Baculovirus gp64 Envelope Glycoprotein Is Sufficient To Mediate pH-Dependent Membrane Fusion

GARY W. BLISSARD* AND JOHN R. WENZ

Boyce Thompson Institute at Cornell University, Tower Road, Ithaca, New York 14853-1801

Received 18 June 1992/Accepted 11 August 1992

The baculovirus gp64 envelope glycoprotein is a major component of the envelope of the budded virus (BV) and is involved in BV entry into the host cell by endocytosis. To determine whether gp64 alone was sufficient to mediate membrane fusion, the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus gp64 protein was transiently expressed in uninfected insect cells. Cells expressing the baculovirus gp64 protein were examined for membrane fusion activity by using a syncytium formation assay under various conditions of exposure to low pH. Cells expressing the gp64 protein mediated membrane fusion and syncytium formation in a pH-dependent manner. A pH of 5.5 or lower was required to induce membrane fusion. In addition, exposure of gp64-expressing cells to low pH for as little as 5 s was sufficient to induce gp64-mediated syncytium formation. These studies provide direct evidence that gp64 is a pH-dependent membrane fusion protein and suggest that gp64 is the protein responsible for fusion of the virion envelope with the endosome membrane during BV entry into the host cell by endocytosis.

Baculoviruses are large double-stranded DNA viruses that are transcribed and replicate in the nuclei of host cells. Baculoviruses infect invertebrates exclusively, with most baculoviruses isolated from insects. The large DNA genome (approximately 80 to 150 kbp) is packaged into rod-shaped nucleocapsids that assemble in the nucleus. Two distinct virion phenotypes are produced in the infection cycle (for reviews, see references 3, 7, and 16). Virions of one phenotype, known as the budded virus (BV), are produced when nucleocapsids bud from the plasma membrane. The BV virions are highly infectious to most tissues of the host and in tissue culture (11, 22). Later in infection, nucleocapsids acquire an envelope in the nucleus and the resulting virions are subsequently occluded in large proteinaceous occlusion bodies termed "polyhedra". Virions enveloped and occluded in the nucleus are referred to as polyhedron-derived virus (PDV). Virions of these two phenotypes (BV and PDV) are identical in nucleocapsid structure and DNA content but differ in the source of virion envelopes and relative infectivities for different cell types in the insect (8, 11, 22).

During infection, the gp64 envelope glycoprotein is synthesized and transported to the plasma membrane (21). Thus, as BV virions bud from the plasma membrane, they acquire a plasma-membrane-derived envelope containing the gp64 protein. The gp64 protein is not detected in PDV virions (2, 10, 18, 23), which are assembled in the nucleus. The gp64 protein is phosphorylated, acylated, and glycosylated and contains inter- and intramolecular disulfide bonds (15, 18, 19, 21). In the membrane, the gp64 protein is present as an oligomer which consists of homotetramers of the gp64 protein (19). In several studies (10, 11, 14, 20, 21), it has been demonstrated that some monoclonal antibodies (MAbs) directed against the gp64 envelope glycoprotein are capable of neutralizing the infectivity of BV virions. Such studies indicate that the gp64 protein plays an essential role in the infection process. While some enveloped viruses fuse with the cellular plasma membrane at the cell surface, others

enter by endocytosis. In the best-studied example of viruses that enter cells by endocytosis (the influenza virus), fusion activity of the hemagglutinin protein is activated by the acidification of the endosome, resulting in fusion of the viral envelope with the endosome membrane. Studies of baculovirus BV entry into host cells in the presence of lipid soluble bases (which prevent acidification of the endosome) show that BV entry into cells is primarily by endocytosis (20). While it is not known whether gp64 is involved in cell receptor recognition, studies of one neutralizing MAb (AcV1) demonstrate that gp64 is involved in entry of the virus by endocytosis at some step beyond the initial binding of the virus to the host cell (20). Because previous studies of the gp64 envelope protein have been performed with whole BV virions (which contain additional viral structural proteins with unknown contributions to infectivity), it has not been possible to determine the precise functional role of gp64 in the infection process. However, it has been speculated that gp64 may be involved in membrane fusion (20).

The gene encoding the gp64 envelope glycoprotein has been identified and sequenced in two baculoviruses, *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (Op MNPV) and *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) (2, 23). Comparisons of the two baculovirus gp64 genes reveal that the two envelope glycoproteins and their genes are highly conserved, showing overall amino acid and nucleotide sequence similarities of 78 and 74%, respectively (2). Furthermore, the predicted ectodomains (domains on the surface of the virion) are approximately 83% conserved in amino acid sequence. Potential N-glycosylation sites, cysteine residues, and potential acylation sites are also conserved (2, 15). Recently, Morse et al. (13) reported a striking similarity between the baculovirus gp64 proteins and the putative envelope proteins from two tick-borne arboviruses. The Thogoto (THO) and Dhori (DHO) viruses are unclassified tick-borne arboviruses that share morphological and biochemical properties with the orthomyxoviruses. While several gene products from the THO and DHO viruses have amino acid sequence and other similarities with proteins from the influenza A, B, and C viruses, the putative envelope glycoproteins of the THO and

* Corresponding author. Electronic mail address: Gary.Blissard@qmrelay.mail.CORNELL.EDU.

FIG. 1. Construction of a plasmid for transient expression of gp64 in uninfected insect cells. A strong baculovirus early promoter was constructed upstream of the OpMNPV gp64 ORF by reconstructing a previously described promoter deletion mutation (4). Plasmid p64HB-3.3 contains the OpMNPV gp64 ORF and approximately 1,500 bp of upstream sequence (a region of intensely repeated sequence is indicated by a hatched line). gp64 sequences upstream of the *Mlu*I site were removed from plasmid p64HB-3.3 and replaced with 82 bp of the regulatory sequences from a deletion subclone (p64CAT-166) (4) as described in the text. The resulting construct (p64-166) contains the gp64 ORF and 166 nucleotides of the gp64 upstream sequence. Relative locations of the TATA box and conserved start site sequence (CAGT) are indicated in the bottom segment. The location of the transcription start site is indicated by the arrow above the CAGT. B, *Bam*HI; Bs, *Bst*EII; H, *Hind*III; Hp, *Hpa*I; MI, *Mlu*I; Xh, *Xho*I.

DHO viruses appear to be unrelated to any influenza virus envelope proteins. However, the putative envelope glycoproteins from the THO and DHO viruses show remarkable similarities to the gp64 envelope glycoproteins of the OpMNPV and AcMNPV baculoviruses. The similarities include large regions of significant amino acid sequence identity, a similar distribution of cysteine residues (61% conservation), three conserved sites for potential *N*-glycosylation, and similarities in overall hydropathy profiles and size (13). The similarities in primary structure of these arbovirus and baculovirus envelope glycoproteins are likely related to the fact that both baculoviruses and the THO and DHO arboviruses infect invertebrate hosts and suggest that the two groups may utilize similar mechanisms for entry into the host cell.

In the present study, we examined the role of the baculovirus gp64 protein by studying its function under conditions in which gp64 was isolated from other viral proteins. Using the OpMNPV gp64 gene under the control of a strong eukaryotic promoter, gp64 was expressed in uninfected cells and examined for membrane fusion activity under various pH conditions. In these studies, we show that the gp64 protein mediates membrane fusion in a pH-dependent manner. These data suggest that the gp64 protein is a membrane fusion protein required for entry of the virus by endocytosis.

Construction of plasmids for expression of gp64 in uninfected cells. In the present study, gp64 was expressed from a plasmid construct that contained the entire gp64 open reading frame (ORF) (including sequences encoding signal and

membrane anchor domains) under the control of an optimized gp64 early promoter. The promoter construct used in this study was characterized in a previous study (4). Regulatory sequence elements upstream of the OpMNPV gp64 TATA box were isolated from promoter construct p64CAT-166 (4) by digesting plasmid p64CAT-166 with *Xho*I and *Mlu*I, gel purifying the 82-bp *Xho*I-*Mlu*I fragment, and cloning the 82-bp fragment upstream of the gp64 TATA box and ORF (Fig. 1). A plasmid containing the entire OpMNPV gp64 gene on a 3.3-kbp *Hind*III-*Bam*HI fragment from the OpMNPV genome (2) was digested with *Hind*III and *Mlu*I to remove upstream sequences. The 4.3-kbp *Mlu*I-*Hind*III fragment, containing pBS vector sequences and the gp64 gene, was gel purified and ligated to the 82-bp *Xho*I-*Mlu*I fragment at the *Mlu*I site. The *Hind*III and *Xho*I sites were filled in with deoxynucleoside triphosphates and Klenow polymerase and ligated. The resulting construct, p64-166, contains 166 nucleotides of sequence upstream of the gp64 ORF, the gp64 ORF, and approximately 288 nucleotides of sequence downstream of the ORF (including the gp64 polyadenylation signal) (Fig. 1). Methods for insect cell culture, plasmid purification, transfection, infection, virus production, and virus titration were described previously (1, 2, 4, 5, 17).

Optimization of gp64 expression in uninfected insect cells. To optimize conditions for expression of gp64 in uninfected cells, plasmid constructs were transfected into insect cells and analyzed for production of gp64 by Western blot (immunoblot) analysis using the anti-gp64 MAbs AcV5 (10), which was previously shown to cross-react with the OpMNPV

FIG. 2. Western blot analysis of gp64 expression in uninfected *L. dispar* cells. *L. dispar* cells were transfected with 1.5 μ g of plasmid p64-166 and incubated for various times posttransfection. The times posttransfection (24 to 96 h) when cells were harvested for Western blot analysis are indicated below the Western blot. Each group of four lanes represents transfections that were performed in quadruplicate. Relative levels of gp64 detected at each time posttransfection are indicated by the graph above the Western blot.

gp64 protein (2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed essentially as described previously (2, 5). For Western blot analysis, 1.9×10^5 *Lymantria dispar* (Ld652Y) cells were plated in each well of a 24-well plate and transfected with various amounts of plasmid DNA (see below). Time zero was defined as the time when plasmid DNAs were added to the cells. Cells from each well were harvested by removing the medium, washing the cells with $1 \times$ phosphate-buffered saline, and solubilizing the cells in 250 μ l of $1 \times$ SDS-PAGE disruption buffer (125 mM Tris [pH 6.8], 2% SDS, 5% β -mercaptoethanol, 0.001% bromophenol blue, 10% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) for a final concentration of 7.6×10^5 cells per ml. Samples were immediately transferred to ice, heated to 100°C for 5 min, and stored at -80°C. For Western blot analysis, 15 μ l of the protein solution from the transfected cells was run on each lane of an SDS-PAGE (10% polyacrylamide) gel, blotted to Immobilon-P filters (Millipore), and reacted with mouse ascites fluid containing the AcV5 MAbs (1:20,000 dilution). Blots were subsequently washed and incubated in a 1:7,500 dilution of goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Promega). Immunoreactive proteins were detected by incubation in a substrate solution of 330 μ g of nitroblue tetrazolium and 165 μ g of 5-bromo-4-chloro-3-indolylphosphate (Promega) per ml. Relative levels of gp64 were determined by scanning and densitometric analysis of Western blots with the Image 1.4 software package (National Institutes of Health).

To optimize conditions for the accumulation of maximal levels of gp64 in uninfected insect cells, we examined two parameters: (i) the quantities of plasmid DNA transfected into cells and (ii) the times posttransfection when steady-state levels of gp64 were highest. Cells were transfected with increasing quantities of plasmid p64-166 (0.2 to 5 μ g of DNA per 1.9×10^5 *L. dispar* cells) and harvested at 48 h posttransfection. Analysis of band intensities from Western blots indicated that transfection of 1.9×10^5 *L. dispar* cells with 0.2 to 2.5 μ g of plasmid p64-166 resulted in significant levels of gp64 production, with the highest steady-state

levels of gp64 observed from transfections of 1.0 to 2.0 μ g (not shown). Next, we examined steady-state levels of gp64 at various times posttransfection. *L. dispar* cells (1.9×10^5 cells) were transfected with 1.5 μ g of the p64-166 plasmid and incubated for 24, 36, 48, 72, or 96 h, and then the cells were harvested for Western blot analysis of gp64 levels. Relative levels of gp64 detected at 24 and 96 h appear to be lower than levels detected at 36 through 72 h posttransfection (Fig. 2). On average, little increase in the relative level of gp64 was observed after 36 h. Maximal steady-state levels of gp64 were reached and maintained between 36 and 72 h posttransfection. Thus, for the purpose of assaying gp64 function, we determined that the optimal transfection conditions consisted of 1.0 to 2.0 μ g of plasmid DNA per 1.9×10^5 *L. dispar* cells (approximately 5 to 10 pg of DNA per cell) and an incubation period of 36 to 72 h posttransfection.

gp64-mediated membrane fusion. To determine whether gp64 (expressed in the absence of other viral proteins) was sufficient to induce membrane fusion, *L. dispar* cells were plated in six-well tissue culture dishes (35-mm-diameter wells), transfected with the p64-166 plasmid, and examined for fusion under various conditions with low pHs. Cells were plated at a density (1.2×10^6 *L. dispar* cells per well) sufficient to produce a monolayer in which most cells were in contact with adjacent cells after a 48-h incubation. Cells in each well were transfected with 10 μ g of p64-166 plasmid DNA (approximately 8.3 pg of DNA per cell). For controls, 1.2×10^6 *L. dispar* cells were either transfected with equal amounts of the pBS plasmid or infected with OpMNPV at a multiplicity of infection of 1. For infections, cells were incubated with virus for 1 h, the virus was removed, and fresh medium was added. Cells were incubated at 27°C in normal growth medium (TNM-FH supplemented with 10% fetal bovine serum at a pH of 6.0) (17) for 48 h. For both transfection and infection, time zero was defined as the time point when virus or plasmid DNA was added to cells. After a 48-h incubation, medium was removed and replaced with Grace's medium plus 10% fetal bovine serum adjusted to either pH 5.0 or 6.0. The low pH value (5.0) was selected because similar values have been reported for eukaryotic endosomes after acidification (9). Cells were observed for syncytium formation at various times after shifting to pH 5.0. Although some membrane fusion was observed at earlier times, a substantial degree of fusion had occurred by 4 h after shifting of the pH. Fused cells were distinguished by the loss of the normal plasma membranes between adjacent cells and the formation of polynucleate syncytia (polykaryon formation). Figure 3 shows the results of an experiment to examine gp64-mediated membrane fusion in transfected cells and in infected-cell controls. As a control for the effect of pH treatment on mock-transfected cells, *L. dispar* cells were transfected with the pBS plasmid (which does not contain the gp64 gene) and similarly shifted to pH 5.0 at 48 h posttransfection. No cell fusion was observed in pBS-transfected cells (Fig. 3B). To determine whether gp64-mediated fusion was low-pH dependent, *L. dispar* cells transfected with p64-166 were exposed to media of either pH 5.0 or pH 6.0. No cell fusion was observed in cells expressing gp64 and remaining at pH 6.0 (Fig. 3A). In contrast, cells transfected with p64-166 (expressing gp64) and shifted to pH 5.0 exhibited widespread membrane fusion resulting in obvious syncytium formation (Fig. 3C). These data from transfected cells expressing gp64 in isolation from other viral proteins show that the gp64 protein is sufficient to mediate membrane fusion and that fusion is dependent on low pH.

The gp64 protein is synthesized and transported to the

FIG. 3. gp64-mediated membrane fusion and pH dependence of syncytium formation in cells expressing gp64. *L. dispar* cells were transfected with plasmid p64-166 (A and C) or a control pBS plasmid (B). After transfection, cells were transferred to TNM-FH medium. At 48 h posttransfection, cells were shifted to Grace's medium at pH 6.0 (A) or 5.0 (B and C) and incubated for 4 h. Polynucleate syncytia were widespread in cells expressing gp64 and shifted to pH 5.0 (C). Aggregations of nuclei within syncytia are indicated by arrowheads in panel C. As a positive control, *L. dispar* cells were infected with OpMNPV and then shifted to pH 6.0 (D) or 5.0 (E) at 48 h postinfection. When infected cells were shifted to pH 5.0, aggregates of nuclei (arrowheads) are observed where syncytia have formed by cell fusion (E). All micrographs show cells at 4 h after the pH was shifted (phase-contrast; magnification, $\times 950$; bar = 20 μm).

surface of infected cells, where budding nucleocapsids acquire this envelope protein. Previous studies show that the gp64 protein is readily detected at the surface of baculovirus-infected cells (21) and is abundant in OpMNPV-infected *L. dispar* cells at 48 h postinfection (2, 5). Therefore, we used *L. dispar* cells infected with OpMNPV as a positive control for gp64 fusion activity. Infected cells (48 h postinfection) were incubated in Grace's medium adjusted to a pH of either 5.0 or 6.0 and observed for syncytium formation. Cell fusion was readily observed in OpMNPV-infected *L. dispar* cells shifted to pH 5.0 (Fig. 3E) but was not observed when the pH was maintained at 6.0 (Fig. 3D).

pH and exposure requirements for membrane fusion. For viral fusion proteins such as the influenza hemagglutinin protein, membrane fusion is initiated by a pH-induced conformational change that results in the activation of the fusion protein (for reviews, see references 12 and 24) followed by a rapid induction of fusion activity. The maximal rate for hemagglutinin-mediated fusion with cellular membranes occurs at pH 4.9 to 5.2 (6). To determine the requirements for gp64-mediated membrane fusion, we examined the pH range required for fusion and the length of time at low pH required to induce fusion. In the studies described above, we determined that the gp64 protein was inactive (for membrane fusion) at pH 6.0 but active at pH 5.0. Although cells were

normally incubated for 4 h before being scored for syncytium formation, we hypothesized that a shorter exposure to low pH might be sufficient to induce membrane fusion. To determine the exposure time required to induce gp64-mediated membrane fusion, we exposed cells expressing gp64 to Grace's medium at pH 5.0 for the following lengths of time: 5, 10, 20, 30, and 60 s; 2, 3, 10, 15, 20, and 30 min; and 1, 1.5, 2, 3, and 4 h. For each exposure period, four replica wells of cells were shifted to pH 5.0 for the times indicated above and then washed with neutral saline and shifted to Grace's medium at pH 6.0. Cells were examined for fusion 4 h after the beginning of the shift to pH 5.0. All periods of exposure to pH 5.0 resulted in unambiguous syncytium formation in all wells, even when cells were exposed for the shortest period (5 s). This indicates that the low-pH activation of gp64 fusion activity occurs rapidly and does not require prolonged exposure to low pH.

To determine more precisely the pH requirement for activation of gp64-mediated membrane fusion, individual wells containing p64-166-transfected cells were shifted to a range of pH values for 5 min and then returned to a pH of 6.0. For each pH treatment, duplicate wells of *L. dispar* cells (1.9×10^5 cells per well; 15.5-mm-diameter wells) were each transfected with 1.5 μg of p64-166 DNA and incubated for 48 h. Cells were washed three times with neutral saline and then

TABLE 1. pH requirement for gp64-mediated syncytium formation^a

pH	Syncytium formation
6.0.....	—
5.7.....	—
5.6.....	—
5.5.....	+
5.4.....	+
5.3.....	+
5.2.....	+
5.0.....	+
4.9.....	+
4.8.....	+
4.7.....	+
4.6.....	+
4.5.....	+
3.0 (10 s).....	+

^a Duplicate wells of transfected cells were exposed to Grace's medium at the indicated pH values for 5 min and then returned to pH 6.0 and scored for fusion 4 h after the pH shift. Exposure of cells to pH 3.0 was for only 10 s.

were shifted to Grace's medium at a specific pH value ranging from 4.5 to 6. After a 5-min incubation at the indicated pH value (Table 1), cells were washed with neutral saline and placed in Grace's medium at pH 6.0. At 4 h after the initiation of the pH shift, wells were scored for syncytium formation (Table 1). Five-minute exposures of cells to pH values of 5.5 or below resulted in obvious and unambiguous cell fusion (syncytium formation). No fusion activity was detected at pH values of ≥ 5.6 (Table 1). Interestingly, we previously found that when cells expressing gp64 were incubated at pH values below approximately 4.7 for long periods (4 h), fusion was not readily observed (data not shown). This is presumably due to adverse effects on the cell caused by prolonged exposure to very low pH. In contrast, a brief exposure (10 s) to an even lower pH value (pH 3.0) resulted in readily detectable syncytium formation (Table 1). The pH requirements for gp64-mediated syncytium formation are consistent with values reported by Volkman and Goldsmith (20) using whole BV for hemolysis assays with human erythrocytes. This suggests that the effects observed in those studies reflect the activity of the gp64 protein and not other virion structural proteins.

Summary. Baculovirus virions of the BV phenotype enter cells by endocytosis. Inhibition studies with an anti-gp64 MAb (AcV1) previously demonstrated that the gp64 virion envelope protein is involved in the entry of virions by endocytosis (20). In addition, it was shown that the AcV1 MAb was capable of neutralizing BV-induced hemolysis of erythrocytes, leading to the suggestion that gp64 may be involved in membrane fusion. For the present studies, we developed a system for transiently expressing the gp64 protein in uninfected cells and examining the function of the gp64 protein in the absence of other viral proteins. Using this system, we provide direct evidence that the gp64 envelope protein is a pH-dependent membrane fusion protein and suggest that gp64 is responsible for membrane fusion during endocytosis of the baculovirus. While additional functions have not yet been demonstrated, it is possible that like membrane fusion proteins from other virus groups, the gp64 protein may play additional roles during infection. Possible roles include the initial recognition and binding to host cell receptors at the cell surface as well as participation in the maturation and budding of BV virions.

We thank Rosalind Wei for excellent technical assistance and Phil Kogan for comments on the manuscript.

This research was supported by Boyce Thompson Institute project 1250 and grant AI31130 from the NIH.

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