Virology **254,** 297–314 (1999)
Article ID viro.1998.9523, available online at http://www.idealibrary.com on IDEAL®

Requirement for GP64 to Drive Efficient Budding of *Autographa californica* Multicapsid Nucleopolyhedrovirus

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Received September 22, 1998; returned to author for revision October 23, 1998; accepted November 16, 1998

Budded virions (BV) of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) contain a major envelope glycoprotein (GP64) that is present on the plasma membrane of infected cells. GP64 is acquired by virions during budding through the plasma membrane, the final step in assembly of the budded virion at the cell surface. Previous studies (S. A. Monsma, A. G. P. Oomens, and G. W. Blissard (1996). *J. Virol.* 70, 4607–4616) showed that insertional inactivation of the AcMNPV *gp64* gene resulted in a virus unable to move from cell to cell and nonlethal to orally infected *Trichoplusia ni* larvae. To determine whether GP64 is involved in virion budding, we measured BV production from Sf9 cells infected with a gp64null virus. Sf9 cells infected with gp64null virus vAc^{64-} were pulse labeled, and progeny BV were isolated on equilibrium sucrose gradients and quantified. BV production from vAc^{64-} was reduced to ~2% of that from wild-type AcMNPV. Thus the GP64 protein is important for efficient virion budding. To determine whether the highly charged 7-amino acid cytoplasmic tail domain (CTD) of GP64 was required for virion production, we generated a series of GP64 constructs containing C-terminal truncations or substitutions. Modified forms of GP64 were analyzed in transfected cells and in recombinant viruses in which the wild-type *gp64* gene was replaced with a modified *gp64.* Deletion of 1–7 amino acids from the CTD did not affect GP64 trimerization, protein transport to the cell surface, or membrane fusion activity. However, deletions of 11 or 14 amino acids, which removed the CTD and portions of the predicted transmembrane (TM) domain, were trimerized but were present at lower levels on the cell surface due to shedding of these truncated proteins. Comparisons of growth curves and quantitative measurements of labeled progeny BV production from recombinant viruses expressing either wild-type or mutant GP64 proteins showed that deletion of the 7-residue CTD only moderately reduced the production of infectious virions (~50%). However, deletions of the C terminal 11 or 14 amino acids had more substantial effects. Removal of the C terminal 11 amino acids reduced titers of infectious virus by 78–96% and labeled progeny virions were reduced by 91–92%. Removal of 14 amino acids from the C terminus resulted in an ~98% reduction in progeny BV and a virus that was apparently incapable of efficient propagation in cell culture. Thus the GP64 CTD is not essential for production of infectious BV, but removal of the CTD results in a measurable reduction in budding efficiency. Deletion of the CTD plus small portions of the transmembrane domain resulted in shedding of GP64, reduced surface levels, and a dramatic reduction in the production of BV. Together, these data indicate that GP64 is an important and limiting factor in BV production.

INTRODUCTION

During the infection cycle, baculoviruses such as the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and the *Orgyia pseudotsugata* MNPV (OpMNPV) produce two types of infectious virus particles that are referred to as virion phenotypes (reviewed in Rohrmann, 1992; Blissard, 1996; Miller, 1997). The two virion phenotypes are termed budded virus (BV) and occlusion derived virus (ODV). Each consists of enveloped nucleocapsids, but the envelopes differ in source and composition (Braunagel and Summers, 1994). BV and ODV serve distinctly different roles in the life cycle of the virus and are assembled by different mechanisms and at different sites in the infected cell. BV are assembled at the cell surface as nucleocapsids bud through the plasma membrane. In contrast, ODV are assembled within the nucleus when nucleocapsids are enveloped within membranes that appear to be derived from the nuclear membrane (Fraser, 1986; Hong *et al.,* 1997). Very late in infection, ODV become embedded within an abundantly expressed viral protein, polyhedrin, to form highly stable occlusion bodies. Animal-to-animal transmission of baculovirus infections is typically by the oral route and is mediated by ODV, which are highly infectious to the epithelial cells of the midgut (Volkman and Summers, 1977). ODV enter and infect host midgut epithelial cells, and infection results in assembly and budding of BV from the basal side of these polarized cells (Granados and Lawler, 1981; Keddie *et al.,* 1989). BV is highly infectious to tissues within the hemocoel and in cell culture (Volkman *et al.,* 1976) and mediates the spread of infection from cell to cell within the animal. BV from viruses such as AcMNPV and OpMNPV have a loosely adhering en-

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0042-6822/99 $30.00

velope that contains a major spike protein known as GP64. On the surface of infected cells and in the BV envelope, GP64 is present as a homotrimer (Oomens *et al.,* 1995). GP64 is thought to serve critical roles during viral entry into host cells. After binding, BV enter cells by endocytosis (Volkman and Goldsmith, 1985), and previous studies have demonstrated that GP64 is necessary and sufficient for low-pH-triggered membrane fusion, a process that is required for release of nucleocapsids from endosomes (Blissard and Wenz, 1992; Leikina *et al.,* 1992; Chernomordik *et al.,* 1995; Monsma and Blissard, 1995; Monsma *et al.,* 1996). During budding and assembly of BV, GP64 concentrates in discrete areas on the plasma membrane (Blissard and Rohrmann, 1989), and these sites appear to represent the sites of BV budding (Volkman *et al.,* 1984). In many cases, viral envelope proteins are important in budding and assembly of progeny virions at the cell surface (Stephens and Compans, 1988). However, the role of the envelope proteins in virion budding may differ substantially in different virus groups. Recent studies of virion budding in two rhabdovirus systems showed that virion budding is severely reduced (~30-fold) in the absence of the major envelope glycoprotein, G protein (Mebatsion *et al.,* 1996; Schnell *et al.,* 1997). In contrast, the budding of some retroviruses is independent of the major envelope protein (Env), requiring only the Gag protein for budding (Ruta *et al.,* 1979; Shields *et al.,* 1978; Gheysen *et al.,* 1989; Gottlinger *et al.,* 1989).

In a previous study, we generated a gp64null AcMNPV baculovirus using a stably transfected cell line to provide the GP64 protein during construction and propagation of the virus (Monsma *et al.,* 1996). Using the gp64null virus, it was demonstrated that GP64 is necessary for cell-to-cell transmission of infection in cell culture and for movement of infection from the insect midgut into the hemocoel. However, the precise mechanism of this defect in the gp64null virus was not known. Possible explanations included (a) a defect in production of BV or (b) production of BV that were not infectious. To address these possibilities in the current study, we used a biochemical approach to examine BV production from a gp64null virus. We show that virion production is almost completely eliminated in the absence of GP64. Our results indicate that GP64 plays an important role in virion budding from the plasma membrane and that the observed defect in cell-to-cell transmission by the gp64null virus results primarily from a defect in virion budding. We also examined the role of the predicted cytoplasmic tail domain (CTD) of GP64 in virion budding from the plasma membrane. A series of GP64 constructs containing deletions or substitutions within the CTD region was generated, analyzed, and used for constructing recombinant viruses in which the wild-type *gp64* gene was replaced with modified forms of *gp64*. We found that the GP64 CTD was not required for either transport to the cell surface, trimerization, low-pH-triggered membrane fusion activity, budding of progeny virions, or infectivity of BV. However, deletions of the CTD and portions of the transmembrane domain resulted in less efficient anchoring of GP64 in the plasma membrane, which was accompanied by GP64 shedding and a decrease in virion budding at the cell surface.

RESULTS

GP64null virus construction

In a previous study (Monsma *et al.,* 1996), we generated a gp64null baculovirus (vAc^{64Z}) in which the *gp64* gene was inactivated by insertion of a *lacZ* cassette into the AcMNPV *gp64* ORF. The vAc^{64Z} virus was generated and propagated in a cell line ($Sf9^{OP64-6}$) that constitutively expresses the OpMNPV GP64 protein. We found that upon prolonged passage of vAc^{64Z} in $Sf9^{OP64-6}$ cells, low-frequency homologous recombination of the virus with the OpMNPV *gp64* gene in the cell line resulted in rescue and generation of a low level of virus expressing a functional GP64 protein. To avoid the potential problem of gp64null rescue by homologous recombination, we generated a second recombinant AcMNPV in which the entire *gp64* ORF was removed. To remove the *gp64* ORF, we generated a transfer vector plasmid containing a *lacZ* cassette (under the control of the OpMNPV IE1 promoter), flanked by sequences found upstream and downstream of the AcMNPV *gp64* ORF (Fig. 1). For generation and propagation of this gp64null virus, we used a Sf9-derived cell line, $Sf9^{Op1D}$ (Plonsky *et al.,* submitted), that constitutively expresses the OpMNPV GP64 protein at higher and more uniform levels than line $Sf9^{OP64-6}$. Cotransfection of the transfer vector and wild-type AcMNPV DNA in $Sf9^{Op1D}$ cells resulted in discrete removal of the AcMNPV *gp64* ORF plus a small amount of flanking sequence and replacement with the lacZ cassette (Fig. 1). The resulting virus (vAc^{64-}), lacking the *gp64* gene and containing a lacZ cassette in the *gp64* locus, was confirmed by PCR and restriction enzyme analysis as described earlier (Monsma *et al.,* 1996). Following extensive passaging of vAc^{64-} in $Sf9^{Op1D}$ cells, we did not detect production of GP64 when Sf9 cells were subsequently infected with vAc^{64-} (data not shown).

Effect of the *gp64* deletion on virion budding

Studies of a previous gp64null virus (vAc^{64Z}) showed that the absence of GP64 expression resulted in a virus unable to move from cell to cell. However, the nature of the defect was not known. Possible explanations included (a) a defect in virion budding or (b) production of virions that were not infectious. To determine whether GP64 was involved in BV budding, we used a biochem-

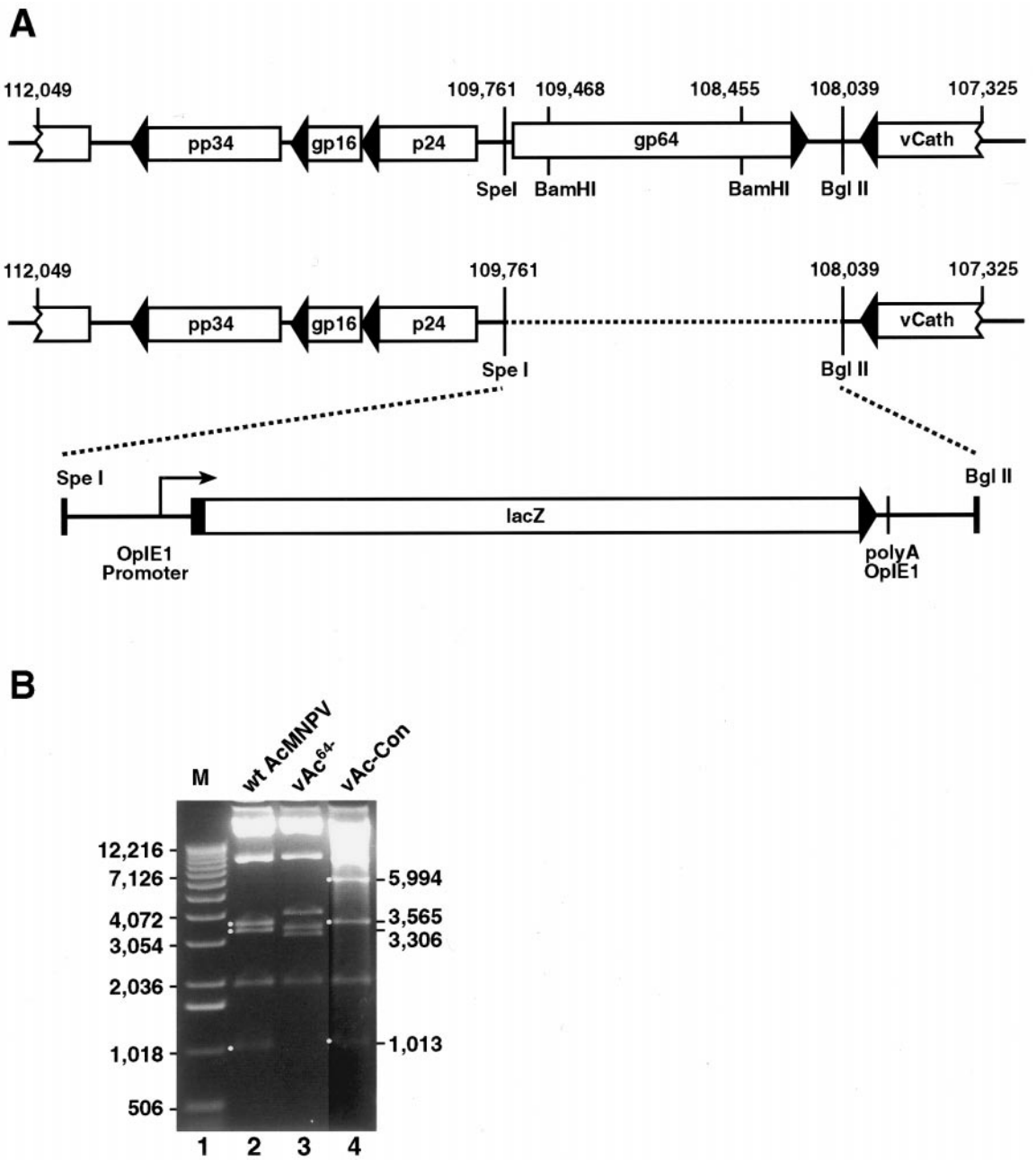

FIG. 1. Construction and analysis of vAc^{64-}, a gp64null virus containing a complete deletion of the AcMNPV *gp*64 ORF. (A) Construction of gp64null virus vAc^{64-}. The top line represents the 4718-bp *Eco*RI-*Sma*I fragment (corresponding to nt 107,325–112,049) from plasmid pAcEcoHΔSma. This fragment contains the AcMNPV *gp64* locus plus flanking genes (open arrows indicate AcMNPV ORFs). The *gp64* ORF was removed from pAcEcoHΔSma as a 1722-bp *Spe*I-*Bgl*II fragment (dashed horizontal line) and replaced with a 4195-bp cassette containing the *Escherichia coli lacZ* ORF under the control of the OpMNPV IE1 early promoter (lower line). The resulting transfer vector was named pAcEcoHΔSmaSpe(Opie1Z+)Bgl and was used to generate gp64null virus vAc^{64-} by cotransfection and homologous recombination with wild-type AcMNPV E2 DNA in Sf9^{Op1D} cells. vAc^{64-} contains a discrete deletion of the AcMNPV *gp64* gene between nt 108,039 and 109,761 and replacement with the 4195-bp *lacZ* expression cassette. (B) Restriction enzyme analysis of genomic DNAs from wild-type and recombinant baculoviruses. Lanes 2–4 show a comparison of *Bam*HI restriction fragment profiles from viral genomic DNA's from wt AcMNPV (Lane 2), gp64null virus vAc^{64-} (Lane 3), and virus vAc-Con (Lane 4). In the wt AcMNPV genome, the *gp*64 gene is represented in three *Bam*HI fragments (lane 2; 3565, 1013, and 3306 bp). All three *gp64* containing fragments are absent from virus vAc^{64-} (lane 3). In virus vAc^{64-}, bp 108,039–109,761 (including the *gp*64 ORF) have been removed and replaced by an OpIE1-βGal cassette. Virus vAc-Con (lane 4) represents a control virus in which a wt *gp*64 gene was reintroduced into virus vAc^{64-} (see experiments described in Fig. 4). *gp64* was reintroduced by cotransfection of vAc^{64-} DNA with transfer vector pΔSmaΔ-GUS, which includes a GUS marker gene cassette downstream of the *gp*64 orf. The *Bam*HI restriction profile of vAc-Con was identical to wt AcMNPV, except for the 3306-bp wt AcMNPV fragment (containing the *gp*64 3′ end), which was replaced with a 5994-bp fragment containing the *gp*64 3′ end and the 2688-bp GUS cassette downstream of the *gp*64 ORF (lane 4). Sizes (bp) of fragments from a DNA ladder (lane 1, M) are indicated on the left and sizes of restriction fragments identified with white dots are indicated on the right.

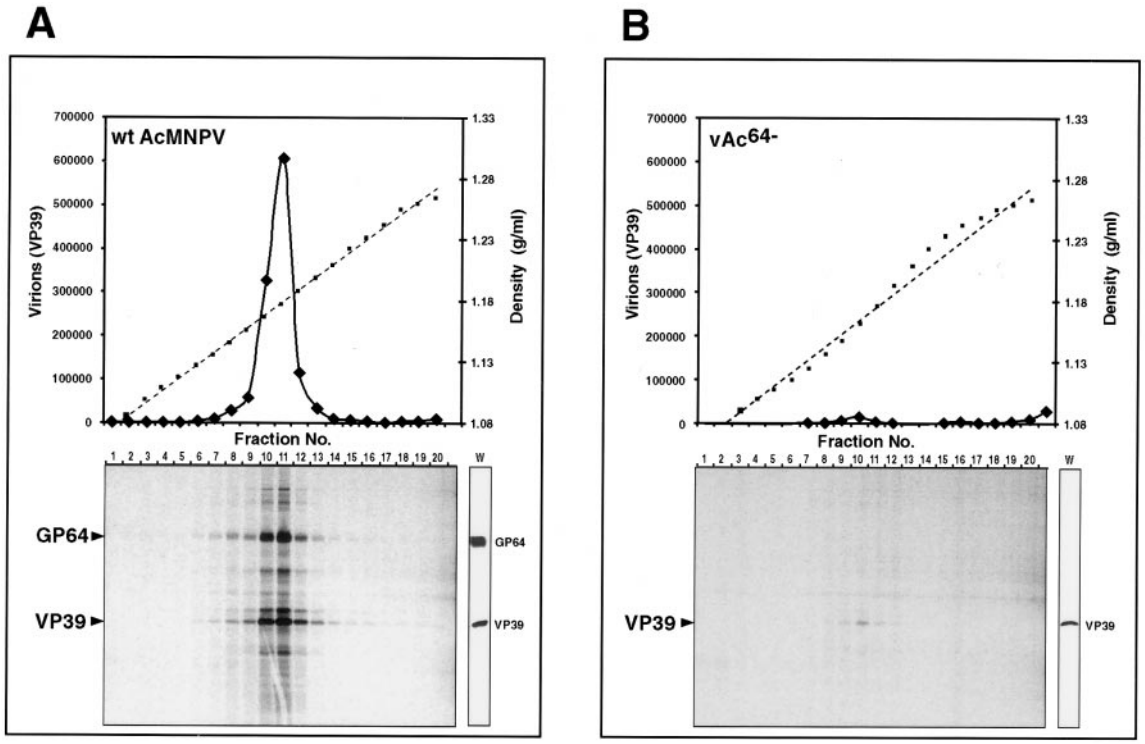

FIG. 2. Quantitative analysis of [^{35}S]methionine-labeled BV production from wild-type AcMNPV (A)- and vAc^{64-} (B)-infected Sf9 cells. Sf9 cells infected with each virus were pulse labeled with [^{35}S]methionine from 16 to 30 h p.i., and labeled progeny virions were isolated from supernatants by purification through a sucrose cushion, followed by separation on equilibrium sucrose density gradients. Gradients were fractionated and the density of each gradient fraction was measured. Equal amounts of gradient fractions were analyzed by electrophoresis on SDS–PAGE gels, and proteins were quantified by phosphorimager analysis (bottom, left). To confirm infection in each experiment, infected cell extracts were examined by Western blot analysis using anti-GP64 and anti-VP39 antibodies (lane W). In each panel (A and B), the top panels show the measured density of each fraction (g/ml, small closed boxes), and a linear curve-fit (dashed line) indicates the linearity of each gradient. The relative quantities of VP39 (large closed boxes, solid line) in gradient fractions were determined by phosphorimager analysis.

ical approach to compare relative quantities of BV production in Sf9 cells infected with either wild-type AcMNPV or the gp64null recombinant virus, vAc^{64-}. Since vAc^{64-} is propagated in Sf9^{Op1D} cells, the virions carry OpMNPV GP64 in their envelope and are therefore infectious in cell culture. However, after infection of Sf9 cells, vAc^{64-} is unable to produce GP64. As an internal control to confirm infection of Sf9 cells by each virus, cell extracts were examined by Western blot analysis, using a combination of anti-VP39 and anti-GP64 antibodies (Fig. 2, bottom, lanes W). Polyhedra formation in infected cells was also monitored (not shown) as a control for productive infection. As expected, the major capsid protein, VP39, was detected from cells infected with both viruses, and GP64 was not detected from Sf9 cells infected with vAc^{64-}, confirming the gp64null virus phenotype. Because the antibody used for these experiments cross-reacts with OpMNPV GP64, these data also show that we could not detect any remaining OpMNPV GP64 from the inoculum.

To distinguish between the virions used to infect cells and potential progeny virions, infected Sf9 cells were pulse labeled with [^{35}S]methionine from 16 to 30 h p.i. and supplemented with unlabeled methionine as a chase at 22 h p.i. Supernatants harvested at 30 h p.i. were used for virion isolation, fractionation, and quantitative analysis. Virions present in supernatants from cells infected with wild-type AcMNPV or vAc^{64-} were pelleted through a 25% sucrose cushion, resuspended, then layered onto a linear 25–60% sucrose gradient and centrifuged to equilibrium. Equal fractions were collected from the gradient, the densities were measured, and proteins were analyzed on SDS–PAGE gels. Relative quantities of labeled virion proteins in each fraction were determined by phosphorimager analysis, using the characteristic polypeptide profile to identify virion fractions. The VP39 capsid protein in virion fractions was used as an internal indicator of the relative number of virions. Figure 2 shows the results of this analysis. Virion fractions isolated from the supernatants of Sf9 cells infected with wild-type AcMNPV were identified by the presence of GP64 and VP39 in standard virion profiles (Fig. 2A; fractions 8–13). Wild-type AcMNPV virions were identified at a peak density of ~1.18 g/ml. Although abundant virion production was detected from wild-type AcMNPV, only trace amounts of the VP39 protein were detected at

similar densities when supernatants from vAc^{64-}-infected Sf9 cells were similarly analyzed (Fig. 2B, fractions 9–11). Long exposures of the gel in Fig. 2B, bottom, showed that other virion proteins were also present in fractions 9–11 (not shown), confirming that the VP39 detected in these fractions resulted from BV. As expected, no labeled GP64 was detected from these progeny virions as the *gp*64 gene was deleted from vAc^{64-} (Fig. 2B). Quantitative measurements of VP39 in all virion-containing fractions indicated that virion production from the vAc^{64-} virus was ~2% of that detected from wild-type AcMNPV. This dramatic reduction in virion production in the absence of GP64 indicates that GP64 is necessary for efficient virion budding. However, the detection of small quantitites of labeled VP39 in virion fractions from vAc^{64-} supernatants also indicates that a very low level of budding occurred even in the absence of GP64.

Construction and analysis of GP64 CTD mutations

The predicted structure of the AcMNPV GP64 protein includes a 20-amino acid hydrophobic N-terminal signal peptide (SP), a large ectodomain of 462 amino acids (present on the cell or virion surface), a hydrophobic transmembrane (TM) domain of 23 residues, and a short hydrophilic cytoplasmic tail domain (CTD) of 7 amino acids (Fig. 3A). By convention, the TM and CTD are defined as follows: the TM is defined as the highly hydrophobic domain near the C terminus, and the CTD is defined as the hydrophilic domain that begins with the first charged residue following the hydrophobic TM domain. Because the cytoplasmic tail of GP64 is the only domain thought to be in direct contact with the cytoplasm and was considered most likely to interact with other viral or cellular proteins during budding, we examined the functional role of the predicted 7-amino acid AcMNPV GP64 CTD. To examine the function of the AcMNPV GP64 CTD, we generated a series of constructs in which amino acid residues were sequentially deleted from the C terminus, with deletions extending through the CTD and into the predicted TM domain (Fig. 3A, C-1 to C-14). For constructs designated C-1 through C-37, numbers represent the number of amino acids removed from the C terminus of AcMNPV GP64. To examine the potential role of a cluster of basic residues within the CTD, we also generated a construct in which each of the three arginine residues was replaced with alanine (Fig. 3A, CΔ3Ra).

Because previous studies showed that a number of GP64 ectodomain modifications resulted in unstable proteins, representative constructs containing CTD and TM mutations were initially examined by transient expression in transfected cells. Expression and trimerization of mutant constructs were compared to that of wild-type GP64 (Fig. 3B; wt *vs* C-1, C-7, C-11, C-14). The two characteristic trimeric forms (Trimer I and II) were identified in the extract of cells transfected with each construct (Fig. 3B, bottom) when examined under nonreducing conditions. All constructs except C-37 were transiently expressed under the control of the wild-type AcMNPV *gp64* early promoter region. Control construct C-37 was expressed from a truncated promoter and was expressed in higher quantities. Because deletions of the CTD and portions of the predicted TM domain might affect protein anchoring, supernatants from transfected cells were also examined for the presence of GP64 that may be shed from the cell surface. Control construct C-37 contains a deletion of the complete CTD and TM and therefore represents a secreted soluble form of GP64 and served as a positive control for secretion or shedding. Supernatants from transfected cells were collected at 48 h post-transfection, and GP64 was immunoprecipitated with an anti-GP64sol polyclonal antiserum. The wt, C-1, and C-7 GP64 proteins were detected in abundance in cell extracts (Fig. 3B, lanes 1–3), and in only trace amounts from supernatants (Fig. 3C, lanes 1–3). In contrast, the GP64 C-11 and C-14 proteins were detected in the supernatants of transfected cells at much higher levels than wt, C-1, or C-7 proteins, suggesting that C-11 and C-14 were shed from the cell surface (Fig. 3C, lanes 4–5). Thus initial analyses of AcMNPV GP64 with CTD and TM truncations showed that the modified proteins were expressed and trimerized in a manner similar to wild-type GP64 and that truncations into the TM domain resulted in less stable anchoring in the membrane.

Construction of recombinant viruses with GP64 CTD mutations

To generate recombinant AcMNPV viruses in which modified forms of *gp64* replaced the wild-type *gp64* gene, viral DNA derived from the gp64null virus vAc^{64-} was cotransfected into Sf9 or Sf9^{Op1D} cells with transfer vectors containing the modified *gp64* genes and sequences flanking the *gp64* locus. A wild-type *gp64* gene was also included for parallel construction of a control virus. Each transfer vector also contained a GUS marker gene under the control of the AcMNPV p6.9 promoter, inserted downstream of the *gp64* gene (Fig. 4A). Because the gp64null virus contains a *lacZ* gene in the *gp64* locus, double recombination events resulted in viruses in which the *lacZ* gene was replaced with the modified *gp64* gene and GUS gene and that exhibited a lacZ^{-}/GUS^{+} phenotype. Virus isolates were identified and plaque purified based on this phenotype and were subsequently examined by PCR analysis, restriction enzyme profiles of genomic DNAs (Fig. 1, lane 4), and sequencing to confirm correct insertion of the wild-type or modified *gp64* gene. Sf9 cells were used to generate viruses containing all constructs shown in Fig. 3A, ex-

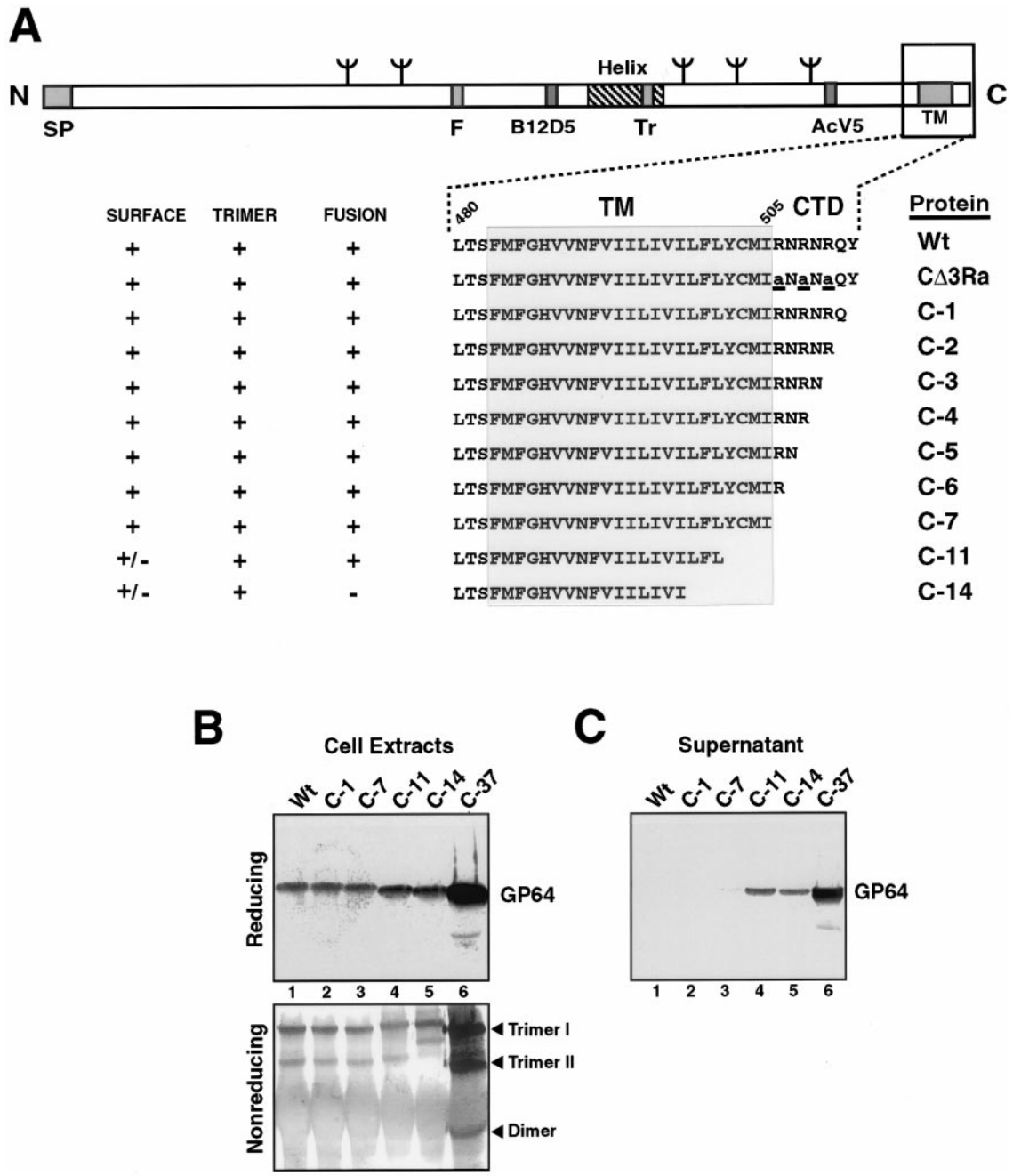

FIG. 3. Construction and analysis of plasmid constructs expressing GP64 proteins with truncations or substitutions in the cytoplasmic tail domain (CTD) and the transmembrane (TM) domain. (A) A schematic representation of the GP64 protein shows the locations of the signal peptide (SP), fusion domain (F), B12D5 and AcV5 epitopes, trimerization domain (TR), transmembrane domain (TM), and cytoplasmic tail domain (CTD). The various deletions and substitutions in the CTD and TM domain used in this study, and their names, are indicated. The indicated constructs were generated in plasmids, examined in transient expression assays, and used to generate recombinant viruses (see Fig. 5). A summary of the properties of each GP64 construct is shown on the left of each construct (see Fig. 5). (B) Expression and trimerization of representative GP64 constructs. Sf9 cells were transfected with plasmids expressing either wild-type GP64 (wt) or modified GP64 (C-1, C-7, C-11, C-14, and C-37), and expression and trimerization of GP64 were examined by Western blot analysis of cell extracts on reducing SDS–PAGE (top) or nonreducing SDS–PAGE (bottom). The positions of GP64 and oligomeric forms (Trimer, Dimer, Monomer) are indicated on the right. (C) Detection of modified GP64 proteins from the supernatants of Sf9 cells transfected with plasmids expressing either wild-type GP64 (wt) or modified GP64 (C-1, C-7, C-11, C-14, and C-37). Supernatants were collected from transfected Sf9 cells examined in (B), and GP64 was immunoprecipitated using an anti-GP64sol antiserum. Plasmid pΔCla-C-37, which expresses a secreted soluble form of AcMNPV GP64 (C-37), was used as a positive control for secretion. All constructs were expressed from the wild-type AcMNPV GP64 early promoter, except C-37 in plasmid pΔCla-C-37, which contains a truncated early promoter.

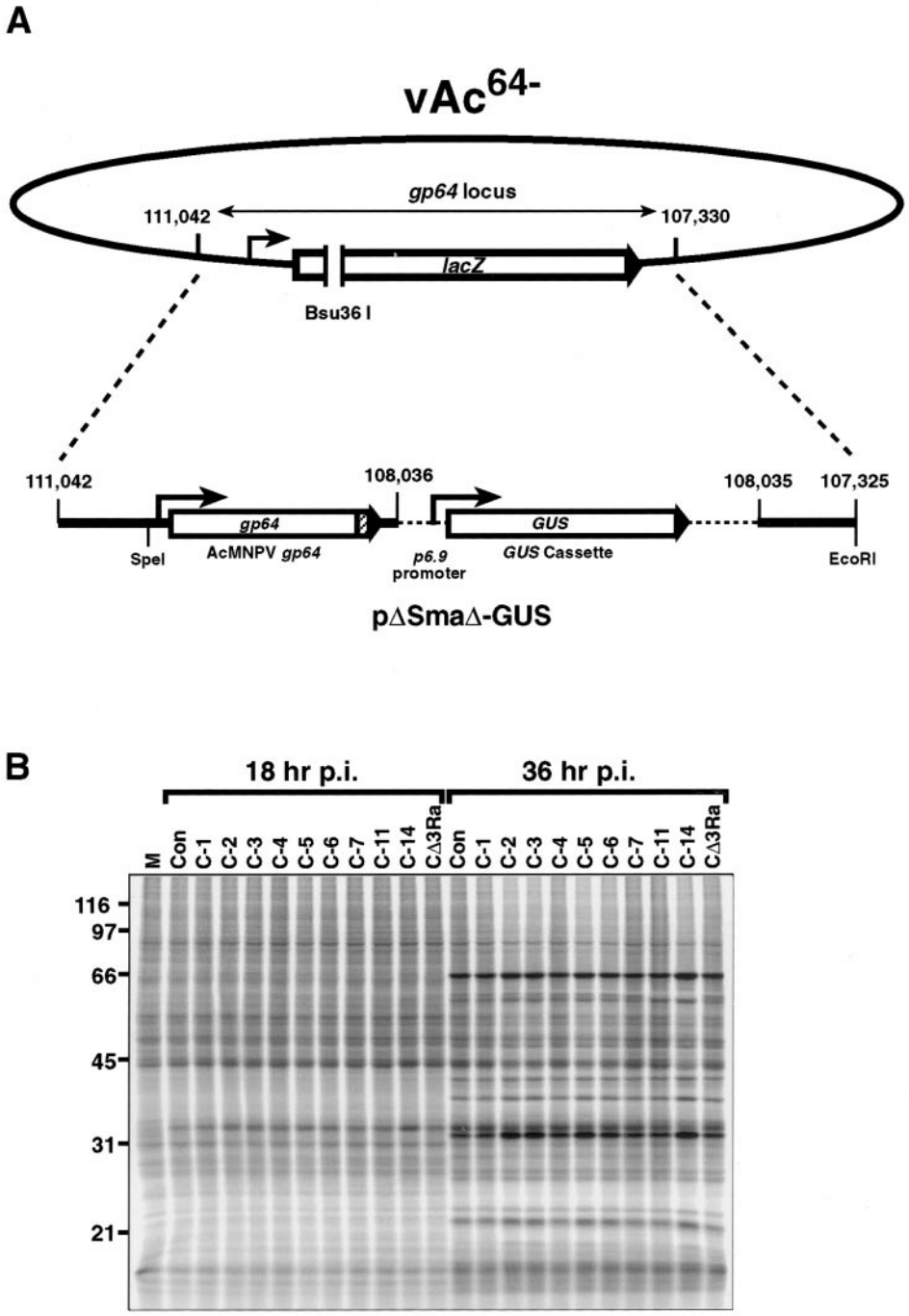

FIG. 4. Construction and analysis of recombinant AcMNPV viruses expressing modified forms of the GP64 protein and GUS. (A) To generate recombinant AcMNPV viruses expressing modified GP64 proteins, gp64null virus DNA (vAc^{64-}, shown as a circle; not drawn to scale) was linearized with unique site Bsu36I (located in the *lacZ* ORF) and cotransfected with transfer vectors containing inserts as indicated below. Each transfer vector contained an AcMNPV *gp*64 gene with modifications of the cytoplasmic tail domain (CTD) and/or transmembrane (TM) domain, a GUS gene cassette containing the GUS gene under the control of the AcMNPV p6.9 promoter, and flanking sequences for recombination into the *gp64* locus (nt 107,325–111,042). Dashed lines represent the boundaries of the area exchanged between vAc^{64-} and transfer vector pΔSmaΔ-GUS by double homologous recombination. This results in replacement of the lacZ cassette of vAc^{64-} with the modified *gp*64 and GUS marker gene. Sequences in the transfer vector derived from the *gp*64 locus of the AcMNPV genome are represented as a solid line, whereas sequences associated with the GUS cassette are represented by a dashed line. Numbers indicated above the sequence represent the standard nomenclature for AcMNPV (Ayres *et al.,* 1994). (B) Pulse labeling of proteins from Sf9 cells infected with viruses expressing wild-type or modified forms of GP64. Cells were infected (m.o.i. of 10) and labeled with [^{35}S]methionine at 18 and 36 h p.i. Extracts from infected cells were electrophoresed on SDS–PAGE gels and examined by phosphorimager analysis. (M, mock infection; Con, Control virus vAc-Con; C-1 to CΔ3Ra, recombinant viruses expressing modified forms of GP64).

cept the C-14 construct. This construct apparently did not produce infectious virions in sufficient quantities to sustain a multiple-round plaque purification procedure. Therefore, $Sf9^{Op1D}$ cells (a line that constitutively expresses wild-type OpMNPV GP64) were used for generation and propagation of the vAc-C-14 virus.

To confirm that the infection cycle proceeded normally in each of the recombinant viruses, viral infections were analyzed by pulse labeling. Sf9 cells were infected with each virus at an m.o.i. of 10, then pulse labeled with [^{35}S]methionine for 1.5 h preceding the indicated times (Fig. 4B, 18 and 36 h p.i.). Cell lysates were then examined for the presence and relative intensities of infected cell specific proteins (ICSPs). Profiles of ICSPs from viruses carrying modified *gp64* genes were similar to those from a control virus (Fig. 4B, lanes Con) in which the wild-type *gp64* gene was inserted in the same manner as modified *gp64* constructs (Fig. 4A).

Analysis of mutant GP64 proteins in recombinant viruses

The expression, localization, and function of wild-type and modified GP64 proteins were examined in infected Sf9 cells. To compare relative quantities of each GP64 protein construct at the cell surface, Sf9 cells infected with each recombinant virus were examined by cell surface ELISA (CELISA) using an anti-GP64 Mab, AcV5. Data from CELISA at 30 h p.i. are shown in Fig. 5A. GP64 levels at the surface of cells infected with viruses expressing constructs C-1 through C-7 and CΔ3Ra were similar to that of wild-type GP64 from either a control virus (Con) or wild-type AcMNPV. The surface levels of GP64 among these constructs ranged from ~80 to 130% of that observed from wild-type GP64 in the control virus, vAc-Con. In contrast, the C-11 and C-14 proteins were detected at substantially lower levels, ~19 and 24%, respectively, relative to the level of wild-type GP64 from the control virus. This is consistent with the earlier data demonstrating increased levels of GP64 in the supernatants of transfected cells expressing the C-11 and C-14 (Fig. 3C). Thus CELISA and immunoprecipitation data indicate that the C-11 and C-14 truncations affected anchoring of these protein constructs to the cell membrane.

To confirm that truncated and modified GP64 proteins were trimerized in Sf9 cells infected with recombinant viruses, infected cell extracts were examined under reducing and nonreducing conditions (Fig. 5B). Previous studies showed that trimer forms I and II both represent homotrimeric forms of GP64 (Oomens *et al.,* 1995) although the physical differences that result in different electrophoretic mobilities are not understood. All constructs were detected from infected cells at levels similar to those observed in the control virus expressing wild-type GP64 and all constructs were trimerized (Fig. 5B, right). We also asked whether low-pH-triggered membrane fusion was affected by the C-terminal truncations or substitutions. Sf9 cells were infected with each of the recombinant viruses, then assayed at 36 h p.i. for membrane fusion activity using a syncytium formation assay. Triggering of fusion was examined over a range of pH values (Fig. 5C). The pH required to trigger membrane fusion activity in the syncytium formation assays was between 5.4 and 5.6 and was similar for constructs C-1 through C-7 and the wt GP64 protein (Con). Interestingly, membrane fusion activity was detected from construct C-11 only at pH 5.2, and no activity was detected from construct C-14. While this effect may be partially explained by lower levels of C-11 and C-14 at the cell surface (Fig. 5A), the levels of cell surface GP64 C-11 and C-14 were similar in CELISA experiments that were performed in parallel (Figs. 5A and 5C). Thus the truncation of additional amino acids in the TM domain in construct C-14 may specifically affect the ability of the protein to mediate fusion. Further studies will be necessary to confirm this preliminary result.

Effects of CTD mutations on infectious BV production

The GP64 protein is necessary for virion entry into host cells by receptor-mediated endocytosis, and studies of a gp64 deletion virus showed that GP64 was important for efficient virion budding. To study the role of the cytoplasmic tail domain (CTD) on production of infectious progeny virus, we examined yields of infectious virions from cells infected with virus constructs containing the truncated or modified GP64 CTDs described above. Titers of virus stocks (Fig. 6A) provided an initial but clear indication that the GP64 CTD was not essential for virus propagation in cell culture. Except for virus vAc-C-14 (which was propagated in $Sf9^{Op1D}$ cells), relatively high titer virus stocks were produced in Sf9 cells from deletion constructs, even when the C-terminus was truncated by 11 amino acids. To examine potential differences in virion production in more detail, we generated viral growth curves for selected viruses (vAc-Con, vAc-C-7, vAc-C-11, and vAc-CΔ3Ra). For these studies, BV production was measured during 6-h periods throughout the course of infection. Sf9 cells were infected with each virus (m.o.i. of 10), cells were washed 6 h prior to each indicated time point, and fresh medium was added. Supernatants containing infectious virions that accumulated in the supernatant (during the subsequent 6-h period) were collected, and infectious BV was quantified by $TCID_{50}$ assay. BV production curves were similar for viruses vAc-Con and vAc-CΔ3Ra (Fig. 6B). The production of infectious BV from vAc-C-7-infected cells was also high but was lower than the control virus (vAc-Con). Significantly lower titers were recorded from vAc-C-7 at a number of time points (18, 36, 48, and 60 h p.i.). Thus

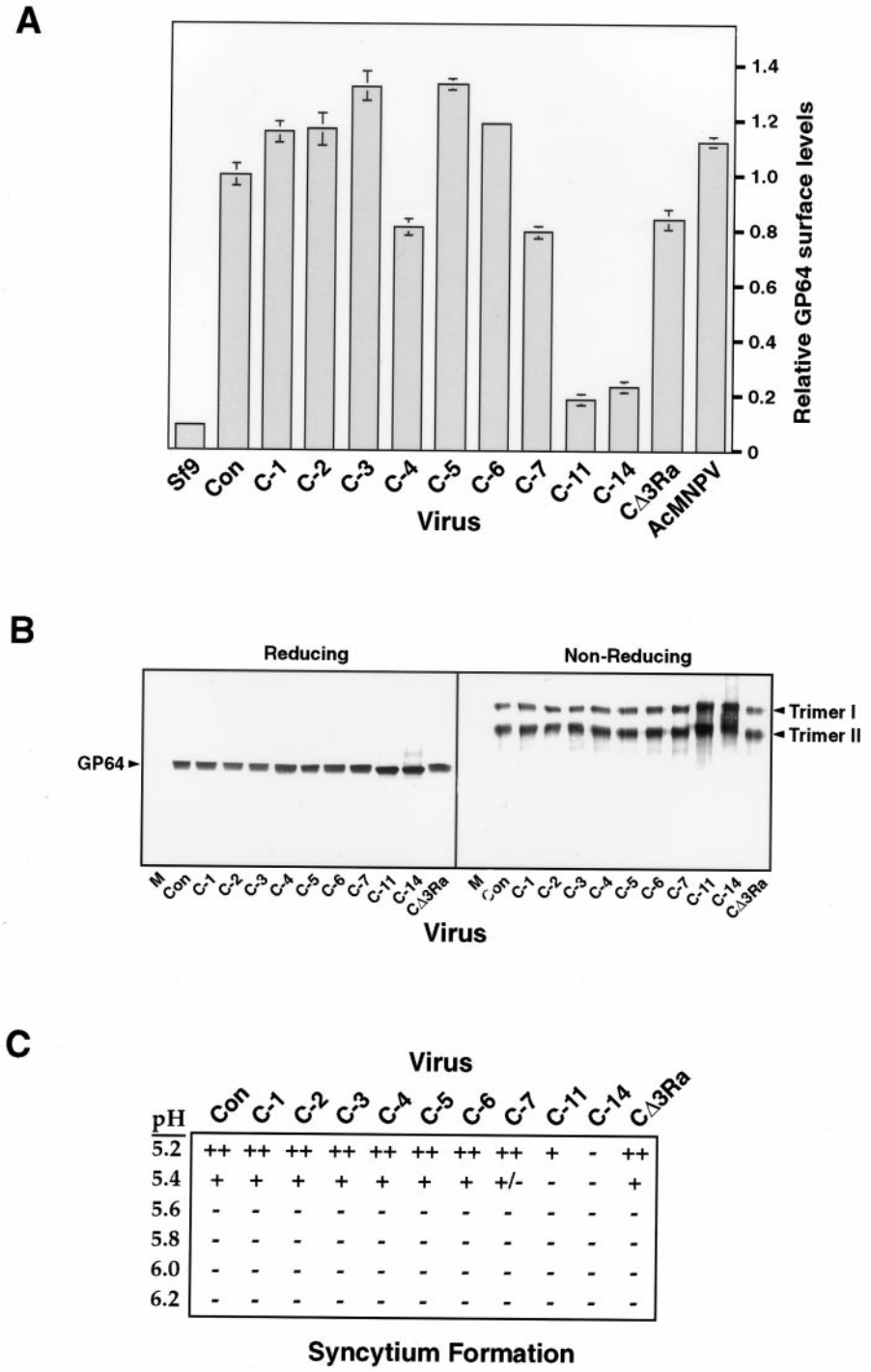

FIG. 5. Expression and functional analysis of wild-type and modified forms of GP64 in infected Sf9 cells. (A) Quantitation of GP64 on the cell surface of Sf9 cells infected with recombinant viruses expressing modified forms of GP64 (C-1 to C-14, and CΔ3Ra) at 30 h p.i. by CELISA. The background CELISA signal from uninfected Sf9 cells is indicated on the left (Sf9). Positive controls are a control virus that expresses the wild-type GP64 protein (Con) and wt AcMNPV. Data points represent triplicate infections and error bars represent standard deviation from the mean. (B) Expression and trimerization of modified forms of GP64 in Sf9 cells infected with the recombinant viruses listed in (A). Western blots of cell extracts (48 h p.i.) electrophoresed under reducing (left) and nonreducing (right) conditions, and detected with Mab AcV5. (C) Syncytium formation assays with recombinant viruses listed in (A). Sf9 cells were infected at a m.o.i. of 10. At 36 h p.i., medium was removed and replaced for 5 min with PBS adjusted to a range of pH values from 5.2 to 6.2, then fresh medium was added and incubated for 2 h prior to scoring for syncytium formation. Syncytium formation requires at least five nuclei in each syncytial mass. (++, syncytia abundant; +, syncytia present but not abundant; ±, syncytia rare but present; −, no syncytia observed.)

A

Virus	Titre	Cells
vAc-Con	5.58×10^8	Sf9
vAc-C-1	4.97×10^8	Sf9
vAc-C-2	2.69×10^8	Sf9
vAc-C-3	2.86×10^8	Sf9
vAc-C-4	8.29×10^8	Sf9
vAc-C-5	1.93×10^8	Sf9
vAc-C-6	3.64×10^8	Sf9
vAc-C-7	1.93×10^8	Sf9
vAc-C-11	1.59×10^8	Sf9
vAc-C-14	3.20×10^7	$Sf9^{Op1D}$
vAc-CΔ3Ra	4.97×10^8	Sf9

B

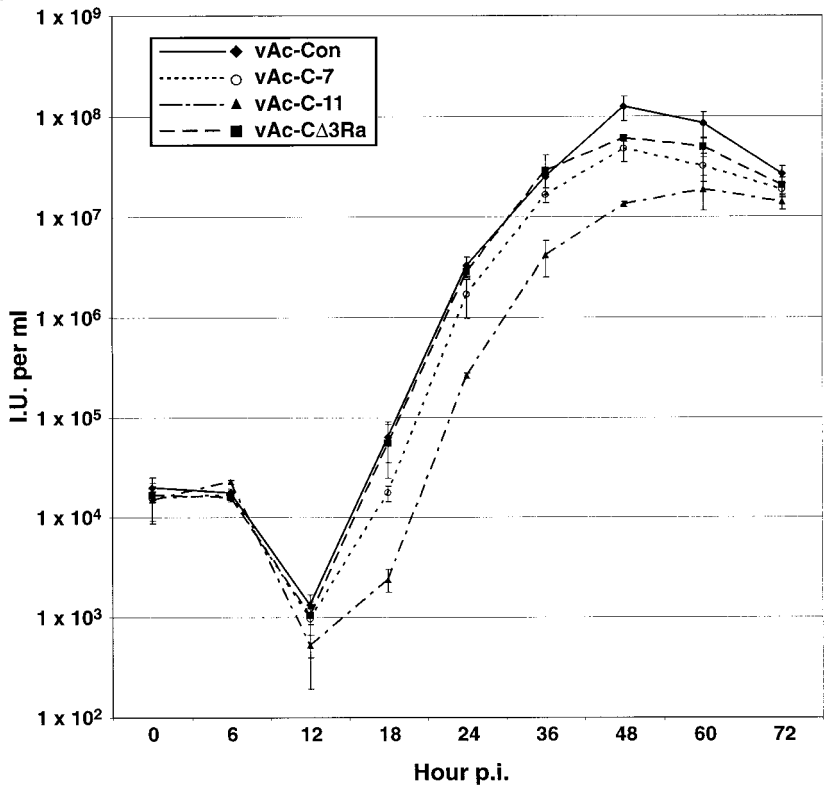

FIG. 6. Production of infectious virions by recombinant viruses expressing wild-type or C-terminally modified forms of GP64. (A) Comparison of virus stock titers. All viruses except vAc-C-14 were titered in Sf9 cells. Virus vAc-C-14 was titered in $Sf9^{Op1D}$ cells which constitutively express OpMNPV GP64. (B) Comparison of virus growth curves for selected recombinant viruses with C-terminal modifications. Viruses expressed either a wt GP64 (vAc-Con), a complete deletion of the GP64 CTD (vAc-C-7), a deletion of the complete CTD plus 4 amino acids of the TM domain (vAc-C-11), or substitutions of all three arginines within the CTD with alanine (vAc-CΔ3Ra). Each data point represents virion production in the preceding 6-h period. Data points represent triplicate infections and titrations and error bars represent standard deviation from the mean.

growth curve data suggested that production of infectious BV from vAc-C-7 was slightly reduced from that of the control virus that expresses a wild-type GP64 protein. Production of infectious BV from virus vAc-C-11 was further reduced. The reduction was consistently ~1 log unit (i.e., a reduction of ~90%) below that measured from the control virus (vAc-Con) at 18–60 h p.i. (Fig. 6B). However, since surface localization of the truncated GP64 from construct vAc-C-11 was reduced (Fig. 5A), we were unable to distinguish between effects caused by the trunction of the TM/CTD sequences and effects caused by lower GP64 levels on the cell surface.

Biochemical analysis of virion production

The reduction in virus titers from cells infected with vAc-C-7, vAc-C-11, and vAc-C-14 could result from either (a) normal production of virion particles but reduced infectivity per virion or (b) a reduction in the quantity of virions budded from the cell surface. To determine whether the observed effects of GP64 truncations C-7,

C-11, and C-14 were due to a reduction in the quantity of progeny virions, we pulse labeled infected cells, then purified and measured the relative quantities of labeled virions that accumulated in cell culture supernatants as described earlier (see also Materials and Methods). The control virus (vAc-Con) used for these studies was generated in parallel with other GP64 replacement viruses and contains a *GUS* marker gene and a wild-type *gp64* gene inserted into the *gp64* locus. Infection and the expression of the GP64 construct from each recombinant virus was confirmed by Western blot analysis of infected cell supernatants (Fig. 7, bottom, lanes W). As before, VP39 in virion fractions was used as an indicator of relative virion quantity. Phosphorimager analysis and comparisons of virion fractions from vAc-C-7- and vAc-Con-infected cells indicated that vAc-C-7 virion production was ~53% of that detected from vAc-Con (Figs. 7A and 7B). These findings parallel the data from growth curves where a small but notable reduction in infectious virus yield was measured for virus vAc-C-7 (Fig. 6B). This indicates that the 7-amino acid GP64 CTD plays a significant role in virion production, although relatively high titers of infectious virions are still produced in its absence (Fig. 6B, vAc-C-7). A more substantial reduction in infectious BV production was observed from growth curve data for virus vAc-C-11, which expresses a more severely truncated GP64 protein. Biochemical analysis of BV production (Fig. 7C) showed that vAc-C-11 produced only ~8–9% of the BV generated from the control virus that expresses wild-type GP64 (Figs. 7C *vs* 7A). Thus the measured reduction by quantitative analysis of virion proteins (91–92%) closely parallels the reduction in infectious BV observed from logarithmic growth curves (78–96%).

We also examined potential virion production from vAc-C-14, which expressed a C-terminal truncation of 14 amino acids. In our studies, insertion of this GP64 mutant into the AcMNPV genome was not sufficient for propagation of a recombinant virus in Sf9 cells (vAc-C-14 was generated and propagated in $Sf9^{Op1D}$ cells). Analysis of virions prepared from supernatants from vAc-C-14-infected Sf9 cells resulted in detection of only trace quantities of BV, ~2% of that detected from the control virus, vAc-Con (Figs. 7D *vs* 7A). These data were similar to earlier results from the gp64null virus, which contained a complete deletion of *gp64* (Fig. 2). Long exposures of gels containing virion fractions from vAc-C-14 revealed that these virions contained the mutant C-14 GP64 protein, albeit in very low quantities.

Effects of GP64 mutations on GP64 incorporation into BV

In addition to the effects of GP64 and the GP64 CTD on virion production, we also analyzed the effects of the CTD and TM truncations on the incorporation of GP64 into virions. As a measure of the concentration of GP64 in each virion, we examined the ratio of GP64 to the major capsid protein, VP39, in virion fractions (Fig. 7; Table 1). The ratio of GP64:VP39 in the peak fraction of the control virus (vAc-Con; Fig. 7, fraction 12) that expresses the wild-type GP64 protein was 0.762 (Table 1). Comparison of this ratio to that from the peak BV factions from viruses vAc-C-7, vAc-C-11, and vAc-C-14 indicated that the GP64:VP39 ratio was substantially lower for each of the viruses containing C-terminal truncations (Table 1, column 3). When data from all fractions containing BV were similarly analyzed, similar results were obtained (Table 1, column 4). Because the mature GP64 protein and the VP39 protein contain different numbers of methionine residues (16 and 9 residues, respectively) and the amount of incorporated label is proportional to the number of methionine residues, protein-labeling data were adjusted to represent relative molar quantities of the two proteins (Table 1, column 5). In all cases, truncation of the GP64 CTD resulted in a reduction (50–63%) in the concentration of GP64 in virions. In the case of C-11 and C-14, reduced quantities of GP64 on the cell surface may account for this difference since cell surface levels of GP64 were reduced by ~81 and 76%, respectively (Fig. 5A). However, in the case of the C-7 protein, cell surface GP64 levels were similar to that from wt GP64 (Fig. 5A), yet the GP64 concentration in the virion was decreased by ~63%. Thus the absence of C-terminal residues and not reduced cell surface levels, appear to result in a reduction in the concentration of GP64 in the virions of vAc-C-7. Together these data indicate that while the CTD influences the incorporation of GP64 into the virion, it is not essential for this process.

DISCUSSION

Using recombinant AcMNPV baculoviruses containing either a deletion of the *gp64* gene or modified forms of *gp64*, we examined the role of the GP64 protein in virion budding. By labeling and quantifying progeny virions from cells infected with either wild-type AcMNPV or a gp64null virus, we showed that GP64 is necessary for efficient production of BV. Therefore, the previously observed defect in cell-to-cell transmission in a gp64null virus (Monsma *et al.,* 1996) is caused primarily by a defect in virus budding. In the current study, we detected only very low quantities of BV in the absence of the gp64 gene, and this result may be explained by one of two possible mechanisms. One possibility is that GP64 is not absolutely required for budding and a low level of budding may occur in the absence of GP64. A second possible explanation is that GP64 is essential for budding and the low level of progeny BV observed in these experiments results from recycling of GP64 present on

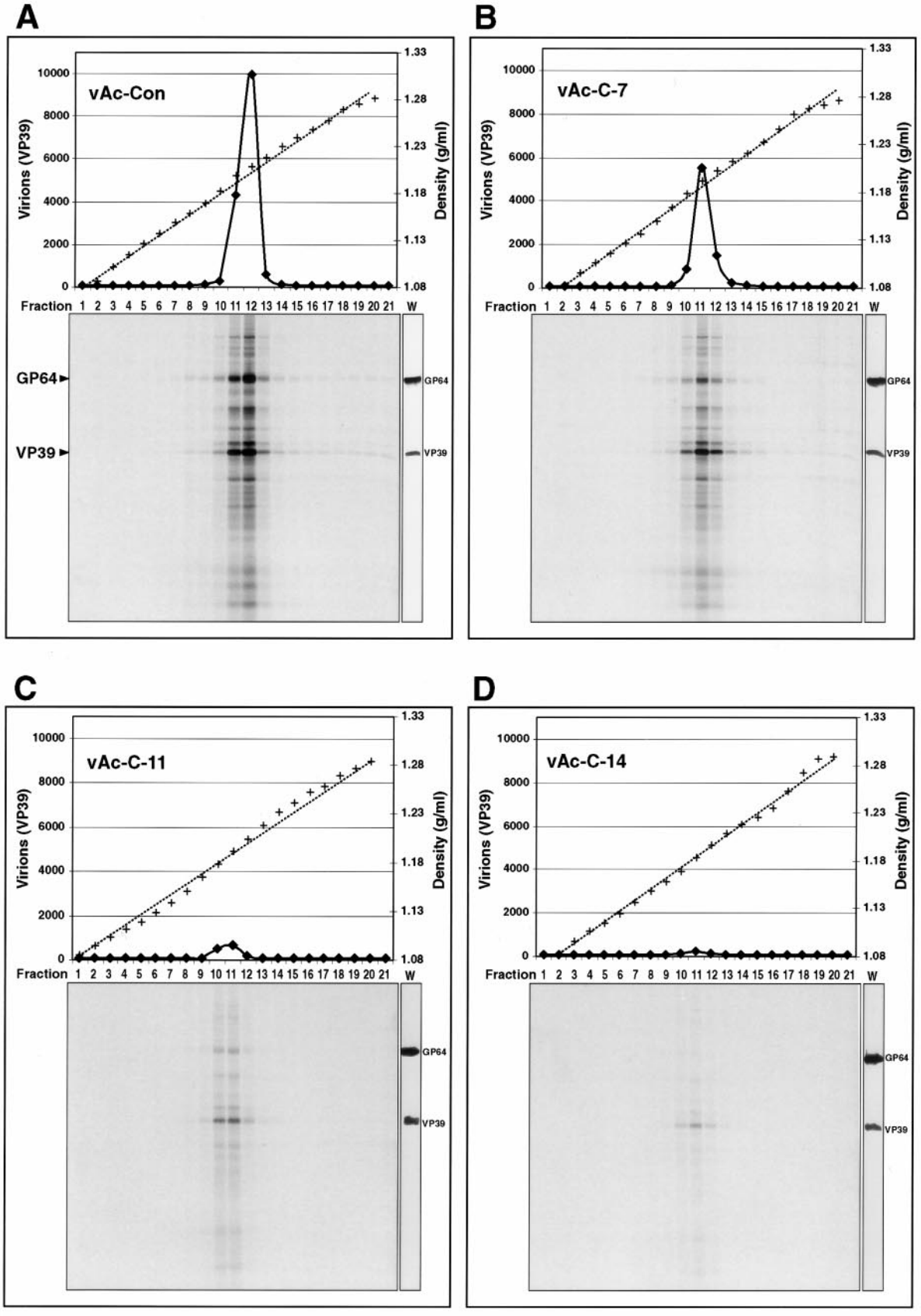
A
vAc-Con
Virions (VP39)
Density (g/ml)
Fraction 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 W
GP64
VP39
B
vAc-C-7
C
vAc-C-11
D
vAc-C-14

TABLE 1

Virion Fractions

Virus	Percent budding	GP64:VP39 ratio		
		Peak virion fraction	All virion fractions	Molar ratios
vAc-Con	100.0%	0.762	0.684	0.129
vAc-C-7	52.5%	0.233	0.255	0.048
vAc-C-11	8.6%	0.233	0.354	0.068
vAc-C-14	1.4%	0.256	0.256	0.051

Note. Percent budding represents a comparison of the [^{35}S]methionine-labeled VP39 capsid protein present in virion fractions from each virus construct (Fig. 7). GP64-VP39 ratios were calculated from phosphorimager data (GP64 and VP39 bands) from either the "Peak virion fraction" or "All virion fractions" for each virus. Molar ratios of GP64:VP39 were calculated for virions of each virus (using data from All virion fractions) based on 16 methionine residues (mature wtGP64), 15 methionine residues (constructs vAc-C-11 and vAc-C-14), or 9 methionine residues (VP39).

infecting gp64null virus particles of the inoculum. Because we were unable to detect recycled GP64 (by Western blots), we currently favor the former explanation. It is not known whether GP64 is recycled or whether it is degraded after virion disassembly during entry. Our observation that the major AcMNPV BV envelope protein is required for efficient budding is similar to results from recent studies of the rhabdovirus G protein. In those studies, virion budding by VSV and Rabies viruses was reduced ~30-fold in the absence of G-protein (Mebatsion *et al.,* 1996; Schnell *et al.,* 1997).

In several cases, it has been proposed that virion budding is driven by interactions between the cytoplasmic domains of viral envelope proteins and capsid or matrix proteins. We therefore examined the role and function of the GP64 cytoplasmic tail domain (CTD) in this process. The predicted CTD of the AcMNPV GP64 protein consists of only 7 amino acids. In comparison, the predicted CTDs of membrane proteins such as Influenza HA and NA, VSV G-protein, and human CD4 are 10 and 6, 29, and 38 amino acids in length, respectively. The small size of the GP64 CTD may be an important characteristic since the small size (7–8 amino acids) is conserved in GP64 proteins identified from *Bombyx mori* NPV (BmNPV), *Choristoneura fumiferama* MNPV (CfMNPV), and OpMNPV (Fig. 8). In addition, the closely related GP75 protein from an arthropod vectored orthomyxovirus (Dhori virus) contains a predicted CTD of only 4 amino acids. In each of these proteins (Baculovirus and Dhori virus), the very short CTD contains two to three arginine residues that confer a basic charge on the CTD. To examine the role of the AcMNPV GP64 CTD, we generated alanine substitutions for the three arginine residues in the CTD and generated a series of sequential deletions through the CTD and into the TM domain. An initial concern was that the modified GP64 constructs might be unstable. In a previous study, a number of GP64 constructs containing insertions and deletions in the AcMNPV and OpMNPV GP64 ectodomain were analyzed, and in many cases, changes in the internal spacing of the ectodomain rendered the protein unstable (Monsma and Blissard, 1995). In the current study, representative GP64 mutants were examined using assays for transport, anchoring, and membrane fusion. We observed no significant differences in expression or stability of GP64 constructs containing deletions or substitutions of the CTD and TM when compared with wild-type GP64 (Figs. 3 and 5). Studies of protein transport and accumulation at the cell surface and membrane fusion identified no apparent effect of the three arginine-to-alanine substitutions (CΔ3Ra) or the deletions of the CTD (C-1 to C-7). Because previous studies have shown that deletions or modifications of the influenza HA CTD can result in modified membrane fusion activity (Melikyan *et al.,* 1997; Ohuchi *et al.,* 1998), we therefore asked whether the AcMNPV GP64 CTD mutations affected triggering of membrane fusion by low pH. No apparent changes were observed for GP64 constructs that were present on the cell surface at or near wild-type levels (C-1 to C-7 and CΔ3Ra). Because we examined only the pH requirement for triggering, we cannot exclude the possibility that the kinetics or magnitude of GP64-mediated pore formation may be affected by changes in the CTD. Whether the GP64 CTD plays a role in fusion pore formation after triggering remains unknown and awaits further more detailed analyses of these constructs. Anchoring of GP64 to the cell membrane did not appear to be substantially affected by the deletion of the 7-amino

FIG. 7. Quantitative analysis of [^{35}S]methionine-labeled progeny BV production from recombinant baculoviruses expressing wild-type (vAc-Con) or C-terminal truncations of GP64 (vAc-C-7, vAc-C-11, vAc-C-14). Sf9 cells infected with each virus were pulse labeled with [^{35}S]methionine from 15 to 40 h p.i., and labeled progeny virions were isolated from supernatants by centrifugation through a sucrose cushion, followed by separation on equilibrium sucrose density gradients, and fractionation. Gradients were fractionated, and the density of each gradient fraction was measured. Equal amounts of gradient fractions were analyzed by electrophoresis on SDS–PAGE gels, and proteins were quantified by phosphorimager analysis (bottom, left). To confirm infection in each experiment, infected cell extracts were examined by Western blot analysis using anti-GP64 and anti-VP39 antibodies (lane W). In each panel (A–D), the top panels show the measured density of each fraction (g/ml, small closed boxes), and a linear curve-fit (dashed line) indicates the linearity of each gradient. The relative quantities of VP39 (large closed boxes, solid line) in gradient fractions were determined by phosphorimager analysis.

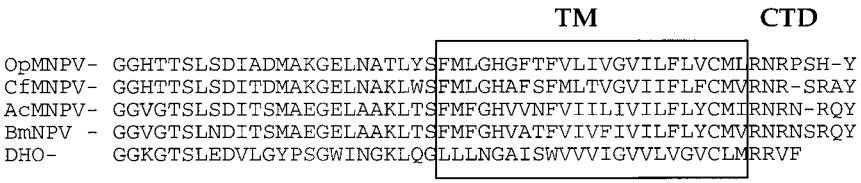

FIG. 8. Alignment of the C-terminal regions of baculovirus GP64 proteins and Dhori virus GP75 of the Orthomyxoviridae. The hydrophobic predicted transmembrane (TM) domain of each protein is indicated by the box, and the cytoplasmic tail domains are aligned to demonstrate the conservation of charged arginine residues and prevalence of the terminal tyrosine residue in baculovirus proteins. (CfMNPV, *Choristoneura fumiferama* MNPV; BmNPV, *Bombyx mori* NPV; DHO, Dhori virus).

acid CTD or by alanine substitutions for three arginine residues in the CTD, as little GP64 was detected in the supernatants of cells transfected with plasmids expressing those constructs. In contrast, cells transfected with constructs C-11 and C-14, which contain deletions of the 7-amino acid CTD plus removal of four or seven additional amino acids from the predicted TM domain, had significantly lower levels of cell surface GP64 (Fig. 3A, C-11 and C-14) and increased levels of GP64 in the supernatants (Fig. 3B; C-11 and C-14). These data support the predicted locations of the boundaries of the TM and CTD. Relatively short deletions into the predicted TM domain (C-11) resulted in rather dramatic effects on anchoring of the protein in the membrane. Thus these data show that the region immediately upstream of amino acid 506 clearly serves as an anchoring domain for the GP64 protein.

We next examined the effects of these C-terminally modified GP64 proteins in the context of viral infection. To accomplish this, we retained wild-type AcMNPV gp64 transcriptional and translational regulatory control for mutant GP64 constructs and used a gp64null virus to insert genes encoding these modified GP64 proteins into the *gp64* locus of the AcMNPV genome (Fig. 4). To examine the ability of modified GP64 constructs to facilitate virion assembly and budding, we analyzed the recombinant baculoviruses for production of infectious virions in growth curve experiments (Fig. 6) and by quantitative analysis of labeled BV production (Fig. 7). The virus carrying the three arginine-to-alanine substitutions in the GP64 CTD (vAc-CΔ3Ra) showed no detectable change in production of infectious virions in growth curve experiments. Virion production was, however, affected when the entire CTD was removed. Separate assays for infectious virions and labeled progeny virions both showed an ~50% decrease in virion production in the absence of the GP64 CTD (Figs. 6 and 7; vAc-C-7). These data indicate that the CTD is important for efficient virus budding but not indispensable. Similar studies have examined the roles of CTDs from the influenza virus HA and NA proteins and the VSV G protein. Deletion of the CTD of either HA or NA does not cause substantial effects on budding and assembly, although altered morphology of virions was observed when the NA CTD was deleted (Jin *et al.,* 1994, 1996; Mitnaul *et al.,* 1996). An influenza virus in which the CTDs were deleted from both the HA and NA proteins resulted in altered particle morphology, and budding was substantially reduced but remained at ~10% of wild-type virus (Jin *et al.,* 1997). Deletion of the majority of the VSV G protein CTD (28 of 29 residues) also results in a severe reduction in virion budding, with virion production reduced to ~11% of that from wt VSV (Schnell *et al.,* 1998). Interestingly, when the VSV G protein CTD and TM domains were replaced with the CTD and TM domains from a heterologous cellular membrane protein (Human CD4), virion production recovered to ~50% of wild-type levels. Although the AcMNPV GP64 protein is necessary for efficient budding, the CTD does not appear to play a critical role in budding since deletion of the entire predicted CTD resulted in only a 50% reduction in budding. The role of the TM domain in virion budding remains unclear since deletion of the CTD plus small portions of the predicted TM resulted in poorly anchored GP64. The correlation of reduced budding with a reduction in cell surface levels of GP64 suggests that cell surface GP64 serves as a limiting factor in budding. However, differences in BV production between vAc-C-11 and vAc-C-14 (Figs. 7C and 7D) cannot be explained by differences in surface levels and therefore suggest a possibly specific role for sequences within the TM. More definitive studies of the function of the TM in virion budding will require replacement of the GP64 TM domain with TM domains from heterologous membrane proteins.

In addition to examining the effect of the CTD on production of infectious BV, we also asked whether the GP64 CTD affected GP64 incorporation into virions. We therefore compared viruses expressing wild-type and truncated GP64 proteins. The GP64:VP39 ratio in virus vAc-C-7 was reduced in comparison to the contol virus expressing wild-type GP64 (Table 1, vAc-Con *vs* vAc-C-7), indicating a lower level of GP64 in each virion. Reduced GP64 in vAc-C-7 virions did not appear to result from a limitation in available GP64 since the level of GP64 measured at the cell surface was similar for vAc-C-7 and the control virus, vAc-Con (Fig. 5A). Therefore the absence of the CTD in vAc-C-7 resulted in both a reduced efficiency of budding and a decreased incorporation of

GP64 into progeny virions. These data suggest that the GP64 CTD promotes efficient budding and is involved in concentrating GP64 within the particle.

In combination, the data presented in this study show that the GP64 protein is necessary for efficient budding of progeny virions. Because we detected low levels of virions containing no labeled GP64 in the absence of a *gp64* gene, we cannot rule out the possibility that incoming GP64 is recycled in these experiments. Therefore it is not known whether the requirement for GP64 in the budding process is absolute. However, quantitative measurements of BV progeny showed a 98–99% decrease in budding in the absence of GP64. We also found that the CTD was not required for budding but appears to moderately affect the efficiency of budding. In comparison, removing the CTD plus small portions of the TM domain resulted in dramatic reductions in budding efficiency, and this appears to result primarily from a limitation in the available GP64 due to the poor retention of these truncated proteins in the membrane. Because GP64 is required for efficient budding, yet removal of the CTD did not reduce budding dramatically, these data suggest that the GP64 protein contains an additional domain that facilitates efficient virion budding and that the domain is located either within the transmembrane portion of the protein or within the ectodomain.

The AcMNPV baculovirus has been developed and used extensively as an expression vector for heterologous proteins, as a biological control agent, and as a model virus system. Recent studies have also suggested that it may be possible to utilize AcMNPV in human gene-therapy applications. If such applications are to be realized and developed, further studies of the function of the GP64 envelope protein will be necessary to understand the requirements for virion entry into host and nonhost cells and the role of this protein in the production of budded virions. For the current studies, we developed and used a recombinant baculovirus system for replacing the essential *gp64* gene with genes expressing modified forms of the GP64 protein. This system permits the insertion of modified GP64 proteins that would otherwise produce a lethal phenotype. The development of this system for examining mutant envelope proteins in the context of the virion and the infection cycle will allow us to address a number of important questions on virus entry, assembly, budding, and infection. Baculoviruses produce two virion phenotypes utilizing what appears to be the same nucleocapsid structure to generate virions in two quite different processes. Although little is known of the process and mechanisms of baculovirus BV assembly and budding, the identification of GP64 as a key component in the process of BV assembly will allow us to further examine and dissect the interactions that occur during this critical process.

MATERIALS AND METHODS

Cells, transfections, and infections

Spodoptera frugiperda (Sf9) cells were used to propagate wild-type (wt) AcMNPV virus as well as most recombinant viruses. Cells were cultured at 27°C in TNM-FH medium (Hink, 1970) containing 10% fetal bovine serum. Viruses without a functional copy of GP64 (vAc^{64-} and vAc-C-14, see below) were propagated in a cell line ($Sf9^{Op1D}$) that constitutively expresses the OpMNPV GP64 protein. Cloning and characterization of cell line $Sf9^{Op1D}$ were described previously (Plonsky *et al.,* in press). Wild-type virus used in these studies was AcMNPV strain E2. AcMNPV nucleotide numbers listed in this study are according to the nomenclature of Ayres *et al.* (1994). For transient transfections, 6 μg of DNA was introduced into 0.6×10^6 cells by $CaPO_4$ precipitation as described earlier (Blissard and Rohrmann, 1991). For viral infections, virus was incubated on cells for a 1-h viral adsorption period. Times postinfection (p.i.) were calculated from the time the viral inoculum was added.

Generation of gp64null virus vAc^{64-}

For studies of virion production from a gp64null virus, the *gp64* gene was deleted from the AcMNPV genome using a modification of a previously described method (Monsma *et al.,* 1996). A transfer vector for recombination into the gp64 locus was constructed as follows: plasmid pOpIE1-βgal-SalI (provided by Dr. D. Theilmann) contains a cassette consisting of a lacZ gene driven by an OpMNPV IE1 promotor and flanked by *Sal*I sites. Linkers (*Spe*I/Sse83871/*Sal*I and *Sal*I/sse83781/*Bgl*II) were inserted into each of the *Sal*I sites to create an *Spe*I site upstream and a *Bgl*II site downstream of the cassette. The *Spe*I/*Bgl*II lacZ cassette was excised and used to replace the wild-type *gp64* ORF in *Spe*I/*Bgl*II-digested plasmid pAcEcoHΔSma (Monsma *et al.,* 1996), creating transfer vector pAcEcoHΔSmaSpe(Opie1Z+)Bgl. This transfer vector contains 2325 bp (nt 109,761–112,049) of sequence from the region immediately upstream of the AcMNPV gp64 ORF and 851 bp (nt 107,325–108,039) of sequence from the region immediately downstream. Wild-type AcMNPV viral DNA (1 μg) and pAcEcoHΔSmaSpe(Opie1Z+)Bgl (2 μg) were cotransfected into 1×10^6 $Sf9^{Op1D}$ cells and overlaid with agarose in TNM-FH. Plaques were picked 3 days after transfection. In sequential plaque assays, plaques were generated in the presence of 120 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal, Labscientific, Inc.), and blue plaques were selected from infected $Sf9^{Op1D}$ cells. A plaque purified viral isolate was named vAc^{64-}. In vAc^{64-}, the complete *gp64* ORF plus an additional 43 bp of upstream and 140 bp of downstream sequence (nt 108,039–109,760) are removed and replaced by the

4195-bp OpIE1-lacZ cassette, with the *lacZ* ORF in the same orientation as *gp*64. Homologous recombination into the *gp*64 locus, replacing the *gp64* gene, was confirmed by PCR analysis as described earlier (Monsma *et al.,* 1996) and restriction enzyme profiles of genomic DNA's.

Generation of GP64 CTD mutations

To generate GP64 proteins containing truncations or single amino acid substitutions near the C terminus, plasmid pAcEcoHΔCla (Monsma *et al.,* 1996), containing the wild-type gp64 gene, was used for site-directed mutagenesis with the MORPH mutagenesis kit (5 prime→3prime, Inc.). Substitution of nt 1514 of the *gp*64 ORF (T) with C created a unique *Bcl*I site at the C-terminal end of the predicted TM domain (conserving isoleucine 505); substitution of nt 1538–1543 (relative to the start of the *gp64* ORF) with CCCGGG created a unique *Sma*I/*Xma*I site at the *gp*64 ORF stop codon, resulting in plasmid pΔCla-BcI/Sma. This modified *gp64* gene was then used to replace the wild-type *gp64* gene in plasmid pΔSmaΔ (a pBS plasmid, containing the *gp*64 locus, AcMNPV nt 107,341–111,046), creating pΔSmaΔ-BcI/Sma. Oligonucleotide linkers corresponding to the various C-terminal deletions or substitutions were then synthesized and cloned into *Bcl*I/*Xma*I digested pΔSmaΔ-BcI/Sma. The resulting plasmids were used to generate transfer vectors for the construction of recombinant viruses (see below).

Budding assay

For a comparison of virion budding from cells infected with either wild-type AcMNPV or vAc^{64-}, Sf9 cells (5×10^6) were infected at a m.o.i. of 5 for 1 h at 27°C, washed once with TNM-FH, and further incubated at 27°C. At 14 h p.i., cells were washed once and starved by incubation in Graces without methionine ($Graces^{-met}$) for 2 h. At 16 h p.i., the medium was replaced with 2.2 ml of $Graces^{-met}$ containing 1 mCi of EXPRE^{35}S protein labeling mix (Dupont, NEN). At 22 h p.i., 0.8 ml of TNM-FH was added. Cells and supernatants were harvested for analysis at 30 h p.i. Cells (5×10^6) were washed once in PBS, then lysed and boiled in 2 ml Laemmli buffer containing a cocktail of protease inhibitors (0.7 μg/ml pepstatin; 1 mg/ml pefabloc; 10 μg/ml leupeptin). Supernatants were cleared of cell debris by brief centrifugation (15 min at 2000 *g,* 4°C), then loaded onto a 25% sucrose cushion and centrifuged at 80,000 *g* for 80 min at 4°C in a SW60 rotor. Virus pellets were resuspended in 300 μl PBS (pH 6.2; incubation at 4°C for 10 min on a shaker platform at low speed). Resuspended virions were loaded onto pre-chilled 12-ml, 25–60% linear sucrose gradients and centrifuged to equilibrium at 96,000 *g* for 16 h at 4°C. Gradients were fractionated at 4°C in a Model 640 Density Gradient Fractionator (Isco), in 0.6-ml fractions at 1 ml/min. A 35-μl sample was removed from each 0.6-ml fraction for density measurements (ABBE-3L refractometer, Spectronic Instruments). The remainder of each fraction was diluted to 1.5 ml with cold PBS pH 6.2, and virions were pelleted by centrifugation at 36,200 *g* for 1.5 h at 4°C. Pelleted virus was resuspended in 30 μl Laemmli buffer containing a cocktail of protease inhibitors (see above) and incubated at 100°C for 5 min. Samples from cell lysates and sucrose gradient fractions were electrophoresed simultaneously on 10% polyacrylamide gels. For cell lysates, ~1.9% (equivalent to ~93,000 cells) of the total sample was loaded per lane. For samples derived from sucrose gradient fractions, the entire sample was loaded in each lane. The portion of the gel containing the cell lysates was transferred to Immobilon-P (Millipore) membrane, and blots were incubated simultaneously with monoclonal antibody (Mab) AcV5 (Hohmann and Faulkner, 1983; Monsma and Blissard, 1995) and Mab 39 (Whitt and Manning, 1988) for simultaneous detection of GP64 and VP39, respectively. The portion of the gel containing gradient fractions was dried, exposed on a phosphorimager screen, and scanned on a Molecular Dynamics phosphorimager. Quantification of individual bands was performed with the Imagequant software package (Molecular Dynamics, Inc.).

For a comparison of virion budding from cells infected with recombinant viruses containing C-terminal truncations and substitutions, the following modifications to the above protocol were used: For metabolic labeling, infected cells were washed in $Graces^{-met}$, and label was added at 15 h p.i. TNM-FH was added at 30 h p.i., and cells and supernatants were harvested for analysis at 40 h p.i.

CELISA, Western blots, and fusion assays

Western blots and fusion assays were carried out as described previously (Blissard and Wenz, 1992). For detection and quantification of GP64 surface levels, a modified cell ELISA (CELISA) protocol was used: cells were fixed in 0.5% glutaraldehyde for 10 min at RT, washed once with phosphate-buffered saline (PBS, pH 7.4), and blocked by incubation in PBS + 1% gelatin for 2 h at 27°C. Cells were then incubated for 45 min at 27°C in Mab AcV5 tissue culture supernatant diluted 1:25 in PBS + 0.5% gelatin. Cells were washed once in PBS for 2 min, followed by incubation for 45 min at 27°C in a secondary goat anti-mouse antibody conjugated to β-galactosidase (GAM-βgal, Fisher) diluted 1:750 in PBS + 0.5% gelatin. Cells were then washed four times (5 min/wash) in PBS, then incubated in 1 mM *o*-nitrophenyl-β-d-galactopyranoside (oNPG) in oNPG substrate buffer (containing 0.01 M of each of the following: Tris base, sodium chlo-

ride, magnesium chloride, β-mercaptoethanol) at 37°C. After addition of the substrate, the OD_{405} was determined at several time points using an ELISA plate reader. Immunoprecipitations were performed as described previously using a polyclonal antiserum prepared against a purified soluble form of OpMNPV GP64 (Oomens *et al.,* 1995).

Generation of CTD recombinant viruses

Plasmids containing genes encoding GP64 C-terminal truncations or substitutions (described above) were used to generate transfer vectors for construction of AcMNPV recombinant viruses by recombination into the GP64 locus of the gp64null virus vAc^{64-}. For that purpose a cassette, containing the β-glucuronidase (GUS) gene driven by the AcMNPV p6.9 promoter and followed by the AcMNPV polyhedrin polyA signal, was cloned as a *Bgl*II fragment downstream of the modified *gp*64 gene into each of the pΔSmaΔ plasmids (described above). The resulting transfer vectors (see Fig. 4) contain 1325 bp (AcMNPV nt 109,717–111,042) of sequence from the region immediately upstream of the AcMNPV *gp64* ORF and 705 bp (AcMNPV nt 107,330–108,035) of sequence from the region immediately downstream. For each recombinant virus, vAc^{64-} viral DNA (1 μg, linearized by Bsu36I digestion) and transfer vector (2 μg) were cotransfected into 1.5×10^6 Sf9 cells, and supernatants were harvested 3 days after transfection. In sequential plaque assays, plaques were selected by blue color in the presence of 120 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (XGluc, Labscientific, Inc.), and viral isolates were named vAc-C-1 to -14, and vAc-CΔ3Ra. Homologous recombination into the *gp*64 locus and replacement of the lacZ cassette was confirmed by PCR analysis and restriction enzyme profiles of genomic DNAs. The mutations in the C-terminal region were confirmed by sequencing the PCR amplified C-terminal region of each recombinant virus.

Pulse labeling

Sf9 cells infected with the CTD recombinant viruses at a m.o.i. of 10 were analyzed by pulse labeling proteins at 18 and 36 h p.i. Two hours before each of these time points, cells were washed once and starved by incubation in Graces without methionine ($Graces^{-met}$) for 30 min, followed by metabolic labeling in $Graces^{-met}$ containing 25 μCi of EXPRE^{35}S protein-labeling mix (Dupont, NEN) per 0.6×10^6 cells, for 1.5 h at 27°C. At the end of the labeling period, cells were washed once in PBS, then lysed and boiled in 350 μl Laemmli buffer containing a cocktail of protease inhibitors (see above). Each sample (45 μl) was electrophoresed on a 12% polyacrylamide gel, dried, exposed to a phosphorimager screen, and scanned on a Molecular Dynamics phosphorimager.

Growth curves

To measure infectious BV production from four of the CTD recombinant viruses, viral growth curves were generated by collecting infected cell supernatants at 6-h intervals at various times postinfection. Sf9 cells (0.65×10^6 cells per well; 12 well plates) were infected at a m.o.i. of 10 for 1 h at 27°C. For each time point postinfection and each virus, triplicate samples were generated, and all wells were infected simultaneously to minimize variability. After infection, cells were washed once with 0.8 ml TNM-FH and incubated in 0.9 ml TNM-FH. At 6 h before each timepoint (except time point 0), cells were washed once and incubated in fresh TNM-FH for 6 h, followed by harvesting of the supernatant. All data therefore represent BV produced during the 6-h time period preceeding the indicated time point. Supernatants were carefully removed from the infected cells, centrifuged at 20,800 *g* for 5 min to remove debris, and titered by $TCID_{50}$ (Summers and Smith, 1987; O'Reilly *et al.,* 1992). X-Gluc, the substrate for the GUS marker gene present in these viruses, was included in the $TCID_{50}$ assay at 120 μg/ml, and cells were examined 8 days p.i. for blue color and polyhedra formation.

ACKNOWLEDGMENTS

The authors thank Dr. D. Theilmann for providing plasmid construct pOpIE1-βgal-Sal, Dr. J. Manning for antibody Mab 39, Dr. P. Faulkner for antibody AcV5, and Dr. S. Monsma for construction and preliminary analysis of vAc^{64-}. This work was supported by National Institutes of Health Grant AI-33657.

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