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Autographa californica multiple nucleopolyhedrovirus GP64 protein: Analysis of domain I and V amino acid interactions and membrane fusion activity

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ABSTRACT

The *Autographa californica* multiple nucleopolyhedrovirus GP64 is a class III viral fusion protein. Although the post-fusion structure of GP64 has been solved, its pre-fusion structure and the detailed mechanism of conformational change are unknown. In GP64, domain V is predicted to interact with two domain I segments that flank fusion loop 2. To evaluate the significance of the amino acids involved in these interactions, we examined 24 amino acid positions that represent interacting and conserved residues within domains I and V. In several cases, substitution of a single amino acid involved in a predicted interaction disrupted membrane fusion activity, but no single amino acid pair appears to be absolutely required. We identified 4 critical residues in domain V (G438, W439, T452, and T456) that are important for membrane fusion, and two residues (G438 and W439) that appear to be important for formation or stability of the pre-fusion conformation of GP64.

Introduction

Membrane fusion is essential for entry of enveloped viruses into their host cells (Plemper, 2011). The process of membrane fusion is typically executed by one or more viral fusion proteins that are anchored in the envelope of the virus particle. Viral fusion proteins have been categorized into three distinct structural classes (Class I, II, and III) (Li and Modis, 2014; White et al., 2008). Even though viral fusion proteins within each class may have substantially different structures and show significant structural divergence, the proteins within each class appear to catalyze membrane fusion via a common mechanism (Martens and McMahon, 2008; White et al., 2008). Following triggering by receptor binding and/or low pH (or perhaps other mechanisms), the typically trimeric fusion proteins extend and expose a hydrophobic domain (a fusion peptide or fusion loops), which then associates with or inserts into the cellular membrane bilayer. The viral fusion protein next folds back onto itself and thereby draws the fusion peptide (and associated host membrane) into close proximity to the transmembrane domain of the fusion protein. The two separate membranes are thus brought into close proximity, facilitating the union of viral and cellular membranes. During the conformational changes that catalyze membrane fusion, the viral fusion protein forces the two membranes to progress through several stages of close contact and interaction. One experimentally distinct intermediate step is referred to as hemifusion (White et al., 2008). Hemifusion is a partial fusion that results from the merging of the outer leaflets of the two adjacent membrane bilayers, while the inner leaflet of each bilayer remains undisturbed. Hemifusion is followed by the rapid opening and closing of a small fusion pore which subsequently enlarges until a stable pore forms (White et al., 2008). Further expansion of the fusion pore completes the process, ultimately releasing the nucleocapsid core of the virus particle into the cell cytoplasm during viral entry.

Baculoviridae is a family of enveloped, double-stranded DNA viruses with circular genomes of approximately 80–180 kbp. Baculoviruses are infectious only to invertebrates, and viruses classified within this group have been isolated from species within the insect Orders Lepidoptera, Diptera, and Hymenoptera (Rohrmann, 2013). The most intensively studied baculovirus is *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Rohrmann, 2013). Budded virions (BVs) of AcMNPV enter host cells by clathrin-mediated endocytosis (Long et al., 2006). The major envelope glycoprotein of AcMNPV BVs, GP64, plays essential roles in virus attachment and fusion during entry, and is also important in virion egress by budding (Hefferon et al., 1999;

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Oomens and Blissard, 1999). GP64 is a classical type I membrane protein that is both necessary and sufficient for mediating pH-dependent membrane fusion during viral entry (Blissard and Wenz, 1992). The transmembrane domain and the pre-transmembrane domain (PTM)/stem region of this protein are critical for membrane fusion and virus infectivity (Li and Blissard, 2008, 2009a, b). GP64 has receptor binding activity (Hefferon et al., 1999), and the receptor binding domain was mapped to the N-terminal 160 aa (Zhou and Blissard, 2008b). Recently, a cholesterol recognition amino acid consensus domain and a pH-sensitive heparin-binding motif were both found to facilitate binding of GP64 to mammalian cells (Luz-Madrigal et al., 2013; Wu and Wang, 2012). Based on the crystal structure of the low-pH (post-fusion) form of AcMNPV GP64, GP64 proteins are classified as class III viral fusion proteins (Kadlec et al., 2008). Class III fusion proteins also include rhabdovirus envelope proteins (such as vesicular stomatitis virus G and rabies virus G), glycoprotein B or gB from herpesviruses, and the GP75 proteins from Thogotovirus-like orthomyxoviruses (Backovic and Jardetzky, 2011; Backovic et al., 2009; Heldwein et al., 2006; Kadlec et al., 2008; Roche et al., 2006; Roche et al., 2007). In addition, computational predictions also suggest that the Bornavirus envelope protein G may also be a class III fusion protein (Garry and Garry, 2009). Although class III viral fusion proteins belong to unrelated viral families and have no apparent sequence similarities, they share common structural features that are essential for membrane fusion, including a long-central helix that forms a triple-stranded coiled-coil at the heart of the trimer, and internal fusion loops (Backovic and Jardetzky, 2011). The hydrophobic residues within the fusion loops (loop 1 and 2) of GP64 are critical for membrane interaction and the progression of different stages of membrane fusion (Dong and Blissard, 2012; Li and Blissard, 2011).

In their post-fusion structures, class III fusion proteins are composed of five structural domains (Domains I–V) (Backovic and Jardetzky, 2011). Unlike the post-fusion structures of class I fusion proteins , in which a 6-helix bundle is proximal to the host and viral membrane-interacting end of the trimer, the post-fusion structure of GP64 has no 6-helix bundle and the long central triple-stranded coiled coil is separated from the fusion loops and transmembrane domain by the entirety of domain I and a portion of domain V (Backovic and Jardetzky, 2011; White et al., 2008) (Fig. 1A and B). GP64 domain V (Fig. 1A, DV) contains an alpha helix (helix D), and a downstream linker region that connects helix D to the pre-transmembrane (PTM) domain (Backovic and Jardetzky, 2011). Helix D and the adjacent downstream linker region of domain V interact extensively with two regions adjacent to fusion loop 2 in domain 1 (referred to as fusion loop 2-proximal segments 1 and 2, or FL2PS1 and FL2PS2) (Kadlec et al., 2008) (Fig. 1B a and b). We hypothesize that these interactions between domains I and V may collaborate to create and/or to stabilize the post-fusion structure, following the pH-triggered rearrangement of the protein trimer. To examine this hypothesis, we substituted residues that make contacts between domain V and domain I and investigated the effects of those substitutions on GP64-mediated membrane fusion.

Results

Construction and expression of modified GP64 proteins

To determine which GP64 residues within the C-terminal domain V portion of GP64 (Fig. 1A; DV in red) are important for membrane fusion activity, we first performed an analysis of the GP64 post-fusion (low pH) structure (Kadlec et al., 2008) to predict amino acid side-chain contacts between residues within the C-terminus of domain V and residues from other domains. Contacts were examined using the WHAT IF molecular modeling package (http://swift.cmbi.ru.nl/whatif/). In the crystal structure of the post-fusion GP64 trimer, helix D and the linker region (from domain V) are predicted to interact extensively with two segments (amino acids residues 144–149 and 163–189) that flank fusion loop 2 of Domain I (Fig.1A, C). Most of these interactions are predicted to occur within the same protomer (monomer) of GP64. These include interactions between the following residue pairs: L189-W439, K170-S437, R163-Q445, and L144-T452 (Fig. 1B). Other predicted interactions include: K146-M453, Q148-K457 and N149-G459 which are between the linker region downstream of helix D (in domain V) and residues flanking the FL2 loop region (Fig. 1B b and d). In contrast to those predicted interactions within each protomer, we also identified additional predicted interactions (K446-S447 and K146-E454), which are found between two separate protomers (Fig. 1B c and d). We performed a sequence alignment of these interacting regions of GP64 proteins from different baculoviruses, as well as the GP64-family homolog from *Thogotovirus*, GP75 (Morse et al., 1992) (Fig. 1C). Based on structural analysis of AcMNPV GP64 (above) and sequence conservation among these GP64 family proteins, we experimentally examined a number of selected amino acid positions. The selected positions include the predicted interacting residues described above (Fig. 1C, closed circles), and several of the highly conserved residues within helix D and the downstream linker region (Fig. 1C, open circles). All of the residues selected for analysis were either individually substituted with alanine, or pairs of interacting residues were substituted with alanines. Alanine was selected for substitutions as alanine is a small non-polar amino acid that contributes only minimally to hydrophobicity and thus represents a neutral substitution. Since substitutions within amino acid positions 432-440 may abolish the AcV5 epitope of GP64 (Hohmann and Faulkner, 1983; Monsma and Blissard, 1995), a c-Myc epitope tag was inserted between the signal peptide and the mature ectodomain of GP64 (Zhou and Blissard, 2008a) in constructs Myc-S437A, Myc-G438A, Myc-W439A, Myc-S440A, Myc-K170A/S437A, and Myc-L189A/W439A. It was previously demonstrated that GP64 containing a c-Myc tag at the N- terminus of the mature ectodomain is expressed and efficiently transported to the cell surface (Zhou and Blissard, 2008a), and we show here that fusion activity by the epitope tagged WT GP64 is similar to that of WT GP64 (see below).

Expression of wild-type and modified GP64 proteins in Sf9 cells was carried out by transient transfection and was driven by the promoter of the AcMNPV *ie1* gene. At 36 h post transfection, GP64 proteins from cell lysates were examined by Western blot analysis under either reducing or non-reducing conditions for SDS-PAGE (Fig. 2A). Non-reducing conditions were used to detect the GP64 trimer, in which monomers are covalently linked by disulfide bonds (Li and Blissard, 2010; Oomens et al., 1995). The same oligomeric forms (trimer I, trimer II and dimer) that are typically observed in infected cells, were detected from all GP64 proteins containing substitution mutations, except construct Myc-K170A/S437A (Fig. 2A). The intensities of bands corresponding to the WT and modified (amino acid substituted) GP64s were similar, indicating that the single or double substitution mutations did not substantially alter the expression, stability, or oligomerization of these GP64 proteins (Fig. 2A). The single exception to this observation was the double substitution construct Myc-K170A/S437A, which was not detected on either reducing or non-reducing gels (Fig. 2A), implying that the interaction between these two residues may be critical for the folding or stability of GP64.