A Cholesterol Recognition Amino Acid Consensus Domain in GP64 Fusion Protein Facilitates Anchoring of Baculovirus to Mammalian Cells

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Baculoviridae is a large family of double-stranded DNA viruses that selectively infect insects. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-studied baculovirus from the family. Many studies over the last several years have shown that AcMNPV can enter a wide variety of mammalian cells and deliver genetic material for foreign gene expression. While most animal viruses studied so far have developed sophisticated mechanisms to selectively infect specific cells and tissues in an organism, AcMNPV can penetrate and deliver foreign genes into most cells studied to this date. The details about the mechanisms of internalization have been partially described. In the present study, we have identified a cholesterol recognition amino acid consensus (CRAC) domain present in the AcMNPV envelope fusion protein GP64. We demonstrated the association of a CRAC domain with cholesterol, which is important to facilitate the anchoring of the virus at the mammalian cell membrane. Furthermore, this initial anchoring favors AcMNPV endocytosis via a dynamin- and clathrin-dependent mechanism. Under these conditions, efficient baculovirus-driven gene expression is obtained. In contrast, when cholesterol is reduced from the plasma membrane, AcMNPV enters the cell via a dynamin- and clathrin-independent mechanism. The result of using this alternative internalization pathway is a reduced level of baculovirus-driven gene expression. This study is the first to document the importance of a novel CRAC domain in GP64 and its role in modulating gene delivery in AcMNPV.

Most viruses have developed, over many years of evolution, sophisticated mechanisms to internalize in the host and take control of the protein synthesis machinery in order to generate thousands of new progeny viruses. Not surprisingly, most viruses possess highly selective mechanisms for internalization into the host cell (1). Many such mechanisms involve the participation of a specific receptor at the host cell surface. These selective internalization mechanisms result in the tropism shown by most viruses toward specific tissues (2).

Baculoviridae is a large family of viruses that selectively infect insects (3). These double-stranded circular DNA viruses possess rod-shaped capsids, giving the family its name. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-studied baculovirus. AcMNPV has been extensively used as an efficient gene expression vector in insect cells for massive protein production (4, 5). Since the initial studies conducted many years ago, it is well established that AcMNPV can transduce mammalian cells with a suitable promoter (6).

Unlike most viruses studied so far, most interestingly, AcMNPV can enter a wide variety of cells from different organisms and drive the expression of foreign genes under the control of mammalian promoters (7, 8, 9).

The fact that baculovirus can enter a wide variety of mammalian cells poses interesting questions. For instance, is AcMNPV using the same mechanism to enter insect and mammalian cells? Is there a receptor involved in this process? Is the same putative receptor present in insects and mammals?

In spite of many years of baculovirus research and dozens of studies showing baculovirus-driven gene expression in mammalian cells, these conundrums remain unsolved to date.

In insect cells, the glycoprotein GP64, a major envelope fusion

protein, is essential for virus budding from the cells, virus internalization into a new host cell, and virus escape from the early endosome inside the infected insect cell (10).

Different studies have demonstrated that GP64 is essential for baculovirus transduction of mammalian cells (11). In mammalian cells, it has been shown that electrostatic interactions, heparan sulfate, and phospholipids are necessary for baculovirus binding to the mammalian cell surface (12).

An elegant recent study highlights the role of cholesterol- and dynamin-dependent endocytosis as the mechanism for virus internalization into mammalian cells (13). However, other studies have shown that baculovirus internalization into mammalian cells also involves dynamin-independent macropinocytosis (14, 15). Many studies, however, have positioned GP64 as an essential element for virus internalization into mammalian cells (13).

Baculovirus GP64 forms trimers in its pre- and postfusion states, similar to vesicular stomatitis virus (VSV) G and herpes simplex virus type 1 (HSV-1) gB glycoproteins. All three proteins resemble each other, belonging to the domain III fusion proteins (16).

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An *in silico* analysis performed for this study identified three putative cholesterol recognition amino acid consensus (CRAC) domains in GP64, which we called Ch1 247-257 (amino acids 247 to 257 of GP64), Ch2 309-317, and Ch3 499-506. According to the structure of GP64 (16), one of the CRAC domains is found in domain II (Ch1 247-257), the second in domain III (Ch2 309-317), and the last in the single transmembrane domain (Ch3 499-506) of GP64.

Besides the role of cholesterol in the endocytosis of baculovirus, dynamin has also been implicated in this process (13). Dynamin is a GTPase that is responsible for endocytosis in eukaryotic cells. Dynamin is responsible for the scission of newly formed vesicles at the plasma membrane and their targeting to (and fusion with) other compartments (17). Clathrin is another protein participating in endocytosis. Clathrin forms trimers and coats vesicles, facilitating the formation of round vesicles (18, 19). Many pathogens utilize the clathrin-dynamin pathway to enter the host cell (20).

Our results suggest that the baculovirus can enter host cells via two independent mechanisms: (i) a dynamin- and clathrin-dependent mechanism utilized by baculovirus when the host cell membranes contain high levels of cholesterol and (ii) a dynamin- and clathrin-independent mechanism utilized by AcMNPV when cholesterol is reduced at the host cell plasma membrane. These two internalization pathways lead to completely opposite consequences in terms of transgene expression.

These results reconcile apparently contradictory studies showing dynamin-clathrin-dependent (13) and -independent (14, 15) mechanisms for baculovirus internalization into mammalian cells while highlighting the complexity of this process. Furthermore, our results position cholesterol as an important element that anchors AcMNPV at the plasma membrane of the host cell, facilitating its internalization via clathrin-coated vesicles.

MATERIALS AND METHODS

Reagents and solutions. All salts were analytical grade and were purchased from Sigma (St. Louis, MO). The FM-464 fluorescent dye, brefeldin conjugates, and CM-Dil were purchased from Invitrogen (Molecular Probes; Invitrogen, Carlsbad, CA). Dehydroergosterol (DHE) was purchased from Santa Cruz Biotechnology (Dallas, TX). Methyl- β -cyclodextrin (M β CD) was purchased from Sigma (St. Louis, MO). The VP39 monoclonal antibody was a very generous donation from Loy Volkman (University of California, Berkeley, CA). HEK293T cells were purchased from Life Technologies (Grand Island, NY).

Plasmids. pCMV-DsRed-Express vector was purchased from Clontech Laboratories (Mountain View, CA). CMV-clathrin-DsRed and human histone 2 (H2-GFP) plasmids were purchased from Addgene (Cambridge, MA).

Recombinant baculovirus production. The *gp64* gene was cloned from genomic *Autographa californica* DNA (GenBank accession number NC_001623, nucleotides 108,172 to 109,949, containing the *gp64* open reading frame [ORF]). Briefly, a PCR with genomic DNA was carried out using the oligonucleotides *gp64*for3 (forward; 5'-ATTACATTTAATATTGTCTATTACGGTTT-3') and *gp64*rev1 (reverse; 5'-AGTACATGAAGTTGCGCGTG-3'). The PCR product was cloned into the pDrive plasmid (Qiagen, Germany). Thereafter, a PCR was performed using pDgp64 and the oligonucleotides *gp64*for4 (5'-TGTGGGTACCTTAATATTGTCTATTACG-3') and *gp64*sig2 (5'-TATAGTCGACGAGCACTGCAACGCGCAATG-3'), containing KpnI and SalI (underlined sequences), and the resulting 1,470-bp fragment was cloned using the same sites in pBluescript SK+ (Stratagene) to generate pSKgp64C. A second fragment was amplified using pDgp64 and the oligonucleotides *gp64*rev1 (mentioned above)

and *gp64*sig1 (5'-TAATCCCGGGCGCCGCAAAGGCAGAATG-3') to amplify the sequence containing the signal peptide, and the 296-bp fragment was cloned using the restriction sites SpeI (located 50 bp upstream from the *gp64* ORF) and SmaI (underlined) in pSKgp64C to generate pgp64display. Finally, the *eGFP* gene was amplified from pEGFP-N1 (Clontech, Palo Alto, CA) using the oligonucleotides GFPforward (5'-ATATCCCGGGATGGTGAGCAAGGGCGAG-3') and GFPreverse (5'-GCTCAAGCTTCTTGTACAGCTCGTCCATGC-3') and cloned in frame with the signal peptide using the sites SmaI and HindIII (underlined), present in pgp64display. Thus, green fluorescent protein (GFP) was displayed between the signal peptide and the amino terminus of the GP64 protein. The resulting fusion gene (*gp64*-GFP) was cloned into the SacI/KpnI sites of the transfer vector pBlueBac4.0 (Invitrogen) under the strong polyhedrin promoter, which was later used to produce the recombinant baculovirus in Sf9 insect cells, as previously described (9). The same vector was utilized for the production of the Bac-GP64-GFP-CMV-DsRed baculovirus. The fragment from the pCMV-DsRed-Express vector (Clontech Laboratories, Mountain View, CA) was amplified by PCR to isolate the CMV-DsRed (*Dicosoma* sp. red fluorescent protein) sequence from the beginning of the cytomegalovirus (CMV) promoter to the polyadenylation signal. The PCR fragment was cloned into the pDrive plasmid (Qiagen, Germany) before the transfer of this fragment to pBlueBac4.0 to produce the transfer vector required for recombination and baculovirus production.

Generation of *gp64*-null AcMNPV bacmid. To delete the wild-type copy of *gp64* present in the baculovirus genome contained in the bacmid from the Bac-to-bac system, we followed previously published protocols (21). Homologous recombination was carried out using a chloramphenicol resistance cassette flanked by 500 nucleotides at the 3' and 5' ends of the *gp64* sequence. The chloramphenicol-resistant bacterial strains obtained after recombination were isolated, and the bacmid was purified. PCRs were conducted to amplify the chloramphenicol resistance using primers matching the 3' and 5' ends of *gp64*. The resulting PCR product was cloned in the pDrive plasmid (Qiagen, Germany) and fully sequenced. Different constructs containing a wild-type copy of *gp64* or the CRAC mutants fused to GFP were cloned into the BB4 transfer vector used later to produce the recombinant baculovirus described in this study. All fused genes generated were sequenced to confirm the integrity of the sequence. The recombinant baculoviruses were named Bac-GP64-null-Y253A-GFP, Bac-GP64-null-Y311A-GFP, and Bac-GP64-null-Y253A-GFP-Y311A-GFP (the double mutant) to reflect the single-point mutations in the CRAC domain and the fact that the wild-type copy of *gp64* was removed from the viral genome.

Production of VSV G-pseudotyped GP64-null baculoviruses. To produce the recombinant baculovirus Bac-GP64-null-Y311A-GFP pseudotyped with VSV G, a plasmid was generated containing the VSV G gene from the vesicular stomatitis virus under the AcMNPV immediate early promoter (IE1). The plasmid was transfected into Sf9 cells 24 h prior to transfecting the cells with a bacmid containing the Bac-GP64-null-Y311A-GFP genome. All transfections were conducted with Cellfectin reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions.

RNA interference studies. HEK293T cells were transfected with small interfering RNAs (siRNAs) against clathrin (stock keeping unit [SKU] no. SR305398), dynamin 1 (SKU no. SR306774), or the serine/threonine p21-activated kinase 1 (Pak1) (SKU no. SR303347) purchased from Origene (Rockville, MD). As a negative control, we used the universal scrambled sequence provided by the manufacturer [siRNA(Scramble) herein; SKU no. SR30004]. siRNAs were transfected as previously reported (22). To improve RNA interference (RNAi) efficiency, cells were transfected twice. The second transfection was performed 3 days after the first one. Transfected cells were studied between 48 and 72 h after the last transfection. The transfection efficiency of the siRNAs was determined using a fluorescently labeled siRNA as previously described (22). In all cases, over 85% of the cells were transfected.

Cell culture and transfection. HEK293T cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml ampicillin, and 100 µg/ml streptomycin. Transient transfection of the cells was performed using 0.8 µg total plasmidic DNA (using the different plasmids indicated in Results). Briefly, the plasmid used in each case (see Results) was combined with 4 µl of Plus reagent (Invitrogen, Carlsbad, CA) in 20 µl Opti-MEM (Invitrogen, Carlsbad, CA). After 15 min of incubation at room temperature, 1 µl Lipofectamine (Invitrogen, Carlsbad, CA) in 24 µl Opti-MEM was added to the mixture and incubated for an extra 15 min. The mixture was then added to cells plated in 200 µl Opti-MEM in 24-well plates. After 3 to 6 h, Opti-MEM was replaced with normal growth medium. Cells were studied 24 h after transfection.

Approximately 100,000 viral particles from the different recombinant baculoviruses were applied to the bath solution in 35-mm petri dishes containing HEK293T cells at 60 to 70% confluence. The efficiency of transduction and number of viral particles utilized were determined by cell cytometry, as previously described (9), from a viral stock containing 10^9 viral particles as determined by plaque assay.

FISH studies. We produced DNA fragments by PCR, using as the template the BB4 transfer vector containing genes required for baculovirus production with the Bac-to-Bac recombination system (Life Technologies, Grand Island, NY). The DNA fragments were labeled with Alexa Fluor 647 using a fluorescence *in situ* hybridization (FISH) tag kit (Life Technologies, Grand Island, NY). DNA labeling was conducted following the manufacturer's instructions. HEK293T cells exposed to recombinant baculovirus for 24 h or control cells (not exposed to the baculovirus) were fixed and permeabilized for FISH studies. All cells were transfected with plasmid H2-GFP (to identify the nucleus after conducting the FISH). We followed the instructions provided by the manufacturer for the DNA hybridization protocol. Cells not exposed to baculovirus were utilized to set the background fluorescence levels, indicated in the figures. The emission spectra of H2-GFP and Alexa Fluor 647 could be easily separated using a spectral confocal microscope FV1000 from Olympus (Japan).

Ig-TIRFM. To track single viral particles in real time, we utilized the light guide-based total internal fluorescence microscopy (Ig-TIRFM) apparatus (TIRF Labs, Inc., Cary, NC) (23). The system was used with an inverted IX81 Olympus microscope and a high-numeric-aperture (1.45) 100× oil immersion Plan Apo objective (Olympus, Japan). The microscope was part of a FV1000 confocal microscope (Olympus, Japan). Using this configuration, we could alternate between multicolor TIRFM and confocal microscopy in seconds. Virus attachment to the cell plasma membrane was monitored in TIRFM mode and then rapidly switched to confocal microscopy mode to continue tracking the single particle with greater depth control.

To measure Förster resonance energy transfer (FRET) in real time in combination with Ig-TIRFM (Ig-TIRFM-FRET), we utilized the sensitized emission method as previously described (24). Briefly, the levels of fluorescence of both donor (DHE) (25) and acceptor (enhanced GFP [eGFP]) were recorded sequentially (26). For excitation, we used a 405-nm solid-state laser coupled to the Ig-TIRFM system via fiber optics (23). As a negative FRET control, we measured the emissions of cytosolic eGFP (expressed as a cytosolic protein via the pEGFP-N1 plasmid) and DHE at the plasma membrane. The FRET efficiency ($FRET_{eff}$) calculated under these conditions was $1.2\% \pm 0.5\%$ ($n = 24$). This value was considered background or baseline for real FRET. Manders' overlap coefficients (R) were calculated from colocalization channels between GFP and DHE using the ImageJ Manders' Coefficients plug-in (27). In all experiments, virus particles were added to the cell medium and incubated for 3 to 5 min before initiating image recording. Images were acquired at a rate of 1 image/5 s. Each image had an integration time of 500 milliseconds.

Image analysis. All images acquired by confocal microscopy or Ig-TIRFM were analyzed using Amira version 5.4 (FEI, Hillsboro, OR). Time-lapse videos (see the supplemental material) were generated with the Amira movie creation plug-in. The Protein Data Bank crystal struc-

ture used for GP64 was PDB 3DUZ (16). The structure was loaded into USCF Chimera (28). All images of crystal structures (see Fig. 3D) and video S4 in the supplemental material were generated with Chimera.

Flow cytometry. Either HEK293T cells incubated with recombinant baculoviruses displaying GFP fused to GP64 protein or purified baculoviruses were introduced into the sorting chamber of a fluorescence-activated cell sorting (FACS) apparatus (FACSCalibur; BD Biosciences). For purified baculoviruses or cells, event counting was terminated at 10,000 events. The fluorescence signals were collected at 525 nm (for GFP) or 650 nm (DsRed) and plotted in logarithmic scale. Counts or events reflect single viral particles or single cells. These measurements were utilized to calculate the percentages of GFP- or DsRed-positive cells and, for single viral particles, the amount of GFP displayed on the surface of purified baculoviruses (29). Baculoviruses were purified from sf9 cell cultures using ultracentrifugation as previously described (9).

Peptide microarray studies. For peptide microarrays, we utilized a modified version of Ig-TIRFM (TIRF Labs, Inc., Cary, NC), as previously described (30). Briefly, peptide spots were printed on conventional microarray slides coated with poly-L-lysine (Sigma, St. Louis, MO). All peptides were purchased from JPT Peptide Technologies (Berlin, Germany). Peptide array spots were printed manually using a TIRF MicroArrayer (TIRF Labs, Cary, NC). The Ig-TIRFM-1000 system utilized for the microarray studies was mounted on a custom platform, which was used to position an iXon electron-multiplying charge-coupled device (EMCCD) camera (Andor Technology, South Windsor, CT). The camera was equipped with a Schneider low-magnification objective (Kreuznach, Germany). This objective facilitated the visualization of the entire microarray. The camera acquired images at a rate of 1 per second. Image analysis was conducted with Igor pro software (Wavemetrics, Portland, OR).

RESULTS

Baculovirus attaches to mammalian host cells in cholesterol-enriched hot spots. In order to follow AcMNPV attachment and internalization into the mammalian host cell (using HEK293T human cells), we developed a recombinant baculovirus carrying an extra copy of GP64 in which the enhanced green fluorescent protein (eGFP) was fused to the N terminus of GP64 for display purposes (see Materials and Methods). We will refer to this recombinant baculovirus as Bac-GP64-GFP.

To track Bac-GP64-GFP attachment to mammalian cells with high spatial and temporal resolution, we developed a novel form of total internal reflection fluorescence microscopy (Ig-TIRFM) (23). Ig-TIRFM provides enhanced signal-to-background ratio by reducing light scatter and background fluorescence while improving surface selectivity (23). These characteristics facilitated the observation and tracking of single viral particles.

Since TIRFM provides information about only a narrow optical section near the plasma membrane (typically less than 100 nm) (23), we have combined Ig-TIRFM with confocal microscopy. With this configuration, we can switch between TIRFM and confocal mode in a few seconds and continue the tracking of viral particles into the cell at greater depths.

Figure 1A shows a typical Ig-TIRFM experiment, illustrating the attachment of Bac-GP64-GFP particles to the plasma membrane of a mammalian cell (see video S1 in the supplemental material for real-time tracking and video S2 for confocal 3-dimensional [3-D] reconstruction of viral particles attached to the cell plasma membrane). Figure 1C integrates the Bac-GP64-GFP particles attaching to the cell in a 45-min period. When integrating the fluorescence signal for long periods, we observed that many viral particles were attached at well-defined areas at the cell membrane. We named these areas hot spots. The areas are more evident

FIG 1 Baculovirus attaches to mammalian host cells in cholesterol-enriched domains. (A) Time course of the association of baculovirus Bac-GP64-GFP with the plasma membrane of HEK293T cells, visualized with confocal microscopy. Panels show the first 5, 10, and 15 min after virus application. White arrowheads point to single viral particles. Red shows the FM-464 labeling of the plasma membrane. (B) Superposition of transmitted-light and lg-TIRFM imaging, illustrating the tracking of single viral particles in TIRFM mode. (C) The tracking of a single viral particle for 45 min is illustrated in a single image, using the third dimension to project the fluorescence over time. The pseudocolor image indicates the intensity of fluorescence over time, using the scale shown to the right (AU, arbitrary units). (D) Sagittal plane of the image in panel C. For illustrative purposes, only the first 15 min are shown.

when the image is rotated 90 degrees to illustrate the sagittal plane (Fig. 1D).

Since cholesterol is an important component of lipid rafts and rafts, in turn, are important signaling centers involved in microdomain signal transduction (31, 32) and given the fact that cholesterol plays a key role in GP64-pseudotyped virus internalization into mammalian cells (13), we decided to explore further the role of GP64 in the context of baculovirus for the cholesterol attachment of Bac-GP64-GFP to the hotspots.

GP64 associates with cholesterol prior to virus internalization into the cell. Methyl- β -cyclodextrin (M β CD) is a cyclic oligosaccharide that binds to cholesterol, facilitating its removal from the cell plasma membrane (33). Because oligosaccharides are not membrane permeable, it is generally accepted that M β CD exerts its effect only at the plasma membrane level (33).

In an attempt to reduce cholesterol from the plasma mem-

brane to evaluate its effects on Bac-GP64-GFP anchoring at hotspots, the cells were incubated for 45 min with 10 mM M β CD prior to virus application.

Most surprisingly, M β CD treatment altered the attachment of Bac-GP64-GFP to the cell membrane. Time-resolved lg-TIRFM shows that Bac-GP64-GFP attached at hotspots in the majority of the cells (Fig. 2A); however, M β CD treatment resulted in random attachment throughout the plasma membrane, as evidenced by the 3-dimensional reconstruction of Bac-GP64-GFP over time when monitoring host cell membrane attachment over a 5-min period (Fig. 2B).

Since cholesterol appeared to play a role in defining the attachment of Bac-GP64-GFP to hotspots, we explored the association of Bac-GP64-GFP with cholesterol via Förster resonance energy transfer (FRET) in combination with lg-TIRFM (lg-TIRFM-FRET). For this purpose, we used the blue-fluorescing form of

FIG 2 GP64 associates with cholesterol at the plasma membrane of host cells. (A) Fluorescence obtained from three representative cells illustrating the tracking of baculovirus Bac-GP64-GFP for 5 min. (B) Control cell and a cell treated with methyl- β -cyclodextrin (M β CD) for 45 min prior to adding the baculovirus Bac-GP64-GFP. AU, arbitrary units. (C) Ig-TIRFM-FRET experiments tracking the FRET between a single particle of baculovirus Bac-GP64-GFP and DHE. Scale to the right shows the FRET efficiency (FRET_{eff}) in pseudocolor values.

cholesterol (dehydroergosterol [DHE]), which we have previously shown to form a good FRET pair with eGFP (26). DHE is a fluorescent sterol that has been shown to be structurally and functionally similar to cholesterol (25).

In this study, we first decorated the host cell plasma membrane with DHE by incubating the cells for 20 min (see Materials and Methods and reference 26) and then proceeded to apply the Bac-GP64-GFP to the bath solution while monitoring FRET between the recombinant baculovirus and DHE using Ig-TIRFM-FRET.

The single-viral-particle studies using Ig-TIRFM-FRET provide evidence of robust FRET between Bac-GP64-GFP and DHE (Fig. 2C). These results unveil an intimate association (less than 100 Å) between GP64 and DHE during Bac-GP64-GFP attachment to the host cell. Furthermore, virus attached to the cell membrane remained within a constrained region of the membrane, as is evidenced by time lapse video (see video S3 in the supplemental material). This assumption is consistent with our observation that viral particles are attached to identifiable hotspots at the cell membrane (Fig. 1C and D).

Identification of a CRAC domain in GP64 essential for cholesterol association. Given the results evidencing a direct contact between virus particles (GP64) and DHE, we performed an *in silico* analysis of GP64 in an attempt to identify cholesterol binding motifs. The most-studied cholesterol binding motif is the cholesterol recognition amino acid consensus (CRAC) domain (34). CRAC domains are essential for cholesterol binding in a variety of

proteins that associate with cholesterol, and they have also been implicated in virus internalization and morphogenesis (35–38). This CRAC motif is characterized by the pattern L/V-X_(1–5)-Y-X_(1–5)-R/K, where X represents any amino acid present in a sequence of between 1 and 5 [X_(1–5)] before the next conserved amino acid (34).

Using pattern identification software (26), we identified 3 putative CRAC domains in GP64, one in domain II, a second in domain III, and the last in the transmembrane domain (Fig. 3A). We named these putative CRAC domains Ch1 247–257, Ch2 309–317, and Ch3 499–506, showing their amino acid location in the GP64 sequence and their localization in the different domains from the GP64 crystallographic structure (16).

To determine which of these domains was capable of binding cholesterol, we implemented a novel form of peptide microarray analysis in which small peptides containing the putative CRAC domains were printed on typical microarray coverslips coated with poly-L-lysine (see Materials and Methods and reference 30). Then, using Ig-TIRFM in the microarray configuration (30), we utilized DHE in binding assays to determine which peptide was capable of binding DHE.

Figure 3B presents the results of a typical peptide microarray cholesterol-binding assay, showing that Ch1 247–257 failed to bind DHE but Ch2 309–317 showed a strong fluorescence signal, reflecting the binding of DHE to this peptide. We cannot discard at this time the idea that a defective conformation in the Ch1

FIG 3 Microarray peptide analysis identifies a CRAC domain in GP64. (A) Diagram illustrating the different domains found in GP64 protein, based on its crystallographic structure (16). The numbers indicate the amino acids in GP64. Protein domains I to V from the crystallographic study are indicated by different colors. For details on the different domains, please refer to reference 16. The position of the transmembrane domain (TM) is shown. Green arrows point to the locations of the three putative CRAC domains identified by *in silico* analysis. (B) Representative results of peptide microarray studies using Ig-TIRFM, showing the binding of DHE by Ch2 but not Ch1. RCh1 and RCh2 indicate the scrambled sequences used as negative controls for the two CRAC domains. Actual CRAC amino acids are presented in red. The 10 spots showing fluorescence signals (labeled 1 to 10) represent replicates. (C) Alanine scanning of all amino acids from the putative CRAC domain Ch2. One by one, all amino acids were replaced with alanine and spotted in the microarray to test DHE binding. The top row shows the wild-type sequence (Ch2). Red shows the CRAC domain amino acid sequence, and green shows the alanine substitutions. The fluorescence intensity resulting from DHE binding to each microarray spot was rated using a qualitative scale, with the results denoted by symbols ranging from +, indicating a low signal, to +++, indicating the strongest fluorescence signal, whereas – indicates no signal above the background level. (D) Three-dimensional representation of the GP64 crystallographic structure obtained from the Protein Data Bank (PDB 3DUZ [16]). The trimer image (left) shows each of the three monomers in a different color. On the right is a zoomed image of the central part, where the Ch2 CRAC domain is located. Yellow shows the Ch2 CRAC domain and its amino acid side chains. Notice that the side chains point outward (please refer to video S4 in the supplemental material for a detailed illustration of the locations of the side chains in the crystal structure of GP64). Rendering of structures was conducted with UCSF Chimera (28).

peptide may prevent binding to DHE. However, such a possibility is remote, based on the FRET and mutagenesis studies we will describe below.

Using an alanine-scanning method, we proceeded to identify which amino acids from the CRAC domain Ch2 309–317 were relevant for DHE binding. We conducted one-by-one replacements of single amino acids with alanine throughout the peptide sequence. The fluorescence intensity resulting from DHE binding to each microarray spot was rated using a qualitative scale (Fig. 3C). As a negative control, we used a peptide containing the same amino acids found in Ch2 309–317 but scrambled (RCh2).

The alanine-scanning microarray method identified L309, Y311, L316, and K317 as essential for DHE binding. Replacing M310 with A significantly reduced DHE binding but did not abolish it (Fig. 3C). The rest of the substitutions had no significant effect on DHE binding (Fig. 3C).

The location of the CRAC domain in the tridimensional struc-

ture of GP64 (obtained from the crystallographic study in reference 16) is illustrated in Fig. 3D. Notice that the second CRAC domain lies at the beginning of domain III from GP64. The figure illustrates the location of the domain in the monomer and trimer forms of GP64.

Structural evidence has shown that the tyrosine from the CRAC domain interacts directly with the polar 3'OH-group of cholesterol, while leucine/valine interact with the hydrophobic side chain and arginine/lysine form the cholesterol binding pocket from the CRAC domain for the human beta-adrenergic receptor (39). Our binding results are in perfect agreement with these structural observations (39), with the addition of L316, which in our study also affected DHE binding but has not been identified as essential for other CRAC domains (39). This Leu may be specific for this particular CRAC domain in GP64.

For the present study, because we were interested in studying the domains from GP64 involved in host cell attachment and in-

FIG 4 Mutation in a CRAC domain prevents GP64-cholesterol interactions. (A) Single viral particles were studied using lg-TIRFM-FRET to study the time course of baculovirus Bac-GP64-GFP particle association with the cell membrane and FRET with DHE. The images are representative of experiments with wild-type baculovirus Bac-GP64-GFP and two recombinant viruses carrying the mutations Y253A and Y311A. (B) Means $\pm$ standard deviations from at least 25 independent FRET measurements. Notice that only mutation Y311A affected FRET between the recombinant baculovirus and DHE.

ternalization, we did not explore further the Ch3 499-506 cholesterol binding domain, which lies in the transmembrane domain from GP64. The Ch3 domain may play a role in the proper incorporation of cholesterol into budding viruses, efficient transport to the plasma membrane during virus replication, or similar roles. Future studies are needed to determine whether this Ch3 CRAC domain is functional and come to conclusions about its role in baculovirus physiology.

Mutation in the CRAC domain prevents GP64-cholesterol interactions. We explored next whether mutations of the CRAC domain may affect the interaction of the baculovirus with cholesterol at the plasma membrane of living cells.

To assess this, we prepared three recombinant baculoviruses carrying GP64 mutants. It is relevant to highlight that the baculovirus genome carried a wild-type copy of GP64, and thus, all mutations described below were conducted on a second copy of GP64. In the first GP64 mutant, we introduced a single amino acid replacement in the second CRAC domain (Fig. 4A), in which Y311 was replaced by alanine (Bac-GP64-Y311A-GFP). We have shown in the peptide array that this mutation abolishes DHE binding (Fig. 3C). The second mutant was aimed at disrupting the first CRAC domain (Fig. 4A). In this case, Y253 was replaced by alanine (Bac-GP64-Y253A-GFP). The third mutant was a combination of the two described above, carrying both mutations, Y311A and Y253A (Bac-GP64-Y311A-Y253A-GFP). All GP64 mutant copies displayed eGFP, in order to assess GFP-DHE FRET in lg-TIRFM-FRET experiments.

Figure 4A shows the results of representative FRET experiments monitored with lg-TIRFM-FRET, using baculovirus carrying the wild-type copy of GP64 displaying eGFP (WT) and two of the mutant baculoviruses described above.

Figure 4B shows the mean FRET values obtained from at least 35 independent single-baculovirus lg-TIRFM-FRET measurements. As illustrated in this figure, only the Y311A mutation altered FRET between the recombinant baculovirus and DHE. As expected, the double mutant (Bac-GP64-Y311A-Y253A-GFP) showed results similar to those for the single Y311A mutation, indicating that only this mutation altered FRET between GP64 and DHE in living cells. As a negative FRET control, we used cytosolic eGFP and measured the FRET efficiency with DHE at the plasma membrane (see Materials and Methods). Using cytosolic

eGFP to assess FRET with DHE resulted in a FRET_{eff} of $1.2 \pm 0.5\%$ ($n = 24$, data not shown). This value was not significantly different from the values of $1.6 \pm 0.6\%$ obtained with Bac-GP64-Y311A-GFP or $0.4 \pm 0.9\%$ with the double mutant Bac-GP64-Y311A-Y253A-GFP. On the other hand, robust FRET was observed between the baculovirus displaying a wild-type copy of GP64 fused to eGFP, with a FRET_{eff} of $15.3 \pm 4.9\%$. The FRET_{eff} of Bac-GP64-Y253A-GFP was $14.6 \pm 5.1\%$, indicating that this mutation did not affect the interaction with cholesterol, in agreement with the peptide microarray results (Fig. 3B).

These results strongly suggest that the second CRAC domain found in GP64 (Ch2) is essential for cholesterol association during baculovirus attachment to the host mammalian cell while suggesting that the first CRAC domain (Ch1 247-257) may not be functional. These results reinforce the peptide microarray data, strongly suggesting that the Ch1 247-257 domain is not functional.

Cholesterol determines the type of vesicle utilized by baculovirus to enter the cell. Since clathrin has been previously identified as important for baculovirus internalization into mammalian cells (13), we expressed a fusion protein of human clathrin with red fluorescent protein driven by the cytomegalovirus early promoter (CMV-clathrin-DsRed).

Cells expressing CMV-clathrin-DsRed were exposed to Bac-GP64-GFP to determine whether this baculovirus enters the cell via clathrin-coated vesicles (as assessed by colocalization studies using confocal microscopy). As illustrated in Fig. 5A, we observed a strong colocalization between Bac-GP64-GFP and clathrin-DsRed in control cells (cells not treated with M β CD), with a Manders' overlap coefficient (R) of 0.9. However, the colocalization was strongly reduced in the cells treated with M β CD for 45 min (Fig. 5B). In this case, the calculated R coefficient was 0.1.

Figure 5C shows the means $\pm$ standard deviations from at least 23 independent observations. The data show that the number of Bac-GP64-GFP-positive cells was not altered by M β CD, only the Bac-GP64-GFP and clathrin-DsRed colocalization. This result indicates that cholesterol reduction at the plasma membrane results in baculovirus internalization into the cell via vesicles devoid of clathrin.

In order to differentiate between baculovirus internalization and baculovirus-driven gene expression, we prepared a recombi-

FIG 5 Host cell plasma membrane cholesterol content determines the type of vesicle utilized by baculovirus for cell internalization. (A) Representative image from studies of colocalization of Bac-GP64-GFP and clathrin-DsRed measured with confocal microscopy. The magnified image on the right shows the vesicles in yellow, indicative of high levels of colocalization between Bac-GP64-GFP and clathrin-DsRed. This observation was confirmed by the high Manders' overlap coefficient ($R = 0.9$). (B) Experiments similar to those for which a representative image is shown in panel A were conducted, but cells were treated for 45 min with MβCD prior to adding the baculovirus to the bath solution. Notice the reduced colocalization between Bac-GP64-GFP and clathrin-DsRed, as measured by the low Manders' overlap coefficient ($R = 0.1$). (C) Determination of the GFP-DsRed colocalization and the percentages of cells positive for GFP (cells containing baculoviruses). Notice that the treatment with MβCD reduced the colocalization between the baculovirus and clathrin but did not alter the amount of cells containing recombinant baculoviruses inside the cytosol. (D) The numbers of cells expressing DsRed were quantified and compared to the numbers of cells carrying the recombinant baculovirus in their cytosol, as measured by immunocytochemistry using a monoclonal antibody specific for VP39 (see Materials and Methods). The expression of DsRed was driven by the CMV promoter in the recombinant baculovirus Bac-GP64-GFP-CMV-DsRed. As shown by the data, altering the clathrin-dynamain pathway results in reduced DsRed expression without affecting the number of viruses inside the cells. Values are the means $\pm$ standard deviations from at least 18 independent measurements.

nant baculovirus that displayed eGFP as a fusion protein with GP64 (similar to Bac-GP64-GFP) but carried a mammalian expression cassette for the red fluorescent protein (DsRed) controlled under the CMV promoter (Bac-GP64-GFP-CMV-DsRed).

We utilized this recombinant baculovirus in control cells and cells treated with MβCD for 45 min and measured the expression of DsRed fluorescent protein 24 h after exposing the cells to Bac-GP64-GFP-CMV-DsRed. As a control for expression, we transfected cells with the plasmid pCMV-DsRed-Express (see Materials and Methods).

Figure 5D correlates the percentage of cells containing Bac-GP64-GFP-CMV-DsRed (as a measurement of baculovirus internalization into the cell) with the percentage of cells expressing DsRed driven by the CMV promoter present in the recombinant baculovirus. Since GP64 is part of the virus coating and is lost during endosome exit, we conducted additional experiments to localize the virus capsid using a monoclonal antibody against VP39, the main capsid protein (see Materials and Methods). Whether we measured GP64-GFP fluorescence (Fig. 5C) or VP39 fluorescence (using a specific monoclonal antibody) (Fig. 5D), we

observed similar results regarding the percentage of cells containing Bac-GP64-GFP-CMV-DsRed.

To explore further the role of the clathrin-dynamain pathway during baculovirus internalization into mammalian cells, we utilized siRNAs to reduce the contents of clathrin and dynamain proteins in the host cells. We also utilized the cell-permeable inhibitor of dynamain, dynasore, to corroborate the siRNA experiments.

As illustrated in Fig. 5D, none of the treatments described above significantly altered the amount of recombinant baculovirus (Bac-GP64-GFP-CMV-DsRed) inside the host mammalian cells as evaluated by the percentage of VP39-positive cells. However, baculovirus-driven gene expression (as assessed by cells expressing DsRed) was strongly inhibited by siRNA(Clathrin), siRNA(Dynamain), and dynasore (50 μM) but not by siRNA(Scramble), used as a negative control. Likewise, the amount of expression induced by transfecting the plasmid CMV-DsRed Express was unaltered by RNAi(clathrin), as expected since plasmid internalization is controlled not by clathrin but by the lipids used for transfection (see Materials and Methods).

These results strongly suggest that baculovirus internalization

into mammalian cells is not affected by altering the clathrin-dynamin pathway but that baculovirus-driven gene expression is significantly reduced by alterations in this pathway. This is in agreement with our observation that cholesterol reduction drives baculovirus internalization via a clathrin-dynamin-independent pathway.

To assess the role of the two CRAC domains described above in baculovirus infectivity in insect cells, we conducted experiments using Sf9 insect cells with these mutants. Not surprisingly, we observed no effect on baculovirus internalization or infectivity in insect Sf9 cells (data not shown). The lack of effect is the result of the baculovirus carrying a wild-type copy of GP64 in its genome (in addition to the mutant copy introduced by us).

In order to evaluate the role of the different CRAC domains in baculovirus infectivity in insect cells, we removed the wild-type copy of the *gp64* gene from the baculovirus genome by recombination methods (see Materials and Methods). We utilized a baculovirus genome lacking the wild-type copy of the *gp64* gene to introduce the copies of *gp64* carrying the mutations of the CRAC domains described above, which also displayed GFP (Y253A, Y311A, and the double mutation Y253A Y311A). These new recombinant baculoviruses were named Bac-GP64-null-Y253A-GFP, Bac-GP64-null-Y311A-GFP, and Bac-GP64-null-Y253A-GFP-Y311A-GFP.

Mutation of the first CRAC domain (Ch1 Y253A) had no effect on virus infectivity or multiplicity of infection (data not shown). However, mutation of the second CRAC domain (Ch2 Y311A) resulted in viruses that could not be amplified. After many attempts, we could not get a useful viral stock. This result is difficult to interpret accurately, but since GP64 is essential for baculovirus infectivity in insect cells, this suggests that mutations in the second CRAC domain destroy GP64 function, resulting in replication-incompetent viruses.

To further confirm that the replication-incompetent baculoviruses were the result of the single-point mutation in GP64 (Y311A), we conducted rescue experiments using the VSV G protein to pseudotype the recombinant baculovirus Bac-GP64-null-Y311A-GFP. For these experiments, we expressed VSV G under the baculovirus immediate early promoter (IE1) contained in a plasmid, as previously described (40). At 24 h after the plasmid transfection, insect cells were transfected with the bacmid for Bac-GP64-null-Y311A-GFP.

Pseudotyped Bac-GP64-null-Y311A-GFP baculoviruses could be amplified similarly to wild-type baculoviruses, resulting in equivalent viral titers. Flow cytometry experiments using purified viruses demonstrated that all recombinant baculoviruses (including the Bac-GP64-null-Y311A-GFP pseudotyped with VSV G) displayed similar GFP fluorescence levels (Fig. 6A). Furthermore, Western blot analysis showed similar levels of GP64-GFP protein content in all recombinant baculoviruses, and only the VSV G-pseudotyped Bac-GP64-null-Y311A-GFP showed slightly higher levels of GP64-Y311A-GFP protein content (Fig. 6B). Notice that with this recombinant baculovirus, the wild-type copy of GP64 could not be detected in the Western blot, as expected since the *gp64* wild-type copy was deleted from the genome (Fig. 6B).

Adding an extra copy of wild-type *gp64* also rescued the GP64-null-Y311A-GFP baculovirus (data not shown). Similar results were obtained by adding an extra copy of *gp64* containing the mutation of the first CRAC domain (data not shown). These results suggest that the Ch2 CRAC domain is required for baculovi-

rus infectivity in insect cells, while destroying the Ch1 CRAC domain had no effect on virus infectivity in insect cells.

To further evaluate the capacity of GP64 null-pseudotyped baculoviruses to drive the expression of foreign genes in mammalian cells, we prepared a new recombinant baculovirus carrying a mammalian expression cassette for DsRed, using as the backbone the bacmid lacking the wild-type copy of the *gp64* gene (Bac-GP64-null-CMV-DsRed). This baculovirus was pseudotyped with VSV G as described above.

Most interestingly, Bac-GP64-null-Y311A-GFP pseudotyped with VSV G could drive high levels of expression of DsRed that were not affected by M β CD treatment (Fig. 6C and D). The foreign-gene expression driven by the recombinant baculovirus carrying a wild-type copy of the *gp64* gene (Bac-GP64-GFP-CMV-DsRed) was severely affected by M β CD (Fig. 6C and D), as we showed above (Fig. 5D).

The fact that the VSV G-pseudotyped recombinant baculovirus (Bac-GP64-null-Y311A-GFP+VSV G) was not altered by M β CD treatment is the result of baculovirus entry into the cells being mediated by VSV G, which has been shown to be independent of cellular cholesterol (10).

To further explore the clathrin-dynamin-independent pathway, we performed additional experiments directed at identifying the key players in this mechanism of internalization. Macropinocytosis is one of the main internalization mechanisms utilized by many viruses and pathogens (for a review, see reference 41). Thus, we decided to explore this possibility first.

Macropinocytosis depends on the concerted action of several kinases (41). The serine/threonine p21-activated kinase 1 (Pak1) modulates cytoskeleton mobility and is required during all of the steps of macropinocytosis (41). To assess the role of this kinase in baculovirus internalization, we conducted experiments using RNA interference to reduce the Pak1 protein content in cells before adding the Bac-GP64-GFP-CMV-DsRed.

Since we observed reduced baculovirus-driven gene expression in cells exposed to M β CD, we conducted experiments to determine how much of the viral genetic material reached the nucleus under the different experimental conditions. For this purpose, we performed fluorescence *in situ* hybridization (FISH) studies (see Materials and Methods).

Figure 7A shows the cellular localization of the baculovirus genetic material in a control cell (in the absence of M β CD). Figure 7B shows a representative cell illustrating the localization of the baculovirus genetic material. As indicated in the figures, nuclear colocalization was attained in the absence of M β CD (human histone 2 [H2]-GFP was used to identify the nucleus). Most interestingly, cells exposed to M β CD for 45 min showed most of the baculovirus DNA labeling around the nucleus but not inside (Fig. 7B). The means $\pm$ standard deviations from at least 35 independent observations are illustrated in Fig. 7C. To determine the amount of baculovirus entering the cell, we utilized an antibody directed against VP39 (see Materials and Methods).

Altering the clathrin-dynamin pathway [with RNAi(clathrin), RNAi(dynamin), or dynasore] prevented the baculovirus genome from reaching the nucleus, as indicated by diminished H2-GFP-DNA-Bac colocalization (Fig. 7C). Interfering with macropinocytosis [RNAi(Pak1)] in the absence of M β CD had no effect on the percentage of cells positive for VP39 or the colocalization of H2-GFP-DNA-Bac. This is in agreement with our previous observations indicating that, in the presence of cholesterol, baculovirus

FIG 6 Assessment of GP64-GFP content on the surface of recombinant baculoviruses and baculovirus-driven foreign-gene expression. (A) Purified single viral particles were introduced into the sorting chamber of a FACScalibur fluorescence-activated cell sorting (FACS) apparatus. The GFP fluorescence signal was acquired at 525 nm, and events were counted until they reached 10,000 individual viral particles for all conditions. The data for Bac-WT-GP64 illustrate the autofluorescence measured from baculoviruses carrying a wild-type copy of GP64 without GFP. This level was considered the background signal. Fluorescence intensity in arbitrary fluorescence units is plotted in logarithmic scale. The shaded (gray) area indicates the GFP-positive signal (GFP+). (B) Western blot analysis of GP64 proteins obtained from purified recombinant baculoviruses, using an anti-GP64 antibody or an anti-GFP antibody as indicated (see Materials and Methods). Notice that the anti-GFP antibody only recognizes GP64-GFP. Notice also that no wild-type copy of GP64 is found in the GP64 null recombinant baculovirus (Bac-GP64-null-Y311A-GFP) pseudotyped with VSV G (5th lane from the left; only the band representing GP64-GFP is observed). (C and D) Baculovirus-driven DsRed expression assessed with HEK293T cells exposed to recombinant baculoviruses in the absence (C) or presence (D) of MβCD. Notice that DsRed expression from Bac-GP64-null-CMV-DsRed pseudotyped with VSV G was not altered by MβCD; only the expression from Bac-GP64-GFP-CMV-DsRed was severely reduced by MβCD treatment. DsRed fluorescence was collected at 650 nm.

internalization is via the clathrin-dynamin pathway (Fig. 6). However, in cells treated with RNAi(Pak1), not only did exposure to MβCD for 45 min prevent baculovirus internalization (evidenced by reduced VP39 labeling inside the cells) but the genetic material did not reach the nucleus (shown by the level of H2-GFP–DNA–Bac colocalization) (Fig. 7C). As indicated in the figure, this was the only treatment that reduced baculovirus internalization.

These results indicate that baculovirus can enter the mammalian cell via at least two different pathways, one of which is the clathrin-dynamin pathway, which results in efficient baculovirus-driven gene expression. The other pathway is clathrin-dynamin independent and Pak1 dependent and results in poor baculovirus-driven gene expression and the accumulation of baculoviruses in the cell cytosol. When the Pak1-dependent pathway is utilized, the baculovirus genetic material does not reach the host cell nucleus. Cells containing normal levels of cholesterol at the plasma membrane favor baculovirus internalization via the clathrin-dynamin pathway, whereas cells treated with MβCD favor the Pak1-dependent pathway (most likely macropinocytosis).

A model for baculovirus internalization and baculovirus-driven gene expression in mammalian cells. Figure 8 shows a

model summarizing all the findings described in this study. The model presents the two independent pathways for baculovirus internalization into mammalian cells. One pathway is clathrin-dependent endocytosis, which results in baculovirus reaching the nucleus of the host cells and, thus, efficient foreign-gene expression. The other pathway is clathrin-dynamin independent and Pak1 dependent. This internalization pathway prevents the baculovirus genetic material from reaching the nucleus of the host cell and, thus, results in reduced baculovirus-driven gene expression. The results of Pak1 RNAi in this second pathway suggest that this alternative pathway is most likely macropinocytosis. The amount of cholesterol on the plasma membrane of the host cell serves as a checkpoint to determine which pathway will utilize the baculovirus. However, cholesterol reduction does not interfere with baculovirus internalization, it only determines which pathway will be used during host cell internalization. The fact that the genetic material of baculoviruses using the macropinocytosis pathway does not reach the cell nucleus suggests that baculovirus cannot escape efficiently from this type of vesicle (although this is only one feasible explanation). If that is the case, our results suggest that cholesterol might also be needed for baculovirus escape from

FIG 7 MβCD treatment prevents the baculovirus genetic material from reaching the host cell nucleus. Fluorescence *in situ* hybridization (FISH) studies to identify the intracellular localization of the baculovirus genome were conducted with HEK293T cells expressing the human histone H2 (H2-GFP), used to identify the nucleus. (A and B) Representative control cell (not exposed to MβCD) (A) and representative cell exposed to MβCD (B) for 45 min prior to addition of recombinant baculovirus. Red shows the FISH signal and blue the fluorescence from H2-GFP, used as the nuclear marker. The nucleus in panel A was rendered using 50% transparency to facilitate the visualization of the FISH signal inside the nucleus. (C) Percentages of colocalization between the FISH signal (DNA-Bac) and nucleus (H2-GFP) in control cells and cells exposed to MβCD. Note that the percentage of VP39-positive cells illustrates the number of cells with viral capsids inside, but the DNA-Bac/H2-GFP colocalization shows the number of cells with viral DNA inside their nuclei. Data show also the effects of interfering with the clathrin-dynamin pathway [RNAi(Clathrin), RNAi(Dynamin), and dynasore] in the absence of MβCD. Finally, to explore the involvement of macropinocytosis, the effects of RNAi(Pak1) in control and MβCD-treated cells are shown. Bars show means $\pm$ standard deviations from at least 35 independent observations. The red horizontal line shows the level of background fluorescence obtained in FISH control studies with HEK293T cells not exposed to baculovirus.

the endosome, which is an essential step in order for the baculovirus genome to reach the nucleus. One could speculate that the CRAC domain from GP64 might play a role in endosome escape also. However, more studies are required to explore this hypothesis in depth.

DISCUSSION

AcMNPV are replicative only in insect hosts; however, they can penetrate a wide variety of cells from different organisms. These viruses can enter mammalian cells from different origins with diverse degrees of efficiency in foreign-gene expression (6, 9, 42). The differences in baculovirus-driven gene expression observed with different cell types pose an enigma, especially when the same strong promoter has been used in many of the studies (9).

Several studies highlight the role of lipids in baculovirus attachment and internalization into insect (11) and mammalian cells (13); however, the mechanism associating baculovirus internalization and lipids remained obscure for many years.

In the present study, we have identified 3 putative cholesterol recognition amino acid consensus (CRAC) domains in GP64, the main envelope protein of baculoviruses. This protein forms part

of a novel family of fusion proteins sharing structural motifs with the vesicular stomatitis virus (VSV) G and herpes simplex virus type 1 (HSV-1) gB glycoproteins. All three proteins resemble each other and belong to the family of proteins known as γ -penetrenes (16, 43).

Most interestingly, we have shown in this study that the CRAC domain located in domain III of GP64 is essential for cholesterol interactions. Using a novel method of massive peptide screening, we have shown that this CRAC domain (used as an isolated peptide) binds the blue-fluorescing cholesterol analog DHE. Furthermore, extensive alanine scanning identified tyrosine (Y311) as an essential amino acid for DHE binding. Additionally, the Y311A mutation resulted in the elimination of FRET between the recombinant baculovirus and the DHE decorating the plasma membrane of living cells.

Finally, we have shown that cholesterol works as a checkpoint to determine the mechanism of baculovirus internalization into mammalian cells. With cholesterol present at the plasma membrane of the host cell, recombinant baculoviruses enter the host cell via a clathrin-dynamin pathway. This internalization pathway facilitates the delivery of the genetic material from the baculovirus into the host nucleus, resulting in efficient baculovirus-driven gene transfer.

FIG 8 A model for baculovirus internalization and baculovirus-driven gene expression in mammalian cells, summarizing the results obtained in the present study. On the left is the pathway utilized by baculovirus to enter mammalian cells when normal levels of cholesterol are present at the plasma membrane. In this case, one of the first events observed is the intimate association between GP64 and cholesterol, as observed with FRET and peptide microarray studies. This intimate association presumably occurs via the Ch2 CRAC domain found in GP64. This internalization mechanism depends on clathrin and dynamin. On the right is the scenario when cholesterol at the plasma membrane is reduced in the host cell. In this case, baculoviruses enter the cell via a clathrin-dynamin-independent but Pak1-dependent mechanism. This results in the baculovirus genetic material not reaching the cell nucleus, resulting in poor baculovirus-driven gene expression. Based on the Pak1 requirement, this internalization mechanism is most likely macropinocytosis. The triangle below indicates that, with high cholesterol content, baculovirus-driven gene expression is efficient. On the other hand, with reduced cholesterol content (tip of the triangle at the right), baculovirus-driven gene expression is significantly reduced (thus pointing towards the macropinocytosis pathway at the right).

On the other hand, with reduced cholesterol at the plasma membrane, baculoviruses enter the host cell via a Pak1-dependent pathway (most likely macropinocytosis). This alternative internalization pathway depends on the presence of the serine/threonine kinase Pak1. The result of choosing this internalization pathway is reduced gene delivery into the host cell nucleus and reduced baculovirus-driven gene expression.

Cholesterol and lipid rafts play key roles in virus assembly and budding in a wide variety of animal viruses (44). Semliki forest virus (SFV) E1 was the first viral fusion protein identified as specifically binding cholesterol at the moment that the fusion peptide in E1 interacts with the plasma membrane (45, 46, 47), similar to what we have found here for baculovirus GP64 with the Ch2 CRAC domain.

Most surprisingly, an *in silico* analysis from the two other members of the class III fusion proteins, the vesicular stomatitis virus (VSV) G and herpes simplex virus type 1 (HSV-1) gB glycoproteins (16), revealed the presence of putative CRAC domains in both proteins.

What was more striking from the analysis was the fact that the two putative CRAC domains identified in VSV G lay in domain III at exactly the same location as Ch2 309-317 from GP64. The first CRAC domain from VSV G is amino acids 318 to 324, and the second putative CRAC domain is amino acids 339 to 348.

The two putative CRAC domains found in HSV-1 gB glyco-

protein (amino acids 686 to 691 and 699 to 676) lie immediately after domain III in the coil-arm complex, which has recently been identified as a domain that contributes to the refolding during the fusion step of virus internalization (48).

Future studies will be needed to determine whether the putative CRAC domains from VSV G and HSV-1 gB are indeed functional and required for virus attachment to the host cell membrane via cholesterol interactions. Notably, M β CD appears to have no effect on the virus internalization mediated by VSV G (13).

A similar CRAC domain has also been identified in the GP41 protein from human immunodeficiency virus (HIV) (49). This CRAC domain is found in the ectodomain, preceding the transmembrane domain of the protein (35). Other viral proteins copurify with cholesterol, for instance, M2 from the influenza A virus. Thus, cholesterol plays key roles in virus internalization and morphogenesis in a variety of animal viruses (35).

In spite all the information available about cholesterol and lipid rafts in virus physiology, very little is known about the molecular mechanisms associating cholesterol with selective virus internalization, sorting, and genetic material transfer during virus infections.

What is the mechanism that drives baculovirus to select a clathrin-dynamin internalization pathway when cholesterol is present? This question poses a complex challenge that we aim to explore in future studies. However, we can speculate about it.

There is a well-established relationship between the clathrin-dynamin endocytic pathway and lipid rafts (50). For instance, dynamin 2, clathrin, and lipid rafts play a key role during the endocytosis of the Na/K/2Cl cotransporter (51). The internalization of human HMI.24 and α -adaplin is mediated by clathrin present in cholesterol-enriched plasma membrane domains (52).

Thus, it is feasible that cholesterol (via GP64-cholesterol interactions) may facilitate the association of baculovirus with clathrin (at the plasma membrane), resulting in the subsequent internalization of the baculovirus in clathrin-coated vesicles. Baculovirus can exit the clathrin-coated vesicles via interactions with GP64 (53). Once liberated from the vesicles, the baculovirus genetic material can reach the host cell nucleus and drive efficient foreign-gene expression (54).

Our results suggest that baculovirus cannot escape efficiently from vesicles devoid of clathrin, and thus, its genetic material does not reach the host cell nucleus. The result is reduced baculovirus-driven gene expression.

The CRAC domain described here might represent a general internalization mechanism, since baculovirus with the Y311A mutation in GP64 cannot replicate in Sf9 cells (Bac-GP64-null-Y311A-GFP). Only when a wild-type copy of the *gp64* gene is present in the baculovirus genome can we obtain infective viral particles. Most interestingly, the mutation of the first CRAC domain (Ch1 Y253A) had no effect on baculovirus infectivity in Sf9 insect cells, even when the wild-type copy of *gp64* was removed from the baculovirus genome (Bac-GP64-null-Y253A-GFP). Furthermore, the Ch2 Y311A mutant could be rescued by introducing an extra copy of *gp64* carrying the Ch1 Y253A mutation or by introducing an extra copy of wild-type *gp64*. Similarly, the Bac-GP64-null-Y311A-GFP recombinant baculovirus could be rescued by pseudotyping the virus with VSV G. All these results point to the Ch2 CRAC domain as essential for GP64 function during baculovirus infectivity.

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Conflict of interest: Alexander Asanov is the CEO of TIRF Labs, Inc., Cary, NC. Ig-TIRFM, Ig-TIRFM-FRET, and the use of microarray peptides with Ig-TIRFM-1000 are the property of TIRF Labs, Inc.

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