The Autographa californica Multicapsid Nucleopolyhedrovirus (AcMNPV) GP64 Protein: Analysis of Transmembrane (TM) Domain Length and Sequence Requirements

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GP64, the major envelope glycoprotein of the Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) budded virion, is important for host cell receptor binding and mediates low-pH-triggered membrane fusion during entry by endocytosis. Previous TM domain replacement studies showed that the transmembrane (TM) domain serves a critical role in GP64 function. To extend the prior studies and examine specific sequence requirements of the TM domain, we generated a variety of GP64 TM domain mutations. Mutations included 4-8 amino acid deletions as well as single and multiple point mutations. While most TM domain deletion constructs remained fusion competent, those containing deletions of 8 amino acids from the C-terminus did not mediate detectable fusion. Addition of a hydrophobic amino acid (A, L, or V) to the C-terminus of construct C8 (a construct that contains a TM domain deletion of 8 amino acids from the C-terminus) restored fusion activity. These data suggest that the membrane fusion function of GP64 is dependent on a critical length of the hydrophobic TM domain. All GP64 proteins with a truncated TM domain mediated detectable virion budding with dramatically lower levels of efficiency than WT GP64. The effects of deletions of various lengths and positions in the TM domain were also examined for their effects on viral infectivity. Further analysis of the TM domain by single amino acid substitutions and 3-alanine-scanning mutations identified important but not essential amino acid positions. These studies showed that amino acids at positions 485-487 and 503-505 are important for cell surface expression of GP64, while amino acids at positions 483-484 and 494-496 are important for virus budding. Overall, our results show that specific features and
amino acid sequences, particularly the length of the hydrophobic TM domain, play critical roles in membrane anchoring, membrane fusion, virus budding and infectivity.
Introduction

In typical infections of eukaryotic cells by enveloped viruses, viral entry is mediated by the fusion of viral and cellular membranes in a process that is directed by membrane-associated viral fusion proteins. In the best models of membrane fusion, two hydrophobic domains of the viral fusion protein are critically important in fusion: the fusion peptide and the transmembrane (TM) domain. The fusion peptide is a hydrophobic domain that inserts into the target cellular membrane, thereby attaching the fusion protein to the target membrane (14). By insertion of the fusion peptide into the target membrane and anchoring of the envelope protein in the viral envelope via the TM domain, a bridge is formed between the two membranes. Subsequent structural rearrangements in the envelope fusion protein bring the viral and cellular membranes into close proximity and culminate in merger of two bilayers, and the subsequent opening of a fusion pore. The TM domain may serve several roles in this overall process. In addition to anchoring the envelope protein in the membrane, the TM domain may play a crucial role in the transition from the initial merger of the outer leaflets of the two membranes (hemifusion) to complete membrane merger and pore formation. Evidence of a more direct role of the TM domain in this process comes from studies in which the proteinaceous TM domains of viral fusion proteins were replaced by lipid (GPI) anchors or protein sequences that altered their structures. Such modifications of the TM domain may lead to partial or full arrest of fusion at the hemifusion step (22). In addition, it was recently proposed that a direct interaction between the fusion and TM peptides of hemagglutinin HA may be required to open the fusion pore (50).
The virus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is a member of the group I NPVs within the Alphabaculovirus genus and is the type species for the family Baculoviridae (17). Budded virions of AcMNPV appear to enter cells via a clathrin-mediated, low-pH-dependent endocytic pathway (25). During entry by endocytosis, the major envelope glycoprotein GP64 mediates low-pH-triggered membrane fusion (5). The GP64 proteins are very highly conserved in the group I NPVs and appear to be closely similar in structure and function only to GP75 proteins from Thogotoviruses, a subgroup of the Orthomyxoviridae. Recent structural studies also indicate that GP64 should be classified as a class III fusion protein, along with proteins such as VSV G and Herpesvirus gB (21). GP64 is a type I integral membrane protein that is present on the infected cell surface and in the virion as a disulfide-linked homotrimer (38). GP64 has host cell receptor-binding activity (16) and a domain associated with receptor binding was recently mapped to an N-terminal region of the ectodomain (60). GP64 is both necessary and sufficient for mediating pH-dependent membrane fusion during viral entry (5, 55, 61). In addition to its essential role in virus entry, GP64 is also necessary for efficient budding and production of infectious virions (36, 37). The AcMNPV GP64 protein is posttranslationally modified by palmitoylation and by glycosylation at multiple sites. However, neither palmitoylation nor any single glycosylation site is necessary for GP64 synthesis, transport, BV production, infectivity, or membrane fusion activity (20, 59). GP64 is also posttranslationally modified by phosphorylation (30, 54) and little is known of the structural or functional implications of this modification. Two important hydrophobic regions were identified in the GP64 ectodomain and examined by
mutagenesis. One region is located between amino acids 220 and 230 of the closely
related *Orgyia pseudotsugata* (Op)MNPV GP64, and substitutions in that region
disrupted normal membrane fusion activity. The second region is located between
amino acids 327 and 335 and is important for oligomerization and cell surface
expression (35). The later region lies within a highly conserved 4-3 heptad repeat (a
leucine zipper from residues 299 to 346) that is predicted to form an amphipathic
alpha helix (35), and near the top of a long central helix in the post-fusion structure
(21). Mutations that disrupted the predicted helix or reduced the hydrophobicity along
the hydrophobic face of the predicted OpMNPV GP64 leucine zipper motif were
sufficient to disrupt fusion activity (35).

GP64 proteins have very short predicted cytoplasmic tail domains (CTD) ranging
from approximately 3 to 8 amino acids (aa) in length. Earlier experimental analysis
showed that the 7 aa CTD of AcMNPV GP64 is not essential for production of
infectious BV, but removal of the CTD results in a measurable reduction in budding
efficiency (37). Baculovirus GP64 proteins have a predicted TM domain ranging from
approximately 16 to 23 amino acids. Unlike the highly conserved ectodomain amino
acid sequences (which are app. 80% identical among baculovirus GP64 proteins),
sequences of the TM domains are more variable in sequence conservation (13-
100%) and the extent of the biological function(s) of the GP64 TM domain remains
unknown. In previous studies, we demonstrated that replacing the 23-amino-acid
AcMNPV GP64 TM domain with corresponding sequences from a range of viral or
cellular type I membrane proteins or with a glycosylphosphatidylinositol (GPI)
addition sequence had, in many cases, severe effects on fusion activity and virus
infectivity (23). This suggested that the specific amino acid sequence of the GP64 TM domain is critical for the function of GP64. In the current study, we further investigated the function of the GP64 TM domain by examining a series of GP64 proteins with modified TM domains. TM domain modifications included truncations, deletions, 3-alanine-scanning mutations, and single and multiple amino acid substitutions. These studies revealed that a) the hydrophobic length of the GP64 TM domain is critical for GP64-mediated membrane fusion activity, b) no specific residue of the TM domain was required for the membrane fusion activity, and c) several regions within the TM domain are important for cell surface localization or virus budding.
Materials and Methods

Cells, transfections, and infections

*Spodoptera frugiperda* (Sf9) cells and the cell line Sf9<sup>Op1D</sup> (that constitutively expresses the OpMNPV GP64 protein (42)) were cultured at 27° in TNMFH medium (18) containing 10% fetal bovine serum (FBS). Transfections were carried out using CaPO<sub>4</sub> precipitation as described earlier (4). For viral infection, budded virus was incubated on cells (MOI 5) for 1 h, and then cells were washed once in TNMFH. Times post infection (pi) were calculated from the time the viral inoculum was added.

Mutagenesis and construction of plasmids and bacmids

The plasmid pGEM3ZGP64 (23) which contains the promoter region and open reading frame (ORF) of AcMNPV GP64, was used as a target for mutagenesis. The C-terminal truncation mutants were generated by PCR. The N-terminal and central region TM deletion mutations as well as the substitution mutations and alanine-scanning mutations were generated using the SLIM site-mutation method (9). The sequences of the primers used are available upon request. The modified GP64 ORFs were subcloned into the XbaI/EcoRI sites of pBiepA vector which contains the promoter region of AcMNPV *ie1* and the poly (A) signal of AcMNPV *gp64* (23). Plasmids were prepared for transfections using a DNA Maxiprep kit (Marigen Biosciences, Inc.). In order to construct recombinant baculoviruses expressing the modified GP64 proteins, GP64 constructs in pGEM3ZGP64 were digested with *KpnI* and *EcoRI* to excise the fragment containing the promoter and GP64 ORF and the fragments were subcloned into the *KpnI* and *EcoRI* sites of the pFastBac1 plasmid (Invitrogen), resulting in removal of the AcMNPV polyhedrin promoter. The modified
gp64 genes were then inserted into the polyhedrin locus of an AcMNPV gp64-null bacmid (vAc64-) by Tn7-mediated transposition as described previously (27). Constructs were confirmed by restriction enzyme digestion and DNA sequencing.

cELISA, syncytium formation, and fusion assays

For analysis and comparisons of relative levels of cell-surface-localized GP64 proteins, we used a cell surface enzyme-linked immunosorbent assay (cELISA). Sf9 cells were transfected with plasmids and incubated for 24 h to permit expression and cell surface localization, then cells were fixed in glutaraldehyde so that cells were not permeabilized. Cell surface localized GP64 was then detected in a cELISA assay by using MAb AcV5 as described previously (59). Briefly, transfected cells in 24-well plates (Corning Inc.) were rinsed once in phosphate-buffered saline (PBS, pH 7.4) and fixed in 0.5% glutaraldehyde for 10 min at room temperature. Fixed cells were washed once with PBS and blocked by incubation for 2 h in PBS containing 1% gelatin at 27°. Cells were then incubated in MAb AcV5 (hybridoma cell culture supernatant diluted 1:25 in PBS containing 0.5% gelatin) for 45 min at 27°. Cells were washed three times in PBS and then incubated in a secondary goat anti-mouse antibody conjugated to Beta-galactosidase (GAM-Beta-gal; diluted 1:750 in PBS containing 0.5% gelatin) for 45 min at 27°. Cells were washed five times in PBS and then incubated at 37° in 2 mg/ml Chlorophenolred-beta-D-galactopyranoside (CPRG) in a solution of PBS containing 2 mM MgCl₂ and 1% BSA. After addition of the substrate, the absorbance of the supernatant (optical density 570 nm) was determined at various intervals using an ELISA plate reader.
For membrane fusion activity assays, Sf9 cells in 24-well plates were transfected with plasmids encoding WT or modified forms of GP64 as described above. At 24 h post-transfection (pt), TNMFH medium was removed, and cells were washed once with PBS at pH 7.4. The PBS at pH 7.4 was then replaced with PBS at pH 5.0. After a 3 min incubation period, cells were washed again with PBS at pH 7.4 then returned to TNMFH. After 4 h incubation at 27°, cells were fixed with methanol for 10 min, stained using a HEMA3 stain kit (Fisher Scientific Company L.L.C.) and the number of nuclei found in syncytia was scored. The criterion for identification of a syncytial mass was the presence of at least five nuclei in a fused cell mass. Five randomly selected representative fields were evaluated for each construct. For calculations of relative levels of fusion activity, the number of nuclei in syncytia was divided by the number of total cells in a field. Those percentages were then normalized to parallel syncytium formation data from wild type GP64 that was localized to the cell surface at equivalent levels.

Labeling of RBCs with R18 and calcein-AM

Sheep RBCs (RBCs, HemoState Laboratories) were co-labeled with the lipid probe octadecyl rhodamine B chloride (R18) and the aqueous dye calcein-AM (Molecular Probes, Invitrogen) as described previously (52) with a minor modification (23). In brief, 10 µl of R18 (2 mM in ethanol) was added to RBCs (1% packed cell volume (PCV) in 20 ml PBS) while gently shaking. The mixture was incubated for 30 min at room temperature (RT) in the dark, then 20 ml of 7.5% FBS/DMEM was added to the suspension to absorb unbound probe. After a 20 min incubation at RT in the dark, the RBC suspension was washed five times in 40 ml of PBS and resuspended...
in 1.25 ml PBS. A 5 µl aliquot of calcein-AM (4 mM in dimethyl sulfoxide) was added to 1 ml R18-labeled RBCs and the suspension was incubated for 1 h at 37° in the dark. The unbound calcein-AM was also absorbed using 20 ml of 7.5% FBS/DMEM for 20 min and the colabelled cells washed four times with PBS as described above. The double-labeled RBCs were suspended in 200 ml of PBS and used within 1 h.

Hemifusion and Pore Formation Assay

To analyze hemifusion and pore formation by WT GP64 and GP64 constructs containing TM domain mutations, an R18 and calcein transfer assay was performed. At 24 h pt, transfected Sf9 cells (8x10^4 cells/well) were washed once with PBS (pH 7.4) and then incubated with R18- and calcein-labeled RBCs (0.4 ml 0.1% PCV/well) for 20 min at RT for binding. Unbound RBCs were removed by three washes with PBS (pH 7.4), and the Sf9 cells were then incubated with PBS at pH 5.0 for 3 min at RT. Sf9 cells were then washed in PBS at pH 7.4 and transferred into TNMFH medium. After incubation for 20 min at 27°, hemadsorption and the transfer of fluorescence were observed by phase-contrast and epifluorescence microscopy, respectively. Five randomly selected fields were scored for dye transfer. For each dye-labeling experiment, the efficiency of dye (R18 or calcien-AM) transfer was estimated by dividing the number of dye-containing Sf9 cells displaying mutant GP64 proteins by the number of dye-containing Sf9 cells displaying wt GP64.

Immunofluorescence analysis of cell-surface GP64

To confirm cell-surface localization of GP64, Sf9 cells were plated in 24-well plates (8x10^4 cells/well), and transfected with plasmids expressing either WT or modified GP64 proteins. After 24 h pt, cells were fixed with 4% paraformaldehyde in
PBS (pH 7.4). Cells were washed with PBS and then incubated with a blocking buffer (1% gelatin in PBS, pH 7.4) at 27°C for 2 h. After washing with PBS, the cells were incubated with a primary anti-GP64 monoclonal antibody (AcV1, 1:25 dilution in PBS) at 27°C for 1 h. Cells were washed three times with PBS (pH 7.4) and incubated for 1 h at 27°C with Alexa fluor 488-goat-anti-mouse (Molecular Probes, Invitrogen) diluted 1:750 in PBS. After washing five times with PBS (pH 7.4), fluorescence was observed with an Olympus IX70 epifluorescence microscope.

**Budding assay and measurement of GP64 incorporation into virions**

For analysis of virus budding efficiency and the relative amounts of GP64 incorporation into BV, we performed the following assays. The TM-truncated and TM-deleted viruses were amplified and titred in Sf9 cells. Viruses containing the alanine-scanning mutants were amplified and titred in Sf9 cells. The titred viruses were used to infect Sf9 cells (5x10^6) at an m.o.i. of 5. After inoculation and incubation for 1 h, the virus inoculum was removed and cells were washed once with TNMFH then incubated at 27°C. At 15 h pi, cells were washed once and starved by incubation in 2 ml methionine-free Grace's medium (Grace's MET, Invitrogen) for 1 h. At 17 h pi, the medium was replaced with 2.2 ml of Grace's MET, containing 200 µCi of ^35^S-EasyTag Express protein labeling mix (1175.0 Ci/mmol, Perkin-Elmer Life Sciences). At 30 h pi, 0.8 ml of TNMFH was added. The supernatants were harvested at 40 h pi and cleared of cell debris by brief centrifugation (10 min at 3000 x g, 4°C), then loaded onto a 25% sucrose cushion and centrifuged at 80,000 x g for 90 min at 4°C in a SW60 rotor. Virus pellets were resuspended in 200 µl Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue, 0.125 M Tris, pH 6.8)
containing a cocktail of protease inhibitors (Complete; Roche Applied Science) and electrophoresed on 10% polyacrylamide-SDS (SDS-PAGE) gels. Dried gels were exposed on phosphorimager screens and scanned on a Molecular Dynamics phosphorimager. Quantification of individual bands was performed using the ImageQuant TL software package (Amersham, GE).

**Western blot analysis**

Reducing and non-reducing SDS-PAGE was performed in 6% or 10% polyacrylamide gels as described previously (37). Following transfer to PVDF membrane (Millipore), blots were blocked in a 4% milk TBST solution as previously described (59). For detection of GP64, MAb AcV5 was used at a dilution of 1:1000. Immunoreactive proteins were visualized using alkaline phosphatase-conjugated goat anti-mouse IgG antibody and NBT/BCIP (Promega) as described previously (59).
Results

Expression and intracellular processing of GP64 deletion mutants

In a prior study (23), we found that substitution of the GP64 TM domain with heterologous TM domains from other viral and cellular membrane proteins had substantial negative effects on GP64 function, indicating an important role for the TM domain beyond simple anchoring of GP64 in the viral envelope. To examine the requirements of the GP64 TM domain for membrane fusion activity and viral infectivity, we generated and examined a series of truncation, deletion, and amino acid substitution mutations in the GP64 TM domain. For the initial analysis, N-terminal, central, and C-terminal portions of the hydrophobic TM domain were truncated (Fig.1A). Although the very short (7 amino acid) cytoplasm tail domain (CTD) was previously shown to be non-essential, several of the larger deletion constructs were generated in both the presence and absence of the CTD (Fig.1A).

The GP64 proteins with modified TM and CTD domains were transiently expressed from plasmids in Sf9 cells. At 24 h post transfection, GP64 proteins from cell lysates were examined by Western blot analysis using either reducing or non-reducing conditions for SDS-PAGE (Fig. 1B). Because GP64 trimers are associated by disulfide bonds, non-reducing conditions were used to assess GP64 oligomerization (16, 38). Oligomeric forms typical of trimer I, trimer II, and dimer were detected from most or all GP64 mutants. However, electrophoretic migration patterns of most constructs were altered from those of WT GP64. Band migration patterns were retarded for trimers of constructs with C-terminal truncations (C4, C5, C7, C8, and C8CT) while the constructs with truncations of the N-terminal and central portion of
the TM domain had trimer migration patterns similar to that of WT GP64 (Fig. 1B).

Examination of protein from each culture supernatant for evidence of protein shedding from the cell surface revealed that reduction in the TM domain length resulted in GP64 shedding. While the WT and control (C0) GP64 proteins were detected in the supernatant in only trace amounts, GP64 constructs containing TM domains of reduced length were detected at substantially higher levels in the supernatant (Fig. 1B, lower panel). Also, inclusion of the short hydrophilic cytoplasmic tail domain at the C-terminus of constructs N8, M8, and C8 did not eliminate or appear to substantially reduce the shedding of GP64 proteins with truncated TM-domains (Fig. 1B, lower panel, lanes 7 vs. 8, 10 vs. 11, 13 vs. 14, super).

**Cell surface localization and conformation**

Next, we asked whether the TM-truncated GP64 proteins were transported to and localized at the cell surface. Using a cELISA protocol, we measured the cell surface level of each protein construct relative to that detected from WT GP64. Deletion of the short 7 amino acid GP64 cytoplasmic tail (CT) dramatically increased the level of the GP64 C0 construct detected at the cell surface. In contrast, most constructs containing truncations of the TM domain resulted in decreased surface levels of GP64 (Fig. 2A). For example, when the TM domain was truncated by removing 4 or 5 amino acids from the C-terminus, surface expression levels decreased to approximately 25-30% of that from WT GP64. Further truncations of 7 or 8 amino acids from the C-terminus resulted in further reductions in cell surface levels with constructs C7 and C8 detected at approximately 9.7% and 7.4% of that
from WT GP64. Deletion of 7 or 8 amino acids from the N-terminus or central region of the TM domain also resulted in substantially decreased cell surface levels of the modified GP64 proteins relative to WT GP64 levels. Addition of the cytoplasmic tail (CT) domain to the C-terminus of truncated protein constructs increased cell surface levels in most cases. Constructs C8CT and N8CT were expressed at the cell surface at substantially higher levels than that of C8 and N8, respectively (Fig. 2A). To confirm the presence of GP64 constructs at the cell surface and to also examine the conformation of the surface-localized GP64 constructs, we used indirect immunofluorescence with a conformation-specific monoclonal antibody (MAb AcV1) that recognizes the native, neutral pH pre-fusion conformation of GP64 (19, 61). The results indicated that like WT GP64, the TM-truncated GP64 constructs were surface-localized and were in the native pre-fusion conformation (Fig. 2B).

Fusion activity of GP64 truncation mutants

We evaluated the membrane fusion activity of each TM-truncated GP64 construct by measuring fusion efficiency in a semi-quantitative syncytium formation assay. Because surface levels of different GP64 constructs varied, the fusion activity of each modified GP64 construct was compared with the activity from WT GP64 that was localized to the cell surface at the same level. While surface levels of some short C-terminal truncations of the TM were reduced (Fig. 2A, C4 and C5), the normalized membrane fusion activities for those constructs were only marginally decreased. Fusion activities detected for constructs C4 and C5 were app. 70-80% of that from wild type GP64 (Fig. 2D, C4 and C5). Further deletion of 7 or 8 amino acids from the C-terminus of the TM domain resulted in substantially reduced or no detectable
fusion activity (Fig. 2D, C7 and C8). Note: In a prior study (37), fusion activity was not detected from a construct equivalent to construct C7 from the current study. This difference results from differences in the pH used for triggering. We found that while no fusion activity was detected from construct C7 exposed to pH 5.2 (data not shown), as in the prior study, exposure to pH 5.0 resulted in a low level of detectable syncytium formation or fusion activity. The absence of detectable fusion activity from construct C8 may have resulted from poor anchoring in the membrane. To examine that possibility, we included the hydrophilic cytoplasmic tail in construct C8CT (Fig. 1A). While inclusion of the short hydrophilic cytoplasmic tail domain resulted in higher levels of this construct at the cell surface (Fig. 2A, C8CT vs. C8), fusion activity was not restored (Fig. 2D, C8CT vs. C8). Overall, GP64 constructs that contained 4-5 amino acid truncations of the C-terminus of the TM domain remained highly functional for membrane fusion but a larger TM truncation (C7) substantially reduced fusion activity and further truncation of the TM domain (C8) completely abolished detectable fusion activity. Addition of the hydrophilic CTD to the C8 deletion resulted in greater accumulation of GP64 at the cell surface but fusion activity was not restored.

A similar analysis was performed by generating deletions of 7-8 amino acids from the N-terminus of the TM domain. Similar to the 7 amino acid deletion from the C-terminus of the TM domain, a 7 amino acid deletion from the N-terminus resulted in only approximately 14% of the fusion activity of wild type GP64 (Fig. 2D, N7). Unlike the C-terminal deletions, a deletion of 8 amino acids from the N-terminus of the TM domain (N8) resulted in extremely low but detectable fusion activity (Fig. 2D, N8).
And in the case of the N8 construct, addition of the hydrophilic CT domain resulted in a decrease in fusion activity (Fig. 1A and 2D, N8CT). Similar results were obtained when 7 or 8 amino acids were deleted from the central or mid-portion of the TM domain (Fig 1 and 2; M7, M8). As with the N8 construct described above, addition of the CT domain to the M8 construct (M8CT) resulted in increased fusion activity (Fig. 2D, compare M8 and M8CT). In total, these results suggest that when the length of the 23 amino acid hydrophobic TM domain was reduced to less than 16 amino acids, the fusion activity of the mutant protein construct was either lost (C8) or very low (N8 and M8). Overall, adding the hydrophilic cytoplasmic tail increased the levels of protein at the cell surface (Fig. 2A, C8, N8, and M8 vs. C8CT, N8CT, and M8CT).

When fusion activity was detected, it appears that addition of the CT domain resulted in an increase in the normalized membrane fusion activity (Fig. 2D, N8 and M8 vs. N8CT and M8CT). However, addition of the CT domain did not restore activity of the fusion-deficient C8 construct.

Since control comparisons with WT GP64 indicated that the fusion defects of C8 and C8CT did not result from low levels of these proteins at the cell surface, we asked whether fusion was blocked prior to the hemifusion intermediate or pore formation steps. RBCs were co-labeled with a membrane dye (R18) and a soluble cytosolic dye (Calcein-AM) and bound to Sf9 cells expressing various GP64 constructs. Fusion was then induced by low pH treatment (pH 5.0 for 3 min) (see Materials and Methods). Although we observed R18 dye transfer in cells displaying construct C8 (Fig. 3), the R18 transfer efficiencies were very low (less than 1% of that from WT GP64; data not shown). We did not observe transfer of R18 or Calcein-AM.
from cells expressing C8CT (Fig.3). Thus by comparison with wild type GP64, constructs containing deletions of 8 amino acids from the C-terminus of the TM domain appear to be severely compromised or defective in the first step in membrane fusion, the merger of the outer leaflet of the lipid bilayer.

**Extending the hydrophobic length of a truncated TM domain restores fusion activity of GP64**

The C8 truncation of the GP64 TM domain was defective for membrane fusion and addition of the hydrophilic cytoplasmic tail did not restore fusion activity to the resulting GP64 construct (C8CT). Therefore, we next asked whether addition of a single hydrophobic amino acid to the C-terminal end of the C8 TM domain was sufficient to restore fusion activity (Fig. 4A). For this analysis we selected hydrophobic amino acids (A, V, and L), and as controls we added hydrophilic amino acids (N and R) (Fig. 4A). GP64 constructs containing these TM modifications were transiently expressed in Sf9 cells and detected by Western blot analysis of cell lysates (Fig.4B). Under both reducing and non-reducing conditions, the profiles of the modified GP64 constructs were similar but not identical to that of WT GP64. The cell surface levels of all constructs except C8A were similar, and were less than 10% of that from WT GP64 (transfected and expressed under the same conditions, Fig. 4C).

In contrast to the other constructs, C8A was detected at the cell surface at a higher level, corresponding to approximately 22% of that from WT GP64 (Fig. 4C, C8A, upper graph). Analysis of membrane fusion by these constructs showed that all constructs containing an additional (heterologous) hydrophobic amino acid (C8A, C8L, and C8V) induced syncytium formation. However, the detected fusion activity
was low and in all cases fusion activity was less than 10% of that from WT GP64 (Fig. 4C, lower graph). No fusion activity was detected for constructs C8, C8N, and C8R by syncytium formation assay so we examined these fusion-negative constructs for membrane merger (hemifusion) and pore formation, as described above. Constructs C8N and C8R showed detectable but low efficiency R18 transfer that appeared similar to that of C8, and lower than 1% of that detected with WT GP64 (Fig. 4D and data not shown). No cytosolic dye transfer was observed for these same constructs. These results combined with prior data (Fig. 3), indicate that the length of the hydrophobic TM domain (≥16aa) is critically important for fusion activity of AcMNPV GP64.

**Effects of TM-domain deletions on infectious BV production**

To examine the effects of GP64 TM truncations on virus infectivity, each *gp64* construct (under the transcriptional control of the WT GP64 promoter) was inserted into the *polyhedrin* locus of a *gp64*-null bacmid using standard Tn7-based transposition (27, 28). In addition to the *gp64* gene construct, the donor plasmid used for transposition also encoded a GUS reporter gene under the control of the AcMNPV *p6.9* promoter. As a positive control, the wild type AcMNPV *gp64* gene was inserted into the same donor plasmid and used to rescue the *gp64*-null bacmid. A similar donor plasmid containing no *gp64* gene was used to generate a negative control bacmid. Following the generation of a bacmid encoding each *gp64* gene construct, bacmid DNA was used to transfect Sf9 cells in a transfection-infection assay (28). Transfected and infected cells were scored for GUS activity, which is a) an indirect indicator of viral replication when detected after transfection, and b) an indicator of
infectious virion production when detected after. Transfection of Sf9 cells with gp64-null bacmids carrying the gp64 TM-deletion constructs C0, C4, C5, and C7 resulted in rescue of gp64-null AcMNPV and production of infectious virions (Fig. 1). In contrast, the gp64-null bacmids carrying the other TM-truncated gp64 genes did not rescue viral infectivity (Fig. 1; C8, C8CT, N7, N8, N8CT, M7, and M8CT).

Interestingly our previous analysis showed that gp64 protein constructs N7, N8, N8CT, M7, and M8CT were capable of mediating membrane fusion (Fig. 2C and D) but they did not rescue viral infectivity (Fig. 1). These results suggest that the TM amino acid sequence requirements for membrane fusion and infectivity are distinct.

To ensure that all the gp64 protein constructs were expressed in Sf9 cells after transfection of the bacmids, lysates of the transfected Sf9 cells were analyzed by Western blot analysis. We detected expression of all of the gp64 protein constructs in the bacmid-transfected cells (data not shown). In addition, to confirm that any defects detected were not due to other lethal mutations in the bacmid, all bacmids encoding modified gp64 genes were transfected into Sf9\textsuperscript{Op1D} cells, which constitutively expresses the OpMNPV GP64 protein. In each case, infectious virions were produced (data not shown), indicating that the lack of virion production in Sf9 cells was due to lack of a functional GP64 protein and not to a second-site mutation.

**Budding efficiency by modified GP64 constructs**

To examine possible effects of the TM domain on virion budding, we examined virion budding from viruses expressing each GP64 TM-truncation construct. Each bacmid expressing a GP64 TM-truncation construct was amplified as a virus and titred in Sf9\textsuperscript{Op1D} cells, which express WT OpMNPV GP64. The virus produced in
Sf9<sup>op1D</sup> cells (displaying OpMNPV GP64 on the virion) was then used to infect Sf9 cells where only the modified GP64 construct was expressed. To determine if expression of only the TM-truncated GP64 affected virion budding, the infected Sf9 cells were pulse labeled with <sup>35</sup>S-methionine and the labeled progeny virions were collected and purified. The relative quantities of virions that accumulated in cell culture supernatants were estimated by quantifying the <sup>35</sup>S-methionine label from the major virion capsid protein, VP39, as described previously (37) (see Materials and Methods). Comparison of each virus with a control virus expressing WT GP64 revealed that removal of the cytoplasmic tail, or truncation or deletion of the putative TM domain of GP64 resulted in substantially decreased virus budding (Fig. 5B). The virus budding efficiencies measured for viruses expressing GP64 constructs C0, C4, and C5 were approximately 24-30% of that from a virus expressing WT GP64. With the exception of construct N8, deletion of 7, or 8 amino acids from the TM domain appeared to result in progressive reduction in virion budding. However, because background virion budding in the absence of GP64 may range from approximately 3-10% of that from WT AcMNPV (37) (and unpublished observations), and because only the bacmid expressing the C7 construct rescued infectivity, it is unclear whether the low levels of virions detected from bacmids expressing constructs C8, C8CT, N8CT, and M8CT, represent background budding or low levels mediated by the GP64 construct. In addition, comparison of larger TM deletions (with and without cytoplasmic tail domains) suggests that the addition of the cytoplasmic tail in constructs C8CT and N8CT did not detectably augment budding efficiency.
Analysis of single and multiple point mutations in the GP64 TM domain

We previously used TM domain replacements (23) and deletions to examine functional requirements of the TM domain. To further investigate the role of the TM domain, we generated and examined the effects of small 1-4 amino acid substitution mutations within the AcMNPV GP64 TM domain. We used two strategies for these studies. First, we performed an alanine scan of the GP64 TM domain, substituting 2 or 3 alanines for amino acids of the TM domain sequence (Fig. 6A). Next, we examined a number of specific amino acid positions based on sequence conservation among the GP64 and related proteins, and on current models of membrane fusion protein function.

For alanine-scanning mutagenesis of the GP64 TM domain, we replaced each 2 or 3 amino acids in the TM domain, with alanine residues (Fig. 6A) and inserted each construct into a recombinant GP64-null virus of AcMNPV as described above. All GP64 constructs were initially examined by reducing and non-reducing gel electrophoresis and Western blot analyses to confirm that the substitution constructs were expressed and trimerized in a manner similar to that of WT GP64 in virus-infected Sf9 cells (Fig. 6B). Analysis of cell surface localization for these alanine-scanning constructs showed that all constructs were expressed and localized at the cell surface with surface levels ranging between approximately 45 and 185% of that measured from WT GP64 (Fig. 6C, upper panel). Two constructs (485-487 and 503-505) resulted in reduced surface levels of GP64 while surface levels of all others were similar to or even greater than that of WT GP64. To determine if specific TM amino acid positions were required for the fusion function of GP64, the alanine-
scanning constructs of GP64 were examined in a syncytium formation assay. All alanine-scanning constructs retained fusion activity and the fusion activity mediated by these constructs was very similar to that of WT GP64, ranging from approximately 70 to 110% of that from WT GP64 (Fig. 6C, lower panel). In addition, analysis of one-step growth curves from viruses expressing the alanine-scanning substitution constructions revealed that the infectious virus production from all viruses was similar to that from the control virus expressing the WT GP64 protein, with no dramatic differences from 6 h to 120 h post infection (data not shown). To further examine possible effects of the TM domain alanine-scanning substitutions on virion budding or incorporation of GP64 into virions, infected Sf9 cells were pulse-labeled with $^{35}$S-methionine, progeny virions were purified, and relative levels of labeled progeny virions were estimated by measuring relative quantities of label in the major capsid protein, VP39, as described earlier. As shown in Fig. 6D, all of the GP64 constructs containing alanine-scanning mutations in the TM domain were assembled into virions. The budding efficiencies of mutants 483-484 and 494-496 were decreased to around 50% of that of from WT virus, while the budding efficiencies measured for other constructs were similar to that of WT virus (Fig. 6E, top panel). Analysis of GP64 incorporation into virions appeared to be similar to (or in one case only slightly less) that that observed from WT GP64. Thus, analysis of alanine-scanning mutations identified no amino acid positions (or small regions) in the GP64 TM domain that were absolutely required for expression, trimerization, cell surface localization, membrane fusion, virion budding, targeting to virions, or viral infectivity.
However, we identified two regions that affected cell surface localization, and two regions that affected virion budding efficiency (Fig. 6C and E).

The GP64 protein is very highly conserved within the group I NPVs of the Baculoviridae and this high level of conservation includes the TM domain (Fig. 7A). Because our initial studies showed that the GP64 TM domain could not be functionally replaced by TM domains from other (non-baculovirus) viral or cellular membrane proteins, we concluded that the TM domain sequence encoded important functions beyond that of simply anchoring the protein in the membrane. Previous studies revealed that glycine residues within the TM domain of the VSV G protein play a critical role in membrane fusion and appear to function in the transition from hemifusion to complete membrane fusion (10). Indeed glycine residues appear to be over-represented in the TM domains of viral fusion proteins (2, 46). Where glycines are not present in such TM domains, it has been proposed that methionine residues may functionally substitute. In addition, more recently it was reported that phenylalanine residues in combination with glycine residues contribute to transmembrane domain interactions (53). Because the GP64 TM domain contains only a single conserved glycine residue and multiple phenylalanine residues, we generated alanine-substitution mutations in single or multiple positions to examine the potential function of the glycine or phenylalanine residues in GP64 function (Fig. 7). Substitutions of the Met residue at the N-terminus of the TM domain and the single charged histidine residue were also examined either alone or in combination with other substitutions. The single and multiple substitution mutations are shown in Figure 7B. All modified GP64 constructs were inserted into recombinant viruses...
(replacing native GP64) and examined for trimer formation, localization at the cell surface, fusion activity (syncytium formation efficiency), and virus infectivity (Fig. 7B). In viruses containing the GP64 TM domain substitutions of single and multiple amino acids (Fig. 7B), the GP64 proteins behaved similar to WT GP64 with no substantial differences observed. A minor difference observed was that levels of cell surface GP64 were reduced moderately for some of the substitution mutations. Substitution of Met 484, Gly 486, His 487, or Phe 500 resulted in decreased cell surface levels (40-75% compared with WT GP64 (Fig. 7B, Surface %) although the detected fusion activity remained high. Thus, consistent with the alanine-scanning analysis, analysis of single and multiple amino acid substitutions at conserved residues or those identified as important in other viral membrane fusion proteins did not identify amino acids that were absolutely required for GP64 function in membrane fusion or other critical functions.
For baculoviruses that encode a GP64 protein, GP64 is essential for the entry of the budded form of the virus. Compared with the highly conserved ectodomains of GP64 proteins, the TM domains are more variable in length and amino acid sequence similarity. This suggests the possibility that TM domains of GP64 proteins have conserved structural characteristics that are not apparent from an analysis of the primary amino acid sequence. The recently described structure of the post-fusion form of GP64 (21) does not include the TM domain, and TM domain structures are generally rare. Recently, we found that replacement of the predicted 23 amino acid GP64 TM domain with corresponding TM domain sequences from a range of viral or cellular type I membrane proteins or with a GPI anchor addition sequence, resulted in defects in transport, membrane fusion, virion budding, and virus infectivity (23). Only TM domains from two related viral membrane proteins (OpMNPV GP64 and Thogotovirus GP75) functionally substituted for the AcMNPV GP64 TM domain sequence. These observations indicate that the TM domain is critical for the function of GP64.

To examine potential length or sequence requirements for the biological functions of the AcMNPV GP64 TM domain, we generated GP64 proteins containing truncated forms of the TM domain by removing 4-8 amino acids from the N-terminal, C-terminal, or central regions of the TM domain. Although the cytoplasmic tail domain has been shown to be dispensable for fusion activity (37), we also generated GP64 constructs containing the cytoplasmic tail domain since addition of a cytoplasmic tail may force a truncated TM domain to span the bilayer membrane and promote
membrane fusion (3). All GP64 proteins with truncated TM domains were synthesized and oligomerized in transfected cells (Fig. 1B), however, the trimer profiles of some constructs containing C-terminal deletions of the TM domain appeared to differ from that of wt GP64. The GP64 trimer is typically observed as two distinct electrophoretic forms. While the two trimer forms (I and II) have different migration rates on nonreducing SDS-PAGE, the two forms appear to have the same mass, as determined by mass spectrometry (38). It has been suggested that the two trimer forms may represent disulfide isomers (26) but it is not known whether they differ in any functional properties. The cell surface levels of TM-truncated GP64 constructs were dramatically reduced (≥67%) and appeared to decrease with successive reduction in length of the TM domain (Fig. 2). Similar results were previously reported for Human Immunodeficiency Virus (HIV) envelope glycoprotein and Vesicular Stomatitis Virus glycoprotein (VSV G) constructs that contained truncated TM domains (1, 39). Recently, studies of TM model peptides suggest that peptide-membrane interactions depend on peptide hydrophobicity and on the matching of hydrophobic peptide length with membrane thickness. A negative hydrophobic mismatch occurs when the hydrophobic stretch of a peptide is too short with respect to the membrane thickness (11, 13, 45, 49, 57). The observed shedding of GP64 proteins with truncated TM domains (Fig. 1B) may result from such a negative hydrophobic mismatch. Addition of the cytoplasmic tail significantly increased the cell surface levels of C8CT and N8CT, and also moderately increased surface levels of M8CT (Fig. 2A) suggesting that addition of the CT domain may force the truncated TM domain to adapt its structure or orientation to overcome the
mismatch as suggested for some model TM peptides (12, 15, 47). The length of the GP64 TM domain also had an effect on its fusogenicity. With successive truncations, the level of membrane fusion activity also decreased successively when compared with wild-type GP64 (normalized activity). Compared with normalized WT GP64, we found that the fusion activity decreased moderately (15-20%) for GP64 constructs C4 and C5, and dramatically for C7 and C8 (Fig. 2D). No fusion activity was detected for C8, a construct containing only 15 amino acids of the TM domain. The N-terminal and middle regions of the TM domain were similarly sensitive since deletion of 7 or 8 amino acids from those regions also resulted in dramatically reduced fusion activity (77-90% reductions). While addition of the CTD increased the fusion activities of N8CT and M8CT in comparison to N8 ad M8, the fusion activities of N8CT and M8CT were still reduced by more than 90% compared with WT GP64. Addition of the CTD did not rescue fusion activity of C8, which was fusion negative. Because the C-terminal portion of the TM domain is somewhat more hydrophobic than the N-terminal region (as measured by hydrophobicity plots), removing the most hydrophobic portion of the TM domain may account for both the lack of detectable fusion activity in C8, and failure of C8CT to rescue fusion activity. Thus, these data suggested that GP64-mediated fusion requires a critical TM domain length of 15-16 amino acids. The critical nature of the hydrophobic length was confirmed by experiments in which a single hydrophobic amino acid (A, L, or V) was added to the C-terminus of mutant C8, resulting in restored fusion activity (Fig. 4C). Addition of hydrophilic or charged amino acids did not restore activity. Interestingly, the fusion activities of the C8 mutants extended with L or V were considerably less than that of
the C7 mutant that contains an I at the same position. It is of interest that in case of the wild type C7 sequence or C8L, C8V, and C8A (which restored fusion), the relative level of fusion correlates with the degree of hydrophobicity of the respective C-terminal amino acid: I, L, V, and A.

We also asked which step in membrane fusion was affected by truncations in the TM domain. We used lipid and cytosolic dyes to separately examine outer leaflet merger (the hemifusion intermediate) and pore formation, focusing on the fusion deficient construct, C8 (a 15 amino acid TM domain). Using membrane and cytosolic dye labeling, we found that construct C8 induced only approximately 1% lipid dye transfer efficiency (hemifusion) when compared with WT GP64. Addition of the 7 amino acid hydrophilic cytoplasmic tail did not restore fusion activity by the C8 mutant and appeared to eliminate the very low level of hemifusion. These results differ from results reported for the influenza HA protein (3). In that case, truncation of 12 amino acids from the C-terminal end of the HA TM domain (resulting in a 15 amino acid TM domain) did not substantially affect lipid dye transfer (hemifusion) but reduced pore formation to approximately 5% of that of the WT HA. A similar result was obtained when the deletion was from the N-terminal end of the HA TM domain. Thus, HA proteins containing TM domains of ≤ 15 amino acids were unable to mediate complete fusion (pore formation) but the first step (membrane merger or hemifusion) was not substantially affected. In contrast, the GP64 TM domain containing only 15 amino acids (constructs C8 and C8CT) was severely defective in the first step, membrane merger. In a recent study (23) we found that replacement of the TM domain of GP64 with either the 21 aa TM domain of OpMNPV F protein or the 23 aa
TM domain of Influenza virus HA, resulted in chimeric GP64s that induce low efficiency hemifusion, but not pore formation. While those data suggested that the hemifusion step was largely independent of the TM domain, our current observation that TM-truncated constructs C8 and C8CT are severely compromised or defective in their ability to induce hemifusion further suggests that a critical hydrophobic length may be important for that initial hemifusion step.

Some differential effects were also observed when truncations were made from the N-terminal or middle regions of the GP64 TM domain. In those cases (N8, N8CT, M8, M8CT), very low levels of membrane fusion were observed and fusion efficiency increased upon addition of the CT domain (Fig. 2D).

Another consideration in the interpretation of results in studies such as this is the contribution or interaction of the specific lipid bilayer of the host cell membrane. In several cases, it has been demonstrated that the lipid composition of the membrane can alter peptide interactions and fusion activity (13, 40, 48, 56). The lipid composition in Sf9 cell membranes differs significantly from that of mammalian cells (29) and these differences may be associated with the lower temperatures typical of insect cell culture or insect growth. Differences in lipid compositions of the membranes may explain the observed differences in fusion by the truncated GP64 and HA constructs. Another possible explanation for the observed differences is a difference in the mechanics of protein-mediated membrane fusion. Plonsky et al. (43) found that unlike HA-mediated fusion, AcMNPV GP64-induced fusion pores open rapidly, irreversibly, and do not flicker. In addition, the initial fusion pores formed by GP64 appeared to be larger than those of HA (43). Interestingly, the minimal TM
domain length for fusogenicity is in the same range for different TM peptides: 17 residues for HA (3), 16 residues for GP64 TM domain, and between 14-18 residues for TM model peptides (26). Effects of the TM domain length on the fusion process was also described previously for other fusion proteins such as those from HIV (39), Foamy virus (41), Murine Coronavirus (6), MuLV (44), and for SNARE proteins (58).

In addition to membrane fusion, we also examined the potential roles of the TM domain in virion assembly during budding. An analysis of virus budding efficiency revealed that virus budding efficiency decreased 70-75% upon deletion of the cytoplasmic tail. Truncation of an additional 4-5 amino acids from the C-terminal end of the TM domain (constructs C4 and C5) resulted in a similarly reduced budding efficiency. Further truncation or deletions of 7-8 amino acids of the TM domain resulted in even more dramatically reduced virus budding efficiency (reductions of 86-96% compared with WT GP64) (Fig. 5B). While the reduced budding efficiency of truncated GP64 constructs appeared to correlate with reduced surface levels of the GP64 constructs, the addition of the cytoplasmic tail domain (which restored surface levels and fusion activity; Fig. 2A and D) did not rescue the budding defects. We also examined the capacity of GP64 proteins with truncated TM domains to substitute for wild type GP64, using a transfection-infection assay in SF9 cells. Infectivity was rescued by constructs containing 16 amino acids from the N-terminal portion of the TM domain, but not by constructs containing 16 amino acids from other parts of the TM domain (i.e. constructs containing deletions from the N-terminal or middle regions were not able to substitute; Fig. 1, C7 vs N7 and M7). By comparing fusion assay results to results from virus infectivity studies, we can conclude that the sequence
requirements for infectivity are more stringent than those for membrane fusion, which
in turn are more stringent than the requirements for membrane anchoring and
intracellular transport.

To further examine the GP64 TM domain and identify specific residues important
for GP64 function, we generated and analyzed single and multiple amino acid
substitution mutations in the TM domain. We specifically focused on conserved
methionine (M484), glycine (G486), histidine (H487), and phenylalanine (F483, F487,
F491, and F500) residues of the TM domain of GP64 (Fig. 7B). We also examined
small regions of 2-3 amino acids using an alanine scan (Fig. 6A). The glycine at 486
is conserved among almost all baculovirus GP64 proteins and the more distantly
related Thogotovirus GP75. Previous studies (10) suggested that at least one of the
two glycine residues of VSV G protein plays a significant role in the transition from
hemifusion to complete fusion. Furthermore a study of Japan HA, which contains two
glycines within the TM domain, demonstrated that substitution of the more N-terminal
glycine to a leucine (G250L) caused a restricted hemifusion phenotype (33). It has
been proposed that these important glycine residues may function as helix breakers
and thereby distort the bilayer, promoting membrane fusion. This concept is
supported by the fact that a conserved proline residue (also a helix breaker) is found
in the TM domains of Foamy Virus and Murine Leukemia Virus envelope proteins and
is essential for fusion function in both instances (41, 51). In the current study,
substitution of the conserved glycine with alanine or leucine resulted in reduced cell
surface levels of those GP64 constructs (Fig. 6, 485-487; Fig. 7B) but the fusion
activities of those constructs (485-487, G486A, and G486L) were similar to WT GP64
or only slightly decreased. Similar results were found in Semliki forest virus E1 protein. Indeed neither of the two conserved glycine residues nor any of the five total glycine residues within the TM domain of E1 are required for fusion activity (24).

Since many or most of the Gly-less TM domains of fusion proteins possess internal methionine residues (10), we also examined the conserved methionine at the position 484. Substitution of Met 484 with alanine resulted in similar or decreased levels of the GP64 construct at the cell surface, but only subtle effects on membrane fusion (Fig. 6, 483-484; Fig. 7, M484A). We also examined the histidine (H487) based on the reported role of a charged residue within the TM domain of Foamy Virus envelope protein (41). Pietschmam et al. (41) found that an evolutionarily conserved positively charged amino acid K959 within the putative TM domain of foamy virus appears to regulate fusion activity. We found no substantial effect from two substitution approaches for the GP64 TM domain histidine residue (Fig. 6, 485-487; Fig. 7B, H487A). Recently, Unterreitmeier et al (53) used a randomized library to biologically select TM domains that self-interact and found that that higher affinity TM domain sequences were enriched in phenylalanine. In addition, phenylalanine is frequently found associated with GxxxG motifs in TM domains of self-interacting proteins. Notably, disruption of the FxxGxxxG motif of the VSV G protein by substitution of phenylalanine or glycine residues resulted in reduced TM-TM associations (53).

Substitutions of the conserved phenylalanines within the TM domain of GP64 (singly or in various combinations with H487) did not cause dramatic effects on fusion activity although the cell surface levels were somewhat reduced for GP64 substitution construct F500A (Fig. 7B).
Using the 2-3 alanine-scanning substitutions (Fig. 6), we found two regions 485-487 and 503-505 that were important for cell surface localization of GP64. Substitutions of either of these two regions with alanines resulted in the cell surface levels that were decreased more than 50% (Fig. 6C). In two additional regions, 483-484 and 494-496, substitution mutations resulted in virus budding that was reduced by more than 50%.

While many reports describe critical roles for the transmembrane domains of viral fusion proteins, no uniform theme has emerged from these studies. In the case of influenza virus HA, it is clear that a transmembrane anchor is required for full fusion activity (32, 34) but a variety of transmembrane sequences will substitute for the native transmembrane domain (33). In contrast, specific sequence requirements appear to be encoded within the transmembrane domains of other fusion proteins, such as the envelope proteins from human immunodeficiency virus type I (39), murine leukemia virus (51), foamy viruses (41), coronavirus (6, 8), VSV (10), Newcastle disease virus (31), and measles virus (7). In the case of the AcMNPV GP64 protein, prior studies indicated that like the later cases, the TM domain sequence encodes critical functions (23). In the present more detailed study of the TM domain sequence, we reached two broad conclusions. First, we found that the TM domain of the GP64 protein requires a critical minimum hydrophobic length of 16 amino acids. The requirement for this length is related not only to protein anchoring, but more importantly this represents a requirement for the function of GP64 as a fusion protein. Second, our analysis of TM domain sequences by substitution mutations in conserved positions and alanine-scanning show that no single amino
acid in the TM domain is absolutely required for GP64 function in any of its critical roles in the virus. Combined with prior studies of substitutions of the entire TM domain, these studies suggest that either a) the GP64 TM domain may encode critical amino acids or sequence elements that are separate but redundant, or b) the overall sequence encodes a higher order structure that is not disrupted by single substitutions or our alanine-scanning approach, yet cannot be replaced by an unrelated heterologous TM domain. Future studies aimed at examining the higher order structure of the GP64 TM domain may provide insight into the relationship between TM domain structure and the complex and critical process of protein-mediated membrane fusion.

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Figure Legends

Figure 1

Construction and expression of GP64 proteins with truncated TM domains.

(A) Schematic representation of the WT GP64 protein with the truncations of the GP64 transmembrane (TM) domain illustrated below. The schematic at the top shows the positions of several major features of the GP64 protein, including the signal peptide (SP), predicted glycosylation sites (fork symbols), monoclonal antibody epitopes (AcV1, B12D5, and AcV5), an amphipathic alpha-helical domain (Helix), an acylation site (wave symbol), and the transmembrane (TM) and cytoplasmic tail (CTD) domains. The name of each GP64 construct is shown on the right of the sequence of the TM domain region. Results from trimerization, surface localization, membrane fusion (syncytium formation), and infectivity assays are summarized on the left. (ND: not done)

(B) Analysis of expression and oligomerization of TM-truncated GP64 constructs. Sf9 cells were transfected with plasmids encoding wild-type and modified GP64 constructs. Cells were lysed at 24 h pt under reducing (top panel) or nonreducing (middle panel) conditions. GP64 proteins were resolved by SDS-PAGE and detected by Western blotting using monoclonal antibody AcV5. The positions of oligomeric forms of GP64 (Trimer I and II, Dimer) are identified on the right. The supernatants from transfected cells were also analyzed under reducing conditions by Western blot analysis (bottom panel).
Figure 2

Analysis of cell surface levels and membrane fusion activity of TM-truncated GP64 constructs. (A) Relative cell surface levels of GP64 constructs were measured by cELISA, using monoclonal antibody AcV5. Each mutant GP64 construct was expressed by transfecting cells with 0.5 µg of the appropriate plasmid DNA. Values represent the means from triplicate transfections, and are normalized to that of cells transfected with 0.5 µg of the plasmid DNA expressing WT GP64. Error bars represent the standard deviation from the mean and the amount of DNA per transfection is indicated below the graph.

(B) Immunofluorescence analysis of cell surface levels of GP64 mutants using MAb AcV1. Cell surface GP64 was detected on transfected Sf9 cells fixed with 4% paraformaldehyde.

(C) Analysis of membrane fusion by syncytium formation assays. Sf9 cells were transfected with plasmids expressing wild type (WT) or TM-truncated GP64 proteins. At 24 h post transfection, cells were exposed to pH 5.0 for 3 min, then incubated for 4 h and observed and photographed under phase-contrast microscopy (x 200). Arrows indicate syncytial masses.

(D) Analysis of syncytium formation efficiency. For calculations of relative levels of fusion activity, the number of nuclei in syncytia was divided by the number of total cells in a field. Those percentages were normalized to parallel syncytium formation data from wild type GP64 that was localized to the cell surface at equivalent levels. For each sample, 5 fields were examined. The means and standard deviations of triplicate transfections are shown.
Figure 3
Analysis of hemifusion and fusion-pore formation by fusion-deficient GP64 proteins.
Red blood cells (RBCs) that were dual labeled with membrane restricted (R18) and
cytosolic (Calcein-AM) dyes were bound to Sf9 cells transiently expressing mutant
GP64 proteins. The bound cells were exposed to acidic PBS (pH 5.0) for 3 min to
induce fusion and examined after a 20 min incubation period. Membrane dye transfer
(R18, left panels) or cytosolic dye transfer (Calcein-AM, right panels) were monitored
by fluorescence microscopy. Each mutant GP64 construct (or control) used for
transfection is indicated on the left.

Figure 4
Analysis of the fusion requirements for GP64 construct C8. A single hydrophobic (A,
L, or V) or hydrophilic (R or N) amino acid was added to the C-terminus of the TM
domain of the fusion-defective GP64 construct C8, and each resulting construct was
then analyzed for restoration of membrane fusion activity.
(A) Construction of GP64 constructs. The sequence of the TM domain of each
construct is indicated below that of the WT GP64 TM domain. The TM domain of the
fusion-defective C8 construct is shown above. Amino acids added to the C-terminus
of the TM domain of C8 are indicated in bold and the construct name is indicated on
the left.
(B) Expression and trimerization of modified GP64 proteins. Sf9 cells were
transfected with plasmids expressing either wild-type GP64 (WT) or modified GP64
constructs (C8A, C8L, C8V, C8N, and C8R), and expression and trimerization of GP64 were examined by Western blot analysis of cell extracts on reducing (top) and nonreducing SDS-PAGE (bottom). The positions of oligomeric forms (Trimer and Dimer) are indicated on the right.

(C) Analysis of relative cell surface levels (top panel) and fusion activities (bottom panel) of GP64 constructs. Relative cell surface levels of GP64 constructs were measured by cELISA (as described in the legend to Fig. 2). Each mutant GP64 construct was expressed by transfecting 0.5 µg plasmid DNA encoding the modified GP64 construct. Relative fusion activity was evaluated using efficiency of syncytium formation calculated from representative random fields and normalized to parallel syncytium formation data from wild type GP64 that was localized to the cell surface at equivalent levels, as described earlier. For each sample, 5 fields were examined. The means and standard deviations of triplicate transfections are shown.

(D) Analysis of hemifusion and fusion-pore formation by GP64 protein constructs. Fluorescence micrographs show either membrane dye transfer (R18, left panels) or cytosolic dye transfer (Calcein-AM, right panels). Each mutant GP64 construct (or control) used for transfection is indicated on the left and assays were performed as described in the Materials and Methods section and Figure 3.
Figure 5

Analysis of virion budding efficiency by viruses expressing TM-truncated GP64 constructs.

(A) Analysis of labeled progeny virions. Viruses expressing TM-truncated GP64 constructs were pulse labeled with $^{35}$S-methionine and progeny BV purified as described in the Materials and Methods section. Labeled viral proteins were analyzed by SDS-PAGE and phosphorimager analysis. GP64 constructs are indicated above the lanes and the positions of GP64 and VP39 proteins are indicated between the panels. Controls include an AcMNPV virus expressing wild type GP64 (WT) and an AcMNPV virus containing a GP64 knockout (Ko). Each lane represents virus purified from an equivalent volume of the cell culture supernatant.

(B) Budding efficiencies of TM-truncated GP64 constructs. Relative budding efficiencies were determined by comparing the quantity of $^{35}$S-methionine label in the major capsid protein (VP39) band from each virion preparation to that from a virus expressing WT GP64.

Figure 6

Analysis of alanine-scanning mutations in the GP64 TM domain.

(A) Schematic representation of the positions of GP64 alanine scanning mutations. Sets of 2 or 3 amino acids in the TM domain were replaced with alanines as indicated (periods represent positions with no change). The name of each alanine-scanning GP64 construct is indicated on the left (numbers represent amino acid modified positions in the AcMNPV GP64 sequence). The two regions important for cell-surface expression of GP64 are indicated by heavy lines above the WT GP64 TM domain.
sequence and two regions important for virus budding are similarly indicated by heavy lines below the sequence. (ED: ectodomain, TM: transmembrane domain, CT: cytoplasmic tail)

(B) Expression and trimerization of GP64 constructs with alanine scanning mutations in the TM domain. Sf9 cells were infected with viruses expressing wild-type GP64 (WT) or the above alanine-scanning constructs of GP64. Expression and trimerization of GP64 were examined by Western blot analysis of cell extracts on reducing SDS-PAGE (top panel) or nonreducing SDS-PAGE (bottom panel). The positions of GP64 and oligomeric forms (Trimer, Dimer) are indicated on the right.

(C) Relative cell surface levels and fusion activities of GP64 constructs. Relative cell surface levels (top panel) of GP64 constructs were measured by cELISA of infected Sf9 cells, using monoclonal antibody AcV5. Relative fusion activity (bottom panel) from each modified GP64 construct was evaluated by measuring the efficiency of syncytium formation from infected Sf9 cells. For each sample, 5 fields were examined. The means and standard deviations of triplicate infections are illustrated.

(D) Analysis of budding efficiency and incorporation of modified GP64 constructs into budded virions. Viruses expressing modified GP64 constructs were pulse labeled with $^{35}$S-methionine and BV purified and processed as described in the Materials and Methods section. The positions of GP64 and VP39 proteins are indicated on the right. Each lane represents virus purified from an equivalent volume of the cell culture supernatant.

(E) Relative budding efficiencies and incorporation of GP64 into budded virions. Relative virion budding efficiency (upper panel) was calculated by comparisons of
measurements of labeled major capsid protein (VP39) in each virus. All data were normalized to that of a virus construct expressing WT GP64 (100%). The efficiency of GP64 incorporation into virions (lower panel) was calculated for each virus expressing a modified GP64 protein with an alanine scanning mutation. Molar ratios of GP64:VP39 were calculated for virions of each virus based on 15 methionine residues (mutant 503-505) and 16 methionine residues (mature WT GP64 and all other mutants) or 9 methionine residues (VP39). The means and standard deviations determined from triplicate experiments are shown.

Figure 7

Analysis of GP64 conservation and single and multiple amino acid substitutions in the TM domain revealed no amino acid positions essential for membrane fusion or viral infectivity.

(A) Sequence alignment of the transmembrane domains of baculovirus GP64 proteins and Thogotovirus GP75. Sequence alignment was performed using the program Clustal W. The position of the highly conserved glycine is boxed. Conserved methionine, histidine, and phenylalanine positions are shown in bold. The GenBank accession number is listed for each virus, with abbreviations as follows: Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV), Rachiplusia ou (Ro)MNPV, Bombyx mori (Bm)NPV, Choristoneura fumiferana (Cf)MNPV, Orgyia pseudotsugata (Op)MNPV, Hyphantria cunea (Hycu)NPV, Antheraea pernyi (Anpe)NPV, Plutella xylostella (Plxy)MNPV, Maruca vitrata (Mavi)MNPV, Choristoneura fumiferana
defective (CfDef)NPV, *Epiphyas postvittana* (Eppo)NPV, *Anticarsia gemmatalis* (Ag)NPV, *Anagrapha falcifera* (Anfa)NPV, Thogotovirus (THOV).

(B) Construction and summary of effects of single and multiple site substitution mutations in the AcMNPV GP64 TM domain. The results of trimerization assays are shown as +/- . Relative cell surface levels were measured by cELISA and relative fusion activities were evaluated using efficiency of syncytium formation from virus infected Sf9 cells, as described in the Materials and Methods section. The means and standard deviations of triplicate infections are shown. Infectivity was determined by transfection-infection assays as described previously (28).
Figure 1

A

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</table>

B

- Trimer I
- Trimer II
- Dimer
- Super
Figure 2

A

Relative Cell Surface Level (% of WT0.5)

DNA (µg)

0.5 0.1 0.05 0.005 WT C0 C4 C5 C7 C8 C8CT N7 N8 N8CT M7 M8 M8CT Mock

B

Mock WT0.5 WT0.005 C0 C4 C5 C7 C8 C8CT N7 N8 N8CT M7 M8 M8CT

C

Mock WT0.5 WT0.005 C0 C4 C5 C7 C8 C8CT N7 N8 N8CT M7 M8 M8CT

D

Normalized Fusion Activity (%)

0 20 40 60 80 100 120 C0 C4 C5 C7 C8 C8CT N7 N8 N8CT M7 M8 M8CT Mock
Figure 3
Figure 4

A

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<td>C8V</td>
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<tr>
<td>C8N</td>
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<td>C8R</td>
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B

Reducing
Non-reducing

Trimer I
Trimer II
Dimer

C

Relative Cell Surface Level (% of WT 0.5)

DNA (µg) 0.5, 0.1, 0.05, 0.005

D

R18
Calcein-AM

Mock
WT
C8
C8A
C8L
C8V
C8N
C8R

Detected from http://jvi.asm.org on August 30, 2017 by guest
Figure 5
Figure 6
Figure 7

A

Amino Acid Sequence Conservation in the GP64 TM domain

<table>
<thead>
<tr>
<th>Virus</th>
<th>Ectodomain</th>
<th>Transmembrane domain</th>
<th>Cytoplasmic tail</th>
<th>Genbank accession</th>
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B

Amino Acid Substitutions in the GP64 TM domain

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<tr>
<th>Construct</th>
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<th>Trimer</th>
<th>Surface(%)</th>
<th>Fusion(%)</th>
<th>Infectivity</th>
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<td>121.7 ±7.2</td>
<td>85.5 ±11.9</td>
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<td>56.6 ±0.7</td>
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<td>59.2 ±2.9</td>
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<td>205.8 ±8.8</td>
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