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The *Lymantria dispar* Nucleopolyhedrovirus Enhancins Are Components of Occlusion-Derived Virus

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Enhancins are metalloproteinases, first identified in granuloviruses, that can enhance nucleopolyhedrovirus (NPV) potency. We had previously identified two *enhancin* genes (E1 and E2) in the *Lymantria dispar* multinucleocapsid NPV (LdMNPV) and showed that both were functional. For this study, we have extended our analysis of LdMNPV *enhancin* genes through an immunocytochemical analysis of E1 and E2 expression and localization. E1 and E2 peptide antibodies recognized proteins of ~84 kDa and 90 kDa, respectively, on Western blots of extracts from *L. dispar* 652Y cells infected with wild-type virus. The 84- and 90-kDa proteins were first detected at 48 h postinfection (p.i.) and were present through 96 h p.i. E1 and E2 peptide antibodies detected E1 and E2 in polyhedron extracts, and the antibodies were shown to be specific for E1 and E2, respectively, through the use of recombinant virus strains lacking the *enhancin* genes. E1 and E2 were further localized to occlusion-derived virus (ODV). The enhancins were not found in budded virus. Immunoelectron microscopy indicated that E1 and E2 were present in ODV envelopes and possibly in nucleocapsids. Fractionation studies with several detergents suggested that the enhancins were present in ODV envelopes in association with nucleocapsids. In contrast, enhancins in granuloviruses are located within the granulin matrix. The presence of LdMNPV enhancins within ODV provides a position for the proteins to interact directly on the peritrophic membrane as ODV traverses this host defense barrier.

Baculoviruses are viruses that are pathogenic for invertebrates and include two genera, the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs). Both groups of viruses produce virions that are similar in both structure and the pathology of infection, although NPVs have members that can package virions singularly or in groups and GVs mostly package virions singularly (3, 4). Another distinction between these viruses is that NPVs produce occlusions, termed polyhedra, that contain many viral particles and GV occlusions (granules) mostly contain single viral particles. Larvae are infected by baculoviruses upon ingestion of the polyhedron or granule and release of the viral particles in the alkaline environment of the midgut.

Enhancins are proteins, first found in GV occlusion bodies, that have the ability to enhance the infection of some NPVs. Also referred to as viral enhancing or synergistic factors, enhancins were first identified and isolated by Tanada and colleagues (26, 28; see reference 27 for a review). The enhancin protein of *Pseudaletia unipuncta* GV (PuGV) was found to enhance the infection of *P. unipuncta* NPV (PuNPV) only when both the PuGV protein and PuNPV were inoculated orally, indicating that the midgut is the site of the enhancin activity (29). The enhancin protein purified from *Trichoplusia ni* GV (TnGV) enhanced *Autographa californica* multinucleocapsid NPV (AcMNPV) infection in *Helicoverpa zea*, *Spodoptera exigua*, *P. unipuncta*, and *T. ni* by a factor of 2- to 14-fold, depending on the host species (33). When the TnGV enhancin was engineered into tobacco plants, AcMNPV infection was increased 10-fold, although little enhancement of *S. exigua* NPV was seen (8). Enhancin genes have been found in several GVs, including *Helicoverpa armigera* GV (HaGV) (23), PuGV (23), TnGV (6), *Xestia c-nigrum* GV (XcGV) (9), *Agrotis segetum* GV (AsGV) (GenBank accession no. AY522332), and *Choristoneura fumiferana* GV (CfGV) (GenBank accession no. AAG33872). The first GV genome to be completely sequenced, that of XcGV, was found to have four different *enhancin* genes (9). In contrast, the *Plutella xylostella* GV (7), *Cydia pomonella* GV (17), *Adoxophyes orana* GV (34), *Cryptophlebia leucotreta* GV (13), and *Phthorimaea operculella* GV (GenBank accession no. AF499596) genomes lack *enhancin* genes.

The proposed function of GV enhancins is to disrupt the peritrophic membrane, thereby facilitating viral passage through this barrier to gain access to the midgut cells. The TnGV enhancin was found to damage the peritrophic membrane lining the larval midgut, thereby exposing the gut wall to viral infection (2). Peritrophic membranes exhibited increased permeability to AcMNPV after treatment with TnGV enhancin in an in vitro assay (19). Bioassay results for *T. ni* larvae infected with AcMNPV and various concentrations of the TnGV enhancin demonstrated that the major effect of enhancin appears to be an increase in infection efficiency caused by the disruption of the insect peritrophic membrane (5). This enhancin was later found to be a metalloproteinase, which degrades mucin, a major protein constituent of the peritrophic membrane (14, 32).

Lymantria dispar MNPV (LdMNPV) is a baculovirus that is pathogenic to *L. dispar*, the gypsy moth, a forest and urban

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tree-defoliating pest in the Northeastern and some Midwestern states. The first GV enhancin homologue found in LdMNPV was the *enhancin 1* (E1) gene, which was also the first *enhancin* gene found in NPVs (1). A second *enhancin* gene (E2) was identified in LdMNPV when the entire genome of isolate 5-6 was sequenced (12). Recently, an enhancin gene was found in a second NPV, *Mamestra configurata* NPV-A (MacoNPV-A) (16). As seen with the GV enhancins, when the MacoNPV-A enhancin gene was expressed in a recombinant AcMNPV, an increase in viral infectivity was found in comparison to wild-type AcMNPV (16). Sequencing of a second MacoNPV (MacoNPV-B) revealed the presence of an enhancin gene (15). MacoNPV-B is closely related to MacoNPV-A but is proposed to be a separate species. A fourth NPV enhancin gene has recently been found in the spruce budworm baculovirus *Choristoneura fumiferana* MNPV (CfMNPV) (GenBank accession no. AF512031).

The LdMNPV E1 and E2 proteins exhibit approximately 29% and 26% amino acid identity, respectively, to the deduced amino acid sequences of TnGV, PuGV, and HaGV, and both contain a conserved zinc-binding domain characteristic of metalloproteases (1, 20). *E1* and *E2* gene transcripts are expressed at late times after infection from a consensus baculovirus late promoter. A viral isolate lacking a functional *E1* gene was constructed to investigate enhancin function. A potency analysis revealed that the *E1*-negative viral strain was approximately two- to threefold less potent than wild-type viruses, suggesting that the LdMNPV enhancin affects viral potency (1). The LdMNPV *E2* gene was also shown to be functional through bioassays (20). Deletion of the *E2* gene caused a decrease of about twofold in viral potency. The deletion of both *enhancin* genes caused a 12-fold drop in potency. These results suggest that each enhancin protein can partially compensate for the lack of the other protein. In this study, we have extended our studies of the LdMNPV enhancins through analyses of *enhancin* gene expression and the localization of E1 and E2 with polyclonal antibodies.

MATERIALS AND METHODS

Cell culture and virus. *L. dispar* 652Y (Ld652Y) cells were grown as monolayers at 27°C in ExCell 420 medium (JRH Scientific) supplemented with 6.25 mM glutamine (Gibco BRL) and 5% fetal bovine serum (Atlanta Biologicals). LdMNPV isolate A21-MPV (25) and the recombinant viruses E1cat (1), E2del, and E1delE2del (20) were used for this study. E1cat contains the chloramphenicol acetyltransferase (*cat*) gene and does not produce *E1* gene transcripts, E2del lacks most of the *E2* gene and does not produce *E2* gene transcripts, and E1delE2del lacks most of the *E1* and *E2* genes and does not produce *enhancin* gene transcripts (20).

Preparation of cell extracts, polyhedra, ODV, and BV. For analyses of enhancin expression by recombinant viruses, Ld652Y cells were infected at a multiplicity of infection (MOI) of 10 50% tissue culture infective dose ($TCID_{50}$) units per cell with the viral isolates A21-MPV, E1cat, E2del, and E1delE2del. At 96 h postinfection (p.i.), the cells were washed from the flasks and pelleted by low-speed centrifugation. The cell pellets were washed with phosphate-buffered saline (PBS) and pelleted a second time. The pellets were then resuspended in 2× cell lysis buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 10% 2-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue). For an analysis of the onset of enhancin protein expression, Ld652Y cells were infected at an MOI of 10 $TCID_{50}$ units per cell with the LdMNPV isolate A21-MPV. At various times p.i. (0 to 96 h), the cells were harvested, washed twice with PBS after centrifugation, and resuspended in PBS. The cell suspensions were taken through three freeze-thaw cycles, adjusted to 0.1 M Na_2CO_3, and incubated at 37°C for 1 h. After incubation, 2× cell lysis buffer was added. For the isolation of occlusion-derived virus (ODV) and budded virus (BV), Ld652Y cells were infected at an MOI of 1 $TCID_{50}$ unit per cell and harvested at 8 days p.i. ODV and BV were isolated using standard techniques (1). An aliquot of BV was further purified by centrifugation through a 25 to 56% sucrose gradient at 80,000 × *g* for 1.5 h at 4°C. For analyses of E1 and E2 localization at 0 to 72 h p.i., 1×10^6 Ld652Y cells were infected with isolate A21-MPV at an MOI of 5 $TCID_{50}$ units per cell. At 0, 24, 48, and 72 h p.i., the flasks were harvested and the cells were pelleted by low-speed centrifugation. The cell pellets were washed with PBS, pelleted a second time, and resuspended in PBS, and an equivalent volume of 2× cell lysis buffer was added. BV was harvested from the medium by centrifugation and resuspended in PBS, and an equivalent volume of 2× cell lysis buffer was added. The cell preparations, polyhedra, ODV, and BV containing cell lysis buffer were incubated at 100°C for 5 min, and the polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Treatment of ODV with detergents. Polyhedra were isolated by centrifugation from Ld652Y cells infected with the LdMNPV isolate A21-MPV at 8 days p.i., the pellets were resuspended in PBS, and the preparations were heat treated at 70°C for 20 min (30). Heat-treated polyhedra were dissolved by the addition of 1 M sodium carbonate to a final concentration of 0.1 M, and the preparations were adjusted to 5 mM NaCl, incubated for 10 min at 60°C, and then chilled on ice. Cell debris and undissolved polyhedra were pelleted by a low-speed spin (2,500 × *g*) for 10 min in a microcentrifuge, and the supernatant containing ODV was removed. ODV aliquots were then subjected to treatment with several detergents (based on the methods described in references 24 and 30). The following detergents were added to ODV aliquots to the indicated final concentrations to achieve critical micelle concentrations: SDS, 0.5%; deoxycholic acid (DOC), 2.1 mg/ml; cetyltrimethylammonium bromide (CTAB), 0.4 mg/ml; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 4.9 mg/ml; octyl-β-D-glucoside (OGL), 3%; and Nonidet P-40 (NP-40), 3%. For each detergent, one aliquot of ODV was incubated with the detergent of interest and a second aliquot was incubated with the detergent and 1% β-mercaptoethanol, except for the NP-40 treatment, where 0.1% β-mercaptoethanol was used. Mixtures were incubated for 5 min at 60°C, cooled to room temperature, and then pelleted at 14,000 × *g* for 10 min. The supernatants were removed, and the pellets were resuspended in 10 mM Tris (pH 7.5). As a control, an aliquot of ODV was spun at 14,000 × *g* for 10 min, and the pellet was resuspended in 10 mM Tris (pH 7.5). All samples were adjusted to a final NaCl concentration of 30 mM. The polypeptides in the supernatant and pellet fractions were resolved by SDS-PAGE.

Generation of polyclonal antibodies and immunoblot analysis. Peptides homologous to the regions from amino acids 279 to 298 of E1 (DERQRDASVY ENGNRDRVER) and 506 to 525 of E2 (DRRYDITIPDNRFNDKGNG) were produced and used for the generation of polyclonal antibodies in rabbits by the SynPep Corporation (Dublin, CA). Peptides were purified by high-performance liquid chromatography and conjugated to keyhole limpet hemocyanin, the activated carrier protein. New Zealand White rabbits were injected five separate times every 2 weeks and a final time approximately 6 weeks later with either the E1 or E2 peptide conjugate. Rabbits were bled after the first battery of injections and after the final injection. The two bleeds were combined, and E1 and E2 polyclonal antibodies were affinity purified.

Vertical-slab SDS-PAGE was performed using standard techniques. After electrophoresis, the gels were stained with Coomassie brilliant blue and dried or electroblotted to a polyvinylidene difluoride membrane (0.2 μm; Bio-Rad) using a Bio-Rad electrophoretic transfer apparatus. Western blotting was performed using standard techniques. Blots stained with the E1 and E2 peptide antibodies were blocked with 5% bovine serum albumin (BSA) and 5% dried milk, respectively. A 1:1,000 dilution of the E1, E2, or both E1 and E2 peptide antibodies was used, and bound primary antibodies were visualized using standard techniques.

Immunoelectron microscopy. Immunoelectron microscopy was performed essentially as described by Hong et al. (10). Polyhedra were isolated from larvae infected per os with E1cat, E2del, or A21-MPV. The sections were blocked for 30 min with Tris-buffered saline (TBS) containing 1% BSA (for E1 peptide antibodies) or 5% dried milk (for E2 peptide antibodies). The sections were incubated with TBS containing 1% BSA or 5% dried milk and a 1:1,000 dilution of the E1 or E2 peptide antibody for 1 h at 4°C and then washed in TBS. The primary antibody was detected using an anti-rabbit immunoglobulin G gold-conjugated antibody. The sections were visualized with a JEOL JEM-1010 transmission electron microscope.

RESULTS

Temporal analysis of enhancin protein expression. To analyze the temporal expression of E1 and E2, extracts from

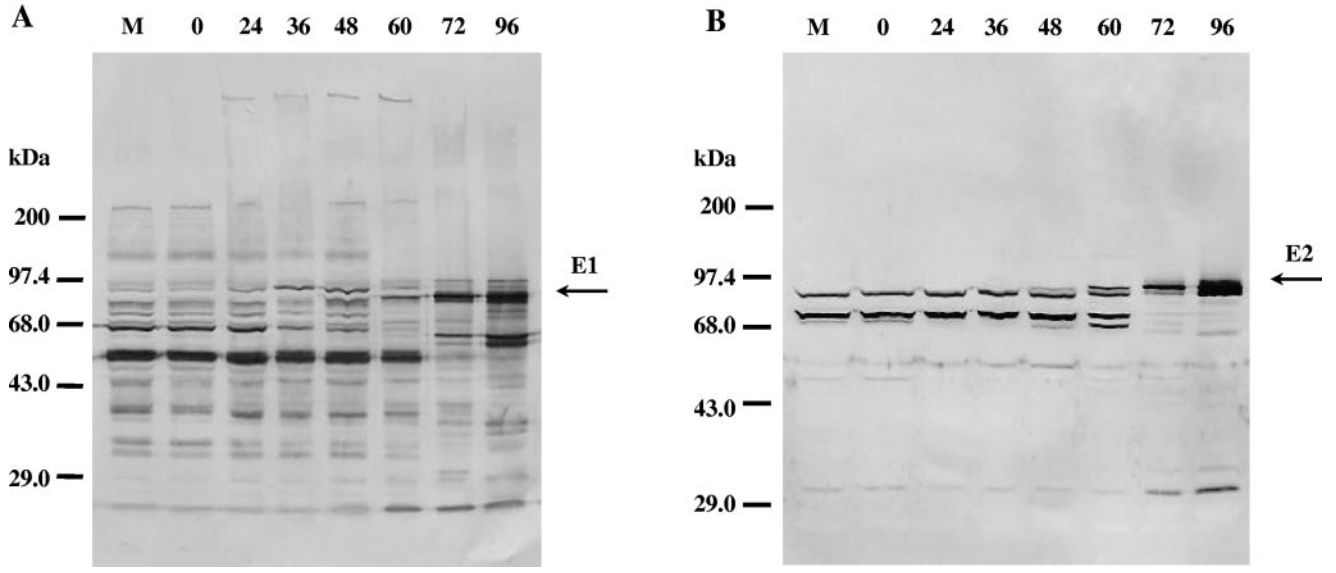

FIG. 1. Temporal analysis of E1 and E2 expression. Cells were infected with A21-MPV, and protein extracts were prepared at the h p.i. shown at the top of the panels. The locations of protein standards are shown on the left, and the locations of E1 and E2 are shown on the right. The blot in panel A was stained with E1 peptide antibodies, and the blot in panel B was stained with E2 peptide antibodies.

Ld652Y cells infected with wild-type virus (A21-MPV) were examined by Western blot analysis at various times p.i. using E1 or E2 peptide antibodies. Proteins of approximately 84 and 90 kDa were first detected with E1 and E2 peptide antibodies, respectively, at 48 h p.i., and the intensity of staining continued to increase at least up to the last time assayed, at 96 h p.i. (Fig. 1A and B). At 72 h p.i., the 84-kDa protein was the most intensely stained protein present, and this protein is close in size to the predicted molecular size of E1 (89.2 kDa). At 72 and 96 h p.i., the 90-kDa protein was the most intensely stained protein present, and this protein is close in size to the predicted molecular size of E2 (88.4 kDa).

E1 peptide antibodies stained several host cell proteins from 0 to 60 h p.i. By 72 h p.i., most of the most prominently stained host proteins were no longer evident (e.g., proteins of ~65, 54, and 51 kDa; Fig. 1A). Infected cell-specific proteins of ~82, 61, and 58 kDa were visible at 72 h p.i. and were prominent at 96 h p.i. A protein of approximately 25 kDa was present throughout the time course and was present in proteins isolated from mock-infected cells. However, the intensity of staining was significantly greater from 60 to 96 h p.i., which may suggest that a virus-specific protein of about 25 kDa was also being stained at late times p.i. E2 peptide antibodies prominently stained host cell proteins of ~82 and 72 kDa (Fig. 1B). At 72 and 96 h p.i., these proteins appeared to be largely absent. Infected cell-specific proteins of ~88 and 31 kDa appeared at 72 h p.i. and were prominent at 96 h p.i. At 96 h p.i., the intensities of the 82- and 84-kDa bands stained with E1 peptide antibodies were about equal, and the intensities of the 88- and 90-kDa bands stained with the E2 peptide antibodies at 96 h p.i. were approximately equal. The lower-molecular-weight proteins stained with the E1 and E2 peptide antibodies at 72 and 96 h p.i. could be proteolytic or degradative products of the E1-staining 84-kDa and E2-staining 90-kDa proteins.

Another blot containing extracts isolated at 96 h p.i. from cells infected with A21-MPV was generated and stained with both E1 and E2 polyclonal antibodies to confirm the apparently different molecular sizes of E1 and E2. Proteins of ~90, 88, 84, and 82 kDa were intensely stained with a mixture of E1 and E2 peptide antibodies (data not shown). Lower-molecular-size proteins of ~61, 58, 31, and 25 kDa were also stained.

Assessment of E1 and E2 antibody specificity. For the time course expression analysis of E1 and E2, the sample from 96 h p.i. was composed largely of polyhedra. The intense staining of the 84- and 90-kDa proteins at the 96-h time point by E1 and E2 peptide antibodies, respectively, suggested that the enhancin proteins were present in polyhedra. Because of the probable location of the enhancins, polyhedra were used to assess the specificity of E1 and E2 peptide antibodies. Protein extracts from polyhedra isolated from Ld652Y cells infected with A21-MPV, E1cat, E2del, and E1delE2del were examined by Western blot analysis using E1 and E2 peptide antibodies. The recombinant virus E1cat does not express the *E1* gene, E2del lacks the *E2* gene, and E1delE2del lacks both *enhancin* genes (Fig. 2). E1 peptide antibodies stained an 84-kDa protein in extracts from polyhedra produced by the wild-type virus and E2del but not in extracts from polyhedra produced by E1cat and E1delE2del (Fig. 3A). Several lower-molecular-weight proteins were weakly stained from extracts of wild-type and E2del polyhedron extracts, but not from E1cat and E1delE2del polyhedron extracts. A protein of ~38 kDa from E1cat and E1delE2del polyhedra weakly stained with E1 peptide antibodies; however, this protein was absent from extracts from wild-type and E2del polyhedra, suggesting that it would cause little background staining in the analysis of polyhedra containing E1. E2 peptide antibodies stained a 90-kDa protein in extracts from polyhedra generated by the wild-type virus and E1cat but not in extracts from polyhedra produced by E2del and E1delE2del (Fig. 3B). A protein of approximately 50 kDa exhibited very weak staining only in extracts from wild-type

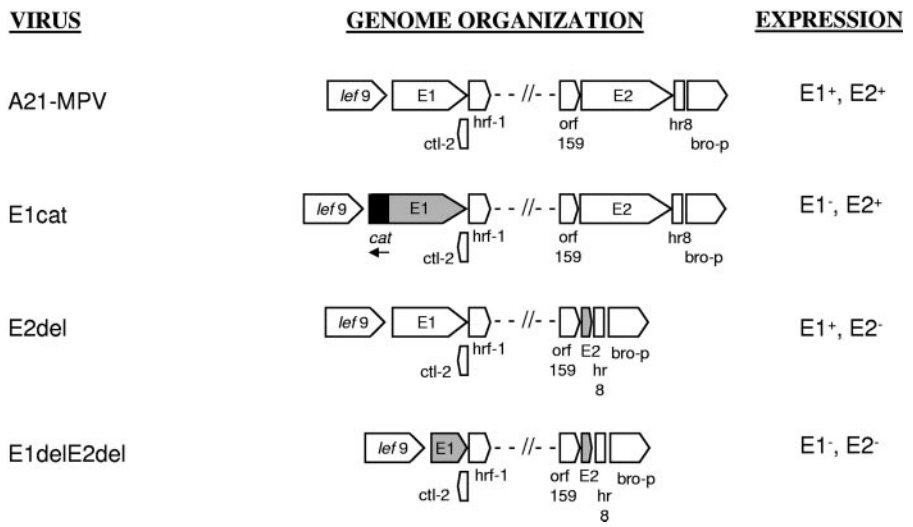

FIG. 2. Schematic representation of A21-MPV and recombinant enhancin viruses. Arrows show the locations and directions of open reading frames in the regions of the *E1* and *E2* genes (12). The shaded open reading frames are those where the *enhancin* gene has either been deleted or disrupted. The *cat* gene is indicated by a black box. The expression of the *enhancin* gene products is indicated on the right for each virus.

and E1cat polyhedra, suggesting that this may be a degradation product of E2. No staining of proteins from extracts using E1 or E2 peptide preimmune serum was found (data not shown).

Localization of enhancins 1 and 2 within viral structures. To determine if the enhancins are components of viral structures, proteins from BV, ODV, and occlusion bodies were analyzed by Western blot analysis. Proteins of 84 and 90 kDa were found in polyhedron-plus-BV extracts stained with E1 and E2 antibodies, respectively (Fig. 4A and B, respectively, lanes P+B). Protein extracts were prepared from the same number of polyhedra that was used to generate the protein extracts in Fig. 4, lanes P+B, and were stained with E1 and E2 antibodies. Proteins of 84 and 90 kDa were stained, and the amount of enhancin protein present appeared to be similar to that present in the polyhedron-plus-BV extracts (Fig. 4A and B, lanes P). ODV was isolated from the same number of polyhedra used to generate the extracts described above, and protein extracts were prepared and stained with E1 and E2 antibodies. Proteins of 84 and 90 kDa were stained, and the amount of enhancin protein present appeared to be similar to that present in the polyhedron-plus-BV and polyhedron extracts (Fig. 4A and B, lanes ODV). Very weakly stained bands of approximately 84 and 90 kDa were present in Western blots containing proteins from pelleted BV after staining with E1

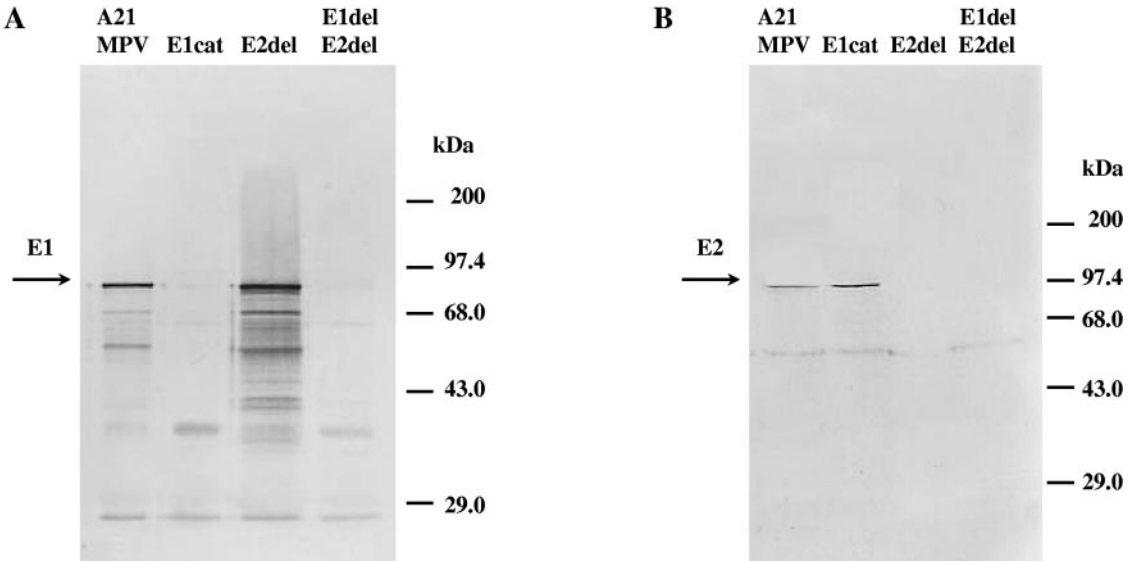

FIG. 3. Western blot analysis of the specificity of E1 and E2 peptide antibodies against proteins within polyhedra. Polyhedra generated by A21-MPV, E1cat, E2del, and E1delE2del were solubilized by alkaline treatment, and the proteins were separated by SDS-PAGE. The locations of protein size standards are shown to the right of the panels, and the locations of E1 and E2 are indicated with arrows. The blot shown in panel A was stained with E1 peptide antibodies, and the blot in panel B was stained with E2 peptide antibodies.

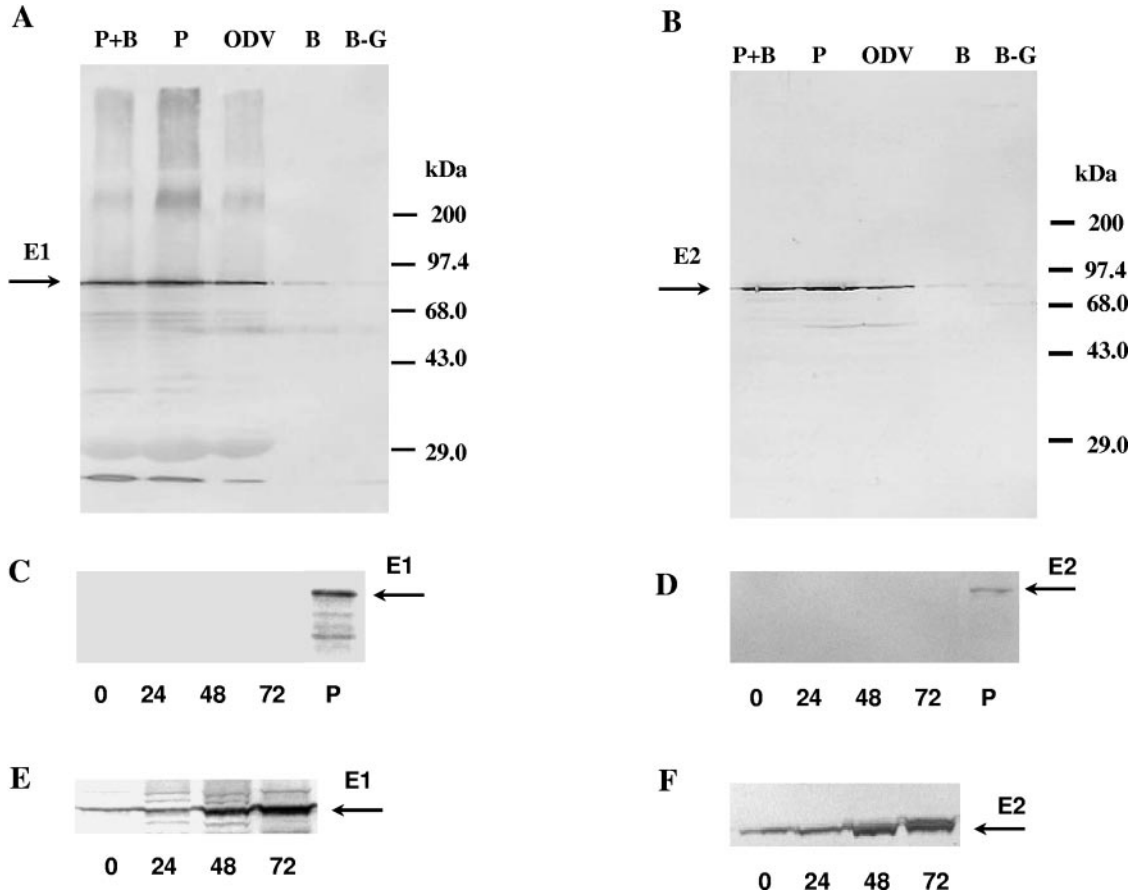

FIG. 4. Western blot analysis of E1 and E2 within polyhedra, ODV, BV, and cell extracts/polyhedra. For panels A and B, protein extracts were prepared from polyhedra and BV (P+B), polyhedra (P), ODV, BV (B), and gradient-purified BV (B-G). Equivalent amounts of polyhedra (1×10^7/lane) were used for the P+B, P, and ODV extracts, and an equivalent amount of BV (5×10^6 $TCID_{50}$ units/lane) was used for the P+B, B, and B-G extracts (the P+B lane contained 1×10^7 polyhedra and $5 \times 10_6$ $TCID_{50}$ BV units). For panels C and D, protein extracts were prepared from BV (an equivalent amount of sample was used in each lane, and the 72-h sample contained about 5×10^6 $TCID_{50}$ units of BV) isolated from 0 to 72 h p.i. and from polyhedra (P). For panels E and F, protein extracts were prepared from infected cells/polyhedra isolated from 0 to 72 h p.i. (the extract from 5×10^5 cells was loaded in each lane). The blots shown in panels A, C, and E were stained with E1 peptide antibodies, and the blots in panels B, D, and F were stained with E2 peptide antibodies. Stained E1 and E2 proteins are indicated by arrows. The locations of protein size standards are shown to the right of panels A and B.

and E2 peptide antibodies, respectively (Fig. 4A and B, respectively, lanes B). These proteins were barely perceivable after staining with E1 and E2 peptide antibodies of protein preparations from BV isolated after gradient centrifugation (Fig. 4A and B, lanes B-G). The appearance of E1 and E2 within BV preparations may be an artifact due to free E1 and E2 nonspecifically associating with BV as a consequence of isolating BV at 8 days p.i. At this time point, infected cells would have lysed and free E1 and E2 may have been present in the cell culture medium along with BV. To address this possibility, BV and cell/polyhedron extracts were prepared from 0 to 72 h p.i. and analyzed for the presence of E1 and E2. Most BV produced by LdMNPV is present by 72 h p.i. under the infection conditions employed (22). Western blots containing equal proportions of proteins from the total amount of BV produced and proteins from infected cells/polyhedra were generated and stained with E1 and E2 peptide antibodies. No stained bands were found in extracts from BV preparations isolated from 0 to 72 h p.i. after staining with E1 and E2 peptide antibodies (Fig. 4C and D, respectively). Bands of 84 and 90 kDa were stained in control lanes P, which contained proteins from polyhedra. Stained bands of 84 and 90 kDa were found in cell/polyhedron extracts from infected cells harvested at 48 and 72 h p.i. after staining with E1 and E2 peptide antibodies (Fig. 4E and F, respectively).

Localization of E1 and E2 within ODV structures. Dissolution studies of ODV and immunoelectron microscopy were performed to investigate the locations of E1 and E2 within ODV structures. ODV were isolated from polyhedra and subjected to several detergent treatments to dissolve ODV envelopes, and the presence of E1 and E2 was determined by Western blot analysis. Briefly, ODV were liberated from polyhedra, undissolved polyhedra and debris were removed by centrifugation, and the supernatant was removed. Various detergents were added to the supernatant, and after an incubation period, the contents were centrifuged and separated into supernatant and pellet fractions. Intact nucleocapsids, if present, would be in the pellet fraction, and the components of nucleo-

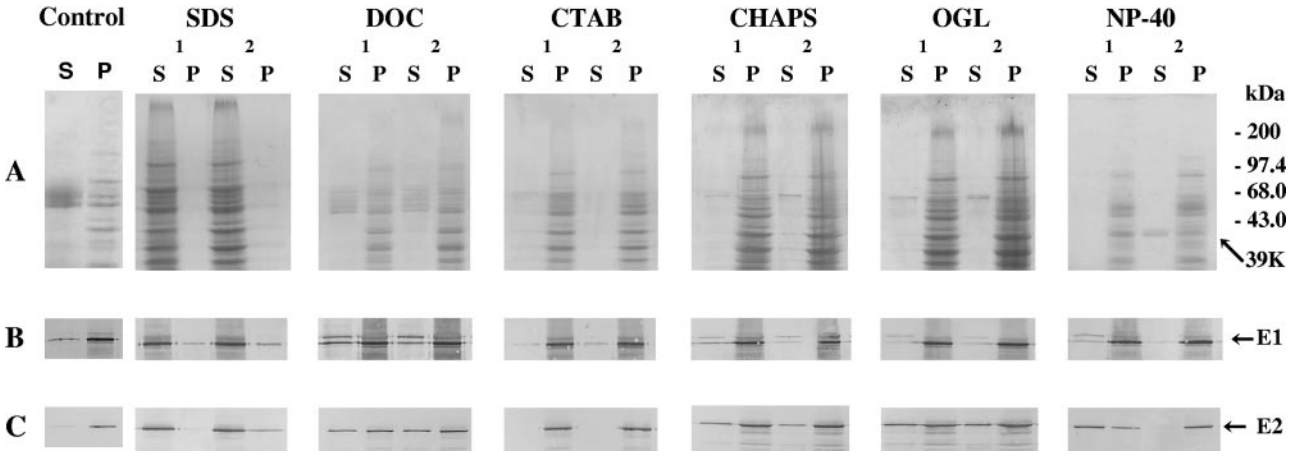

FIG. 5. Coomassie staining and Western blot analysis of envelope and nucleocapsid fractionated proteins. ODV were isolated, treated with the indicated detergents, and then separated into supernatant (S) and pellet (P) fractions. The S and P fractions in lanes 1 were not treated with 2-mercaptoethanol, and those in lanes 2 were. The control lanes were not treated with a detergent. The gels were generated in triplicate. The gels in panel A were stained with Coomassie blue, the blots in Panel B were stained with E1 peptide antibodies, and the blots in panel C were stained with E2 peptide antibodies. The locations of protein size standards are shown to the right of the panels, and the locations of E1, E2, and the viral 39K protein are indicated by arrows.

capsid envelopes would be in the supernatant. The pellet (P) and supernatant (S) fractions were analyzed by Western blot analysis with antibodies to E1 and E2 peptides (Fig. 5B and C, lanes 1), and a duplicate SDS-PAGE gel (Fig. 5A, lanes 1) was stained with Coomassie blue to determine the location of the nucleocapsid proteins. A duplicate set of preparations was made, and 2-mercaptoethanol was added (lanes 2) in addition to the detergents to reduce thiol linkages, if present.

Nucleocapsid proteins, E1, and E2 were predominately present in the pellet fraction in the non-detergent-treated control, as expected (Fig. 5, control panel). Very small amounts of E1 and E2 were found in the supernatant, indicating that the starting polyhedron fraction contained E1 and E2 and/or that the preparations/treatments may have disrupted some ODV envelopes. Treatment with SDS was performed as a control to confirm that dissolution of the envelope and nucleocapsid caused E1, E2, and capsid proteins to be present in the supernatant fraction (Fig. 5, SDS panel). The pellet fraction contained small amounts of capsid proteins, E1, and E2, indicating that most, but not all, ODV and/or polyhedra were dissolved during the treatments. The supernatants contained the envelope and capsid proteins, including a major protein of 39 kDa, indicating that the treatment dissociated nucleocapsids as well as capsid envelopes. The 39-kDa protein was likely the p39 capsid protein since it was present as a major protein in ODV preparations. An antibody to the AcMNPV p39 capsid protein was used in a Western analysis to attempt to confirm that the observed 39-kDa protein was the conserved capsid protein, since an antibody to the LdMNPV p39 capsid protein does not exist; however, it failed to stain any proteins present in an ODV extract.

Initially, the treatment of ODV was performed with the nonionic detergent NP-40 to remove virion envelopes (24). After the treatment of ODV with NP-40, a small amount of E1 was found in the supernatant fraction (Fig. 5B). To address the possibility that detergents differ in the ability to disrupt virion envelopes, additional detergents were used for this investigation. The treatment of ODV with the anionic, weakly denaturing detergent DOC caused about 50% of E1 to be present in the supernatant fraction (Fig. 5B). In contrast, capsid proteins were still present in the pellet fraction, indicating that the capsid structure was not disrupted (Fig. 5A). Similar to the results obtained with NP-40, the treatment of ODV with the zwitterionic detergent CHAPS and the nonionic detergent OGL caused the movement of a small amount of E1 into the supernatant. Approximately half of the liberated E1 protein appeared as a higher-molecular-weight form after treatment with DOC, and a small amount of this form was also present after treatments with CHAPS, OGL, and NP-40. The higher-molecular-weight E1 protein may be an artifact due to a strong association of the detergents with E1, resulting in an altered mobility during electrophoresis.

The treatment of ODV with DOC, CHAPS, OGL, and NP-40 caused about 25 to 50% of the E2 to be present in the supernatant fractions (Fig. 5C). In contrast, capsid proteins predominately remained in the pellet fractions (Fig. 5A). Identical results for E1 and E2 were obtained with and without 2-mercaptoethanol after treatment with the detergents, with one exception, suggesting that thiol linkages are not present between E1 or E2 and a capsid protein(s). Treatment with NP-40 and 2-mercaptoethanol caused E2 to be present in the pellet fraction and resulted in the presence of a protein of approximately 39 kDa in the supernatant fraction. The treatment of envelopes with the cationic detergent CTAB did not liberate E1 or E2 from the envelope or failed to disrupt the envelope.

To further examine the location of E1 and E2 within ODV, wild-type, E1cat, and E2del polyhedra were examined by immunoelectron microscopy. E1 and E2 peptide antibodies predominately stained envelopes of occluded virions within polyhedra produced by the wild-type virus (Fig. 6A and B). Staining with E1 and E2 peptide antibodies occurred both at the periphery and within virion envelopes proximal to nucleocapsids. Staining with E1 and E2 antibodies also occurred on

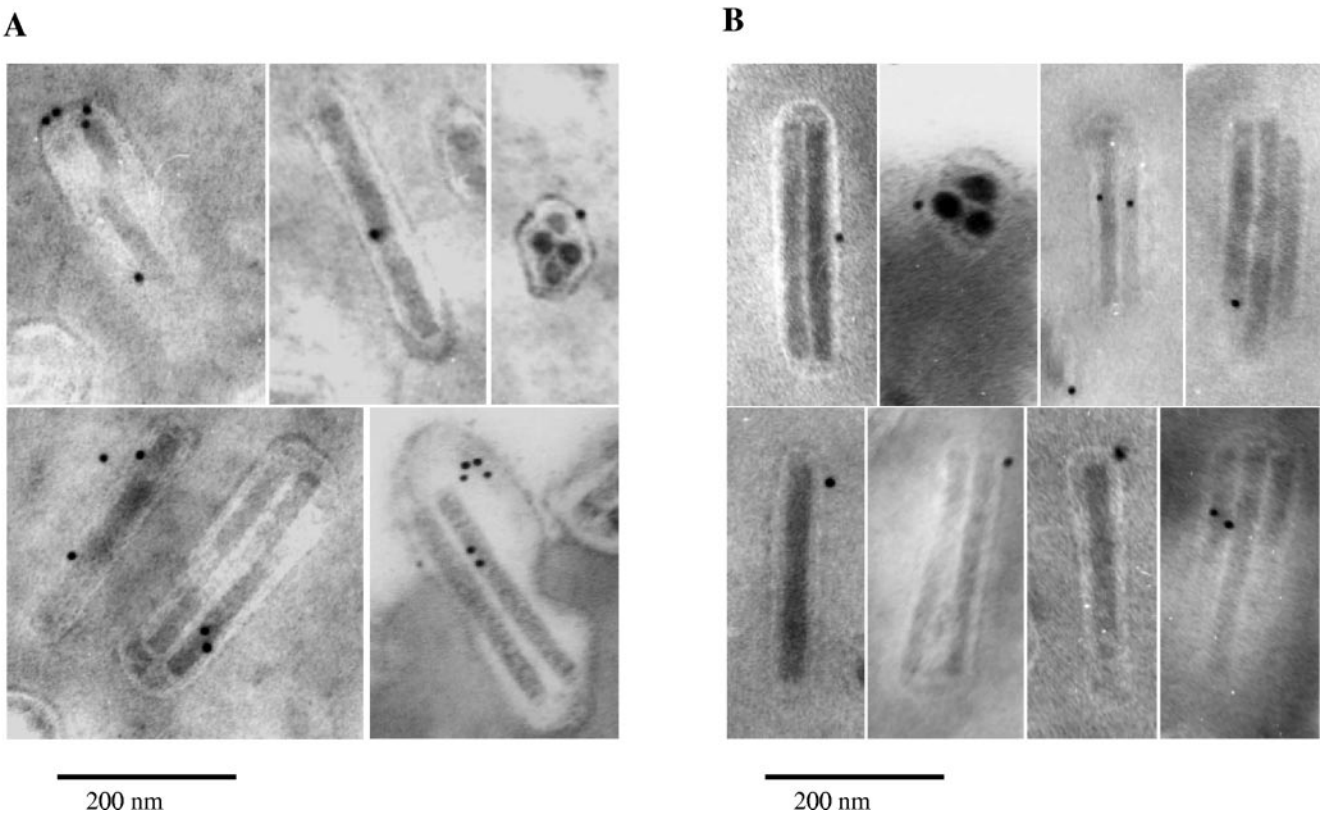

FIG. 6. Immunogold localization of E1 and E2 within polyhedra. A21-MPV polyhedra were stained with E1 (A) or E2 (B) peptide antibodies. Bar, 200 nm.

nucleocapsids. The distribution of gold particles within the envelopes/nucleocapsids and at the envelope periphery appeared to be random. Very little labeling occurred in other areas within polyhedron cross sections, and the amount that did occur was similar to the amount that occurred when E1cat polyhedra were labeled with E1 peptide antibodies and when E2del polyhedra were labeled with E2 peptide antibodies (data not shown). In addition, very little immunogold labeling of ODV occurred in E1cat and E2del polyhedral sections stained with E1 and E2 peptide antibodies, respectively, and where labeling did occur, it was randomly distributed throughout ODV structures (data not shown). In a Western analysis of polyhedral and ODV extracts, a protein of ~28 kDa was weakly stained by E1 peptide antibodies. However, since immunogold labeling of E1cat polyhedra by E1 peptide antibodies was slight and randomly distributed throughout the cross sections, the labeling of the 28-kDa protein would not compromise the finding of E1 within ODV envelopes.

DISCUSSION

In previous investigations, the function of the LdMNPV enhancin genes was investigated through an analysis of the impact of deletions of these genes on viral potency (1, 20). Deletions of the enhancin genes decreased viral potency, indicating that the enhancins are involved in an event that is a component of the initial infection process, such as to facilitate viral entry into the host. To extend our investigations into the mode of LdMNPV enhancin action, we have investigated the expression and localization of E1 and E2 through analyses with polyclonal antibodies.

Since E1 and E2 exhibit an amino acid identity of approximately 28%, it is unlikely that polyclonal antibodies generated against the full-length proteins would be specific for E1 and E2. In an effort to generate E1- and E2-specific antibodies, areas of the proteins that exhibited traits of being highly antigenic in one enhancin and exhibiting low antigenic traits in the other enhancin were sought. A peptide homologous to the region from amino acids 279 to 298 of E1 was used to generate an antibody because it exhibited a relatively high hydrophilicity value (3.0), a high surface probability (0.75), and a high antigenic index (0.5), in contrast to the same region of E2 (hydrophilicity value, 1.5; surface probability, 0.5; and antigenic index, 0.1). E1 and E2 exhibit 50% amino acid identity in this region. A peptide homologous to the amino acid region from residues 506 to 525 of E2 was used to generate an E2 antibody. This region of E2 exhibits a hydrophilicity value of approximately 3.0, a surface probability of 0.7, and an antigenic index of 0.5. The region from amino acids 506 to 525 of E1 exhibits a hydrophilicity value of approximately 1.5, a surface probability of 0.35, and an antigenic index of 0.13. E1 and E2 proteins exhibit an amino acid identity of 15% within the region of amino acids 506 to 525.

Western blot analysis with the E1 and E2 peptide antibodies first detected unique proteins of ~84 and 90 kDa, respectively, at 48 h p.i. in infected cell extracts prepared from 0 to 96 h p.i.

The 84- and 90-kDa proteins are close to the predicted sizes of E1 (89.2) and E2 (88.4), and their appearance at 48 h p.i. occurred at the same time that E1 and E2 transcripts were first detected in an earlier study (1, 20). The sizes of these proteins and their detection at 48 h p.i. are consistent with the 84- and 90-kDa proteins being E1 and E2, respectively. The findings that the E1 and E2 polyclonal antibodies stained different-sized proteins and that they stained proteins specific to cells infected with wild-type and E2del viruses and with wild-type and E1cat viruses, respectively, indicate that these antibodies were specific for E1 and E2. The absence of stained 84- and 90-kDa proteins from E1cat and E1delE2del and from E2del and E1delE2del polyhedra, respectively, was expected since the E1cat and E2del recombinant viruses do not generate any *E1* or *E2* gene transcripts, respectively, and the E1delE2del virus does not produce either *enhancin* gene transcript (1, 20). Overall, these results indicate that the E1 and E2 peptide antibodies can be used for independent analyses of *E1* and *E2* gene expression and protein localization.

E1 and E2 were found in polyhedra and were further localized to ODV (Fig. 4A and B). The amounts of E1 and E2 isolated from polyhedra and from ODV isolated from the same number of polyhedra were approximately the same, suggesting that most, if not all, E1 and E2 present in polyhedra were located within ODV. No staining of any BV proteins occurred when BV isolated from 0 to 72 h p.i. was analyzed, indicating that E1 and E2 are not components of BV (Fig. 4C and D). Within ODV, only one form of E1 and E2 was found, in contrast to the case with protein lysates prepared at 96 h p.i. A comparison of protein lysates from cells prepared at 72, 96, and 120 h p.i. and from ODV revealed that only the 84- and 90-kDa E1 and E2 proteins were present within polyhedra (data not shown). These results support the suggestion that the lower-molecular-weight proteins detected at 96 h p.i. were likely degradative products of the E1 and E2 proteins.

To determine the location of E1 and E2 within the ODV structure, ODV was treated with several detergents to disrupt the virion envelope. The treatment of ODV with the anionic detergent DOC released about 50% of the E1 and E2 into the soluble fraction, and treatments with CHAPS (zwitterionic), OGL (nonionic), and NP-40 (nonionic) caused the release of about 25 to 50% of the E2 present in ODV into the soluble fraction while leaving the nucleocapsids intact, suggesting that E1 and E2 were present in ODV envelopes (Fig. 5). Since there was E1 and E2 in the pellet fraction, the enhancins may also be associated with or be components of nucleocapsids. The use of detergents to localize a protein to ODV envelopes does not always give a definitive result. The treatment of *Orgyia pseudotsugata* MNPV ODV with NP-40 caused the release of about 50% of the p25 envelope protein; however, the remainder of this protein was found in the capsid fraction (24). In contrast with E2, very little E1 was released into the soluble fraction after treatments of ODV with CHAPS, OGL, and NP-40. This difference may suggest that E1 has a stronger association with the nucleocapsid than does E2. The finding that the anionic DOC detergent had the greatest effect on liberating the enhancins from the envelope/association with nucleocapsids may suggest that the enhancins have an ionic bond with the nucleocapsid through positively charged amino acids. It is interesting that the cationic CTAB detergent had essentially no impact on the liberation of the enhancins from the nucleocapsid fraction.

The location of E1 and E2 within ODV was further investigated through electron microscopy. Immunogold labeling of polyhedron sections resulted in the labeling of mostly ODV envelopes, either at the outside edge or within the envelope (Fig. 6). Staining did occur over ODV nucleocapsids; however, since the sectioning plane cannot be defined, labeling may have occurred within the envelope and not on the nucleocapsid. Staining of transversely sectioned nucleocapsids did occur but was infrequent, indicating that the enhancins were also located in association with the nucleocapsid. Overall, immunogold labeling clearly showed that E1 and E2 were present within ODV envelopes; however, the enhancins also appeared to be associated with nucleocapsids. The detergent fractionation and immunogold labeling results suggested that E1 and E2 were located within the ODV envelope and may be in association with the nucleocapsid. However, these results do not rule out the presence of enhancins within the nucleocapsid.

Analyses of E1 and E2 for the presence of transmembrane alpha-helices by the dense alignment surface method (http://www.sbc.su.se/~miklos/DAS/), the PRED-TMR algorithm (http://o2.db.uoa.gr/PRED-TMR), the TMHMM method (www.cbs.dtu.dk/services/TMHMM/), MEMSAT2 (http://bioinf.cs.ucl.ac.uk/psipred/), and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) all predict the presence of a transmembrane alpha-helix in LdMNPV E1 in the amino acid region from about positions 738 to 761 and in E2 in the amino acid region from about positions 747 to 771 (Fig. 7A). The TMHMM method further predicts that amino acids of E1 from positions 1 to 737 and of E2 from positions 1 to 746 are located on the outside of the membrane structure, residues 738 to 760 (E1) and 747 to 769 (E2) span the membrane, and amino acids 761 to 782 (E1) and 770 to 788 (E2) are located on the inside of the membrane structure (i.e., next to the nucleocapsid). These predictions suggest that the carboxyl-terminal regions of E1 and E2 are anchored within the ODV envelope and that the majority of the proteins extend beyond the envelope surface. An interesting correlation is that many of the gold particles were found at the outside edge of ODV envelopes after immunogold labeling (Fig. 6), which is consistent with the E1 and E2 epitopes (regions 279 to 298 and 506 to 525, respectively) being located on the outside of ODV envelopes. If this orientation were present, the LdMNPV enhancins would be positioned to interact with the peritrophic membrane within the host midgut. It is interesting that the regions from positions 761 to 782 of E1 and 770 to 788 of E2 each contains six basic amino acids (Fig. 7B). These basic residues may bind to the nucleocapsids, and if so, could be the reason why the anionic detergent DOC was most effective at liberating the enhancins from ODV.

Analyses of the MacoNPV-A, MacoNPV-B, and CfNPV enhancins for transmembrane alpha-helices with the programs described above identified this feature in all three proteins by all of the programs. MacoNPV-A, MacoNPV-B, and CfNPV enhancins contained a single transmembrane region located near the carboxy terminus (Fig. 7A). In addition, MacoNPV-A and -B enhancins contain seven and five basic residues, respectively, between the end of the transmembrane region and the carboxy terminus (Fig. 7B). An analysis of GV enhancins for

A

Viral or bacterial Enhancin Gene	# AA	TMHMM	MEMSAT	PRED-TMR	DAS	SOSUI
LdNPV E1	782	1 (738-760)	1 (743-760)	1 (744-761)	1 (738-761)	1 (738-760)
LdNPV E2	788	1 (747-769)	1 (748-771)	1 (750-768)	1 (750-771)	1 (749-771)
MacoNPV-A	847	1 (804-826)	1 (804-823)	1 (804-823)	1 (804-825)	1 (803-825)
MacoNPV-B	848	1 (806-828)	1 (805-824)	1 (805-824)	1 (805-828)	1 (803-825)
CfNPV	758	1 (719-741)	1 (720-741)	1 (720-740)	1 (718-741)	1 (719-741)
XcGV E1	824	0	0	1 (345-362)	0	0
XcGV E2	867	0	0	0	0	0
XcGV E3	898	0	0	0	0	1 (137-159)
XcGV E4	856	0	0	1 (357-376)	0	0
HaGV	902	0	0	1 (357-373)	0	1 (137-159)
PuGV	901	0	0	0	0	0
TnGV	901	0	0	0	0	0
CfGV	901	0	0	0	0	0
AsGV	1004	0	0	0	0	0

B

LdNPV E1: 761: VN**RRG**R**Q**SP**K**AAE**R**APPPLQ**R**V
LdNPV E2: 770: TIA**RRA**K**R**DDA**R**PPSVI**K**A
MacoNPV-A: 827: SPS**KK**QVIT**K**E**K**P**K**PVI**K**SI**K**
Maco-NPV-B: 828: PNAIETII**K**E**K**P**K**TNI**K**SI**K**
CfNPV: 742: **K**NMATPNTS**H**NLAPNIS

FIG. 7. Analysis of NPV and GV enhancins for transmembrane alpha-helices and of the carboxy termini. The number of amino acids present in each enhancin protein is shown (# AA), and the number of transmembrane alpha-helices identified is given, with the location of the domain shown in parentheses, below each analysis method (A). The amino acid sequence of each NPV enhancin gene, from the end of the transmembrane domain to the carboxyl terminus, is shown in panel B, with the basic residues shown in bold. The GenBank accession numbers for the *enhancin* gene sequences are as follows: LdMNPV E1, AF019971; LdMNPV E2, AF081810; MacoNPV-A, AF467808; MacoNPV-B, AF539999; CfNPV, AF512031; XcGV E1-4, AF162221; HaGV, D28558; PuGV, D14871; TnGV, D12617; CfGV AAG33872; and AsGV, AY522332.

the presence of transmembrane alpha-helices detected either no domains or the presence of a region by only one method, which is consistent with their localization to granules, as discussed below (Fig. 7A).

The presence of LdMNPV E1 and E2 within the envelope would be consistent with their mode of action of increasing viral potency (1, 20). Studies of GV enhancins have shown that these metalloproteinases disrupt the peritrophic membranes within lepidopteran larvae (2, 19). Investigations of GV enhancins indicated that the proteins are located within granules but are not structural components of envelopes or nucleocapsids (2). The granulin fraction, but not the virion fraction, of TnGV granules exhibited proteolytic activity against high-molecular-weight glycoproteins within the peritrophic membrane. Enhancins within TnGV occlusion bodies are estimated to comprise approximately 5% of the occlusion body mass (27). In contrast, the results of fractionation and immunoelectron microscopy studies in this investigation indicate that the LdMNPV enhancins are not components of the polyhedral matrix. The different locations of GV and LdMNPV enhancins may indicate that GVs and NPVs that contain enhancins employ different mechanisms for the use of these proteins. The GVs appear to release enhancin through dissolution of the granule, thereby allowing the protein to act on the peritrophic membrane. In contrast, by locating within ODV envelopes, the LdMNPV enhancins are able to act on the peritrophic membrane as the nucleocapsids move through the barrier, thereby facilitating movement to gain access to the midgut cells. Whether GVs and NPVs are divergent in the localization of enhancins will require analyses of the locations of enhancins in additional NPVs.

The recent sequencing of the genomes of *Bacillus cereus* (11), *Bacillus anthracis* (21), *Bacillus thuringiensis* (accession no. NC 005957), and *Yersinia pestis* (18) revealed the presence of homologs to baculovirus *enhancin* genes. We performed a phylogenetic analysis of all currently known enhancins to investigate their relationships (Fig. 8). The LdMNPV enhancins exhibit amino acid identities with the enhancins from MacoNPV-A, MacoNPV-B, CfMNPV, *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *Y. pestis* of approximately 18%, 20%, 23%, 17%, 17%, 17%, and 21%, respectively, for E1 and 18%, 18%, 21%, 19%, 19%, 19%, and 20%, respectively, for E2. Interestingly, the phylogenetic analysis grouped the LdMNPV, MacoNPV-A, MacoNPV-B, CfMNPV, *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *Y. pestis* enhancins together and all of the GV enhancins in a second group, with the exception of AsGV. This may suggest that the GVs acquired enhancin genes relatively early after their divergence from the MNPVs. The *B. cereus*, *B. anthracis*, and *B. thuringiensis* and MacoNPV-A and MacoNPV-B enhancins exhibit short divergent distances (0.017, 0.023, and 0.014 and 0.093 and 0.093, respectively) within the phylogenetic tree, suggesting a common origin of

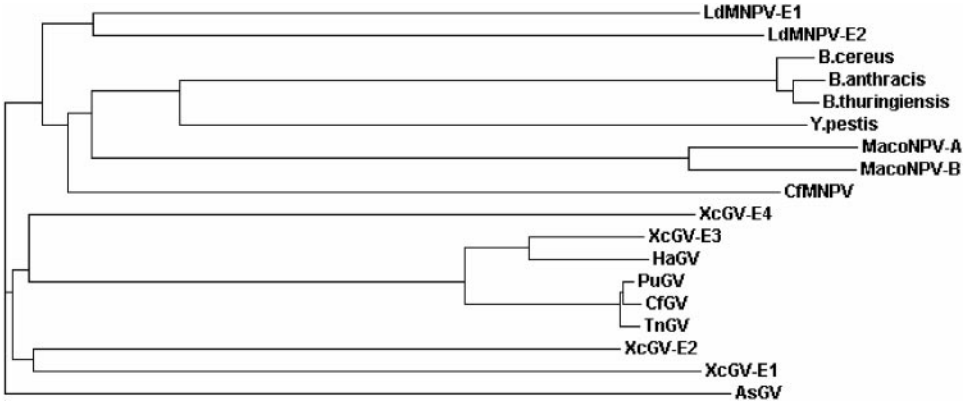

FIG. 8. Phylogenetic tree of known enhancin proteins. The branch lengths in the tree are proportional to the calculated genetic distances. The tree was generated using enhancin protein sequences and the Phylogenetic Tree function of the ClustalW program (31; http://www.ebi.ac.uk/clustalw/#). The GenBank accession numbers for the bacterial *enhancin* gene sequences are as follows: *B. anthracis*, AE016879; *B. cereus*, AE016877; *B. thuringiensis*, NC 005957; and *Y. pestis*, AL590842.

their enhancin genes. In contrast, the remaining members in this grouping exhibit long divergent distances (LdMNPV E1, 0.34; LdMNPV E2, 0.37; *Y. pestis*, 0.35; MacoNPV-A/B, 0.33; CfMNPV, 0.40; and *B. anthracis*/*cereus*, 0.34), which may suggest that the enhancins in these MNPVs and bacteria had independent origins. In contrast, the XcGV-E3, HaGV, PuGV, CfGV, and TnGV enhancins exhibit short divergent distances, suggesting a common origin of their enhancins. The remaining GVs in this group exhibit long divergent distances (XcGV E4, 0.37; XcGV E2, 0.33; and XcGV E1, 0.37), and as noted above, AsGV (0.40) is separate from the bacterial/NPV and GV groups. It is interesting that the conserved HEXXH zinc-binding site of metalloproteinases present in the other enhancins is lacking in the XcGV E1 (HQXXH), XcGV E4 (QKXXD), and AsGV (HVXXH) enhancins. This may suggest that these enhancins are pseudogenes and hence would no longer be under evolutionary pressure to conserve the protein sequence.

The grouping of NPV and bacterial enhancins suggests a common or related origin of their enhancin genes. The *B. cereus* group contains the closely related organisms *B. cereus*, an opportunistic pathogen of humans; *B. anthracis*, a mammalian pathogen; and *B. thuringiensis*, an insect pathogen. The presence of enhancin genes in *B. cereus* and *B. anthracis* led to the suggestion that these organisms evolved from an ancestor of the *B. cereus* group that was an opportunistic insect pathogen (11, 21). The subsequent finding of an enhancin gene in *B. thuringiensis* provides further support for this hypothesis. If the *B. cereus* ancestor resided in the guts of insects that were NPV hosts, there may have been an opportunity for an exchange of genetic material between these bacteria and NPVs. The enhancin in *Y. pestis*, the causative agent of the disease referred to as plague, may aid its colonization of the flea. The *Y. pestis* enhancin gene is flanked by a tRNA gene and transposase fragments, which may suggest that this bacteria obtained its enhancin gene via horizontal transfer.

In summary, we have shown that the LdMNPV E1 and E2 proteins are components of ODV envelopes and may also be associated with nucleocapsids. The results of this study suggest that enhancins are not present in BV and are not components of the polyhedral matrix. Studies are in progress to address the impact of LdMNPV enhancins on the *L. dispar* peritrophic membrane.

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