Spindles of an Entomopoxvirus Facilitate Its Infection of the Host Insect by Disrupting the Peritrophic Membrane[▽]

Wataru Mitsuhashi,* Hiromu Kawakita,† Ritsuko Murakami, Yutaka Takemoto, Tomoaki Saiki,‡
Kazuhisa Miyamoto, and Sanae Wada

National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan

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The mode of action by which entomopoxvirus (EPV) spindles, proteinaceous crystalline bodies produced by EPVs, enhance EPV infection has not been clarified. We fed *Anomala cuprea* EPV (AcEPV) spindles to host insects; subsequent scanning electron microscopy revealed the disruption of the peritrophic membranes (PMs) of these insects. The PM is reportedly a barrier against the infection of some insects by viruses. Quantitative PCR of AcEPV DNA in the ectoperitrophic area revealed that PM disruption facilitated the passage of EPVs through the PM toward the initial infection site, the midgut epithelium. These results indicate that EPV spindles enhance infection by EPVs by disrupting the PM in the host insects. Fusolin is almost exclusively the constituent protein of the spindles and is the enhancing factor of the infectivity of nucleopolyhedroviruses (NPVs) and possibly that of EPVs. Spheroid is another type of proteinaceous crystalline structure produced by EPVs. *Pseudaletia separata* EPV (PsEPV) spheroids reportedly contain considerable amounts of fusolin and enhance NPV infection. We assessed the ability of AcEPV spheroids to enhance EPV infectivity and their effect on the PM and carried out immunological experiments; these experiments showed that AcEPV spheroids contain little or no fusolin and are biologically inactive, in contrasts to the situation in PsEPV.

Entomopoxviruses (EPVs) are poxviruses that infect insects of orders such as Coleoptera, Lepidoptera, Orthoptera, and Diptera. Many EPVs form two types of proteinaceous crystalline bodies: spheroids and spindles. The spheroid contains virions, whereas the spindle does not; thus, spheroids are agents of infection. The major constituent proteins of the spheroid and spindle are spheroidin and fusolin, respectively, and these two are the most abundant proteins among those produced by EPVs. Peroral administration of the spindles of a coleopteran EPV, *Anomala cuprea* EPV (AcEPV), to *Bombyx mori* and *Spilosoma imparilis*, lepidopteran hosts of two species of nucleopolyhedroviruses (NPVs), enhances the infectivity of not only the occluded NPVs of these two species (16, 19) but also of a nonoccluded NPV of *B. mori* (4), although AcEPV itself cannot infect these two insects. However, these are artificial phenomena that occur by experimental combination of the spindles with NPVs; these insects on plants rarely ingest AcEPV spindles that are present in soils, together with the NPVs. Also, this mechanism of enhanced infectivity of NPVs is considered to be worthless for the survival of AcEPVs that cannot infect the insects. NPVs and EPVs are taxonomically distant DNA insect viruses. NPVs belong to the family *Baculoviridae*, whereas EPVs belong to the *Poxviridae*. However, AcEPV spindles also enhance the infectivity of AcEPV in its host insect, the larva of the cupreous chafer *A. cuprea*, strongly suggesting that the natural biological function of EPV spindles is to enhance the infectivity of EPVs (15, 20).

Mitsuhashi and Miyamoto (17) showed that the peritrophic membrane (PM) of *B. mori* larvae was disintegrated by AcEPV spindles. The PM is an acellular membrane that lines the midgut lumen in the form of a tube, extending from the anterior midgut to the hindgut (3). The PM prevents NPV virions from reaching the microvilli of the midgut cells, which are the initial site of infection by the virus in some lepidopteran insects (21, 24–26). Thus, this disintegration of the PM in *B. mori* is thought to be the mode of action by which EPV spindles enhance NPV infection (17). This PM disintegration in the *B. mori* larvae is very dramatic; frequently, the PM was not detected in the dissected *B. mori* larvae under a binocular microscope (17). In contrast, the PM is observed under the binocular microscope to be wholly present when the integument and dorsal epithelium of the midgut of *A. cuprea* larvae are carefully torn open by using a pair of forceps after the larvae have been fed AcEPV spindles. Therefore, the effect of the spindles on the host's PM and mode of action by which EPV spindles enhance EPV infection in their hosts remain unknown. Thus, our first aim in the present study was to determine whether in fact mild alteration occurs in the PMs of *A. cuprea* larvae that had been fed spindles—an alteration that is not recognizable by binocular microscopy but still enhances the infectivity of the EPV, as evidenced by other techniques, including electron microscopy. If the PM is damaged sufficiently for EPV virions to pass from the endoperitrophic to ectoperitrophic space more easily than in a normal PM, then the mode of action of the enhancement of insect-virus infection by AcEPV spindles can be considered to be similar between EPV and NPVs.

Wijonarko and Hukuhara (27) reported that both spheroids and spindles of the lepidopteran EPV, *Pseudaletia separata* EPV (PsEPV), enhanced the infectivity of *Pseudaletia unipuncta* NPV (PuNPV) in the insect, *P. separata*, which is a host for both PuNPV and PsEPV, and to similar degrees. Moreover, PsEPV

* Corresponding author. Mailing address: National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan. Phone: (81) 29-838-6081. Fax: (81) 29-838-6028. E-mail: mitsuhas@affrc.go.jp.

† Present address: Minami 2-25-8, Ushiku, Ibaraki 300-1222, Japan.

‡ Present address: Izumi-chou 4748-10, Izumi-ku, Yokohama 245-0016, Japan.

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fusolin has been identified as the factor of enhancing PuNPV infection and was reported to be present in the virions within PsEPV spheroids, as well as PsEPV spindles (6, 8, 9, 27). AcEPV fusolin produced by a recombinant baculovirus enhanced *Bombyx mori* NPV (BmNPV) infection in *B. mori* larvae (Y. Takemoto, W. Mitsuhashi, R. Murakami, K. Miyamoto, and S. Wada, Abstr. 57th Meet. Kanto Branch Jpn. Soc. Seric. Sci., abstr. 14, 2006); this strongly suggests that AcEPV fusolin in spindles is the factor of enhancing the infectivity of EPV as it does for NPVs, when the spindles are administered with AcEPV spheroids. AcEPV spheroids did not enhance NPV infection, unlike PsEPV spheroids (16, 17). However, it has not been studied whether AcEPV spheroids enhance AcEPV infection. Therefore, our second purpose in the present study was to elucidate whether AcEPV spheroids are able to enhance the infectivity of the EPV and whether the potential factor enhancing EPV infection, fusolin, is present in these spheroids. To determine this, we examined various properties associated with the ability of spheroids to enhance EPV infectivity by observing the PMs in host insects of the EPV that had been fed spheroids, performing bioassays of their infection-enhancing ability, and conducting immunological and biochemical studies on AcEPV spheroids.

We describe here for the first time the mechanism underlying the enhancement of EPV infection by spindles on the basis of the results of experiments using AcEPV and its coleopteran host. We also describe the results of our search to elucidate whether fusolin is present in AcEPV spheroids and whether spheroids have fusolin-associated biological effects. Our findings reveal several discrepancies between AcEPV and PsEPV spheroids.

MATERIALS AND METHODS

Virus and insects. The origin and propagation method of AcEPV have been described by Furuta et al. (4). *A. cuprea* larvae that were the offspring of adults collected in the field in Ibaraki or Chiba Prefecture, Japan, were reared at 25°C in our laboratory on a diet comprising a mixture of leaf mold (Kyodo Jirushi Co., Ltd., Japan), autoclaved black soil, and wheat germ at a ratio of 17:10:1 (wt/wt) until they were used for the study.

Purification of spindles and spheroids. AcEPV spindles and spheroids used in the present study were purified by a modification of the method of Furuta et al. (4). The modification was the omission of potassium iodide density gradient centrifugation. The levels of cross-contamination of resultant highly purified spindles by spheroids and of resultant highly purified spheroids by spindles were examined with a hemacytometer. Various concentrations of the inocula of spindles, spheroids, or spindles combined with spheroids were prepared for ingestion by the larvae by using the preparations of the proteinaceous crystalline bodies and sterile distilled water. In some experiments, before per os administration, part of the spindle or spheroid preparation was heated in Eppendorf tubes in a 75°C water bath for 30 min to eliminate the virulence (infectivity) of spheroids in the inocula.

Method for inoculating larvae with spindles and/or spheroids. The method of per os administration of the inocula of proteinaceous crystalline bodies or sterile distilled water to the larvae in each experiment was performed as follows.

Small pieces (each piece; 2 by 2 by 2 mm) of an artificial diet (Insecta LF; Nihon Nosan Kogyo Co. Ltd., Tokyo, Japan) were placed on wells (one piece/well) of six-well plates (MS-8006R; Sumitomo Bakelite Co. Ltd., Tokyo, Japan), and then the inoculum of the spheroids and/or spindles (or sterile distilled water) was dripped into each piece. The second-instar larvae of *A. cuprea* of the first day were transferred to wells (one larva/well) for administration. Water was dripped into the spaces among the wells to make the atmosphere inside the plate extremely humid, and the plate was wrapped with aluminum foil to prevent light from reaching the wells. The larvae that had consumed the diet completely within a certain period, which varied according to the experiment, were retained for the next steps in the experiments.

Scanning electron microscopy (SEM) of the PMs of larvae after being fed spindles or spheroids. The PM is already present in *A. cuprea* second-instar larvae immediately after the first molt (W. Mitsuhashi, unpublished data). The individual *A. cuprea* second-instar larvae of the first day were each fed a piece of artificial diet containing 4×10^5 or 4×10^6 spindles, 4×10^5 spheroids, or sterile distilled water as a control as described above. Each spindle inoculum contained 0.8% spheroids compared to the number of the spindles, whereas the spheroid inoculum contained 0.5% spindles compared to that of the spheroids. Larvae that had completely fed the diet within half a day were placed for 20 min in cold water containing crushed ice to paralyze them by being chilled and were then placed in phosphate-buffered saline (PBS; 0.01 M Na_2HPO_4 - NaH_2PO_4 , 0.15 M NaCl). The integument was then carefully torn in the direction of the midline using a pair of forceps in the PBS, and the dorsal epithelium of the midgut was torn in a similar manner. The PMs were carefully separated from the insect bodies, rinsed in 0.1 M sodium cacodylate buffer, and then fixed for 2 h at room temperature in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer. Each sample was dehydrated with an ethanol series (50, 70, 90, and 100%) on a SemiPore filter (JEOL, Tokyo, Japan), and the ethanol was then replaced with isoamyl acetate. The resulting samples were critical point dried by use of a dryer (JCPD-5; JEOL), sputter coated with osmium by using an osmium plasma coater (NL-OPC80; JEOL DATUM, Tokyo, Japan) or with platinum using an ion beam sputterer (IBS/e; Southbay Technology, San Clemente, CA), and then viewed by SEM using an JSM-6301F or JSM-7000F (JEOL) scanning electron microscope. The PMs from three larvae from each feeding group were viewed by SEM. In the case of the 4×10^5 spheroid group, the PMs from only two larvae were examined by SEM.

Real-time quantitative PCR of AcEPV DNA in the ectoperitrophic area after feeding of spheroids with or without spindles. The permeability of the PMs of *A. cuprea* larvae to AcEPV was investigated after the larvae had been fed the inocula of spheroids with or without spindles. We measured the initial quantity of AcEPV fusolin gene in the midgut juices present between the PM and midgut epithelium and in part of the midgut epithelium in order to elucidate whether or not a greater number of the AcEPV virions had passed through the PM after feeding of an inoculum of spheroids combined with spindles than after feeding of a spheroid inoculum alone. The spheroid inoculum was administered at 4×10^3 spheroids per larva. The inoculum of spheroids combined with spindles contained 4×10^3 spheroids and 4×10^4 heated spindles per larva; the 4×10^3 spheroids were composed of 3.68×10^3 spheroids and 3.2×10^2 heated spheroids contaminating the 4×10^4 heated spindles.

Six hours after the transfer of the larvae to the wells of the six-well plates, on each of which the inoculum had been placed, as described above, the larvae that had completely eaten the diet on the wells were transferred to cold water containing crushed ice and were left on it for about 20 min for chilling. Each larva was then placed in 200 μl of PBS, and the integument was carefully torn with a pair of forceps. The anterior and posterior regions of the midgut containing the two circles of prominent sac-shaped structures (henceforth referred to as "sac-circles") were then sliced away from the rest of the exposed midgut with a sharp edge (Fig. 1). The sacs of the two separated sac-circles contained midgut juices that had passed through the PM from the endoperitrophic to the ectoperitrophic space. The midgut juices in the sac-circles were the only midgut juices between the PM and midgut epithelium that could be used for this experiment; other ectoperitrophic midgut juices could not be collected without leakage of endoperitrophic content, owing to the inevitable injury of the PM that occurs at the present level of technical expertise in this process. The two sac-circles and attached portions of midgut were then transferred together into 100 μl of PBS as one experimental sample, and the remainder of the midgut (henceforth referred to as the "midgut part") was transferred into 200 μl of PBS as another sample. The partial PM and food bolus were removed from the two kinds of samples by using forceps. The tube (ring)-shaped midgut partial portion(s) of each sample was torn into sheet-like piece(s) by use of forceps, and the adherent tissues (trachea, fat bodies, etc.) were removed from the piece(s) with forceps. The samples were then rinsed four times with 40 μl of fresh PBS. The midgut part and sac-circles samples were transferred into 180 and 45 μl of PBS, respectively. The sacs of each piece were cut into many smaller pieces with a sharp edge in the 45 μl of PBS to release the digestive juices that they contained. The PBS containing the released midgut juices and the many pieces and that containing the midgut part were then frozen until the next use.

We added 135 μl of PBS to each thawed 45- μl sample containing the fragmented sac-circles, and the sample was then pipetted repeatedly to suspend virions and centrifuged to remove tissues at $700 \times g$ for 2 min. The supernatant was collected for DNA extraction. On the other hand, the thawed midgut part in an Eppendorf tube was homogenized with a micropestle for 1 min. Template DNA for real-time quantitative PCR was then extracted from the samples by

FIG. 1. Material from *Anomala cuprea* larvae used for real-time quantitative PCR. (A) Dissected midgut consisting of most of the whole midgut of a first-day second-instar larva (the PM inside has been removed). There is a circle of prominent sac-shaped structures (referred to here as “sac-circles”) on both the anterior and the posterior regions of the dissected midgut. Those on the anterior region of the midgut may be ceca. These two circles with portions of midgut were cut away from the rest of the midgut with a sharp edge (incision lines are indicated in the picture) and were combined into a single sample. (B) Separated sac-circles with a portion of midgut from the rest of the anterior region of the midgut of second-instar larva. Each sac contains digestive juices that passed through the PM from the endoperitrophic to the ectoperitrophic space (viewed from an angle different from that in panel A). (C) Diagram of a second-instar *A. cuprea* larva. Part of the integument has been omitted so that the materials are visible.

using a DNA purification kit (DNeasy tissue kit; QIAGEN Sciences) in accordance with the manufacturer's instructions.

Quantitative PCR was performed with real-time detection of the AcEPV fusolin gene (18) in an ABI-Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA) using the DNA samples. The gene-specific reagents for the AcEPV fusolin gene were as follows: forward primer, 5'-ATTGTGTGAT GCTGTATTTGCA3'; reverse primer, 5'-CAGCTGGTGGAGGAGGTATCA T3'; and TaqMan probe, 5'-CAGACCAGGCCCGATCCAGAA3'. These sequences were designed by using Primer Express software (PE Applied Biosystems). PCR was performed in a 25- μ l reaction mixture that contained 12.5 μ l of TaqMan Universal PCR Master Mix (PE Applied Biosystems), 500 nM concentrations of each primer, 250 nM TaqMan probe (PE Applied Biosystems), template DNA (8.2 μ l of DNA solution from the sac-circles sample or 7.3 μ l of DNA solution from midgut-part samples), and sterile distilled water (3.2 μ l for sac-circles examination or 4.1 μ l for midgut-part examination). Cycle parameters were 50°C for 2 min to allow the UNG AmpErase to prevent amplicon carryover contamination and 95°C for 10 min to activate *Taq* polymerase, followed by 60 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve was obtained from known concentrations of the control AcEPV genomic DNA in *A. cuprea* DNA solution purified from the midgut juices of a pair of sac-circles or from the midgut part of second-instar larva of the first day fed only sterile distilled water in a diet in accordance with the above-mentioned method. Two replicates for each DNA sample were made, and the samples in which the initial DNA was small and thus the DNA was quantified from only one of the two replicates were regarded as AcEPV DNA-undetected samples. AcEPV genomic DNA for standard samples was prepared by a new method of DNA extraction from scarabaeid EPV spheroids: a guanidine-HCl method modified by increasing the amount of 2-mercaptoethanol. Purified spheroids (9.0×10^6) were treated with 245 μ l of buffer (470 mM guanidine-HCl, 0.59 M 2-mercaptoethanol, 490 mg of proteinase K, 8.2 mM Tris-HCl [pH 8.0], 122.5 mM NaCl, 1.6 mM EDTA, 0.82% sodium dodecyl sulfate [SDS]) at 58°C for 1 h. Soon after the completion of the treatment, a phenol-chloroform-isoamyl alcohol extraction and a chloroform-isoamyl alcohol extraction were performed. Then the DNA was collected by isopropyl alcohol precipitation. In each of the experiments using different parts of the insects, the ratio of the number of AcEPV DNA-detected samples to the number of AcEPV DNA-undetected samples was compared between the spindle- and spheroid-inoculated group and the spheroid-only-inoculated group.

Bioassays of infectivity-enhancing ability of spheroids. We carried out bioassays to examine whether spheroids enhance the infectivity of EPV; as described above, it was known only that AcEPV spheroids did not enhance the infectivity of BmNPV. Part of the spindle or spheroid preparation was heated, as described above, to eliminate the infectivity (virulence) of spheroids in the inoculum. This infectivity had to be eliminated because the complex of the infectivity of spheroids and their ability to enhance EPV infectivity, if present, will affect the infection rate of insects with EPVs in bioassays, and the degree of the effect of the infectivity of the spheroids on the rate cannot be estimated. AcEPV fusolin produced in recombinant baculoviruses retains its high level of activity in enhancing NPV infection after being heated at 80°C for 30 min (Y. Takemoto, W. Mitsuhashi, and R. Murakami, unpublished data). In addition, AcEPV spindles

retain their high level of ability to enhance EPV infection after being heated at 70°C for 30 min (15) and their high ability to increase NPV infectivity after being heated at 75 to 95°C for 30 min (W. Mitsuhashi, R. Murakami, and K. Miyamoto, Abstr. 37th Ann. Meet. Soc. Invertebr. Pathol., abstr. 94-95, 2004). Therefore, the heat treatment in the bioassays was unlikely to inactivate potential enhancing activity possessed by fusolin in the spheroids. To confirm this conclusion, we performed bioassays comparing the activities of heated and unheated spindles on EPV infection. In these assays, the inoculum for one larva comprised 4×10^4 heated or unheated spindles mixed with 2.5×10^3 unheated spheroids. Also, for other bioassays (major bioassays), several concentrations of heated spheroid or spindle preparations were mixed with several concentrations of unheated spheroid preparations.

After administration of an artificial diet containing the inoculum, as described above, larvae that had completely consumed the diet within a day were retained and individually reared on leaf mold free of spindles and spheroids in plastic cups (diameter, ca. 50 mm; height, ca. 30 mm; one larva per cup) at 25°C to determine infection. The check for infection was carried out at 45 days after administration as described by Mitsuhashi et al. (20). Briefly, larvae were examined visually for a whitish external appearance, which is a clinical symptom of infection; later, the infection was confirmed by identification of proteinaceous crystalline structures in pieces of fat bodies by use of light microscopy. We compared the infection rates obtained from these bioassays.

Immunoelectron microscopy of spheroids. Immunoelectron microscopy of spheroids was performed to elucidate whether fusolin, which is known to enhance NPV infection and may enhance EPV infection, was present in AcEPV spheroids. Primary antibody (polyclonal antibody against AcEPV fusolin) was produced by immunizing a mouse with fusolin from dissolved and undissolved purified AcEPV spindles.

Purified spindles combined with purified spheroids were embedded in 1.5% agarose gel (NuSieve 3:1 agarose; BioWhittaker Molecular Applications, Rockland, ME). The resultant sample was fixed in 0.5% glutaraldehyde–5% paraformaldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 25 min at room temperature. After being washed in a 50 mM sodium cacodylate buffer, the sample was dehydrated in ethanol solutions of increasing concentrations up to 95% and embedded in LR White resin (London Resin Company Co., Ltd., Hampshire, United Kingdom). Ultrathin sections were made with a diamond knife on an ultramicrotome (LKB 8800 Ultratome III; LKB, Bromma, Sweden).

The ultrathin sections were collected on nickel grids, preincubated in Tris-buffered saline (20 mM Tris-HCl [pH 8.0], 225 mM NaCl) containing 10% normal goat serum and 0.1% bovine albumin, and subsequently floated overnight at 4°C on a drop of anti-AcEPV fusolin antibody diluted 1:600 in Tris-buffered saline containing 0.1% bovine albumin. The grids were washed with Tris-buffered saline containing 0.1% bovine albumin and then incubated for 40 min with 1% goat anti-mouse immunoglobulin G (H+L) conjugated with 20-nm gold beads (British BioCell International, Ltd., Cardiff, United Kingdom). The grids were then washed with Tris-buffered saline, and the specimens were postfixed in 2% glutaraldehyde in Tris-buffered saline for 10 min and washed with Milli-Q water. The sections were stained with uranyl acetate and examined by transmission electron microscopy (JEM-1010; JEOL).

SDS-PAGE and Western blot analyses of spheroid proteins for fusolin detection. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analyses of spheroid proteins were carried out to elucidate whether fusolin was present in AcEPV spheroids. The samples examined were purified AcEPV spheroids (containing AcEPV virions) and purified AcEPV virions. Ten million purified spindles or spheroids were treated with 50 μ l of sample buffer (5% SDS, 0.72 M 2-mercaptoethanol, 0.1 M Na₂HPO₄-NaH₂PO₄, 8 M urea [pH 7.8]) for 10 min at room temperature to completely dissolve spheroid proteins including structural proteins of the EPV virions and spindles. The resultant samples appeared to be completely dissolved, since no pellets were generated after the centrifugation of the samples at $17,600 \times g$ for 5 min. Each dissolved sample was then boiled for 10 min at 100°C. On the other hand, the AcEPV virion sample was purified, and the proteins were dissolved as follows. Spheroids (2.72×10^8) were treated with 100 ml of alkaline reducing buffer (0.8 M Na₂CO₃, 0.1 M sodium thioglycolate) for 2.5 h at room temperature. Undissolved spheroids were then removed from the liberated-virion suspension by centrifugation at $1,000 \times g$ for 5 min, and the resultant supernatants were centrifuged at $91,300 \times g$ for 30 min in a Beckman type 35 rotor to obtain virion pellets. After the pellets were resuspended in 0.1 M Tris-HCl (pH 8.0), the suspensions combined, and its volume was adjusted to 30 ml by adding 0.1 M Tris-HCl (pH 8.0). The suspension was layered on 8 ml of a 40% (wt/wt) sucrose solution. This step was followed by centrifugation in a Beckman SW28 rotor at $103,900 \times g$ for 90 min. The supernatant was discarded, and the pellet was resuspended in 0.1 M Tris-HCl (pH 8.0). The suspended sample was then pelleted by centrifugation in a Beckman TLA55 rotor at $75,500 \times g$ for 30 min. The pellet was dissolved by adding 50 μ l of the sample buffer described above, and the resultant sample was boiled at 100°C for 10 min.

SDS-PAGE was carried out by using a 12% low-bis SDS-polyacrylamide gel and the two types of the sample solution described above, in accordance with the method of Hirano (7). A 10- μ l sample solution was loaded per well of the gel. Bands on the polyacrylamide gel were then detected by Coomassie brilliant blue R-250 staining. For Western blot analysis, the bands separated by SDS-PAGE as described above were transferred onto a microporous polyvinylidene difluoride membrane (ProBlott; Applied Biosystems) by using a semidry transfer system (NA-1512; Nihon Eido, Ltd., Tokyo, Japan) at 15 V for 45 min. The membranes were then incubated in a blocking solution (maleic acid buffer containing 1% [wt/vol] blocking reagent [Roche, Indianapolis, IN]; pH 7.5), for 30 min at room temperature. The membranes were incubated for 1 h at room temperature with a 1:5,000 dilution of the anti-AcEPV fusolin antibody described above and washed three times in Tris-buffered saline containing 0.05% Tween 20. Next, the membranes were incubated with a 1:8,000 dilution of an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Promega, Madison, WI) for 1 h and washed in Tris-buffered saline containing 0.05% Tween 20, and the proteins visualized by using a BCIP-NBT solution kit for alkaline phosphatase stain (Nakalai Tesque, Inc., Kyoto, Japan). In the Western blot analysis, using the same number (10^6) of spindles per lane as that in the SDS-PAGE was excessive for obtaining an appropriately shaped fusolin signal band, so we used 100-fold-diluted samples (10^4 spindles and spheroids per lane) instead.

RESULTS

SEM. An electron microscopic view of the PMs of *Anomala* insects is shown for the first time in Fig. 2. There have been few electron microscopic observations of coleopteran PMs (2). The PMs of the larvae feeding on 4×10^6 or 4×10^5 spindles were damaged (Fig. 2A to C), particularly after receiving a greater dose of inoculum (Fig. 2A and B). There were several types of disruptions. First, we detected a number of areas in which the PM lacked the protein matrix that should have embedded the multilayered network structure and showed the presence of a number of irregularly shaped holes and/or gaps. Some of the holes and gaps (many of which were bigger than AcEPV virions, which are reported to be 440 nm long and 250 nm wide [10]) extended from the endoperitrophic to the ectoperitrophic space. These holes and gaps had been generated by the loss of some or all layers of the network structure in these areas, after the spindles had been fed to the larvae (Fig. 2A to C). Second, in some of the above-mentioned disrupted areas, considerable material that appeared to be agglutinated protein matrix was

present on the networks (Fig. 2A). Third, some layers of the network structure appeared to be separated in many areas, since layers beneath the ectoperitrophic layer were not visible there. If intact, the layers of the PM generally appeared to be compressed. We occasionally observed spindles that appeared to be slightly dissolved on the endoperitrophic surface of the PM, and the protein matrix of the PM around these spindles was missing, strongly suggesting that fusolin in soluted form that had begun to exude from the spindles had detached the proteins (Fig. 2D and E). In addition, the pointed structure of some of these spindles penetrated the network layer of the PM (Fig. 2E). It is therefore possible that disruption of the PM by spindles was due partly to this mechanical destruction by them. In contrast, no damage to the PM similar to that caused by spindle treatment was observed with spheroid treatment and in the control (sterile distilled-water treated) (Fig. 2F and G); the pore sizes were far smaller than the sizes of the virions. In some areas, the protein matrix only partly covered an ectoperitrophic network layer; although some pores in those areas were bigger than those in which the protein matrix was rich, they were still smaller than the AcEPV virions (Fig. 2H). These two presentations of the PM seen in the spheroid treatment and in the control were also observed in undisrupted areas of the PM in the spindle treatments. Similar naked areas have been seen in the ectoperitrophic surface of the normal PM in some of other insect species (2, 5).

Thus, first observations at the electron microscopy level of the PMs of host insects that had been fed spindles revealed various alterations in the PM; previous observations of spindle-associated changes of the PM in *B. mori* larvae were made by using light microscopy. These results strongly suggest that greater numbers of EPV virions could reach the ectoperitrophic space in the spindle-treated insects because they could pass through the disrupted areas that had been generated by the spindles, unlike in the spheroid-treated insects or the controls. Fusolin in the spindles is considered to be responsible for the disruption of the PM.

Real-time quantitative PCR of AcEPV fusolin gene in the ectoperitrophic area. The number of DNA samples of the midgut juices and the midgut part, in which the AcEPV DNA was detected (i.e., $\geq$ the detection limit of AcEPV DNA in standard samples; the detection limit [5.96×10^{-9} μ g/25 μ l in the digestive juices and 1.75×10^{-9} μ g/25 μ l in the midgut part]) and the average initial AcEPV DNA weight per a reaction mixture are presented below. Among larvae inoculated with spheroids, AcEPV DNA was detected in none of the 10 samples of the digestive juices and in only 1 of the 12 samples of the midgut part tested; the amount of AcEPV DNA in that sample was estimated to be 2.75×10^{-8} μ g/25 μ l. Among larvae given spheroids combined with heated spindles, AcEPV DNA was detected in 4 of the 9 samples of the midgut juices (average AcEPV DNA amount = 2.73×10^{-8} μ g/25 μ l) and 6 of the 11 samples of the midgut part (average AcEPV DNA amount = 1.50×10^{-8} μ g/25 μ l). The ratio of the number of samples in which AcEPV DNA was detected to that in which it was undetected differed significantly between the two treatments in both types of insect materials (the chi-square test, $P < 0.02$). The detected DNA amount in the samples corresponded to the number of AcEPV virions. The DNA detection rates in the spindle- and spheroid-treated samples and the

FIG. 2. Scanning electron micrographs of the PM of *A. cuprea* second-instar larvae after they were fed purified spindles or spheroids. The PM is on a SEMpore filter (an aluminum stub in panel C). All PMs were viewed from the ectoperitrophic side. Arrows indicate spindles. (A, B, D, and E) Larvae after being fed 4×10^6 spindles; (C) larvae after being fed 4×10^5 spindles; (F) larvae after being fed 4×10^5 spheroids; (G and H) larvae after being fed only sterile distilled water. Destruction of the PM occurred after spindles were fed. In panels A to C, holes and/or gaps were generated, and the protein matrix was detached in numerous spots (especially in panel C). In panel A, a broad area of some layers was lost, and material that appeared to be agglutinated protein matrix was present on the inner network of the PM. In panels D and E, spindles that appeared to only slightly dissolve are shown, and the proteins around them are missing. The pointed structure of the spindles may have destroyed the PMs in panel E. In panel H, the protein matrix appeared to cover a network just beneath the naked ectoperitrophic network, i.e., the surface of the PM.

TABLE 1. Test of the ability of AcEPV spheroids to enhance AcEPV infection in a host insect (*Anomala cuprea*)^a

Spheroid preparation (no. of spheroids)	Combination with a spheroid preparation		No. of infected larvae: no. of uninfected larvae	Infection rate (%)
	Heated spheroid preparation (no. of heated spheroids)	Heated spindle preparation (no. of heated spindles)		
— ^b	—	4 × 10 ⁴	0:33	0
	4 × 10 ⁵	—	0:37	0
3 × 10 ²	—	4 × 10 ⁴	10:25 ^A	28.6
1 × 10 ³	—	—	0:33 ^B	0
	4 × 10 ⁴	—	0:33 ^B	0
	4 × 10 ⁵	—	1:34 ^B	2.9
2.5 × 10 ³	—	—	3:34 ^α	8.1
	3.2 × 10 ^{2c}	—	2:35 ^α	5.4
	4 × 10 ⁴	—	5:32 ^α	13.5
	4 × 10 ⁵	—	2:34 ^α	5.6
	—	4 × 10 ⁴	18:18 ^β	50.0
6.3 × 10 ³	—	—	4:30 [*]	11.8
	4 × 10 ⁴	—	6:28 [*]	17.6
	4 × 10 ⁵	—	4:33 [*]	10.8

^a The spindle preparation used in this study contained 0.8% spheroids compared to the number of spindles, and the spheroid preparation used in this study contained 0.5% spindles compared to the amount of spheroids. To inactivate the virulence of the spheroids, part of the spheroid or spindle preparation was heated. The number of infected larvae as a ratio of the number of uninfected larvae in the same row followed by the same superscript letter or symbol does not differ significantly at the level of $P < 0.05$ as determined by the chi-square test in each of the three groups (grouped according to the number of spheroids in the spheroid preparation: first group, 3×10^2 and 1×10^3 ; second group, 2.5×10^3 ; and third group, 6.3×10^3).

^b —, None.

^c This inoculum (3.2×10^2 heated spheroids combined with 2.5×10^3 spheroids) was administered to evaluate the enhancing ability of 0.8% spheroid contamination of the spindles (i.e., 3.2×10^2 heated spheroids in 4×10^4 heated spindles).

spheroid-only-treated samples (i.e., the number of samples in which DNA was detected divided by the number tested and then multiplied by 100) were similar to the rate of infection of the larvae administered similar numbers of spheroids with or without spindles in the present bioassays, respectively (Table 1; 2.5×10^3 spheroids combined with 4×10^4 spindles, infection rate = 50%; 2.5×10^3 spheroids, infection rate = 8.1%). Thus, almost all of the insects, in the parts of which AcEPV DNA was detected, might become infected with the EPV. It might occur that when the PM near sac-circles was disrupted by spindles, midgut juices containing AcEPV virions flowed out from the site of the disruption and entered the sac-circles and that the DNA in these virions was then detected by quantitative PCR. These results indicate that greater numbers of AcEPV virions passed through the disrupted sites formed in the PM (see above) by the spindles, and that they reached the ectoperitrophic space and then entered the midgut epithelium. These results also indicate that the PM acts as a barrier against viral infection in coleopteran species, as it does in lepidopteran insects.

Bioassays. The bioassay results of the effects of heat treatment on fusolin activity of enhancing EPV infection were as

follows. Of the 39 larvae given 4×10^4 heated spindles mixed with 2.5×10^3 unheated spheroids, 22 became infected, whereas 26 of the 38 larvae inoculated with 4×10^4 unheated spindles mixed with 2.5×10^3 unheated spheroids were infected. The ratio of the number of infected larvae to that of uninfected larvae did not differ significantly between the two treatments (the chi-square test, $P > 0.2$). In the control, none of 38 larvae that had been fed sterile distilled water as a substitute for above proteinaceous body suspension became infected with the EPVs. Therefore, these results confirm that such heat treatment has little or no effect on fusolin activity, and thus heat treatment of spheroids was judged appropriate for bioassay. Strictly speaking, the inoculum combining unheated spindles with unheated spheroids contained too many unheated spheroids by 3.2×10^2 compared to that in the inoculum combining heated spindles with unheated spheroids, because the purified spindle preparation contained 0.8% contaminating spheroids. These excess spheroids may be responsible for the slightly higher infection rate than that in the bioassay using the inoculum combining heated spindles with unheated spheroids since almost the same number, i.e., 3×10^2 , of spheroids enhanced the infectivity a little (28.6%), as shown in Table 1.

The main bioassay results are shown in Table 1. As a control, 39 larvae that had been fed sterile distilled water were reared, and none of these larvae became infected with the viruses. The heated spindle inoculum retained a high capacity to enhance AcEPV infection (Table 1). We inoculated 3.2×10^2 heated spheroids per larva in order to assess the effect of the 3.2×10^2 heated spheroids contaminating the 4×10^4 heated spindles inoculated per larva, and neither this inoculation nor any inoculation of heated spheroids greater than 3.2×10^2 enhanced the EPV infection. Thus, the enhancement of infectivity by the inocula (4×10^4 heated spindles combined with 3.2×10^2 or 2.5×10^3 spheroids) was considered to be due completely to the heated spindles only. We obtained these results by using a different type of bioassay than that used by Mitsuhashi et al. (20) and Mitsuhashi (15), and our findings support their conclusions that EPV spindles enhance the infectivity of EPV and that this enhancement may be the natural biological function of EPV spindles. Furthermore, our results strongly suggest that the spheroids do not enhance infection. This lack of enhancing ability of spheroids appears to be due to their failure to damage the PM in a way similar to that seen by SEM after inoculation of larvae with spindles.

Immunoelectron microscopy of spheroids. Spindles were immunopositive for anti-AcEPV fusolin antibody, whereas the spheroids were immunonegative for the antibody; far more gold beads occurred within the spindles than within the spheroids, and the frequency of occurrence of the beads within the spheroids was not significantly different from that in the control (Fig. 3). This strongly suggests that there is little or no fusolin in the spheroids. Our results concerning the spheroids were discrepant from a report of experiments using a similar method (immunoelectron microscopy), which showed that PsEPV spheroids contain considerable amounts of fusolin (9).

SDS-PAGE and Western blot analyses of spheroid proteins for fusolin detection. In SDS-PAGE, when the spheroid sample was examined, at least 13 bands were detected by the naked eye in the range of 250 to 25 kDa (Fig. 4A), and at least 11

FIG. 3. Immunoelectron microscopy of AcEPV spheroids. Arrows, spheroids; arrowheads, spindles; bar, 1 μm. (A and B) Incubation with anti-AcEPV fusolin antibody; (C) control (no primary antibody). The rate of occurrence of gold beads within the spheroids incubated with the antibody was not significantly different from that within the spheroids in the control, whereas that within the antibody-treated spheroids was significantly lower than that within the spindles incubated with the antibody.

bands were detected in the same range when the purified AcEPV virion sample was examined (Fig. 4B). Most bands were considered to represent viral structural proteins. However, the fusolin-sized (48 kDa) band was not clearly detected in the former sample (10⁶ spheroids per lane), whereas the

same number of spindles showed a very clear band of fusolin (Fig. 4A). Also, no band of fusolin was detected in the purified AcEPV virion sample (Fig. 4B). A weak signal of fusolin was detected in the Western blot analysis of the spheroids (Fig. 4A). However, the signal was

FIG. 4. SDS-PAGE and Western blot analyses of AcEPV spheroids. The spheroids containing virions and purified virions were searched for the presence of fusolin. WB, Western blot analysis. M, marker (Precision Plus Protein Standards, All Blue; Bio-Rad). S, purified AcEPV spindles; Sh, purified AcEPV spheroids; V, purified AcEPV virions; FUS, fusolin. The figure indicates the number of spindles or spheroids treated per lane. Anti-AcEPV fusolin antibody was used as a probe in the Western blot analysis. The purified spheroids used for the analysis in panel A contained very few (1.3%) contaminating spindles in comparison to the number of the spheroids. In the Western blot analysis in panel A, there was a large difference in the level of detection of positive fusolin signals between the purified spindle samples and the same number of purified spheroid samples, strongly suggesting that the weak fusolin signal was (at least mainly) derived from the spindles contaminating the spheroid samples.

very weak compared to that in the spindle samples, suggesting that the weak signal was derived from the 1.3% contamination of the purified spheroids by spindles. On the other hand, no fusolin signal was detected in the AcEPV virions in the Western blot analysis, which is a far more powerful method than SDS-PAGE for detecting target proteins (Fig. 4B).

These results strongly suggest that there is little or no fusolin in the AcEPV spheroids. In the case of PsEPV, a considerable amount of fusolin was detected in the spheroids by immunological experiments (immunodiffusion tests and Western blot analysis) (27). In addition, the same number of spheroids as spindles enhanced NPV infection to similar extents, suggesting that the amount of fusolin is similar between spheroids and spindles (27). Thus, our results contradict those of experiments that used PsEPV.

DISCUSSION

We revealed here for the first time that EPV spindles enhance EPV infection by disruption of the PM in the host insects. The mode of action by which spindles enhance EPV infectivity is thus similar to that by which spindles enhance NPV infectivity. Our electron microscopy studies of the PMs of the hosts orally inoculated with spindles revealed various alterations in the PM. In addition, we found by SEM that AcEPV spheroids did not disrupt the PM and by bioassay that they did not enhance AcEPV infection and that fusolin was not detectable in spheroids and virions by immunological and biochemical examinations. Fusolin, the constitutive protein of the spindle, is a possible infectivity-enhancing factor of EPVs because it is the enhancing factor of the infectivity of some NPVs (6, 8; Takemoto et al., Abstr. 57th Meet. Kanto Branch Jpn. Soc. Seric. Sci.) and because the disruption of the PM caused by spindles leads to enhanced infectivity of both NPV (17) and EPV (the present study). Thus, the lack of fusolin in the spheroids is considered to be responsible for our SEM observations of the PM after the feeding of spheroids to host larvae and for the results of our bioassays using spheroids. These results concerning spheroids differ from those of the previous studies in that not only PsEPV spindles but also spheroids enhanced PuNPV infection and at almost the same level; the enhancing factor for PuNPV (i.e., fusolin) was present in both the spindle and the spheroid (9, 27).

Here, we developed a new method for extracting scarabaeid EPV DNA from the spheroids. A method we previously used for AcEPV DNA extraction is a standard one, with some modification. Other researchers have used the standard method for lepidopteran, orthopteran, and coleopteran EPV spheroids, and occasionally the standard method with some modification has been used for the latter spheroids. In the standard method, EPV virions are first released from spheroids by use of an alkaline reducing buffer. Proteinase K digestion of virion proteins is then performed, and PCI or CI extraction is conducted. With this method, 10^8 spheroids/ml are treated for 3 to 15 min for spheroid dissolution. However, use of this protocol for AcEPV spheroid dissolution results in very poor dissolution, and only small amounts of DNA are obtained. With our previous modification of the standard method for AcEPV spheroids, the number of treated spheroids was dramatically decreased, and the treatment period was length-

ened. Namely, 10^5 to 10^6 spheroids/ml were treated in an alkaline reducing buffer (0.8 M Na_2CO_3 , 0.1 M sodium thioglycolate) for 1 h (18; W. Mitsuhashi and M. Sato, unpublished data). This modification improved AcEPV DNA yields compared to the standard method of EPV DNA extraction, but the yield was rather low compared to that of the new method used in the present study. It also had a disadvantage in that very large amounts of alkaline reducing buffer were required when the intention was to extract large amounts of DNA for some experiments, such as construction of the physical maps of the genome or gene libraries. We found that the guanidine-HCl method also caused very poor dissolution of AcEPV spheroids, similar to that observed with our previous method. We therefore modified the guanidine-HCl method in the present study. Our new method has dramatically improved spheroid dissolution even in small volumes of buffer compared to the previous methods. It also saves time by executing the dissolution of both spheroids and EPV virion proteins in one simultaneous treatment. Thus, our method is simple and efficient for obtaining large amounts of EPV DNA of coleoptera. We and other researchers have encountered difficulties with dissolution of scarabaeid EPV spheroids with the standard method (12; D.W. Roberts and M. Bergoin, Proc. 4th Int. Colloq. Insect Pathol., p. 381–385, 1970). These difficulties are presumably due to the larger numbers of cysteine residues in the spheroid protein, spheroidin, of scarabaeid EPVs compared to those in the spheroidin of *Betaentomopoxvirus* viruses, since the cysteine residues may make intra- and interchain disulfide bonds that are most likely involved in the formation of the paracrystalline structure of spheroidins.

In the quantitative PCR experiment, the AcEPV DNA likely did not replicate before excision of the midgut, since only about 6 to 7 h had passed after the larvae were transferred to wells on which artificial diet containing spheroids with or without spindles was placed (11). Furthermore, the larvae did not begin to eat the artificial diet in the experiments until at least 2 or 3 h after they had been transferred to the wells. These facts strongly suggest that the quantity of the AcEPV DNA revealed in the midgut part samples indicates the DNA amount of the AcEPV virions that had fused with the microvilli and thus that the difference in DNA amount between the spindle- and spheroid-treated and the spheroid-only-treated samples reflects the differences in the amount of the AcEPV virions that had fused with the microvilli between the two.

The amino acid sequences of fusolins show low but significant similarity to those of GP37s in some baculoviruses (1, 14). These sequences also show distant similarity to those of some bacterial chitin-binding proteins, including Cbp1 from *Alteromonas* sp. strain O-7 and CHB1 from *Streptomyces olivaceoviridis*, to that of CBP from *Streptomyces halstedii* and to those of ChiBs from some bacteria, including *Pseudoalteromonas* sp. strain S9 and *Salinivibrio costicola* (14, 22, 23). Like these bacterial proteins, fusolins and GP37s have conserved regions that constitute a potential chitin-binding domain (14, 22, 23), and the domain in fusolins, GP37s, and some of these bacterial proteins is specifically classified as chitin-binding domain 3 (InterPro database). Indeed, *Spodoptera litura* NPV GP37 showed chitin-binding ability (14). Therefore, fusolin may disrupt the PM by binding with the chitin in it. However, the mechanical force of the pointed structures of spindles before

their disappearance by dissolution may be partially responsible for the disruption of the PM observed in the present study. Further study is required to assess the effect of this mechanical force on PM disruption.

Some granuloviruses (GVs) and NPVs, both belonging to *Baculoviridae*, are known to harbor enhancin, a metalloprotease that disrupts their host insects' PM by degrading the intestinal mucin of the PM and thus enhances NPV infection (13, 24, 26). In the course of their evolution, EPVs and baculoviruses have acquired different tools for the same purpose of overcoming a barrier, the PM, against initial infection in the host midgut. Insect viruses of a wide range of other taxa also may have evolved the other types of gene products for overcoming the PM barrier.

Understanding the strategies involving the possible chitin-binding action and mechanical force that viruses use to overcome the PM barrier in more detail and at a molecular level may lead to the development of more effective methods or tools for overcoming the PM barrier and thus to the development of more effective viral insecticides.

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