Enhanced Fusion of a Nucleopolyhedrovirus with Cultured Cells by a Virus Enhancing Factor from an Entomopoxvirus

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Fusion of *Pseudaletia unipuncta* nucleopolyhedrovirus with an armyworm cell line (SIE-MSH-805-F) was studied by means of three fluorescence assays that are based on the relief of fluorescence self-quenching of octadecylrhodamine B chloride (R18). A gradual increase in fluorescence intensity indicative of virus–cell fusion was observed by spectrofluorometry when R18-labeled polyhedron-derived virus was incubated with cultured cells. The fusion was enhanced by the virus enhancing factor (EF) from *Pseudaletia separata* entomopoxvirus. Lysosomotropic agents had little effect on the virus–cell fusion. The percentage of positively fluorescent cells, as determined by flow cytometry, gradually increased after the addition of labeled virus and was higher in the presence of the EF than in its absence. Confocal microscopy of cultured cells that had been combined with labeled virus showed that the fluorescence appeared first on their surface. The plasma membrane of cultured cells had specific affinity to the EF, as revealed by indirect immunofluorescence microscopy.

Key Words: nucleopolyhedrovirus; entomopoxvirus; cell culture; *Pseudaletia separata*; fluorescence dequenching; lysosomotropic agents; virus enhancing factor; flow cytometry; confocal microscopy; immunofluorescence microscopy.

INTRODUCTION

A virus enhancing factor (EF) that enhances nucleopolyhedrovirus (NPV) infection in the armyworm, *Pseudaletia separata*, has been isolated from the spheroid of *Pseudaletia separata* entomopoxvirus (PSEV) (Xu and Hukuhara, 1992, 1994). The EF is localized in the occluded virion within the spheroid (Hukuhara et al., 1995). It is also present in the nonoccluded virion and the spindle of PSEV (Hukuhara et al., 1995; Wijonarko and Hukuhara, 1998). The identity of the EF with a protein constituting the spindle, called fusolin, has been suggested because of the similarity of the EF gene to the previously reported fusolin genes (Hayakawa et al., 1996).

NPV virions are present in two forms, budded virus (BV) and polyhedron-derived virus (PDV). They have unique envelope compositions and appear to enter cells by different mechanisms (see Williams and Faulkner, 1997). PDV transfers their genome and accessory proteins to the cytosol of target cells by fusion between the viral envelope and the plasma membrane. BV enters target cells by acid-triggered fusion with the endosomal membrane after being internalized by endocytosis (Volkman, 1986). Fusion of BV at the cell surface has also been observed (Volkman et al., 1986). The BV envelope contains a viral-encoded glycoprotein, GP64, that is required for cell-to-cell transmission of infection (Volkman, 1986; Blissard and Wenz, 1992; Monsma et al., 1996; Hefferon et al., 1999).

In an attempt to determine the mode of action of the EF, we have investigated its effect on virus–cell fusion using fluorescence assays that are based on the relief of fluorescence self-quenching (see Hoekstra and Klappe, 1993). The assays involve the exogenous insertion of a lipotropic fluorescent probe in the bilayer of the NPV envelope at such a high concentration as to cause efficient quenching of fluorescence. Fusion of the labeled bilayer with target unlabeled cell membranes dilutes the probe and results in membrane fluorescence dequenching, with a corresponding increase in fluorescence. Thus, the assays provide a measure of the degree of virus fusion. We herein report the enhancement of NPV fusion with cultured cells by the EF.

MATERIALS AND METHODS

Cultured cells. SIE-MSH-805-F (MSH) from *P. separata* was grown in TC/10. IPLB-SF-21AEII (SF) from Spodoptera frugiperda and IPLB-LD-652Y (LD) from Lymantria dispar, were grown in Grace’s medium. The culture media were supplemented with 10%...
fetal bovine serum. They were maintained at 25°C and subcultured at 4- to 7-day intervals.

Preparation of labeled PDV. Polyhedra of the typical strain of P. unipuncta multiple NPV (PsunMNPV) were produced and purified as described previously (Hukuhara et al., 1987). To liberate occluded virions, 1 × 10^{10} polyhedra in 2 ml of distilled water were combined with an equal volume of dissolution buffer (0.2 M glycine, 0.4 M NaOH, pH 10.6) for 5 min. Then, the mixture was diluted by the addition of the same volume of ice-cold distilled water and centrifuged at 1500g for 20 min to remove undissolved polyhedra. The supernatant was placed on top of a 20% (w/w) sucrose cushion. After centrifugation at 55,000g for 1 h, PDV that accumulated in the pellet were suspended in 1 ml of Hepes buffer (10 mM Hepes, 145 mM NaCl, pH 7.4) and mixed with 15 μl of ethanolic solution of octadecylrhodamine B chloride (R18) (Molecular Probes, Inc., Junction City, OR) in the dark for 1 h using a rotary shaker (200 rpm). Labeled PDV was pelleted by centrifugation at 55,000g for 1 h to remove unbound R18, suspended in 1 ml of Hepes buffer to a final concentration of 2 μg/ml, and stored at −30°C until used.

Preparation of the EF. A Chinese isolate of PSEV was propagated in P. separata larvae (Hukuhara et al., 1990). Spindles were purified from larval cadavers as described (Wijonarko and Hukuhara, 1998) and solubilized in a dissolution buffer consisting of 0.6 M Na_2CO_3, 0.015 M sodium thioglycolate, and 0.03 M EDTA (5 × 10^7 spindles/ml). Solubilized spindle proteins were dialyzed overnight against 0.01 M Tris–HCl buffer (pH 8) and applied onto an affinity column (Hi-Trap NHS-activated, Pharmacia Biotech., Uppsala, Sweden) that had been coupled with immunoglobulin from an anti-EF rabbit antiserum (Xu and Hukuhara, 1994). The column was washed with the same buffer to remove unbound proteins. The EF that remained adsorbed was eluted with 0.4 M Na_2CO_3, dialyzed overnight against phosphate-buffered (0.1 M) saline solution (PBS, 0.85%, pH 7.4) at 4°C, and stored at −30°C until used. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of samples at different purification steps was performed as described previously (Xu and Hukuhara, 1994).

Fluorescence assays. The EF was added to suspensions of MSH, SF, or LD cells (5 × 10^5/ml) in the respective culture medium to give the final concentration of 0.2–20 μg/ml and maintained throughout the experiment. After 30-min incubation with the EF, the cells were combined with R18-labeled PDV (final concentration, 2 μg/ml). Control cells to which no EF was added were also combined with labeled PDV in a similar manner. The experiments were conducted at 25°C unless otherwise indicated under Results. Three kinds of methods were employed to access the R18 emission signals. (1) Spectrofluorometry. The assay was performed as described previously (Kozuma and Hukuhara, 1994) using a spectrophotofluorometer (Type FP-920; Nihon Bunko, Tokyo). EF-containing samples were completely solubilized at the end of the assay to obtain 100% dequenching by the addition of Triton X-100 at a final concentration of 0.1%. The fluorescence intensity observed earlier in the assay was represented by relative fluorescence, which was defined as the fraction of the intensity at the maximal dequenching. (2) Flow cytometry. The assay was performed using a Coulter flow cytometer (Elite ESP; EPICS, Hialeah, FL) equipped with an argon laser (exitation line at 488 nm) according to the manufacturer’s manual. Debris smaller than cultured cells were excluded from the analysis by the conventional scatter gating method. Five thousand events per sample were collected in a list mode fashion. R18 emission signals were collected using BP filter at 575 nm. The threshold of positivity for fluorescence intensity was arbitrarily set, based on the negative control cells to which no labeled virions had been added. (3) Confocal microscopy. The wet-mount preparations of a mixture of MSH cells, the EF, and labeled virions were investigated with a confocal laser scanning microscope (TCS-NT; Leica, Heidelberg, Germany). The EF was added to increase the proportion of fluorescent cells and thus to facilitate the analysis. The 568-nm laser line was employed for excitation. R18 emission signals were collected using TRITC filter at 590 nm.

Indirect immunofluorescent staining. MSH cells were incubated for 15 min with 1% normal goat serum in PBS (pH 7.2) to reduce nonspecific staining. The cells were incubated in turn with the EF (0.2 μg/ml) for 30 min, with 5% anti-EF serum in PBS for 15 min, and with 2.5% fluorescein isothiocyanate (FITC)-conjugated goat immunoglobulin against rabbit IgG (The Binding Site Ltd., Birmingham, England) in PBS for 30 min at 25°C. Three washings with PBS were performed after each step. The final preparations were examined with a Leica confocal microscope using the 488-nm laser line for excitation. FITC emission signals were collected using a filter at 515–545 nm.

RESULTS

Solubilized spindle proteins of PSEV were applied onto an affinity column that had been coupled with anti-EF antibodies. Binding, washing, and elution with sodium bicarbonate solution resulted in the purification of a 38-kDa protein, which was presumed to be the EF (Fig. 1). When MSH cells were combined with R18-labeled PDV of PsunMNPV in the presence or absence of the purified EF, the fluorescent intensity as determined by spectrofluorometry started to increase immediately after the virus addition (Fig. 2). The initial rate
of increase of the relative fluorescence was 14% per min in the presence of the EF and 2% per min in its absence. Thereafter, the rates gradually decreased until the relative fluorescence plateaued in 2–3 h. The higher the EF concentration, the higher the plateau level (Table 1). No membrane fluorescence dequenching was observed when labeled virions were added to suspensions of SF or LD cells. The results indicated that the PDV fused specifically with MSH cells and that the virus–cell fusion was enhanced by the EF.

**FIG. 1.** SDS–PAGE patterns of spindles, solubilized spindle proteins unabsorbed to the affinity column, the EF eluted from the column, and the mixture of standard proteins. Lane 1, intact spindles; lane 2, unadsorbed proteins in the washing; lane 3, purified EF in the eluent; lane 4, standard proteins. The numerals on the side represent their molecular weights (kDa). The position of the EF band is denoted with an arrow.

**FIG. 2.** Kinetics of fusion of PsunMNPV and MSH cells at 25°C in the presence (A) or absence (B) of the EF (2 μg/ml), which was added 30 min prior to the addition of R 18-labeled PDV at time zero. The fluorescence intensity was determined at specific time periods and is presented as a fraction of the intensity observed at 100% dequenching.

**FIG. 3.** Fluorescence histograms of an MSH cell sample to which no virus was added (A) and cell samples to which R 18-labeled PDV was added in the absence (B through E) or presence (F through J) of the EF (2 μg/ml). The threshold of positivity was set, based on the autofluorescence of the negative control sample, as indicated by the horizontal bar (A). The percentages represent the fractions of positively fluorescent cells. The time intervals between the virus addition and the measurement are shown in parentheses. Flow cytometry was performed at 24°C.

![SDS–PAGE patterns](image1)

![Kinetics of fusion](image2)

![Fluorescence histograms](image3)
TABLE 1
Effect of Different Concentrations of the EF on the Plateau Level in the Fluorescence Dequenching Assay of Membrane Fusion between PsunMNPV and MSH Cells

<table>
<thead>
<tr>
<th>Concentration of the EF (µg/ml)</th>
<th>Relative fluorescence and SE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>72.82 ± 2.24</td>
</tr>
<tr>
<td>0.20</td>
<td>46.26 ± 1.20</td>
</tr>
<tr>
<td>0.02</td>
<td>33.48 ± 1.61</td>
</tr>
<tr>
<td>0.00</td>
<td>28.04 ± 1.32</td>
</tr>
</tbody>
</table>

* The averages and their standard errors (SE) were calculated from the fluorescence intensities of four replicate samples observed in 90 min following the addition of R18-labeled PDV.

In an attempt to determine the pathway of virus entry, several lysosomotropic agents were added to MSH cells just before the addition of labeled PDV. Subsequent monitoring of the fluorescence intensity by spectrofluorometry indicated that these agents had little effect on the fusion kinetics (data not shown) or the plateau level (Table 2) irrespective of the presence or absence of the EF. The results suggested that the acidification of endosomes was not required for the entry of PDV into MSH cells.

The fluorescence pattern of MSH cells to which R18-labeled PDV had been added was analyzed by confocal microscopy. Shortly after the virus addition, a low proportion of the cells exhibited fluorescence on several sites of the cell surface. The proportion of such cells gradually increased as time lapsed (Fig. 4A). In 60 min following the virus addition, more than half of the cells exhibited fluorescence not only on the cell surface but also in the perinuclear zone of the cytoplasm (Fig. 4B).

To determine the affinity of the EF to MSH cells, cultured cells were incubated with the EF and then processed for indirect immunofluorescent staining with an anti-EF rabbit serum and FITC-conjugated goat immunoglobulin against rabbit IgG. Confocal microscopy of the cells revealed the presence of intense fluorescence on the cell surface (Fig. 4C). No fluorescence was observed in control cells which had not been incubated with the EF or the anti-EF serum. The results indicated that the plasma membrane of MSH cells had specific affinity to the EF.

**DISCUSSION**

Horton and Burand (1993) demonstrated the nonendoctic entry of PDV of Lymantria dispar multiple NPV into a gypsy moth cell line (ILPL-LdEIta) using fluorescence dequenching assay of membrane fusion. Our study has provided three lines of evidence for the entry of PDV of PsunMNPV by fusion with the plasma membrane of the host cell. First, the time course curve of the fluorescence dequenching assay showed no appreciable lag phase, whose presence is typical for viruses requiring an endocytic pathway (see Helenius et al., 1989). Second, agents known to raise the pH of endosomes and thus to block the virus fusion with the endosomal membrane had little effect on the fusion kinetics and the plateau level. Third, confocal microscopy revealed the early occurrence of fluorescence on the cell surface presumably as the result of the dispersion of the fluorescent probe from the virion envelope into the plasma membrane. Later occurrence of fluorescence in the perinuclear zone is difficult to interpret. One possibility is the internalization of R18-labeled lipid of the plasma membrane, as was reported in the case of fluorescently labeled phospholipids (Sleight and Pagano, 1985).

A proteinous factor present in the capsule of the Hawaiian strain of P. unipuncta granulovirus (PsunGV) has been known to enhance the infection of PsunMNPV in armyworm larvae (Tanada and Hukuhara, 1971). It is called synergistic factor (SyF) or PsunGV-H enhancin and is different from the EF in the gene nucleotide sequence (Roelvink et al., 1995; Hayakawa et al., 1996). Ohba and Tanada (1983, 1984a) showed that the factor also enhanced the in vitro infection of several lepidopterous cell lines with Trichoplusia ni multiple NPV (TnMNPV) and Autographa californica multiple NPV (AcMNPV). They suggested that the SyF acted as an enhancer in the attachment of BV to cultured insect cells on the basis of their observation that the SyF attached to certain areas of the plasma membrane and agglutinated the cells (Ohba and Tanada, 1984b). Hukuhara and Zhu (1989) demonstrated the enhanced infection of SF cells by the SyF with both BV and PDV of TnMNPV. Kozuma and Hukuhara (1994) showed that the SyF enhanced the fusion of BV TnMNPV with SF cells using fluorescence dequenching assay, that preincubation of cells, but not of virions, with the SyF affected the fusion kinetics, and that lysosomotropic agents did not inhibit the fusion. They suggested that the SyF was bound to the plasma membrane through the presence of a SyF-receptor.

**TABLE 2**
Effect of Several Lysosomotropic Agents on the Plateau Level in the Fluorescence Dequenching Assay of Membrane Fusion between PDV PsunMNPV and MSH Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Relative fluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>1mM</td>
<td>77.86</td>
</tr>
<tr>
<td>Monensin</td>
<td>1µM</td>
<td>71.43</td>
</tr>
<tr>
<td>Methylamine</td>
<td>5mM</td>
<td>70.37</td>
</tr>
<tr>
<td>Control (no agent)</td>
<td>—</td>
<td>78.57</td>
</tr>
</tbody>
</table>

* The fluorescence intensities were determined in 120 min following the addition of lysosomotropic agents and R18-labeled PDV to MSH cells in the presence or absence of the EF (2 µg/ml).
ceptor and that the binding gave rise to membrane bilayer destabilization leading to merging of the plasma membrane with the viral envelope.

We have demonstrated enhanced virus–cell fusion by the EF in a system involving PsunNPV and MSH. The system is suited for fusion study because it shows affinity to both EF and virus but is not suited for infection study because of abortive PsunNPV infection (T. Hukuhara et al., unpublished). To demonstrate the correlation between enhanced fusion and enhanced infection, it may be necessary to use other NPVs, such as AcMNPV, that replicate well in this cell line. The current literature is replete with reports showing that fusion of enveloped viruses with the cell plasma membrane of permissive cells is mediated by a specific viral membrane "fusion peptide" and cell surface molecules (see Dimitrov, 1997). The best-characterized virus is the human immunodeficiency virus-type 1. Its entry into the host cell may involve the following three major steps: (1) the high-affinity binding of a fusion peptide to a cell receptor induces conformational changes in their complex, resulting in interactions of parts of the fusion peptide with a coreceptor called fusin; (2) the interaction of fusin with the viral envelope helps in the relocation of the fusion peptide to a position in close proximity to the surface of the host cell membrane; and (3) the exposed hydrophobic fusion peptide induces local attraction and destabilization of the cell and viral membrane, resulting in their fusion (Dimitrov, 1996). It is tempting to speculate that the MSH cells have a specific receptor for the EF that acts as a fusion peptide.

Infection of insect larvae with NPVs commonly occurs by oral ingestion of polyhedra. They are solubilized by the midgut digestive juices and released the occluded virions, PDV, into the lumen. The nucleocapsid of the virions enters the columnar cell of the midgut epithelium by fusion of the virus envelope with the microvillus membrane (Kawanishi et al., 1972). We hypothesize that NPV infection in armyworm larvae is enhanced by the EF through enhanced fusion of PDV with the microvillus membrane. It is hoped that further characterization of the mode of action of the EF using the comparative approach will provide a better understanding of the process of NPV entry to host insects.

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REFERENCES


