced *Escherichia coli* cells; Ind, IPTG-induced cells; MBF, purified protein eluted from amylose column with maltose; Cut, purified protein after factor Xa cleavage; MBP, MBP size marker (42 kDa). Arrows indicate the polydnavirus protein (27-kDa PDV), the 42-kDa MBP, and the 70-kDa fusion product. (B) Western blot of CsPDV fusion protein. The purified fusion protein was separated from induced cell lysates, blotted to an Immobilon-P membrane, and probed with polyclonal antiserum raised against the 30-kDa/VHv1.1 fusion protein. Cut, 70-kDa fusion protein, MBP, and CsPDV protein incubated with polyclonal antiserum; MBP, 42-kDa MBP incubated with polyclonal antiserum; Cut-Pre, 70-kDa fusion protein, MBP, and CsPDV protein incubated with preimmune serum.

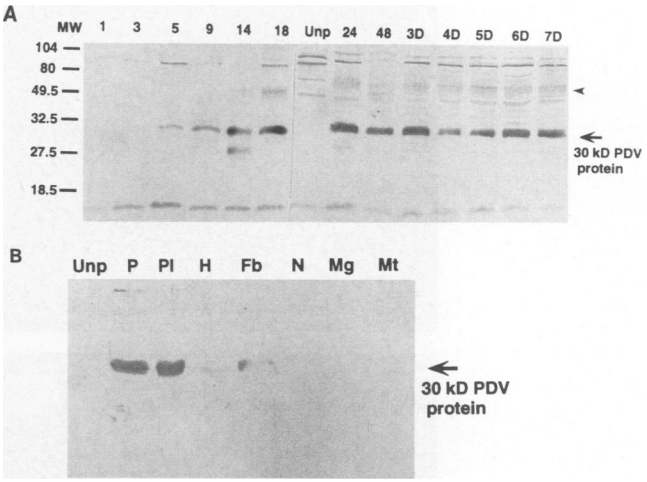

FIG. 2. Time course of VHv1.1 protein expression in the hemolymph and detection of VHv1.1 protein in tissues of *H. virescens* larvae by Western blot. (A) Hemolymph was collected from either parasitized or unparasitized fourth-instar larvae and analyzed with polyclonal antiserum against 30-kDa/VHv1.1 protein. Lanes contain samples taken at various times pp from 1 to 48 h and 3 to 7 days; Unp, unparasitized insect hemolymph. Arrowhead, 50-kDa protein. (B) Parasitized insect tissues, hemolymph, hemocytes, and plasma were collected at 24 h pp as described in Materials and Methods. Ten micrograms of each sample was loaded on an SDS–10% PAGE gel and analyzed by Western blot. Unp, hemolymph of unparasitized insects; P, hemolymph of parasitized insects; Pl, insect plasma; H, hemocytes; Fb, fat body; N, nervous system; Mg, midgut; Mt, Malpighian tubules.

VHv1.1 protein occurring under these conditions. Other hemolymph samples were not subjected to freeze-thaw cycles before analysis. The 30-kDa/VHv1.1 protein was detected predominantly in the hemolymph of parasitized insects. It was also detected, at a lower level, in fat body and hemocytes (Fig. 2B, lanes H and Fb) but was not detected in nervous system, midgut, or Malpighian tubules samples (Fig. 2B, lanes N, Mg, and Mt). Fractionation of the hemolymph into cell-free plasma and hemocytes indicated that the protein was present in both fractions but predominantly in the plasma (Fig. 2B, lane Pl).

Several hemolymph proteins from either parasitized or unparasitized insects reacted nonspecifically to the VHv1.1/MBP antiserum (Fig. 2A) but did not react to the VHv1.1 antiserum (Fig. 2B). Two immunoreactive proteins of 30 and 50 kDa were detected only in parasitized insects with the VHv1.1/MBP antiserum (Fig. 2A). The 30-kDa protein was the major immunoreactive protein in parasitized insects and was, as predicted from sequence analysis, somewhat larger than the protein released from the bacterial fusion construct. Polyclonal antiserum raised against the 27-kDa product (VHv1.1 antiserum) produced by factor Xa digestion had characteristics similar to the VHv1.1/MBP antiserum but higher specificity and did not react nonspecifically to other insect proteins (Fig. 2B). The VHv1.1 antiserum reacted strongly with the 30-kDa protein, and when blots were overdeveloped, the 50-kDa protein was also detected from parasitized insects (data not shown). On the basis of its secretion, molecular weight, and reactivity to the VHv1.1/MBP and VHv1.1 antisera, the 30-kDa protein from parasitized larvae has been identified as the VHv1.1 gene product and is designated 30-kDa/VHv1.1. A 30-kDa immunoreactive protein was also expressed from a VHv1.1 baculovirus construct encoding the entire VHv1.1 cDNA. The VHv1.1 protein expressed from the baculovirus construct cannot be distinguished immunologically from the protein detected in naturally parasitized larvae (Fig. 2B).

The 50-kDa immunoreactive protein (Fig. 2A) may be a related gene product encoded by the 1.4-kb polydnavirus gene. The 1.4-kb mRNA hybridizes strongly to 1.1-kb probes and is thought to encode a closely related protein. The 30-kDa and 50-kDa proteins appear to have a similar distribution in parasitized insects, but the 50-kDa protein is not specifically addressed, as this gene is, at present, uncharacterized.

Immunological localization of the 30-kDa/VHv1.1 protein. Secretion of the 30-kDa/VHv1.1 protein into the hemolymph and detection of the protein in both plasma and hemocytes suggested that the protein may interact directly with hemocytes. To study the nature of interaction between the 30-kDa/VHv1.1 protein and hemocytes, cells from parasitized insects were examined by indirect immunofluorescence labeling with the VHv1.1/MBP antiserum to detect the 30-kDa polydnavirus protein. Parasitized hemocytes reacted nonspecifically with preimmune serum (Fig. 3A). Similarly, the VHv1.1/MBP antiserum did not show specific binding to hemocytes from unparasitized insects (Fig. 3B). Fully spread granulocytes and elongate plasmatocytes were observed, but cell type-specific hemocyte binding was not observed. By contrast, in parasitized insects, specific binding was observed to plasmatocytes and granulocytes but not to other hemocyte types (Fig. 3C). The surface of plasmatocytes showed antibody binding, while

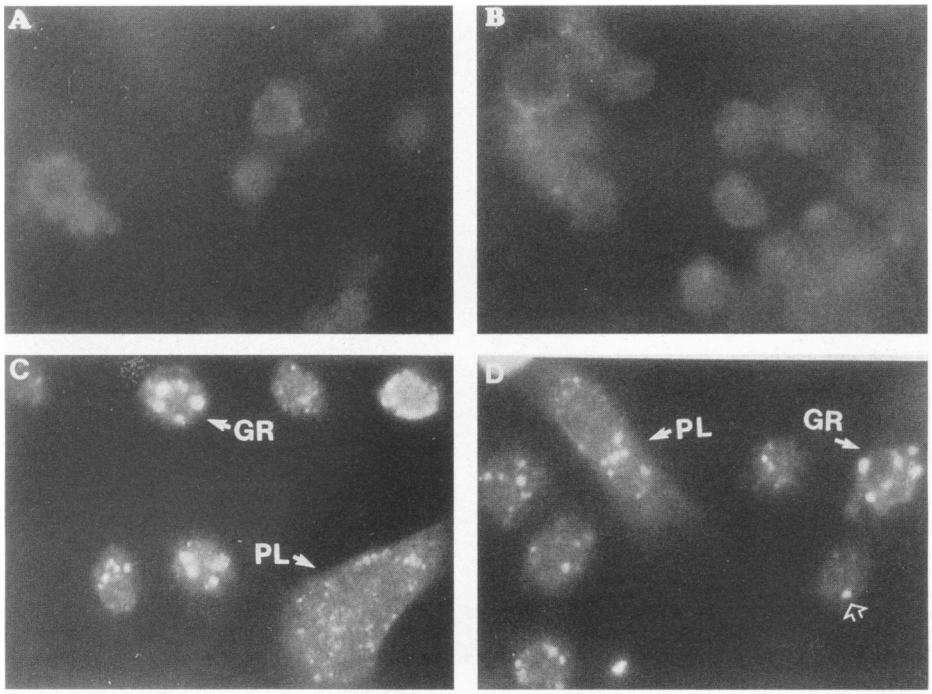

FIG. 3. Immunofluorescence detection of 30-kDa/VHv1.1 CsPDV protein in the hemocytes of *H. virescens* larvae. The cells were spread in vitro for 1 h with Grace's medium and subjected to indirect immunofluorescence labeling as described in Materials and Methods. (A) Hemocytes of parasitized larvae incubated with preimmune serum. (B) Hemocytes of unparasitized larvae incubated with 30-kDa/VHv1.1 antiserum. (C) Hemocytes of parasitized larvae incubated with 30-kDa/VHv1.1 antiserum. (D) Hemocytes of unparasitized larvae overlaid with parasitized plasma and incubated with 30-kDa/VHv1.1 antiserum. Arrows show rounded granulocytes (GRs) and elongate plasmatocytes (PLs). GRs had many immunofluorescent foci, showing that the 30-kDa CsPDV protein was detected on or in the cells (open arrow). PLs had smaller foci and less intense immunofluorescent staining (C and D). This staining was not observed in unparasitized cells (A and B).

brightly stained inclusions were apparent in granulocytes. The granulocyte binding showed heavily stained inclusions, but surface binding was not apparent, perhaps because of morphological differences in these cell types (Fig. 3C). It is possible that the immunoreactivity observed in hemocytes reflected infection of hemocytes with CsPDV, expression of the protein, and detection of the protein prior to its secretion into the hemolymph.

To determine if the protein bound to hemocytes in the absence of viral infection, hemolymph was collected from larvae at 24 h pp. At this time, CsPDV is present in hemocytes but is not detected in the cell-free plasma (Fig. 4). The cell-free plasma does, however, contain the 30-kDa/VHv1.1 protein (Fig. 2B). To apply the protein to hemocytes in the absence of virus infection, 24-h-parasitized cell-free plasma was added to unparasitized hemocytes in vitro. After incubation, the control cells were rinsed, fixed, and assayed for binding of the 30-kDa/VHv1.1 protein. Immunofluorescence labeling was evident and was associated only with granulocytes and plasmatocytes (Fig. 3D). Binding to hemocytes in the overlay experiments could not be distinguished from that observed in parasitized insects. These results demonstrated direct binding of the 30-kDa/VHv1.1 protein to hemocyte types that are involved in the encapsulation response.

Functional assay of the 30-kDa/VHv1.1 protein. To determine if the 30-kDa/VHv1.1 protein is associated with abrogation of the cellular immune response in parasitized insects, a recombinant baculovirus was constructed (Materials and Methods). The VHv1.1/pVL1393 construct allows expression of the 30-kDa/VHv1.1 protein in the absence of other proteins associated with parasitization. The VHv1.1/pVL1393 virus was assayed in cell culture and in vivo for expression of the 30-kDa/VHv1.1 protein. The 30-kDa/VHv1.1 protein was detected beginning at 24 h in the hemolymph of VHv1.1/pVL1393-infected insects, with significant amounts at 30 h and

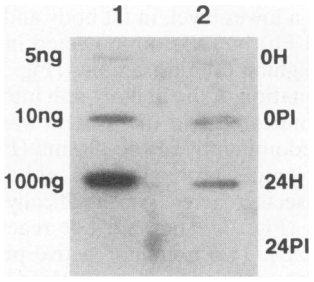

FIG. 4. Hybridization analysis of CsPDV DNA from plasma or hemocytes in parasitized fourth-instar larvae. Hemolymph was collected from 20 parasitized fourth-instar larvae at 0 and 24 h pp. Hemocytes and plasma were separated by centrifugation, and DNA was extracted from hemocytes and plasma separately by proteinase K digestion and phenol-chloroform-isoamyl alcohol extraction. Samples were loaded on a nylon membrane with a 24-well slot-blot and hybridized with the VHv1.1 probe as described in Materials and Methods. Lane 1, CsPDV DNA (control), extracted by the procedure described by Fleming et al. (15). Purified CsPDV DNA at 5, 10, and 100 ng. Lane 2, each sample was collected from 20 parasitized insects at 0 or 24 h pp, as indicated. H, hemocyte DNA; Pl, plasma DNA.

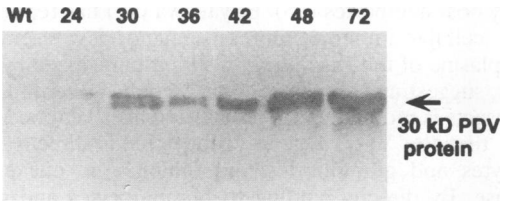

FIG. 5. Time course of VHv1.1/pVL1393 protein expression in Sf9 cells detected by Western blot. A total of 6×10^5 Sf9 cells were infected with either wild-type (Wt) baculovirus or recombinant viruses at a multiplicity of infection of 10, and cultures were collected at various times from 24 to 72 h. Cells were separated from medium by centrifugation (10,000 × *g*, 2 min, 4°C). Two hundred microliters of disruption buffer was added to the each cell pellet. Five microliters of each sample was analyzed on an SDS–10% PAGE gel, transferred to nitrocellulose, and incubated with a 1:1,500 dilution of polyclonal antiserum raised against the fusion protein produced in *E. coli* (see Fig. 4). Wt, wild-type Ac*M*NPV-infected cell lysates at 48 h. VHv1.1/pVL1393 recombinant virus-infected cell lysates were tested at 24, 30, 36, 42, 48, and 72 h.

through 72 h postinfection (Fig. 5). To mimic the introduction of the polydnavirus protein during parasitization, insects were first infected with the recombinant Ac*M*NPV. The infection was allowed to progress for 24 h before injection of washed wasp eggs. The immune response to eggs was then assayed 24 h after egg injection (48 h after baculovirus infection). When washed parasite eggs were injected into uninfected larvae, 69.23% (18 of 26) of the larvae encapsulated one or more of the injected eggs (Table 1). A similar result was observed when parasite eggs were injected 24 h after infection with the E2 wild-type baculovirus (68.96% [20 of 29] of larvae had one or more encapsulated eggs). By contrast, injection of the VHv1.1/pVL1393 recombinant virus reduced the encapsulation response by more than 50% (Table 1; 31% [9 of 29] of larvae have encapsulated eggs), indicating that expression of the 30-kDa/VHv1.1 protein markedly affects the immune system in the absence of other factors from the parasitic wasp. The difference between the encapsulation response of E2- and VHv1.1/pVL1393-infected larvae was statistically significant ($P < 0.005$), demonstrating that the 30-kDa/VHv1.1 protein, when introduced into insect larvae via a recombinant baculovirus, inhibited the cellular immune response to parasite eggs (Table 1).

TABLE 1. Effect of recombinant virus on protection of wasp eggs from encapsulation[a]

Larvae	Recombinant virus	WT virus	Saline (control)	Naturally parasitized
Immunoresponsive	9*,†	20*,‡	18†,‡	0
Nonimmunoresponsive	20*,†	9*,‡	8†,‡	28
Total	29	29	26	28

[a] One microliter of 10^4 PFU of pVL1393:VHv1.1 virus (recombinant) or wild-type (WT) virus was injected into each fourth-instar *H. virescens* larva. One microliter of saline solution was also injected as a control. At 24 h postinjection, 8 to 12 washed wasp eggs were injected into each pretreated larva. The presence or absence of encapsulation was examined 24 h later. Naturally parasitized insects at 24 h pp were also scored as controls. Larvae containing at least one egg with more than 100 attached hemocytes over one-third of the egg's surface were scored as immunoresponsive. Larvae containing eggs with fewer than 20 attached hemocytes (usually none) at 24 h were scored as nonimmunoresponsive. Significance: *, significantly different at $P < 0.005$ ($\chi^2 = 8.344$); †, significantly different at $P < 0.005$ ($\chi^2 = 8.003$); ‡, not significantly different.

DISCUSSION

Polydnaviruses disrupt insect immune and developmental systems after parasitization by insect endoparasites. The primary objective in many studies of polydnaviruses is to determine the mechanisms through which polydnaviruses disrupt insect physiological systems. Molecular and genetic approaches to the study of polydnaviruses have determined that (i) polydnaviruses are integrated into the wasp genome and inherited in a Mendelian manner (14, 30, 41), (ii) viral replication is regulated, occurring only in the female oviduct (30, 33), (iii) viral gene expression is required for successful endoparasite development (1, 8, 12, 13), (iv) viral gene expression differs in the two insect hosts (33), (v) viral genes are spliced (11), and (vi) the viral genome is complex and contains gene families (11). However, the determination of functional activities of polydnavirus genes and gene products has proven more difficult, largely because the insect physiological systems targeted by polydnaviruses are incompletely understood.

To study the functional activity of the VHv1.1-encoded protein, the gene was expressed in a bacterial expression vector as a fusion to the MBP, allowing ready purification of the VHv1.1/MBP fusion protein for use in immunization procedures. The VHv1.1 bacterial construct was not expected to produce a functional protein, as it lacked 28 N-terminal amino acids, including a residue within a potential ATP-binding domain (11). The presence of the cysteine motifs also suggested that the three-dimensional structure of the protein was complex and that proper folding would be required for functional activity. As expected, injection of the VHv1.1 protein cleaved from the MBP fusion protein into *H. virescens* larvae did not significantly affect the growth, development, or immune system of the insects (data not shown).

The production of antisera against the VHv1.1/MBP fusion protein and the cleaved polydnavirus protein produced reagents that recognized a 30-kDa protein found only in parasitized insects. The VHv1.1/MBP also detected a 50-kDa protein that was present only in parasitized insects. Hybridization analyses of CsPDV mRNAs using the VHv1.1 gene as a probe have shown that 1.4- and 2.7-kb mRNAs are also expressed in parasitized insects (3, 39). Presumably, the 50-kDa protein is encoded by one of the cross-hybridizing mRNAs. Interestingly, the 50-kDa protein is detected as a diffuse band, suggesting that it may be glycosylated. By contrast, the 30-kDa protein does not have this appearance. Similar observations were reported for WHv1.0 and WHv1.6, in which the WHv1.0 gene is not glycosylated, while the WHv1.6 gene is glycosylated (3). The specificity of the antiserum to the 30-kDa/VHv1.1 protein was confirmed when the entire VHv1.1 cDNA was expressed under the control of the polyhedrin promoter. The 30-kDa protein produced from the baculovirus system could not be distinguished from the CsPDV protein produced in naturally parasitized insects. Taken together, these experiments demonstrate that the antibodies bind specifically to the 30-kDa/VHv1.1 protein.

The VHv1.1 gene product was detected predominantly in the hemolymph of parasitized insects as early as 5 h and for at least 7 days thereafter. The rapid appearance of the protein in the hemolymph indicated that it could have a role in immunosuppression after parasitization by *C. sonorensis*. However, the observation that unprotected eggs may be completely encapsulated as early as 4 h (36) after parasitization is an indication that additional parasite-derived factors may have an immunosuppressive role during the very early stages of parasitization. *C. sonorensis* ovarian proteins bind to granulocytes and plasmatocytes after parasitization and may contribute to

the rapid inhibition of the encapsulation response (36). At later times, CsPDV, in the absence of the other factors, can interfere with the insect cellular immune response (10, 12, 13, 33). Our results demonstrate that the 30-kDa/VHv1.1 protein binds to hemocytes and inhibits the cellular immune response in the absence of other factors from the parasitic wasp. The encapsulation response is reduced by 50% relative to controls. Clearly, some insects remain immunoresponsive in these experiments, while none of the parasitized insects were able to mount an effective immune response. The partial immunosuppression that is observed in our experiments may be attributed to the absence of other polydnavirus genes, venom, and/or ovarian proteins that are present in parasitized insects. It is likely that other members of the cysteine-rich gene family also contribute to suppression of the cellular immune response. The immunosuppressive role of venom (32) and ovarian proteins has also been demonstrated (20a). In our view, suppression of the cellular immune response after parasitization by *C. sonorensis* is achieved through the interaction of multiple factors, including (but not necessarily limited to) both viral and ovarian proteins. The relative importance of viral, venom, and ovarian proteins may also differ in a single parasitoid when different lepidopteran hosts are parasitized (30a).

In these studies, we have used a recombinant baculovirus as a delivery vehicle and assay system for a single polydnavirus gene. In theory, this allows isolation and functional analysis of individual polydnavirus genes, but the approach is necessarily limited to activities that can be studied in the context of an active baculovirus infection. Preliminary studies indicated that the encapsulation response was not affected by injection of 10^4 PFU of the E2 strain of Ac*M*NPV. During examination of wasp eggs injected into baculovirus-infected *H. virescens* larvae, we observed encapsulated eggs, some of which had capsules containing baculovirus-infected hemocytes. The preliminary studies suggested that hemocytes retain the ability to encapsulate wasp eggs for at least 48 h after baculovirus infection. Experiments with the recombinant VHv1.1 baculovirus demonstrated that encapsulation was significantly reduced relative to control-infected insects. Clearly, the use of recombinant baculoviruses containing polydnavirus genes may be limited and dependent upon the physiological system targeted by the polydnavirus gene product. However, when a robust bioassay can be developed, it may also be possible to assay for functional activity before purifying the protein. An additional advantage of the baculovirus recombinant is that the polydnavirus gene is persistently expressed in infected larvae at levels similar to those in natural parasitization.

The primary insect defense reaction to invading parasites is encapsulation by hemocytes, primarily granulocytes and plasmatocytes (25, 30). For successful parasitization, hymenopteran endoparasites must overcome the host cellular immune response by suppressing or evading encapsulation of eggs and larvae (26, 35). Study of the mechanisms through which the 30-kDa/VHv1.1 protein inhibits encapsulation will likely require purification of the protein. The localization of the protein on the surface of plasmatocytes and within granulocytes suggests that cellular receptors are involved. A *Pseudoplusia includens* polydnavirus gene that induces apoptosis in polydnavirus-infected hemocytes, thereby destroying infected granulocytes and reducing the immunological capabilities of the infected host, *Microplitis demoliter*, has been described (30a). Several other factors from parasitic hymenopterans may contribute to immunosuppression. Ovarian proteins from *C. sonorensis* have been implicated in suppression of the early cellular immune response (36). Venom may directly target and destroy host hemocytes (25). Hayakawa (17) has reported that a host cellular immunosuppressive factor is present in the larval plasma of the *Pseudaletia separata* parasitized by *Cotesia kariyai*, suggesting that the cellular immune system in parasitized insects is modulated by preexisting host factors. We have shown that the VHv1.1 gene product binds directly to plasmatocytes and granulocytes and inhibits the encapsulation response. By directly binding to plasmatocytes and granulocytes, the 30-kDa/VHv1.1 protein may alter the normal function of these cells and contribute to suppression of the host cellular immune response. Studies are in progress to examine the mechanisms through which the VHv1.1 and related polydnavirus genes block the cellular immune response in parasitized insects.

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