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Host Cell Receptor Binding by Baculovirus GP64 and Kinetics of Virion Entry

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GP64 is the major envelope glycoprotein from budded virions of the baculoviruses *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV). To examine the potential role of GP64 as a viral attachment protein in host cell receptor binding, we generated, overexpressed, and characterized a soluble form of the OpMNPV GP64 protein, GP64sol^{Op}. Assays for trimerization, sensitivity to proteinase K, and reduction by dithiothreitol suggested that GP64sol^{Op} was indistinguishable from the ectodomain of the wild-type OpMNPV GP64 protein. Virion binding to host cells was analyzed by incubating virions with cells at 4°C in the presence or absence of competitors, using a single-cell infectivity assay to measure virion binding. Purified soluble GP64 (GP64sol^{Op}) competed with a recombinant AcMNPV marker virus for binding to host cells, similar to control competition with psoralen-inactivated wild-type AcMNPV and OpMNPV virions. A nonspecific competitor protein did not similarly inhibit virion binding. Thus specific competition by GP64sol^{Op} for virion binding suggests that the GP64 protein is a host cell receptor-binding protein. We also examined the kinetics of virion internalization into endosomes and virion release from endosomes by acid-triggered membrane fusion. Using a protease sensitivity assay to measure internalization of bound virions, we found that virions entered Spodoptera frugiperda Sf9 cells between 10 and 20 min after binding, with a half-time of approximately 12.5 min. We used the lysosomotropic reagent ammonium chloride to examine the kinetics of membrane fusion and nucleocapsid release from endosomes after membrane fusion. Ammonium chloride inhibition assays indicated that AcMNPV nucleocapsids were released from endosomes between 15 and 30 min after binding, with a half-time of approximately 25 min.

INTRODUCTION

Baculoviruses are enveloped viruses that contain large circular double-stranded DNA genomes ranging in size from ~80 to 180 kbp (Volkman *et al.,* 1995). Transcription, DNA replication, and nucleocapsid assembly occur within the nuclei of infected host cells. Baculoviruses are characterized by an infection cycle that produces two virion phenotypes, budded virus (BV) and occlusion-derived virus (ODV), which are structurally and functionally distinct (reviewed in Blissard, 1996; Miller, 1997; Rohrmann, 1992). BV is highly infectious in cell culture and mediates cell-to-cell transmission in the infected animal. Virions of the second phenotype, ODV, are embedded within large proteinaceous occlusion bodies that are produced in the very late phase of the infection cycle. Structurally, BV and ODV differ by the origin and composition of their envelopes (Braunagel and Summers, 1994). They also differ in the mechanisms by which they enter host cells. BV enters cells through the endocytic pathway (Volkman and Goldsmith, 1985), whereas ODV appears to enter the midgut epithelial cells via direct membrane fusion at the cell surface (Granados, 1978; Summers, 1971). Although several studies have examined binding properties of the two virion phenotypes (Horton and Burand, 1993; Wang *et al.,* 1997; Wickham *et al.,* 1992), the viral attachment proteins (viral receptor-binding proteins) and host cell receptors have not been identified for either ODV or BV.

The BV envelopes of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* multicapsid nucleopolyhedrovirus (OpMNPV) contain an abundant viral-encoded glycoprotein, GP64, that is not found in the ODV envelope (Blissard and Rohrmann, 1989; Braunagel and Summers, 1994; Whitford *et al.,* 1989). BV examined by electron microscopy contains characteristic spike-like peplomers within the envelope, and these spike proteins appear to be concentrated at the end of the virion that is associated with virion budding. Immunoelectron microscopic studies of virions budding from AcMNPV-infected cells suggest that the spikes are composed of the GP64 protein (Volkman, 1986), and it was recently shown that GP64 is necessary for efficient production of BV (Oomens and Blissard, 1999). Each spike or peplomer is believed to represent a single homotrimer of the GP64 protein (Oomens *et al.,* 1995), and antibodies directed against GP64 have been shown to neutralize infectivity of the BV

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(Hohmann and Faulkner, 1983; Volkman *et al.,* 1984). However, the single neutralizing monoclonal antibody (MAb AcV1) that has been characterized in detail was shown to neutralize virion entry by inhibition of membrane fusion, a step after virion binding (Chernomordik *et al.,* 1995; Volkman and Goldsmith, 1985). MAbs that inhibit virion binding have not been reported. Previous studies showed that the GP64 protein is necessary and sufficient for low pH-triggered membrane fusion activity, the process that occurs during virion entry by endocytosis (Blissard and Wenz, 1992; Leikina *et al.,* 1992), and membrane fusion has been examined in some detail (Chernomordik *et al.,* 1995; Markovic *et al.,* 1998; Monsma and Blissard, 1995; Oomens and Blissard, 1999; Plonsky *et al.,* 1999; Plonsky and Zimmerberg, 1996). By generating and examining a GP64-null virus containing a *gp64* knockout, it was demonstrated that GP64 is an essential viral structural protein of AcMNPV (Monsma *et al.,* 1996). In animals fed occlusion bodies from the GP64-null virus (vAc64Z), midgut cells were efficiently infected by ODV, but the infection was unable to move from the midgut epithelium into cells and tissues of the hemocoel. Similarly, infection by vAc64Z did not spread from cell to cell in tissue culture. Subsequent studies showed that GP64 is required for efficient virion budding from the cell surface (Oomens and Blissard, 1999).

Envelope proteins from animal viruses mediate entry into host cells by facilitating two critical functions: binding to host cell receptors and fusion of the viral envelope with the host cell plasma membrane. In some cases, such as the well studied influenza hemagglutinin (HA) protein, both functions are provided by a single protein. In other cases, such as the parainfluenza viruses, these two functions are segregated into two proteins: hemagglutinin/neuraminidase (HN) and fusion (F) proteins. Viral binding to a host cell receptor may serve two major functions. First, adhesion of the virus particle to the cell may be a necessary prerequisite for subsequent endocytosis. Second, the viral envelope and host plasma membrane must be closely apposed to fuse. In some parainfluenza viruses, it has been demonstrated that the receptor-binding activity of the HN protein is necessary for fusion induced by the F protein (Lamb, 1993). In the case of the baculoviruses OpMNPV and AcMNPV, the GP64 protein was previously demonstrated to encode a low-pH-activated membrane fusion activity (Blissard and Wenz, 1992). However, host cell receptor binding has not been previously assigned to GP64 or any other BV envelope protein. A recent study of GP64 glycosylation mutants showed that decreased infectivity was observed when some glycosylation sites were eliminated, suggesting that the GP64 glycosylation state may affect virion binding (Jarvis *et al.,* 1998). Perhaps most compelling is the observation that a closely related orthomyxovirus homolog of GP64 (the GP75 protein of the Thogoto virus) has hemagglutination activity (Morse *et al.,* 1992; Portela *et al.,* 1992). Because the GP64 protein contains primary sequence similarities across the entire predicted ectodomain of GP75, this suggests that the GP64 protein may also encode a receptor-binding function. In the present study, we examined the role of GP64 in virion binding at the cell surface. To determine whether GP64 was involved in virion binding to host cell receptors, we first generated, purified, and characterized a secreted soluble form of the GP64 protein, GP64sol^{Op}. It was previously demonstrated that GP64sol^{Op} was trimerized in a manner indistinguishable from native membrane-bound GP64 protein (Oomens *et al.,* 1995). In the current study, we used N-terminal sequencing, limited proteolysis, and dithiothreitol (DTT) sensitivity assays to further demonstrate that GP64sol^{Op} is indistinguishable from the predicted ectodomain of native GP64. Using purified GP64sol^{Op} as a competitor for virion binding to *Spodoptera frugiperda* Sf9 cells, we show that GP64sol^{Op} competes for binding of AcMNPV BV at the surface. To further characterize virion entry, we also examined the kinetics of BV entry by measuring the timing of BV internalization into endosomes and release of nucleocapsids from endosomes.

RESULTS

Production of a secreted soluble form of GP64

A secreted soluble form of the OpMNPV GP64 protein was produced in an AcMNPV baculovirus expression vector system by insertion of a modified *gp64* ORF into the polyhedrin locus of AcMNPV. Site-directed mutagenesis was used to insert a stop codon after Leu477, which is located immediately upstream of the predicted transmembrane domain of the OpMNPV *gp64* ORF (Fig. 1A). The resulting construct was placed under the control of the polyhedrin promoter in transfer vector pAcDZ1 (Jansen *et al.,* 1991), and a recombinant baculovirus was generated from BacPAK6 viral DNA. The secreted soluble form of GP64 (GP64sol^{Op}) was purified from supernatant collected from infected BTI-Tn-5B1–4 (High Five) cells at 48 h postinfection (p.i.) as described in Materials and Methods. Quantity and purity of GP64sol^{Op} were assessed by Bradford protein assay and by Coomassie blue and silver staining of GP64sol^{Op} electrophoresed on SDS-polyacrylamide gels, followed by densitometric scanning of stained gels (Fig. 1B). Extensive purification of GP64sol^{Op} resulted in a preparation exceeding (93–95%) purity, which was used for all competition studies. A partially purified preparation of GP64sol^{Op} was used for protease resistance and oligomerization studies. We previously showed that secreted soluble GP64sol^{Op} was trimerized in a manner indistinguishable from native OpMNPV GP64, suggesting that GP64sol^{Op} was identical to the ectodomain of native GP64 (Oomens *et al.,* 1995). To further confirm the native structure and processing of GP64sol^{Op}, we examined GP64sol^{Op} by N-terminal sequencing, endoglycosidase treatment, partial reduction with DTT, and protease treatment (below). For these stud-

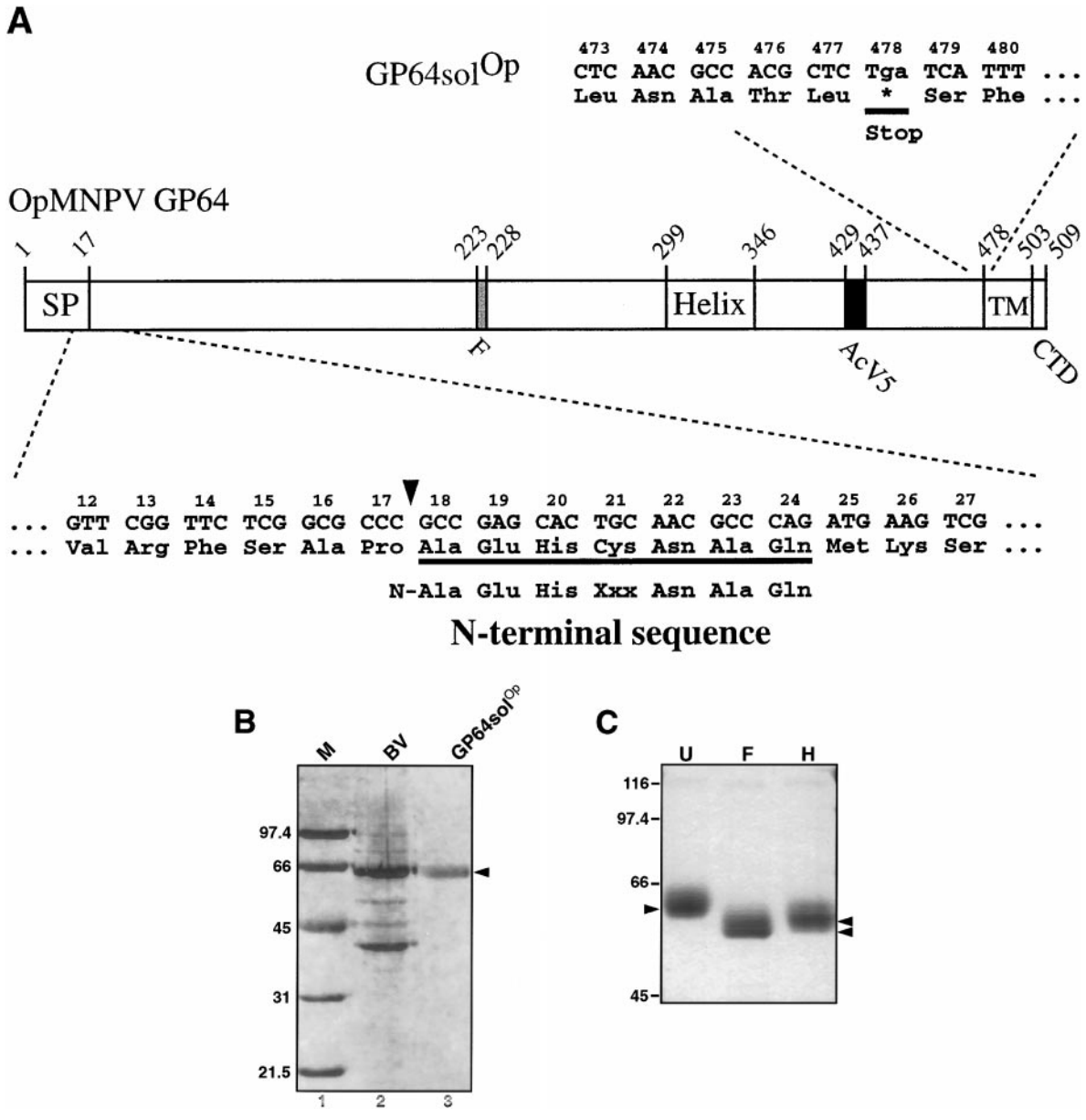

FIG. 1. (A) Construction of GP64sol^{Op}. The OpMNPV GP64 open reading frame is represented as an open bar with protein domains indicated on and below the bar. The gene encoding the secreted soluble form of GP64 (GP64sol^{Op}) contains a stop codon (*, Stop) introduced at Tyr478, immediately upstream of the transmembrane domain (TM). The N-terminal sequence determined from the mature secreted soluble GP64 protein is indicated below the nucleotide sequence and the predicted N-terminal sequence. The signal peptide cleavage site is indicated by an arrowhead. SP indicates signal peptide; F, fusion domain; Helix, predicted amphipathic α-helical region; AcV5, MAb epitope; and CTD, cytoplasmic tail domain. (B) Highly purified GP64sol^{Op} (purified as described in Materials and Methods) was compared with GP64 from OpMNPV budded virions (BV) by SDS–PAGE and Coomassie blue staining. Lane 1 indicates protein markers; lane 2, OpMNPV BV; and lane 3, highly purified GP64sol^{Op}. (C) GP64sol^{Op} glycosylation. Purified GP64sol^{Op} (undigested, U) was compared with endoglycosidase F (F)- or endoglycosidase H (H)-digested GP64sol^{Op} on SDS–polyacrylamide gels stained with Coomassie blue. The positions of protein markers and the mature GP64sol^{Op} (arrowhead) are indicated on the left, and the positions of major products of each digest are indicated on the right.

ies, GP64sol^{Op} was compared with GP64 derived from OpMNPV virions (Figs. 1 and 2).

Posttranslational modifications

The N-termini of baculovirus GP64 proteins are highly hydrophobic although not well conserved in primary sequence (Blissard and Rohrmann, 1989; Hill and Faulkner, 1994; Whitford *et al.,* 1989). This is consistent with the presence of a signal peptide that is cleaved during entry into the secretory pathway. In a previous study (Blissard and Rohrmann, 1989), the signal peptide cleavage site for the OpMNPV GP64 protein was predicted at a posi-

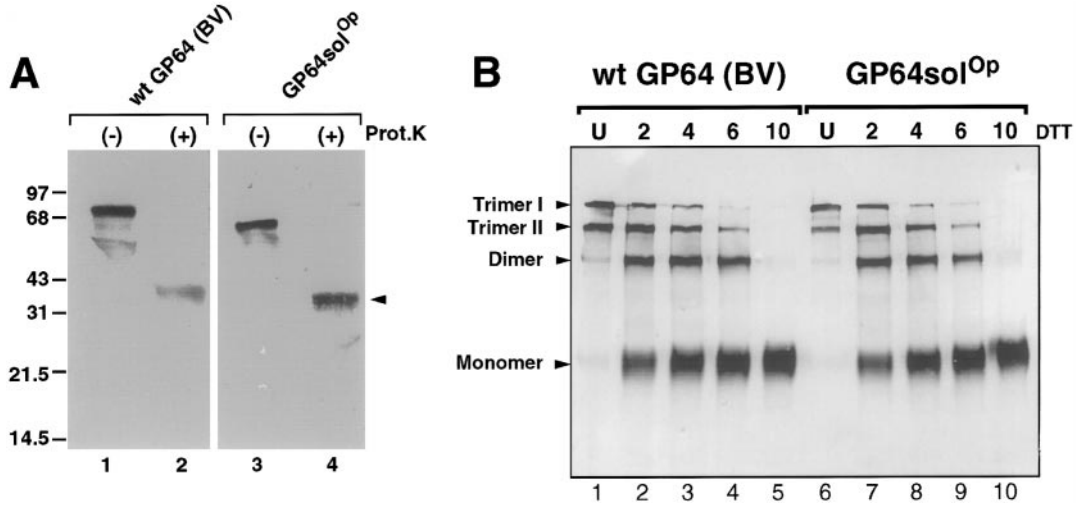

FIG. 2. (A) Protease resistance of OpMNPV GP64 and GP64sol^{Op}. GP64sol^{Op} (0.5 μg) and OpMNPV BV (1 μg) were digested with 0.1 mg/ml proteinase K (Prot.K) for 10 min at 56°C and then examined by Western blot analysis using MAb AcV5. Lane 1 indicates undigested OpMNPV budded virions; lane 2, OpMNPV BV digested with proteinase K; lane 3, undigested GP64sol^{Op}; and lane 4, GP64sol^{Op} digested with proteinase K. The position of the protease resistant C-terminal fragment of GP64 is indicated by the arrowhead. (B) DTT sensitivity of OpMNPV GP64 and GP64sol^{Op}. Purified GP64sol^{Op} and wild-type GP64 (wt GP64) from OpMNPV budded virions were reduced in varying concentrations of DTT at 37°C for 5 min and then prepared under nonreducing conditions and electrophoresed on SDS–polyacrylamide gels. Samples of GP64sol^{Op} (0.5 μg) and OpMNPV BV (1 μg) were treated with increasing concentrations of DTT (0–10 mM). GP64 proteins were identified by Western blot analysis using MAb AcV5. Lanes 1–5 indicate OpMNPV BV; and lanes 6–10, GP64sol^{Op}. Concentrations of DTT (mM) are indicated above each lane of the gel. Oligomeric forms of GP64 (trimers I and II, dimer, monomer) that were characterized previously (Oomens *et al.,* 1995) are indicated on the left.

tion between amino acids 17 and 18 (Fig. 1A), and this corresponds to a similar position (between amino acids 20 and 21) in the AcMNPV GP64 protein. N-terminal sequencing of highly purified GP64sol^{Op} resulted in the following N-terminal sequence, N-Ala-Glu-His-Xxx-Asn-Ala-Gln, demonstrating that the predicted peptide cleavage site is correct (Fig. 1A, arrowhead).

The native AcMNPV and OpMNPV GP64 proteins contain five and seven potential sites for N-linked glycosylation, respectively, and previous studies have shown that the native proteins contain ~6–8 kDa of N-linked carbohydrate per monomer (Blissard and Rohrmann, 1989; Jarvis and Garcia, 1994; Oomens *et al.,* 1995; Whitford *et al.,* 1989). In SDS-PAGE gels, GP64sol^{Op} migrates with a mobility similar to (or slightly smaller than) that of the native GP64 protein (Fig. 1B). Digestion of purified GP64sol^{Op} with endoglycosidase F or endoglycosidase H resulted in increased mobility consistent with that reported from native OpMNPV GP64 (Fig. 1C) (Oomens *et al.,* 1995).

Proteinase K digestion and DTT reduction

To determine whether the secreted soluble form of GP64 retained a native conformation, GP64sol^{Op} was compared directly with GP64 from OpMNPV BV in proteinase K resistance assays and DTT reduction assays (Fig. 2). Previous studies of GP64 from AcMNPV virions showed that proteinase K digestion of native GP64 resulted in protease resistant peptides of ~34–37 kDa (Volkman and Goldsmith, 1988). To examine protease resistance, we used an MAb (AcV5) that recognizes a defined, conserved linear epitope near the C-terminus of the GP64 ectodomain (Monsma and Blissard, 1995). When digested with proteinase K, a ~34-kDa protease-resistant peptide (that encompasses the C-terminal portion of GP64) was identified from both wild-type OpMNPV GP64 and GP64sol^{Op} (Fig. 2A, lanes 2 and 4, respectively). These proteinase resistance data strongly suggest that analogous portions of both proteins are exposed to cleavage by proteinase K and that GP64sol^{Op} is found in the same conformation as native GP64 from BV.

GP64 is present in BV as disulfide-linked homotrimers, and a previous study demonstrated that two electrophoretic forms of trimeric GP64 are observed on nonreducing SDS-PAGE gels (Oomens *et al.,* 1995). The two trimer forms were designated trimer I and trimer II. Trimers I and II were also detected from GP64sol^{Op}. Thus trimers of GP64sol^{Op} were indistinguishable from those of wild-type GP64 derived from virions (see also Fig. 2B, lanes 1 versus 6). To confirm the similarities and further examine GP64 trimerization, both GP64sol^{Op} and wild-type OpMNPV GP64 from BV were incubated in increasing concentrations of the reducing agent DTT, from 2 to 10 mM. In both cases, increasing concentrations of DTT resulted in a gradual loss of trimeric forms. At low DTT concentrations (2–4 mM), trimer I appears to be more susceptible to reduction than trimer II. This was observed for both GP64sol^{Op} and GP64 from OpMNPV BV

(Fig. 2B, lanes 2 and 3 and 7 and 8). At higher concentrations of DTT (10 mM), both GP64s were almost completely reduced to monomers (Fig. 2B, lanes 5 and 10). These similarities in sensitivity to DTT suggest that GP64sol^{Op} and wild-type OpMNPV GP64 are very similar in structure.

Baculovirus BV binding and infectivity

To examine virion binding and entry in subsequent experiments, we used a recombinant AcMNPV virus containing a *lacZ* gene under the control of a promoter (*Drosophila* hsp70) that is active early in the infection cycle. The recombinant AcMNPV marker virus (vAchsZ) (Monsma *et al.,* 1996; Vlak *et al.,* 1990) was used in a single-cell infectivity assay to measure changes in virion binding. To identify assay conditions in which changes in virion binding could be detected and quantified, we first generated a dose-response curve. Sf9 cells (3×10^5) were infected with increasing concentrations of vAchsZ virions (m.o.i. 0.0125–0.2), by binding BV to cells at 4°C for 1 h; then cells were washed, incubated at 27°C for 24 h, and stained for β-galactosidase expression in single infected cells. This resulted in a proportional linear increase in the number of single infected cells detected from increasing concentrations of virions (Fig. 3A). Thus within this range of virion concentrations and under these conditions, virions are not present in saturating concentrations; therefore, competition for virion binding can be more readily detected by this method. Virion concentrations within the linear range of this assay were used in all subsequent experiments.

In these studies, virion entry was synchronized by binding BV to cells at 4°C for 1 h and then shifting the temperature to 27°C to permit endocytosis of virions. To confirm that the vAchsZ BV bound at 4°C remained at the cell surface at the end of the 1-h binding period, virions were bound to cells at 4°C for 1 h and then cells were washed and treated with the protease subtilysin to inactivate or release virions at the cell surface. As a control, virions were bound to cells at 4°C for 1 h, washed in TNM-FH medium, and then incubated at 27°C for 1 h (allowing bound virions to internalize) and subsequently treated with subtilysin. Treatment of cells with subtilysin immediately after the 4°C binding step resulted in a dramatic decrease in numbers of infected cells detected (Fig. 3B, column 2). In contrast, when virions were bound at 4°C and then allowed to enter endosomes by incubating at 27°C for 1 h before subtilysin treatment, no detectable decrease in infectivity was observed (column 3). A control pretreatment of BV with subtilysin before binding (column 4) resulted in little binding and infectivity, as expected. These data indicate that during the 4°C incubation period, virions remain at the cell surface, and that bound virions enter within 1 h after the shift from 4°C

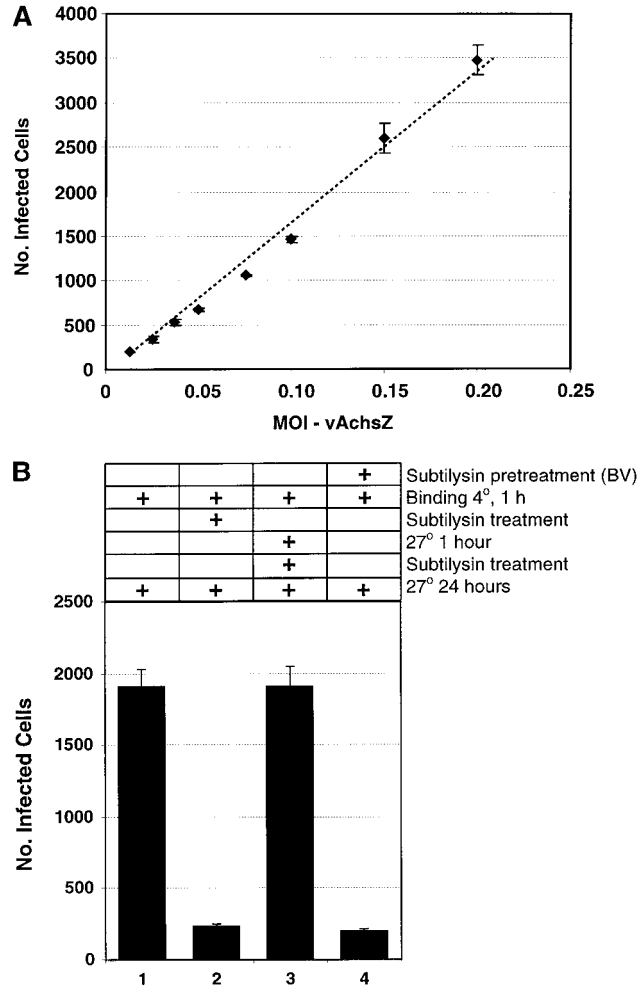

FIG. 3. (A) Dose-response curve for a single-cell infectivity assay with virus vAchsZ. To develop a rapid single-cell infectivity assay for detecting changes in virion binding, a range of virion concentrations were examined in a 1-h viral binding assay. To identify a range of virion concentrations in which the response to increasing virion concentrations was linear, Sf9 cells (3×10^5) were incubated with increasing concentrations of vAchsZ (m.o.i. 0.0125–0.2) for 1 h at 4°C and then washed in TNM-FH and incubated for 24 h in TNM-FH at 27°C. Single cells were scored for expression of β-galactosidase as described in the Materials and Methods. In the dose-response curve, each data point represents the averaged results from three wells, and bars represent standard deviation. (B) Protease sensitivity of virions bound at the cell surface. To determine whether virions remained at the cell surface at the end of a 1-h binding period at 4°C, the sensitivity of surface-bound virions to inactivation or release by treatment with the protease subtilysin was examined in a single-cell infection assay. Virions of vAchsZ were bound to Sf9 cells at 4°C for 1 h and then cells were washed 3× with TNM-FH at 4°C and either not treated (lane 1) or treated with 2 mg/ml subtilysin (lane 2) before shifting to 27°C and incubating for 24 h. As a control, virions were bound at 4°C, cells were washed as above, and then virions were allowed to internalize by shifting to 27°C for 1 h before treatment with subtilysin (lane 3). Control vAchsZ virions were pretreated with subtilysin before binding and analysis in the single-cell infection assay (lane 4). Each data point represents average data from three wells, and bars represent standard deviation.

to 27°C. Thus entry by endocytosis (after binding) is efficiently synchronized in this manner.

Virion and GP64sol^{Op} competition

Because GP64 is the major envelope protein of the BV, we hypothesized that BV binding to the host cell receptor may be mediated by GP64. The OpMNPV and AcMNPV GP64 proteins are highly conserved, showing 84% sequence identity in the ectodomains. In addition, in previous studies (unpublished), we found that OpMNPV BV appears to bind and enter Sf9 cells, although Sf9 cells are not permissive for OpMNPV replication. Thus if the GP64 protein encodes the host cell receptor-binding activity, the OpMNPV GP64 protein may compete for the same binding sites used by AcMNPV or the AcMNPV recombinant, vAchsZ. To determine whether OpMNPV virions compete for the same binding sites used by AcMNPV, psoralen-inactivated virions of AcMNPV and OpMNPV were used as competitors in the virion-binding assay described above. For these competition assays, Sf9 cells were incubated for 1.5 h at 4°C with various concentrations of psoralen-inactivated AcMNPV or OpMNPV virions before the addition and binding of vAchsZ at 4°C for 1 h. Prebinding of psoralen-inactivated AcMNPV or OpMNPV virions at 4°C resulted in a dramatic decrease in the number of cells infected by vAchsZ, as detected in the single-cell infectivity assay (Fig. 4). Results of these competition assays suggest that both AcMNPV and OpMNPV virions competed with vAchsZ for binding sites on Sf9 cells.

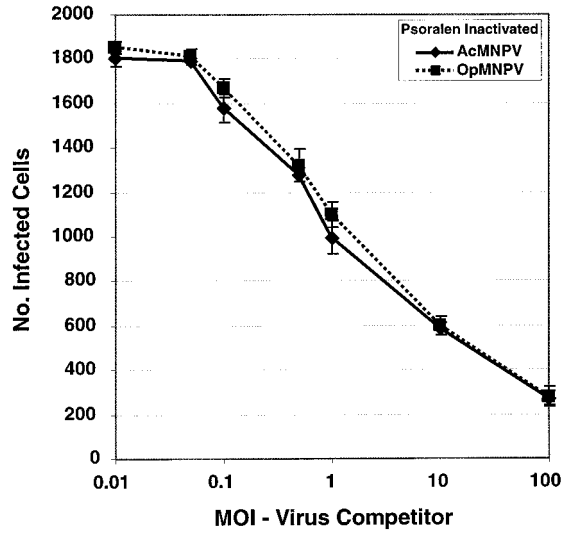

FIG. 4. Competition for virion binding by inactivated AcMNPV and OpMNPV BV. To determine whether OpMNPV and AcMNPV BV utilize the same host receptor-binding sites on Sf9 cells, psoralen-inactivated BV of AcMNPV and OpMNPV was used as competitors for binding of BV from vAchsZ. Sf9 cells were preincubated with increasing concentrations of psoralen-treated AcMNPV and OpMNPV BV (*X*-axis, m.o.i.) at 4°C for 1 h. The *X*-axis represents the quantity of BV corresponding to the indicated m.o.i. before psoralen inactivation. After prebinding of psoralen-inactivated BV (AcMNPV or OpMNPV), vAchsZ was added and allowed to bind for 1 h at 4°C. Cells were washed in TNM-FH, fresh medium was added, and infection was allowed to proceed for 20 h. Numbers of infected cells were determined as described in Materials and Methods. Each data point represents average data from three wells, and bars represent standard deviation.

Competition studies with a soluble GP64 protein

Because both AcMNPV and OpMNPV appeared to compete for the same host cell receptor sites, we next asked whether secreted soluble GP64 (GP64sol^{Op}) would similarly compete for binding to the host cell receptor. To address this question, varying concentrations of GP64sol^{Op} were incubated with Sf9 cells at 4°C for 1.5 h, and then vAchsZ was added and bound to cells for an additional hour at 4°C. As a control, identical molar concentrations of BSA were included in a parallel competition assay. Incubation of cells with increasing concentrations of GP64sol^{Op} resulted in a dramatic decrease in the number of infected cells (Fig. 5). Inhibition of virion binding was first detected at a GP64sol^{Op} concentration of 50 nM and was further inhibited by increasing concentrations of GP64sol^{Op} (100 and 200 nM). Only a minor reduction in the numbers of infected cells was observed when equivalent concentrations of BSA were used as a nonspecific competitor (Fig. 5, dashed line). Competition by GP64sol^{Op} for virion binding was observed in at least six separate experiments. To determine whether native GP64sol^{Op} trimers were required for efficient binding and as a further control to confirm the role of GP64, purified GP64sol^{Op} trimers were reduced by treatment in 10 mM DTT before competition assays. Previous studies showed that these conditions were sufficient to reduce trimeric GP64sol^{Op} to monomers (Fig. 2B). For these experiments, concentrated GP64sol^{Op} was reduced in 10 mM DTT and then diluted to a final concentration of 50 nM GP64sol^{Op} and 0.1 mM DTT for use as a competitor. The treatment of GP64sol^{Op} with 10 mM DTT resulted in a loss of competition by GP64sol^{Op} (Fig. 6). In a mock experiment, the same final dilution of DTT (0.1 mM DTT) added alone to cells did not affect infection in the absence of the competitor (not shown). The inability of reduced GP64sol^{Op} to effectively compete for virion-binding sites suggests that disulfide bonds may be necessary for maintenance of a receptor-binding domain of GP64 or that native trimeric GP64 is necessary for efficient receptor binding by GP64 (Fig. 6). As an additional control, GP64sol^{Op} was also pretreated with chymotrypsin, followed by protease inhibitors, before use in competition assays. Protease-treated GP64sol^{Op} did not compete for binding by vAchsZ (Fig. 6).

Kinetics of virion entry

The timing of BV entry into endosomes was measured by first synchronizing BV binding at the cell surface at

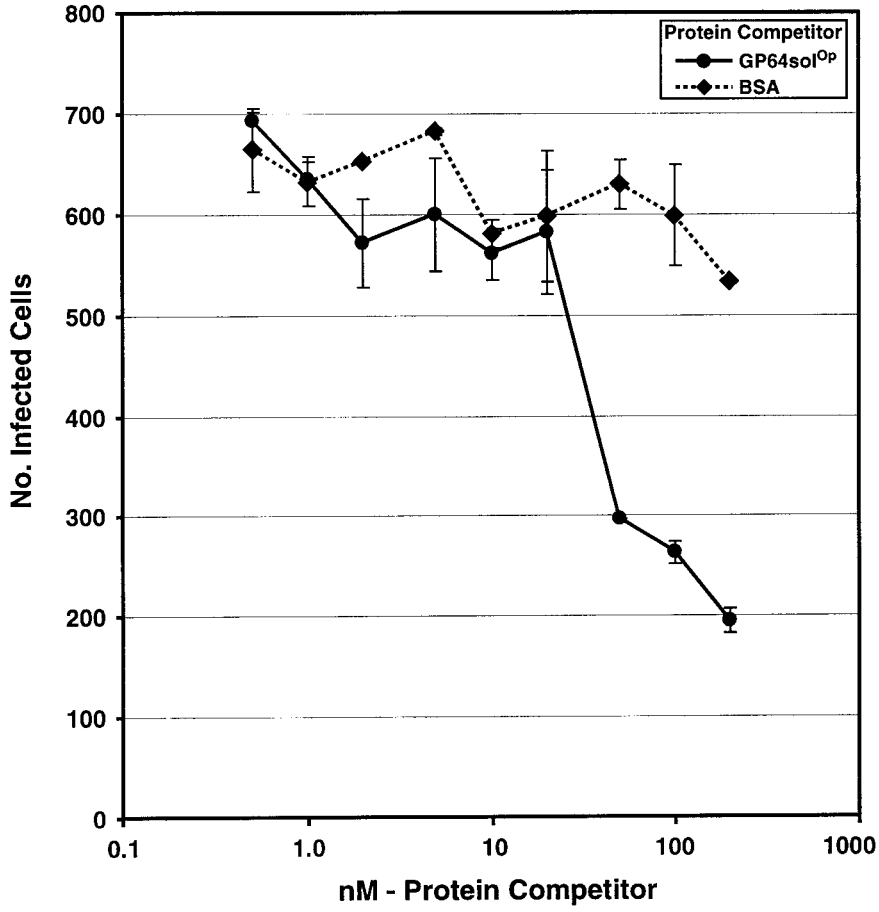

FIG. 5. GP64sol^{Op} competition for AcMNPV binding. GP64sol^{Op} was used as a competitor for virion binding in a single-cell infectivity assay as described earlier. Sf9 cells were preincubated with increasing concentrations (*X*-axis) of either GP64sol^{Op} (solid line) or BSA (dashed line) at 4°C for 1.5 h, and then vAchsZ virions were added and bound for 1 h at 4°C. Cells were washed at 4°C in PBS and then incubated at 27°C for 22 h and assayed for single-cell infectivity. The numbers of infected cells (*Y*-axis) were quantified as described in Materials and Methods. Each data point represents average data from two wells, and bars represent standard deviation.

4°C, incubating for various periods at 27°C to permit endocytosis, inactivating or releasing BV remaining at the cell surface with subtilysin treatment, and then measuring the virus that was internalized and therefore protected from subtilysin treatment. For these studies, we used virus vAchsZ and the single-cell infectivity assay described above. vAchsZ was bound to cells for 1 h at 4°C. and then the temperature was shifted to 27°C for increasing intervals of time (0–90 min). At the end of each time interval, cells were treated with 2 mg/ml subtilysin to inactivate or release BV remaining at the cell surface. Cells were washed and then incubated at 27°C for 24 h, and the numbers of infected cells were determined as described earlier. The protease resistance curve shown in Fig. 7A (solid line) shows that between 0 and 10 min at 27°C, BV were highly sensitive to inactivation by exogenously applied subtilysin. Resistance to inactivation by subtilysin increased dramatically between 10 and 20 min of incubation at 27°C. As shown in Fig. 7A, the measured half-time for internalization of virions into endosomes was ~12.5 min.

Release of BV nucleocapsids from the endosome occurs when the BV envelope fuses with the membrane of the endosome, an event catalyzed by the pH-triggered fusion activity of GP64. Studies using a syncytium formation assay indicate that AcMNPV and OpMNPV GP64s are triggered at pH values of ~5.5–5.7 (Blissard and Wenz, 1992; Leikina *et al.,* 1992). Lipophilic amines such as ammonium chloride or chloroquine buffer the endosomal pH and can be used to inhibit membrane fusion during endocytosis. For precise studies of timing, ammonium chloride appears to be the reagent of choice because ammonium chloride can buffer the pH of the endosome within 1 min after its addition to cells (Greber *et al.,* 1993). To measure the timing of baculovirus nucleocapsid release from the endosome, we used ammonium chloride inhibition in combination with the single-cell infectivity assay. vAchsZ BV binding at the cell sur-

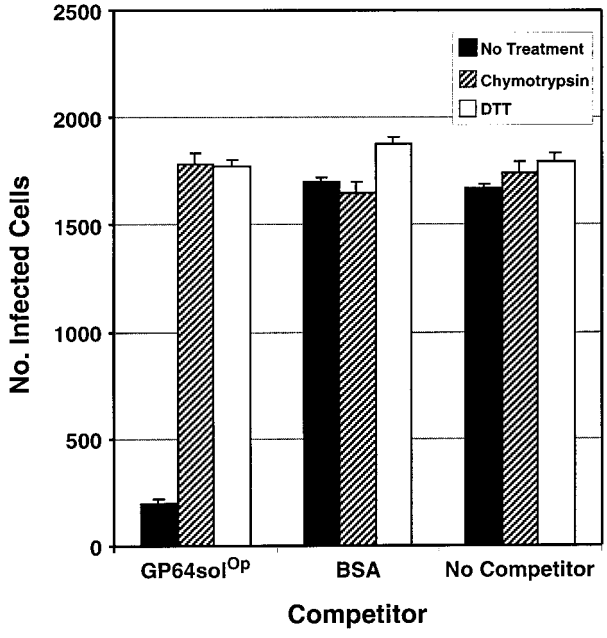

FIG. 6. Reduction in specific competition by GP64sol^{Op} after treatment with protease or reducing agent. GP64sol^{Op} was treated with either 1 mg/ml chymotrypsin (hatched bars) or 10 mM DTT (open bars) before use as a competitor in a virion-binding assay, as in Fig. 5. Competitor (GP64sol^{Op} or BSA) concentrations were 50 nM; a control competitor, BSA, was similarly treated. Treated and untreated protein competitors were preincubated with Sf9 cells at 4°C for 1.5 h before incubation with BV of vAchsZ and detection of single-cell infectivity as described in the legend to Fig. 5 and Materials and Methods. Bars indicate numbers of infected cells, and each bar represents the average data from three wells. Error bars represent standard deviation.

face was synchronized by binding virions at 4°C for 1 h and then shifting the cells to 27°C. After various intervals at 27°C (0–120 min), ammonium chloride was added to a final concentration of 25 mM, and cells were incubated for up to 24 h. At 24 h after shifting to 27°C, plates were scored for infected cells. When ammonium chloride was added immediately after the 4°C BV-binding period, a ~98% inhibition of virion infectivity was observed. With increasing intervals of incubation at 27°C before ammonium chloride addition, the numbers of infected cells that were detected increased (Fig. 7B). Escape from ammonium chloride inhibition was first detected after 16 min and was near maximal levels after ~28–30 min of incubation at 27°C. Within 30 min at 27°C, infectivity had increased to ~90% of that from cells in which no ammonium chloride was added. Thus after binding of BV at the cell surface, the estimated half-time for triggering of membrane fusion and release of BV nucleocapsids from the endosome in Sf9 cells was ~25 min (Fig. 7B).

DISCUSSION

The process of baculovirus entry by receptor-mediated endocytosis may be subdivided into several discrete steps: virion binding at the cell surface, formation of clathrin coated pits and uptake of the virion into a vesicle (endosome), triggering of membrane fusion by low pH, and release of the nucleocapsid into the cytoplasm. Although the role of GP64 in membrane fusion has been examined in some detail (Blissard and Wenz, 1992; Chernomordik *et al.,* 1995; Markovic *et al.,* 1998; Monsma and Blissard, 1995; Monsma *et al.,* 1996; Plonsky *et al.,* 1999, 1997), its role as a potential host cell receptor-binding protein has not been previously explored. Several lines of indirect evidence suggested that GP64 might represent the viral encoded host cell receptor-binding protein. First, the GP64 protein represents the most abundant viral protein identified from the BV envelope, and BV-neutralizing antibodies recognize the GP64 protein (Hohmann and Faulkner, 1983; Roberts and Manning, 1993). Recent studies of GP64 glycosylation also suggested that the glycosylation state of GP64 may affect virion binding to host cells (Jarvis *et al.,* 1998). Second, GP64 homologs (known as GP75) from a small group of arboviruses within the Orthomyxoviridae appear to be involved in receptor binding. The GP75 proteins from the Thogoto and Dhori viruses show remarkable primary amino acid sequence identity with baculovirus GP64 proteins, and Thogoto virus hemagglutination is inhibited by an MAb directed against GP75 (Leahy *et al.,* 1997; Morse *et al.,* 1992; Portela *et al.,* 1992). Finally, the GP64 protein expressed on the surface of transfected insect cells is capable of mediating low pH-triggered membrane fusion (Blissard and Wenz, 1992). Because prior receptor binding (docking) is usually a requirement for membrane fusion, this final observation strongly suggested that GP64 may also function as the host cell receptor-binding protein.

In the current study, we examined the role of GP64 in host cell receptor binding by using a soluble form of OpMNPV GP64 as a competitor for virion binding. To examine competition for virion binding to the host cell, we developed a competition assay in which a recombinant baculovirus (vAchsZ) carrying a marker gene was bound to the cell surface by incubation at 4°C in the presence of competitor proteins. Unbound virions were removed by washing cells before shifting the temperature to 27°C. Successful virus binding was scored by analysis of single-cell expression of β-galactosidase from the recombinant virus. In competition experiments, we showed that both AcMNPV and OpMNPV competed with vAchsZ for binding, suggesting that OpMNPV and AcMNPV utilize the same cellular receptor or receptors. To examine the role of GP64 in virion binding to host cells, we used a highly purified soluble form of GP64 as a direct competitor for virion binding. The purified soluble GP64 (GP64sol^{Op}) was determined to be structurally indistinguishable from the ectodomain of native GP64 from budded virions, based on oligomerization patterns and

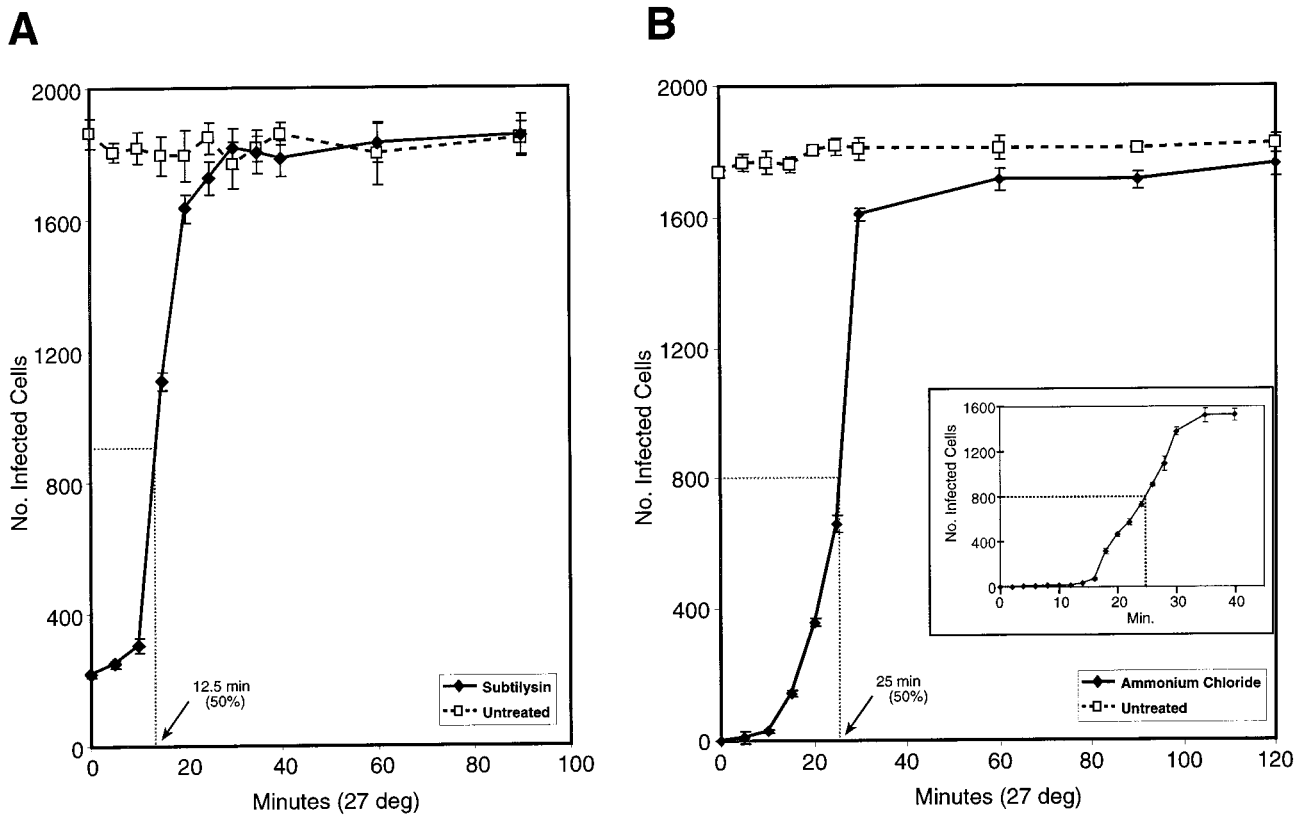

FIG. 7. (A) Kinetics of virion internalization into endosomes. The kinetics of virion internalization into endosomes were examined by synchronously binding vAchsZ BV to the cell surface of Sf9 cells at 4°C for 1 h and then shifting to 27°C for various periods of time and treating with subtilysin. Resistance of internalized BV to inactivation by subtilysin was measured by the single-cell infection assay described earlier. Synchronously bound virions of vAchsZ were incubated at 27°C for time periods of 5–90 min. At the end of each time period, cells were treated with subtilysin, washed, and incubated at 27°C for 24 h. Numbers of infected cells were determined as described in Materials and Methods, and each data point represents the average of three wells. Error bars represent standard deviation. Subtilysin-treated cells (solid line); untreated control cells (dashed line). (B) Kinetics of nucleocapsid release from the endosome. The kinetics of nucleocapsid release from the endosome were examined by measuring the time (after binding) required to acquire resistance to inhibitors of endosomal membrane fusion. Virions of vAchsZ were synchronously bound to Sf9 cells for 1 h at 4°C, and then the temperature was shifted to 27°C for increasing time periods (2–120 min). At the end of each period, medium was replaced with medium containing 25 mM ammonium chloride for the remainder of the assay (24 h). The numbers of infected cells were determined as described above. The inset shows an identical experiment in which ammonium chloride was added at 2-min intervals between 0 and 40 min.

their comparative sensitivities to glycosidase, protease cleavage, and reducing agent DTT. Because the soluble form of GP64 specifically competed for vAchsZ binding to Sf9 cells, these data directly implicate GP64 as a host cell receptor-binding protein. In addition, reduced GP64sol^{Op} did not exhibit any significant competition in this assay, suggesting that disulfide bonds may be important in maintaining a structure capable of efficiently binding the receptor. Although we did not attempt to measure the affinity of GP64sol^{Op} binding to host cells in the current study, it is likely that GP64sol^{Op} binds to host cells with a lower overall affinity than virions, which contain local concentrations of GP64. Such differences in affinity may explain why we were not able to eliminate virion binding completely in this very sensitive assay. However, the observation that GP64sol^{Op} competes for virion binding in this assay provides the most compelling data to date that GP64 serves as a baculovirus attachment protein.

gp64 genes from several baculoviruses (AcMNPV, BmNPV, OpMNPV, and CfMNPV) have been identified, and these proteins are highly conserved, showing approximately 80% amino acid sequence identity between the predicted ectodomains (Blissard and Rohrmann, 1989; Hill and Faulkner, 1994; Majima *et al.,* 1996; Whitford *et al.,* 1989). Because both AcMNPV and OpMNPV are able to enter *Spodoptera frugiperda* cell lines yet are distinctly different viruses with distinct host ranges, the differences in host range may be explained by factors other than virion binding. Indeed, our previous studies have shown that two OpMNPV late promoters are not fully functional in the AcMNPV genome (Garrity *et al.,* 1997). Studies of host range determinants in AcMNPV and other baculoviruses have identified critical host range factors that do

not appear to be associated with virion entry into the host cell (Croizier *et al.,* 1994; Du and Thiem, 1997; Kamita and Maeda, 1993; Lu and Miller, 1996; Maeda *et al.,* 1993; Miller and Lu, 1997). Those studies indicate that baculovirus host specificity can be determined at levels other than virion binding at the cell surface. However, identification of the host receptor for AcMNPV BV will be important for understanding whether viral entry may influence tissue specificity within the infected host and for applications of AcMNPV BV for gene delivery in biotechnological applications such as gene therapy. BV from AcMNPV are infectious to cells of many insect tissues and are highly infectious in insect cell culture. Several early studies showed that BV entered cells of diverse organisms that were nonpermissive for viral replication (Brusca *et al.,* 1986; Carbonell and Miller, 1987), although the mechanisms of entry were not examined. Recent studies of several mammalian cell types showed that AcMNPV BV efficiently entered primary cultures of human hepatocytes and cell lines derived from hepatocytes (Barsoum *et al.,* 1997; Boyce and Bucher, 1996; Hofmann *et al.,* 1995; Sandig *et al.,* 1996). Other recent studies have reported BV entry into a broad range of mammalian cell lines (Condreay *et al.,* 1999; Shoji *et al.,* 1997). The apparently wide array of potential cell types that AcMNPV BV can enter suggests that the BV interaction with the host cell may involve recognition of a host receptor molecule that is highly conserved and present on many cell types or that the interaction may be relatively nonspecific.

In the current study, we also examined the kinetics of two successive steps in baculovirus BV entry by receptor-mediated endocytosis: (1) uptake into the endosome and (2) release from the endosome by membrane fusion. vAchsZ entry was analyzed by synchronizing BV entry by binding virions to cells at 4°C and then using a protease resistance assay to measure virions internalized after various periods of incubation at 27°C. The measured half-time required for virus uptake was 12.5 min. This rate of entry is similar to those reported from influenza virus, adenovirus, and SFV, which have uptake half-times of 12.5, 10, and 5–10 min, respectively (Greber *et al.,* 1993; Marsh and Helenius, 1989; Martin and Helenius, 1991). Release of baculovirus virions from endosomes was measured using an inhibitor of low-pH-triggered membrane fusion. The measured half-time for nucleocapsid release from the endosome was ~25 min. This is similar to release times reported from several enveloped viruses, including influenza, VSV, and West Nile virus (Gollins and Porterfield, 1986; Marsh and Helenius, 1989; Matlin *et al.,* 1982). In an earlier study (Volkman and Goldsmith, 1985), it was estimated that significantly longer times (half-times of 1–1.5 h) were necessary for AcMNPV BV release from the endosome. The differences may result from differences in the assay techniques used in the two studies. In the current study, we used a sensitive single-cell infectivity assay that detects viral entry and early gene expression in single cells, thus isolating virion entry from subsequent steps such as late gene expression, viral replication, and plaque formation. The current data on BV entry and nucleocapsid release correspond well with data reported from other enveloped viruses.

In summary, we identified GP64 as a protein involved in AcMNPV binding to host cell receptors, and we characterized two steps in AcMNPV entry into host cells: internalization into and release from endosomes. Because the GP64 proteins are highly conserved in several baculoviruses and share a common ancestry with the GP75 envelope proteins from several orthomyxoviruses that are transmitted by ticks, it is possible that a common entry mechanism is shared by these disparate groups that both infect arthropods. Future studies that identify and characterize the host cell receptor for baculovirus binding will be important for understanding how these very different viruses are adapted to their invertebrate hosts.

MATERIALS AND METHODS

Cells, viruses, and recombinant virus construction

Spodoptera frugiperda (Sf9) and *Lymantria dispar* (Ld652Y) cells were cultured at 27°C in TNM-FH complete medium (Hink, 1970) containing 10% FBS. Viruses AcMNPV (strain E2) and vAchsZ (Monsma *et al.,* 1996; Vlak *et al.,* 1990) were titered on Sf9 cells, and OpMNPV was titered on Ld652Y cells as described previously (Blissard and Rohrmann, 1989). To construct a recombinant virus expressing soluble GP64, a stop codon was engineered into the OpMNPV *gp64* gene in plasmid p64-166 (Blissard and Wenz, 1992) immediately upstream of the predicted transmembrane domain. A synthetic oligonucleotide (5′-CTCAACGCCACGCTCTgaTCATTTATGCTGGGGCAC-3′) was used to convert the codon for Tyr478 (TAC) to TGA, using site-directed mutagenesis (Deng and Nickoloff, 1992; Ray and Nickoloff, 1992). Next, the synthetic antisense oligonucleotide 5′-ACCATCTGTAGATCTTGTAGTGTT-3′ was used for site-directed mutagenesis to introduce a *Bgl*II restriction site just upstream of the *gp64* ATG, and the resulting plasmid was named p64-BglATG-STOP. The ~1700-bp *Bgl*II–*Spe*I fragment containing the gp64 ORF from p64-BglATG-STOP was cloned into *Bam*HI–*Xba*I-digested transfer vector pAcDZ1, replacing the lacZ ORF and placing the modified *gp64* ORF under control of the very late polyhedrin promoter. The resulting plasmid was named (pAcGP64STOP). A recombinant virus was generated by cotransfection of Sf9 cells with pAcGP64STOP and *Bsu*36I-digested BacPAK6 viral genomic DNA (Clontech), and the virus

was named vAcGP64sol^{Op}. This virus was also referred to as EFP^{sol}C2 in a previous report, and the soluble protein expressed from this virus (GP64sol^{Op}) was also referred to earlier as GP64EFP^{sol} (Oomens *et al.,* 1995). Recombinant viruses were isolated by three rounds of limiting-dilution cloning. Recombinant viral genomes were confirmed by restriction enzyme analysis and by PCR using primers homologous to the polyhedrin promoter and the OpMNPV *gp64* gene.

Purification of GP64sol^{Op}

For biochemical studies requiring high purity GP64sol^{Op} and to confirm biological assays, a highly purified preparation of GP64sol^{Op} was prepared. A four-step protocol was developed to purify GP64sol^{Op} from tissue culture supernatants of vAcGP64sol^{Op}-infected cells. Insect cells (BTI-Tn-5B1–4) were grown in serum-free media (Excell 405; JRH Biosciences) and infected at an m.o.i. of 10 with vAcGP64sol^{Op}. After infection, the cells were diluted to a density of 1×10^6 cells/ml and maintained in spinner culture at 27°C for 48 h. Cells and debris were removed by low-speed centrifugation, supernatants were collected, and sodium azide was added to 0.02% w/v. Sodium azide was present at all times throughout the purification, except for ion-exchange loading, wash, and elution. ConA-agarose beads (Sigma) equilibrated in ConA wash buffer (150 mM NaCl, 20 mM NaH_2PO_4, pH 7.0) were added to the supernatant (~143 mg beads/liter) and mixed gently overnight at ~4 rpm at 7°C in a roller bottle. The beads were collected in a large buchner funnel, transferred to a glass column (3-cm diameter), and washed extensively with ConA wash buffer. Bound glycoproteins were eluted with two bed volumes of 0.5 M glucose, 0.5 M methyl-D-mannopyranoside, and 20 mM NaH_2PO_4, pH 7.0, followed by three bed volumes of ConA wash buffer. The eluate and first bed volume of wash were pooled and dialyzed overnight against 200 mM NaCl and 20 mM NaH_2PO_4, pH 7.0. The dialyzed pool was centrifuged (27,000*g* for 30 min at 4°C) to remove precipitated materia. For hydrophobic interaction chromatography, the supernatant was applied to a column composed of phenyl–Sepharose CL-4B equilibrated with 200 mM NaCl and 20 mM NaH_2PO_4, pH 7.0. After sample application, the column was washed with one bed volume of 200 mM NaCl and 20 mM NaH_2PO_4, pH 7.0, followed by one bed volume of 20 mM Tris–HCl, pH 8.0. The bound proteins were eluted by washing with two bed volumes of distilled H_2O. The bound GP64sol^{Op} eluted as a major peak at the 20 mM Tris–HCl–dH_2O transition, followed by a very broad tail. The fractions were analyzed on SDS–polyacrylamide gels, and the fractions containing GP64sol^{Op} were pooled and dialyzed against 20 mM Tris–HCl, pH 8.0. For anion-exchange chromatography, the pooled fractions were applied to a DEAE-Memsep cartridge (Millipore) equilibrated with 20 mM Tris–HCl, pH 8.0. After sample application, the cartridge was washed with 20 mM Tris–HCl, pH 8.0. Bound proteins were eluted with a linear salt gradient (0–250 mM NaCl) in 20 mM Tris–HCl, pH 8.0. GP64sol^{Op} eluted as several peaks at salt concentrations from 75 to 150 mM NaCl. The fractions containing GP64sol^{Op} were pooled, and Tween 20 was added to 0.1% vol/vol and sodium azide was added to 0.02% wt/vol. The pooled fractions were concentrated with Centriprep 30k MWCO ultrafiltration units. For gel filtration chromatography, the concentrated fractions were applied to a 94-cm × 2-cm column of Sephacryl S400HR equilibrated with 200 mM NaCl and 20 mM NaH_2PO_4, pH 7.0. The peak fractions containing GP64sol^{Op} were pooled and concentrated as above, and aliquots of GP64sol^{Op} were stored at −80°C. The final purity of GP64sol^{Op} was ≥95% as judged by densitometric quantification of Coomassie blue-stained SDS–polyacrylamide gels, and typical expected yields were ~1–2 mg/liter of cells (at 1×10^6 cells/liter). For protease resistance assays, a partially purified preparation of GP64sol^{Op} was prepared and used. Supernatants of cells infected with vAcGP64sol^{Op} were collected as before and incubated with ConA beads as described above; then protein was precipitated in 10% ammonium sulfate. GP64sol^{Op} was further purified by gel filtration chromatography as described above. Purity of partially purified GP64sol^{Op} was estimated at ~60% by Bradford assay and Coomassie-stained SDS–polyacrylamide gels and confirmed by Western blot analysis.

Glycosidase treatment, protease resistance, and reduction of GP64sol^{Op}

For glycosidase digestions, 2 μg of purified GP64sol^{Op} was digested with either 0.5 unit of Endo F (Boehringer) or 0.01 unit of Endo H (Boehringer) for 1 h in 10 mM sodium phosphate buffer, pH 7.0, at 37°C and then electrophoresed on 6% SDS–polyacrylamide gels.

To compare the protease resistance profiles of GP64sol^{Op} and native OpMNPV GP64, 0.5 μg of secreted soluble GP64 (GP64sol^{Op}) and 1.0 μg of OpMNPV budded virions were each treated with 0.1 mg/ml proteinase K for 10 min at 56°C. Digestion products were examined by electrophoresis on SDS–polyacrylamide gels and Western blot analysis with MAb AcV5 (Hohmann and Faulkner, 1983). For comparisons of oligomerization and the susceptibility of oligomers to reduction by DTT, purified GP64sol^{Op} (0.5 μg) or OpMNPV budded virions (1.0 μg) were incubated with increasing concentrations of DTT at 37°C for 5 min. Trimerization and reduction were examined by preparing proteins in 37.5 mM iodoacetamide, 2% SDS (no β-mercaptoethanol), and electrophoresing proteins on a 6% nonreducing polyacrylamide

gel as described earlier (Oomens *et al.,* 1995), followed by Western blot analysis using MAb AcV5.

Binding, infectivity, and competition assays

To measure the effects of GP64sol^{Op} competition on BV binding, we used recombinant virus vAchsZ (which constitutively expresses β-galactosidase in the early phase) for a sensitive single-cell infectivity assay. To demonstrate the linearity of the assay, Sf9 cells (3×10^5) were infected in 24-well plates with varying concentrations of vAchsZ virions (m.o.i. 0.0125–0.2) by incubating virus with cells for 1 h at 4°C. Cells were washed three times in complete TNM-FH medium at 4°C and then incubated for 24 h in complete TNM-FH medium at 27°C, fixed 10 min in 0.5% gluteraldehyde in PBS at room temperature, and stained for β-galactosidase activity by incubation in a solution of 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM $MgSO_4$ in 1 × PBS, pH 7.4, as described previously (Sussman, 1995). For each virus dilution, three wells of cells were infected, and the number of stained cells in each well were counted. Data presented (Fig. 3) represent the average and standard deviation (error bars) of data from three wells. For competition assays, Sf9 cells (3 or 6×10^5) were preincubated with varying concentrations of competitors for 1.5 h at 4°C; then BV of vAchsZ was added and incubated with agitation for 1 h at 4°C, and cells were processed and assayed as described above. For some experiments, higher or lower m.o.i. values of vAchsZ were used, but in all cases, the values fell within the linear range of the assay (as established above, Fig. 3A). Viruses (AcMNPV and OpMNPV) used as competitors in binding assays were inactivated by the addition of psoralen (4′-aminomethyl; Triosalen, Sigma). Psoralen was added to virus stocks ($\sim 1 \times 10^8$ infectious units/ml) to a final concentration of 1 mg/ml followed by cross-linking for 10 min with a UV transilluminator (312 nm); then virus preparations were diluted to the appropriate concentrations and used in competition assays. Inactivation of viral infectivity was confirmed by examining infectivity on Sf9 or Ld652Y cells as described above.

For competition assays, protein competitors consisted of highly purified GP64sol^{Op} and a negative control, BSA (Sigma Chemicals). As additional controls, protein competitors were also treated with chymotrypsin (2 mg/ml) at 37°C for 30 min. Reactions were terminated with 1 μM leupeptin, 1 μM pepstatin A, and 4 μM Pefabloc. Complete proteolysis of GP64sol^{Op} was assessed by electrophoresis and examination on 8% polyacrylamide gels. As an additional control, DTT was added to a final concentration of 10 mM, and samples were incubated at 95°C for 20 min. After treatment in DTT, protein samples were diluted 100× to a final concentration of 0.1 mM DTT and applied to cells as competitors. To confirm that this concentration of DTT did not interfere with infection, cells were also preincubated with 0.1 mM DTT before infection with vAchsZ, in the absence of competition.

BV binding, entry, and endocytosis assays

For analysis of virion binding at the cell surface and entry after binding, BV of virus vAchsZ was bound to Sf9 cells at 4°C for 1 h, and cells were washed in complete TNM-FH at 4°C. In initial experiments, subtilysin was added to a final concentration of 2 mg/ml either before or after shifting cells to 27°C and incubating for 1 h. As controls, BV were also pretreated with subtilysin (2 mg/ml) before binding BV to Sf9 cells. For studies of virion entry, BV of vAchsZ were bound to Sf9 cells at 4°C for 1 h; then the temperature was shifted to 27°C for increasing periods of time. At the end of each time interval at 27°C, cells were treated with 2 mg/ml subtilysin in PBS for 15 min at 37°C to inactivate or remove virions remaining at the cell surface. This concentration of subtilysin was previously determined to inactivate BV when BV were treated before binding to host cells (not shown). Cells were then washed three times with complete TNM-FH medium, incubated for 24 h at 27°C in TNM-FH complete medium, and then fixed and stained as described above and scored for single infected cells. To examine the kinetics of virion release from the endosome, virions were bound to cells for 1 h at 4°C. The temperature was then shifted to 27°C for increasing time intervals as described above. At the end of each interval, media was exchanged for TNM-FH containing 25 mM ammonium chloride and then incubated 24 h at 27°C and assessed for single cells expressing β-galactosidase activity.

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