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The roles of viral protein AC102 in *Autographa californica* multiple nucleopolyhedrovirus late stage infection

By

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ABSTRACT

The roles of viral protein AC102 in *Autographa californica* multiple nucleopolyhedrovirus late stage infection

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Microbial pathogens rely on their host cells to replicate, and thus have evolved a multitude of mechanisms to exploit host cell machinery, divert cellular resources, and remodel cells to form a favorable environment for replication. One global target of microbial pathogens is host actin, a highly conserved protein that is involved in a myriad of cellular functions. Actin is found in the cell in two forms, monomeric G-actin and filamentous F-actin, and is a major component of the eukaryotic actin cytoskeleton. A notable group of pathogens that target host actin are baculoviruses, which are large enveloped DNA viruses that replicate in host cell nuclei. Baculoviruses represent an extreme example of actin subversion by a pathogen, as they use actin at almost every step of the viral replication cycle. Specifically, the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is unique among viruses in its extraordinary augmentation of actin within the nucleus during infection. In particular, the AcMNPV protein AC102 is essential for viral replication and facilitates the nuclear localization of G-actin (termed NLA) during early AcMNPV infection. However, AC102 shares no significant primary sequence similarity with non-baculovirus proteins and has no recognizable sequence motifs, making it difficult to predict how AC102 functions in NLA or what other processes it may be involved in during AcMNPV infection.

Through my dissertation research, I have provided a basic characterization of AC102's expression profile, localization, and protein interactions. My research also reveals several previously unknown roles of AC102 in late stage AcMNPV infection processes. First, my research shows that AC102 is expressed predominantly during late infection. This result was surprising given that the only previously known function of AC102 was during early infection. AC102's late expression suggested that AC102 may also have role(s) during late infection. To further investigate AC102's function(s), I generated ten mutant viruses, each containing a single point mutation in a conserved amino acid residue of AC102, and screened for viruses with replication defects. While most of these viruses exhibited modest 2- to 5-fold reductions in viral titer compared with wild type virus, the AC102-K66A mutant virus produced 10-fold fewer progeny, and was therefore chosen for further analysis.

Characterization of the AC102-K66A mutant virus revealed many defects in late stage viral infection, indicating that AC102 does indeed play an important role in late viral functions.

First, the AC102-K66A mutant virus is defective in nucleocapsid morphogenesis, as the major capsid protein VP39 forms long tubular filaments that are often bundled together in AC102-K66A infected cells instead of properly packaged nucleocapsids. This indicates that AC102 is important for proper nucleocapsid assembly during late infection. Second, the AC102-K66A mutant virus is defective in the assembly of F-actin in the nucleus during late infection. Nuclear F-actin polymerization is mediated through the actions of viral protein P78/83 (an actin nucleation promoting factor) and the host Arp2/3 actin nucleating complex. Interestingly, a tagged version of wildtype AC102 co-purifies with a previously identified nucleocapsid-associated protein complex consisting of viral proteins P78/83, C42, and EC27. This indicates that AC102 is a previously unidentified member of the P78/83-C42-EC27-AC102 complex. AC102's interaction with P78/83 in a nucleocapsid-associated protein complex provides an explanation for AC102's roles in nucleocapsid morphogenesis and nuclear F-actin polymerization. Future research will reveal how AC102 mechanistically contributes to the functions of the P78/83-C42-EC27-AC102 complex and if there is a regulatory link between AC102's roles in early NLA and late nuclear F-actin polymerization.

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CHAPTER 1

Introduction

Microbial exploitation of host actin

Microbial pathogens rely on their host cells to replicate, and thus have evolved a multitude of mechanisms to exploit host machinery, divert cellular resources, and remodel cells to form a favorable environment for replication. A global target of microbial pathogens is actin, a highly conserved protein that is involved in a myriad of cellular functions and is a major component of the eukaryotic actin cytoskeleton. Pathogens use host actin during many stages of their replication cycles, for example, to inhibit cellular processes, promote invasion and uptake, evade the host immune system, and drive intracellular transport to and from sites of replication (Welch and Way, 2013; Haglund et al., 2010; Aktories, 2011; Aktories et al., 2011; Carabeo, 2011; Taylor, Koyuncu, and Enquist, 2011; Vance, Isberg, and Portnoy, 2009; Reed et al., 2014).

Actin dynamics

Actin is a 42 kDa globular protein that is one of the most abundant proteins in eukaryotic cells. Actin is a major component of the actin cytoskeleton and is responsible for a variety of cellular functions including cellular motility, morphology, division, and intracellular trafficking. Two forms of actin exist in the cell: a globular, monomeric form (G-actin) and a filamentous form (F-actin) that is assembled from G-actin monomers. The spontaneous assembly of G-actin is not kinetically favorable, and cells therefore encode actin-nucleating proteins that lower the kinetic barrier to G-actin association to begin the process of actin polymerization. Once nucleation occurs, actin filaments elongate more rapidly from their barbed or (+) ends than their pointed or (-) ends, leading to the generation of long filaments in cells.

Cells encode three classes of actin nucleating proteins: the Arp2/3 complex, formins, and tandem-monomer-binding proteins (Firat-Karalar and Welch, 2011). The Arp2/3 complex is a multiprotein complex that binds to the sides of existing actin filaments and nucleates a new filament to form a Y-branched structure. Two subunits in this complex, Arp2 and Arp3, are structurally similar to actin itself and facilitate the nucleation event. By itself, the Arp2/3 complex is a weak nucleator and requires activation by nucleation promoting factors (NPFs) such as host cell Wiskott-Aldrich Syndrome protein (WASP) family proteins, which bind G-actin and the Arp2/3 complex to facilitate Arp2/3-mediated actin polymerization (Firat-Karalar and Welch, 2011; Goley and Welch, 2006). Formins, in contrast, are multidomain proteins that assemble unbranched actin filaments. Formins both nucleate F-actin assembly and elongate actin filaments by binding to the (+) ends of actin filaments and facilitating the incorporation of actin monomers into the growing actin filament (Chesarone, Dupage, and Goode, 2010). Lastly, tandem-monomer-binding proteins, typified by the protein Spire, contain tandem G-actin binding motifs that bring actin monomers together to nucleate actin polymerization (Qualmann and Kessels, 2009; Firat-Karalar and Welch, 2011). Actin filament polymerization, coupled with filament severing, capping, and depolymerization, work together to remodel the actin cytoskeleton in response to internal or external cues in order to quickly accommodate the ever-changing needs of the cell (Brieher and Bement, 2013; Lee and Dominguez, 2010).

Actin is also transported between the cytoplasm and the nucleus (Clark and Merriam, 1977; Hofmann, 2009). Though once a topic of debate, it is now accepted that a low level of actin is present in the nucleus, mostly in the G-actin form, and is important for proper gene expression regulation (Jockusch et al., 2006). G-actin is actively transported into and out of the nucleus by the actin-specific karyopherins importin-9 and exportin-6, respectively (Stuven,

Hartmann and Gorlich, 2003; Dopie et al., 2012). Dephosphorylated cofilin binds cytoplasmic G-actin and interacts with importin-9 for actin's nuclear import, whereas phosphorylated profilin binds nuclear G-actin and interacts with exportin-6 for actin's nuclear export. Nuclear actin levels can be augmented through the phosphorylation or dephosphorylation of profilin and cofilin, through the degradation or retention of importin-9 or exportin-6 in either the nucleus or cytoplasm, or through direct actin SUMOylation, which leads to the retention of nuclear actin (Dopie et al., 2012; Dopie et al., 2015; Hofmann et al., 2009; Sathish et al., 2004).

In an uninfected cell, nuclear actin is important for nuclear architecture and cellular development, dedifferentiation, transcription, and gene expression regulation (Visa and Percipalle, 2010; Virtanen and Vartiainen, 2017; Misu, Takebayashi, and Miyamoto, 2017). Nuclear G-actin has been shown to affect gene expression through its interactions with all three RNA polymerases, RNPs, chromatin remodeling complexes, and stress response regulating factors such as MRTF (Belin and Mullins, 2013; Kapoor and Shen, 2014; Kwak et al., 2004; Misu, Takebayashi, and Miyamoto, 2017; Miyamoto et al., 2011; Serebryannyy, Cruz, and DeLanerolle, 2016; Visa and Percipalle, 2010; Weissbach et al., 2016). Further research on nuclear actin may shed light on cellular reprogramming during processes such as development and senescence (Kwak et al., 2004; Misu, Takebayashi, and Miyamoto, 2017), and may reveal novel regulatory mechanisms of cellular gene expression.

Baculovirus utilization of host actin

Baculoviruses provide an extreme example of actin subversion by a pathogen, as they use actin at almost every step of the viral replication cycle. Baculoviruses are a diverse group of large, enveloped viruses that infect mostly lepidopteran hosts, replicate in host nuclei, and contain one circular covalently-closed, supercoiled DNA genome (Rohrmann, 2013). The baculovirus replication cycle is divided into four stages: immediate early, delayed early, late, and very late (Clem and Passarelli, 2013; Rohrmann, 2013; Passarelli and Guarino, 2007; Monteiro et al., 2012). In the immediate early and delayed early stages, baculovirus must evade the host immune system and reprogram its host cells for viral replication. During the late stage, nucleocapsids are formed and escape from the host cell, and during the very late stage, virions become occluded in large proteinaceous bodies called polyhedra, which are released into the environment for insect-to-insect transmission.

The most well-studied baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), relies on actin for entry into host nuclei (Ohkawa, Volkman, and Welch, 2010), viral progeny production (Volkman, 1988; Kasman and Volkman, 2000; Goley et al., 2006; Hess, Goldsmith, and Volkman, 1989), and possibly viral egress from host cells (Ohkawa, unpublished). AcMNPV is unique among viruses in its extraordinary augmentation of nuclear actin during infection, which provides an ideal system with which to study the cellular functions of nuclear actin. In particular, the AcMNPV protein AC102 is essential for viral replication and is known to be involved in the translocation of actin to the nucleus during early infection (Ohkawa, Rowe, and Volkman, 2002; Gandhi et al., 2012). This dissertation research focuses on previously unknown functions of AC102 in nuclear actin polymerization and nucleocapsid morphogenesis during late stage infection. AC102's roles in the nuclear translocation of actin, nucleocapsid morphogenesis, and the nuclear polymerization of actin suggest that AC102 links early and late infection events through a nuclear actin-based mechanism.

History of the description and study of baculoviruses

The first accounts of baculoviruses date back to the development of the silk industry, which appeared in China almost 5000 years ago and spread to Japan by around 300 C.E. (Steinhaus, 1954; Benz, 1986; van Oers and Vlak, 2007). Historical accounts in these areas describe "silkworm jaundice", where silkworms swell, become yellowish in color, and eventually die. In the 1800s, with the invention of light microscopy, diseased silkworms were shown to contain light-refractive polyhedron-shaped particles, now known to be characteristic of many baculovirus infections.

More recently, researchers have developed AcMNPV into various biological tools. For example, modified genomes of AcMNPV are excellent protein expression vectors due to AcMNPV's extremely strong promoters (van Oers, 2006; Cox, 2012). In addition, AcMNPV can enter a multitude of cell types, spurring development of mammalian cell transduction technologies (Kost and Condreay, 2002). AcMNPV and other baculoviruses have also been used as biological pesticides (Wood and Granados, 1991; Szewczyk et al., 2006). Although these tools have contributed to many scientific endeavors including vaccine production (Cox, 2012; van Oers, 2006), the focus on biotechnology has diverted attention away from basic research on how baculoviruses interact with their host to establish an infection and replicate.

The baculovirus replication cycle

Successful viral replication depends on baculovirus' ability to control the orderly progression of its viral replication stages and processes. Baculovirus replication, like that of most large DNA viruses, is characterized by an early phase and a late phase, divided by viral DNA synthesis. As noted above, baculovirus replication can be further subdivided into immediate early, delayed early, late, and very late phases of infection (Berretta et al., 2013).

Viral entry and early gene expression

AcMNPV budded virions (BV), the viral progeny form that is responsible for cell-to-cell spread, enter the cell through endocytosis to begin infection (Rohrmann, 2013). BV envelope protein GP64 acts as a membrane fusion protein that allows for nucleocapsid escape from the acidified endosome (Long et al., 2006). The nucleocapsid then undergoes actin-based motility to reach the nucleus (Ohkawa, Volkman, and Welch, 2010), the site of viral replication.

The AcMNPV protein P78/83 is responsible for actin-based motility of nucleocapsids (Ohkawa, Volkman, and Welch, 2010). P78/83 is a nucleocapsid-associated phosphoprotein that has significant homology to actin nucleation-promoting factors (NPFs) (Vialard, 1994; Machesky and Insall, 2001). Phosphorylated P78/83 recruits and activates the host actin nucleating Arp2/3 complex, promoting the polymerization of branched actin filaments behind the nucleocapsid to propel it forward (Goley et al., 2006; Ohkawa, Volkman, and Welch, 2010; Mueller et al., 2014).

Once the nucleocapsid reaches the nucleus, it enters through nuclear pores by a mechanism that is not fully understood (Ohkawa, Volkman, and Welch, 2010; Au, Wu, and Panté, 2013). The virus then uncoats, exposing its DNA genome, which quickly forms

nucleosomes with host histones, allowing for proper viral gene expression (Wilson and Miller, 1986; Wilson and Price, 1988; Volkman, 2007).

Most early genes function to evade the host immune system, form a nuclear replication center called the virogenic stroma (VS), and reprogram the host cell for viral replication (Clem and Passarelli, 2013; Monteiro et al., 2012; Rohrmann, 2013). Immediate early viral genes are expressed immediately upon nucleosome formation and are transcribed by host RNA polymerase II (RNA pol II) (Volkman, 2015; Friesen, 1997; Berretta et al., 2013). This is possible, in part, because these genes have promoters similar to those in eukaryotes and contain cap sites, TATA box motifs, Kozak consensus sequences, and eukaryotic enhancer-like elements (Ayres et al., 1994). Delayed early genes are also transcribed by host RNA pol II, but additionally require the expression of immediate early viral proteins, such as the major viral transactivator IE-1 (Rohrmann, 2013). These factors bind baculovirus transcriptional enhancer sequences called *homologous regions* (*hrs*) to activate delayed early transcription (Guarino, Gonzalez, and Summers, 1986; Landais et al., 2006).

Nuclear localization of actin in early stage infection

During early gene expression, G-actin begins to accumulate in the nucleus. This phenomenon has been termed the Nuclear Localization of Actin (NLA). When Ohkawa, Rowe, and Volkman (2002) treated AcMNPV-infected Sf21 cells with the drug aphidicolin, which inhibits DNA synthesis and thus late viral gene expression, G-actin (GFP-actin) could be observed in the nucleus, suggesting that early gene products were responsible for the NLA phenotype. Ohkawa and colleagues uncovered a set of six early viral genes that were sufficient to induce the NLA phenotype when transfected into uninfected TN-368 insect cells. These viral genes were *ie-1*, *pe38*, *he65*, *ac004*, *ac102*, and *ac152*. Two of these genes, *ac102* and *he65*, were interchangeable in the virus-free transfection system. However, when *ac102* and *he65* were later investigated in the context of viral infection, *ac102* was found to be essential for viral replication (as evidenced by the lack of infectious particle production when *ac102* knock-out viral DNA is transfected into cells), whereas *he65* was determined to be expendable (as evidenced by no observable differences in the kinetics of BV replication for *he65* knockout viruses as compared to wild-type viruses) (Gandhi et al., 2012). Another one of these genes, *ac152*, was only necessary for the expression of *ac102* in transfected cell experiments and was not necessary when *ac102* expression was driven by a heterologous promoter (Ohkawa, Rowe, and Volkman, 2002). Additionally, *ac102* or *he65* had to be expressed one day after heterologously expressed *ie-1*, *pe38*, and *ac004* to produce the NLA phenotype; co-expression of the genes together did not result in G-actin in the nucleus, suggesting that AC102 plays a role at the end of the process leading to NLA and that temporal regulation is important for this phenotype. Gandhi *et al.* (2012) also showed that in the context of viral infection, *ac102* was the only NLA gene that was necessary for G-actin accumulation in the nucleus, though deletion of *pe38* delayed NLA.

While it is known that NLA is a prerequisite for nuclear F-actin polymerization during late-stage infection, a process that is critical for BV formation (Kasman and Volkman, 2000; Goley et al., 2006; Hess, Goldsmith, and Volkman, 1989), nothing is known about the role that this phenomenon plays in early viral infection. Nuclear G-actin is known to play roles in cellular reprogramming and the regulation of gene transcription in uninfected cells (Belin and Mullins, 2013; Kapoor and Shen, 2014; Kwak et al., 2004; Misu, Takebayashi, and Miyamoto, 2017;

Miyamoto et al., 2011; Serebryannyy, Cruz, and DeLanerolle, 2016; Visa and Percipalle, 2010; Weissbach et al., 2016; Olave, Reck-Peterson, and Crabtree, 2002; Morrison and Shen, 2009; Bartholomew, 2013). This could very well be the role of nuclear G-actin in early infection, as cellular reprogramming is a critical process during early viral infection. However, this hypothesis has yet to be tested.

Viral genome replication and the transition to late infection

The transition to the late phase of infection is marked by and requires viral genome replication. Several delayed early genes are late expression factors, which are essential or stimulatory to viral DNA synthesis (Todd, Passarelli, and Miller, 1995). The essential factors consist of virally-encoded DNA polymerase (*dnapol*), helicase (*p143*), primase (*late expression factor-1* or *lef-1*), primase accessory factor (*lef-2*), single stranded DNA binding protein (*lef-3*), and the major transactivator IE-1 (*ie-1*) (Kool et al., 1994; Lu and Carstens, 1991; Lu and Miller, 1995). The expression and buildup of these factors lead to high levels of viral genome replication, which in turn allow for the expression of late genes (Rohrmann, 2013).

Late gene expression

Unlike other DNA viruses, baculoviruses utilize their own virally-encoded RNA polymerase to transcribe late genes. This viral RNA polymerase is a four-subunit protein complex that has limited sequence similarity with eukaryotic and prokaryotic RNA polymerase subunits (Broyles and Moss, 1986; Carstens, Lu, and Chan, 1993; Gross and Shuman, 1998; Guarino et al., 1998; Partington et al., 1990; Passarelli, Todd, and Miller, 1994; Titterington, Nun, and Passarelli, 2003; Todd et al., 1996). Viral RNA polymerase binds to different promoter sequence motifs than host RNA pol II, which allows for selective transcription of viral late genes. Utilizing a virally-encoded divergent RNA polymerase is also thought to allow for host transcriptional shut-down without affecting late gene expression (Rohrmann, 2013; Nobiron, 2003). Most late genes are driven by promoters that contain a TAAG motif that is recognized by the virally-encoded RNA polymerase, and encode virion-associated structural proteins that are highly expressed, providing the building blocks for viral assembly.

Nucleocapsid structure and morphogenesis

Nucleocapsid morphogenesis occurs in the intrastromal regions of the VS, which forms a complex, lobed structure in the center of the nucleus that is filled with viral DNA and functions as the viral replication center (Vanarsdall, Mikhailov, and Rohrmann, 2007). Assembled nucleocapsids consist of a cap-like apical structure, a cylindrical sheath, and a claw-like basal structure (Fraser, 1986; Wang et al., 2016; Federici, 1986; Pearson et al., 1988; Thiem and Miller, 1989).

The cap-like structure, located on the apical end of the nucleocapsid, is pointed and is thought to be involved in DNA packaging (Fraser, 1986). During nucleocapsid morphogenesis and packaging, nucleocapsid precursors of nearly full length associate with the DNA-containing virogenic stroma on their cap-like apical end, where they undergo DNA packaging and maturation (Fraser, 1986). Because empty nucleocapsid precursors exist, it has been proposed that DNA is packaged into these nucleocapsid precursors, which is an energetically unfavorable

process that requires an ATP-driven motor in all known DNA viruses that package DNA in this way (Aathavan et al., 2009; Ortega, Gaussier, and Catalano, 2007; Smith et al., 2001). While it is theorized that a motor involved in baculovirus DNA packaging would interact with the apical cap, no such motor has yet been discovered. Alternatively, it is possible that nucleocapsid proteins assemble around the viral genome to package nucleocapsids. Variation in the length of nucleocapsid sheaths can be seen when the genome size is increased or decreased, suggesting that the sheath architecture of capsid structures can be adjusted to accommodate larger or smaller genomes (Fraser, 1986; Kool et al., 1994). This may support the theory of capsid assembly around the viral genome, as remodeling of preformed nucleocapsid length seems unlikely, and would be energetically intensive, perhaps requiring remodeling machinery such as AAA+ ATPases (Snider, Thibault, and Houry, 2008; Scheele et al., 2011).

The nucleocapsid sheath is made up of the major capsid protein VP39 and assembles into a "helical spring" structure (Wang et al., 2016; Pearson et al., 1988; Thiem and Miller, 1989). The basal claw-like structure of the nucleocapsid contains a complex of proteins consisting of P78/83, C42, and EC27. Yeast-two-hybrid studies show that C42 can directly bind EC27 and P78/83, but that P78/83 and EC27 do not directly bind each other (Braunagel et al., 2001). Additionally, native gel electrophoresis and Western blotting show that C42, EC27, and P78/83 exist in two complexes: one of ~180 kDa and one large complex of ~500-600 kDa (Braunagel et al., 2001). It remains unclear what other proteins interact with C42, EC27, and P78/83 in these complexes. P78/83 promotes actin-based motility (discussed above) as well as late-stage nuclear F-actin assembly (discussed below), which are essential processes in baculovirus entry and BV production, respectively (Ohkawa, Volkman, and Welch, 2010; Goley et al., 2006). C42 binds P78/83 and has a nuclear localization signal, allowing it to import bound P78/83 into the nucleus for nucleocapsid assembly (Wang et al., 2008). The function of EC27 in this complex is not well understood, however, it has been shown to have two differing forms: a 27 kDa form translated from the predicted coding DNA sequence and a larger 35 kDa form that is a product of ribosomal frameshifting during translation, leading to an EC27-E18 fusion protein (Braunagel et al., 1998; Braunagel et al., 1996). BV only contain the 35 kDa form of EC27 (Braunagel et al., 1998; Braunagel et al., 1996; Engelking, 2000; Hou et al., 2012; McCarthy and Theilmann, 2008).

As one might expect, the formation and functionality of the basal region is essential for nucleocapsid assembly. For example, viruses containing knockouts of C42 or EC27 do not produce viral progeny and instead form tubular electron-lucent capsid-like structures in the nucleus (Vanarsdall, Mikhailov, and Rohrmann, 2007), and viruses containing a mutated version of P78/83 cause a 100-fold reduction in viral progeny and produce aberrantly long nucleocapsids (Goley at al., 2006). Though the basal region is known to be essential to nucleocapsid morphogenesis, it has never been isolated from the capsid sheath for further study, and thus, all members of the complex may not have been discovered. My research has revealed that viral protein AC102 is a previously unidentified member of the basal structure protein complex, as described in Chapter 2. In light of AC102's known role in NLA, new questions emerge about the link between nuclear actin and nucleocapsid morphogenesis. How AC102 connects these phenomena and/or orchestrates their coordination is a question worthy of study.

Egress from the host cell

After viral assembly, nucleocapsids escape the nucleus and bud from the plasma membrane through a mechanism that is not fully elucidated. Microtubule transport has been proposed as a mechanism for viral egress (Biswas, Blissard, and Theilmann, 2016; Fang, Nie, and Theilmann, 2009), however, microtubules are not necessary for BV production (Loy E. Volkman and Zaal, 1990), indicating that other critical players must be involved in this process.

Nuclear F-actin in late stage infection

During late phase viral replication, the G-actin brought into the nucleus during NLA is polymerized into nuclear F-actin. This nuclear F-actin mostly resides in the ring zone (RZ) of the nucleus, though some can be seen in the VS (Charlton and Volkman, 1991). Similar to the actin polymerization necessary for actin-based motility, nuclear F-actin polymerization requires the activity of P78/83, which recruits and activates the host Arp2/3 complex to form dynamic, branched actin networks within the nucleus (Goley et al., 2006). The dynamic nature of these networks is essential for BV production, as treatment of infected cells with drugs that either stabilize or destabilize actin leads to an abolition of BV production (Kasman and Volkman, 2000; Goley et al., 2006; Hess, Goldsmith, and Volkman, 1989).

Nuclear F-actin may also play a role in nucleocapsid morphogenesis. Treatment of AcMNPV infected cells with cytochalasin D, an F-actin binding drug that promotes actin depolymerization (Cooper, 1987; Brieher and Bement, 2013), results in no nucleocapsid production. Instead, tubular electron-lucent capsid-like structures are formed in the RZ of the nucleus, and the virogenic stroma appears relaxed or decondensed, with no properly formed electron-dense stroma (Volkman, 1988; Hess, Goldsmith, and Volkman, 1989). However, it was later shown by Oppenheimer and Volkman (1995) that cytochalasin D also leads to the degradation of unphosphorylated P6.9, the viral basic protein that binds viral genomes to prepare them for packaging into capsids, suggesting that it might be a lack of proper viral genome packaging that leads to improper nucleocapsid morphogenesis. Indeed, treating infected cells with other actin-perturbing drugs such as latrunculin B, a drug that binds monomeric actin and prevents incorporation into the nucleocapsid, or jasplakinolide, a drug that stabilizes actin filaments, leads to normal nucleocapsid formation, but no egress out of the nucleus, resulting in no BV production (Goley et al., 2006; Ohkawa, unpublished). Interestingly, however, Ohkawa and Volkman (1999) later showed that infected cells expressing cytochalasin D-resistant actin were able to make wildtype levels of viral progeny, confirming that cytochalasin D does have an actin-related effect on nucleocapsid morphogenesis.

Does nuclear F-actin affect nucleocapsid formation? My research, described in Chapter 2, shows that AcMNPV containing a mutant version of AC102 leads to significantly reduced nuclear F-actin, a 10-fold reduction in BV production, and the formation of capsid-like tubular structures in the nucleus. This indicates that, at least in the case of perturbing AC102 function, a defect in nucleocapsid morphogenesis correlates with reduced nuclear F-actin. Whether a causal relationship exists, however, remains to be determined.

Nuclear F-actin in late stage infection may also serve other purposes. For example, most viruses use microtubules for transport in entry and escape from the host cell (Greber and Way, 2006). Baculoviruses, however, do not require microtubules to produce BV (Volkman and Zaal, 1990), suggesting other means of entry and escape. Dynamic F-actin is known to drive entry of

nucleocapsids into the nucleus (Ohkawa, Volkman, and Welch, 2010) and is therefore also a tempting candidate for nucleocapsid egress, especially given the presence of P78/83-dependent dynamic nuclear F-actin in the nucleus during the timeframe for nuclear escape.

Very late stage infection

In the very late stages of infection, nucleocapsid egress comes to a halt, and nucleocapsids become trapped inside of the nucleus. During AcMNPV infection, trapped nucleocapsids attach to and line up on elongated pieces of nuclear envelope-derived membrane, which eventually wrap around them to form enveloped virions with multiple nucleocapsids (Fraser, 1986; Shi et al., 2015). These virions are called occlusion-derived viruses (ODV) because they become occluded in paracrystalline, polyhedron-shaped structures called occlusion bodies or polyhedra. Occlusion bodies are comprised mostly of the very late protein polyhedrin, encoded by the *polh* gene, and are released into the environment once the cell and insect tissues are broken down, where they can stably persist for long periods of time before being ingested by another insect (Jaques, 1975).

Very late genes generally function to form the ODV-rich occlusion bodies and breakdown insect tissues for occlusion body release into the environment. Very late gene promoters are similar to late promoters, but contain an additional A+T rich "burst sequence" between the late TAAG motif and the translation initiation codon (Passarelli and Guarino, 2007; Rankin, Ooi, and Miller, 1988). Hyperexpression from the very late promoters of *polh* and *p10* has facilitated the development of excellent protein expression vectors from the AcMNPV genome (Cox, 2012). In addition, the late protein VLF-1 is known to be essential for the potent expression of very late genes and acts as a transcriptional activator by binding to very late promoter burst sequences (Todd et al., 1996; Yang and Miller, 1998; Yang and Miller, 1999).

VLF-1 is also a structural protein and associates with one end of the nucleocapsid (Braunagel et al., 2003; Vanarsdall et al., 2006; Li et al., 2005; Yang and Miller, 1998). Interestingly, knockout viruses that do not contain VLF-1 do not produce BV and instead form electron-lucent tubular structures in the nucleus of cells (Vanarsdall, Okano, and Rohrmann, 2004; Vanarsdall et al., 2006). These structures are similar, if not identical, to the tubular structures seen in infected cells treated with cytochalasin D (described above) and in cells infected with viruses lacking certain structural proteins (described below).

The transition from late to very late stage infection seems to correlate with proper nucleocapsid formation, though a causal relationship has not been established. A variety of null viruses that lack nucleocapsid-associated proteins, such as C42, EC27, 49K, 38K, VP1054, AC53, PK-1, and VLF-1 (Guan et al., 2016; Liang et al., 2013; Marek et al., 2013; Vanarsdall et al., 2006; Vanarsdall, Peason, and Rohrmann, 2007; Wu et al., 2006; Wu et al., 2008), do not produce BV, and electron micrographs of transfected cells reveal that they do not have properly formed nucleocapsids and instead contain electron-lucent tubular capsid-like structures. In addition, cells transfected with several of these null mutants lack polyhedra (Liang et al., 2013; Liu et al., 2008; McLachlin and Miller, 1994; Wu et al., 2006). Clearly, a link between nucleocapsid formation and very late infection exists, but has not yet been fully described.

The AcMNPV replication cycle is complex, and there is still much to be learned about its coordination and control. Several links between infection processes are known but not fully understood. For example, NLA is a critical prerequisite for nuclear F-actin polymerization, F-actin polymerization is necessary for BV production, and nucleocapsid morphogenesis is linked

to the transition to very late-stage infection (Charlton and Volkman, 1991; Goley et al., 2006; Liang et al., 2013; Liu et al., 2008; McLachlin and Miller, 1994; Ohkawa and Volkman, 1999; Ohkawa, Rowe, and Volkman, 2002; Volkman, 1988; Wu et al., 2006). Future research will allow us to form a more complete picture of how the multitude of viral processes are regulated and coordinated during AcMNPV infection.

AC102, an understudied protein that is necessary for NLA, nucleocapsid morphogenesis, nuclear F-actin assembly, and the transition to very late stage infection

This dissertation focuses on the viral protein AC102. AC102 is a small 13 kDa protein that is essential for viral replication (Gandhi et al., 2012). AC102 is highly conserved in all alphabaculoviruses except for one (see Chapter 2, Figure 2.2). However, it shares no significant homology with non-baculovirus proteins and has no recognizable sequence motifs (Gandhi et al., 2012; Ohkawa, Rowe, and Volkman, 2002), making it difficult to predict how AC102 functions or what processes it may be involved in. AC102 facilitates the nuclear localization of G-actin (NLA) during early AcMNPV infection and is essential for viral replication and nuclear F-actin polymerization, though it remains unclear if AC102 has a direct effect on nuclear F-actin assembly or is only important in NLA as a prerequisite to the polymerization of nuclear F-actin.

In Chapter 2, I describe my discovery that AC102 is a previously unreported nucleocapsid protein that belongs to the P78/83-C42-EC27-AC102 viral complex, is essential for proper nucleocapsid morphogenesis, affects F-actin assembly during late infection, and is necessary for the transition to very late phase infection. Future implications of AC102's role in these processes are addressed in Chapter 3.

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CHAPTER 2

AC102 is a member of the P78/83-C42-EC27-AC102 nucleocapsid complex and is important for nucleocapsid morphogenesis and nuclear F-actin formation during late-stage AcMNPV infection

INTRODUCTION

Baculoviruses are a diverse group of enveloped viruses that primarily infect lepidopteran insects. They contain circular DNA genomes that are replicated in the nucleus of host cells (Rohrmann, 2013). The type species of alphabaculoviruses, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), is commonly used as a vector for protein expression and mammalian cell transduction, and as a biological pesticide (van Oers, 2006; Kost and Condreay, 2002; Wood and Granados, 1991; Szewczyk et al., 2006). Although baculoviruses have been studied since the 1940s (Bergold and Brill, 1942; Bergold, 1947; Bergold, 1963), many questions remain regarding the mechanisms by which they mobilize and exploit host cell machinery during infection, and how strains with improved properties can be engineered.

AcMNPV is also among a group of diverse microbial pathogens that target the host cell actin cytoskeleton to enable infection (Welch and Way, 2013; Haglund et al., 2010). AcMNPV is unique, however, in the extent to which it uses host actin at all stages of its replication cycle. The AcMNPV replication cycle is divided into immediate early, delayed early, late, and very late stages (Clem and Passarelli, 2013; Rohrmann, 2013; Passarelli and Guarino, 2007; Monteiro et al., 2012). The immediate early phase begins when enveloped viruses fuse with the plasma membrane or are endocytosed and fuse with the endosomal membrane, resulting in the release of viral nucleocapsids into the host cell cytoplasm. AcMNPV then undergoes actin-based motility, which speeds transit to the nucleus, where replication occurs (Ohkawa, Volkman, and Welch, 2010). Immediate and delayed early genes are then expressed to reprogram the host cell for viral replication (Rohrmann, 2013). Expression of early genes also induces the translocation of monomeric actin (G-actin) into the cell's nucleus, a phenomenon referred to as nuclear localization of actin (NLA) (Ohkawa, Rowe, and Volkman, 2002). Interestingly, NLA can also be induced in uninfected cells by expressing only five viral genes: *ie-1*, *pe38*, *ac004*, *ac152*, and either *ac102* or *he65* (Ohkawa, Rowe, and Volkman, 2002). Of these, *ac102* is of particular interest because it must be expressed one day after expression of the others, and is essential for viral replication and the induction of NLA, suggesting it may be an essential effector of the NLA phenomenon. Importantly, the ability of AcMNPV to induce NLA sets it apart from other microbes that manipulate actin, and suggests that studying this phenomenon represents an opportunity to understand how pathogens can affect the shuttling of actin between the cytosol and nucleus.

AcMNPV also mobilizes actin during the late stage of infection, which begins as viral DNA replication commences. In this stage, virion-associated structural proteins are expressed. Viral nucleocapsids containing the DNA genome and capsid components are then assembled in a replication structure called the virogenic stroma (VS), which is located in the center of the nucleus (Passarelli and Guarino, 2007; Rohrmann, 2013; Chen et al., 2013; Xue et al., 2012). During the late stage, G-actin recruited to the nucleus during early infection is polymerized into filamentous actin (F-actin), which is essential for viral progeny production (Volkman, Goldsmith, and Hess, 1987; Hess, Goldsmith, and Volkman, 1989; Charlton and Volkman, 1991; Kasman and Volkman, 2000; Goley et al., 2006). Nuclear F-actin polymerization is dependent on the activity of P78/83, an actin nucleation promoting factor (NPF) that, in its phosphorylated form, recruits and activates the host Arp2/3 complex to facilitate actin polymerization (Goley et al., 2006). Furthermore, P78/83 forms a complex with two other viral proteins: C42 (encoded by *ac101*) and EC27 (encoded by *ac144*) (Braunagel et al., 2001). This complex associates with one end of the nucleocapsid and is necessary for proper nucleocapsid formation (Russell, Funk, and

Rohrmann, 1997; Goley et al., 2006; Vanarsdall, Peason, and Rohrmann, 2007; Li et al., 2010). Progeny nucleocapsids escape the nucleus and become enveloped by budding from the plasma membrane, resulting in the formation of progeny budded virions (BV).

Finally, during very late stage infection, nucleocapsids within the nucleus acquire envelopes originating from the host nuclear envelope to form occlusion-derived virions (ODV). ODV are then occluded in proteinaceous paracrystalline structures called polyhedra, which are composed of the polyhedrin protein (encoded by *polh*) (Blissard and Rohrmann, 1990; King and Possee, 1992). The ODV-rich polyhedra are released into the environment upon liquefaction of the insect and mediate insect-to-insect transmission, completing the cycle of viral replication (Granados and Lawler, 1981; Keddie, Aponte, and Volkman, 1989).

AC102 appears to be a central player in NLA, nuclear F-actin assembly, and BV production (Ohkawa, Rowe, and Volkman, 2002; Gandhi et al., 2012). However, we do not yet understand how AC102 may link together early and late actin-dependent processes. In this study, we further investigate AC102's role during infection. We make the surprising observation that AC102 is primarily expressed late in infection. In addition, we observed that AC102 is required for F-actin polymerization during late infection, as well as for VS morphology and nucleocapsid morphogenesis. Finally, we discovered that AC102 is a nucleocapsid protein that associates with the P78/83-C42-EC27 nucleocapsid complex. Taken together, our findings indicate that AC102 is a previously unidentified member of the P78/83-C42-EC27-AC102 nucleocapsid complex, and is therefore necessary for nucleocapsid morphogenesis and P78/83-dependent F-actin assembly during late infection.

RESULTS

AC102 is predominantly expressed late in infection

Although AC102 was presumed to be an early gene based on its activity in promoting the NLA phenotype, the timing of AC102 expression had not previously been investigated. We therefore began by determining the temporal expression profile of AC102 by Western blotting over a range times post infection with the wild type AcMNPV strain WOBpos (Goley et al., 2006), using a polyclonal antibody we generated that specifically recognizes AC102 (**Fig. 2.1A**). We used a finer time resolution in the window between 8-16 hours post infection (hpi), a time period that includes the transition between early and late infection (Friesen and Miller, 1985; Passarelli and Guarino, 2007). Surprisingly, at early time points (0, 8, and 10 hpi; **Fig. 2.1A**), no detectable AC102 was present. Expression of AC102 was first detected at 12 hpi and protein continued to accumulate through 36 hpi (**Fig. 2.1A**). The timing of AC102 expression matched that of VP39, the major capsid protein and a tightly regulated late factor (Friesen and Miller, 1985; Thiem and Miller, 1989; Todd et al., 1996; Passarelli and Guarino, 2007).

To confirm a lack of significant AC102 expression during early gene expression, infected cells were treated with aphidicolin, a drug that inhibits DNA synthesis and thus prevents the transition from early to late infection. No AC102 was detected by Western blotting after aphidicolin treatment (**Fig. 2.1B**). These data indicate that AC102 is predominantly expressed after the transition to late gene expression.

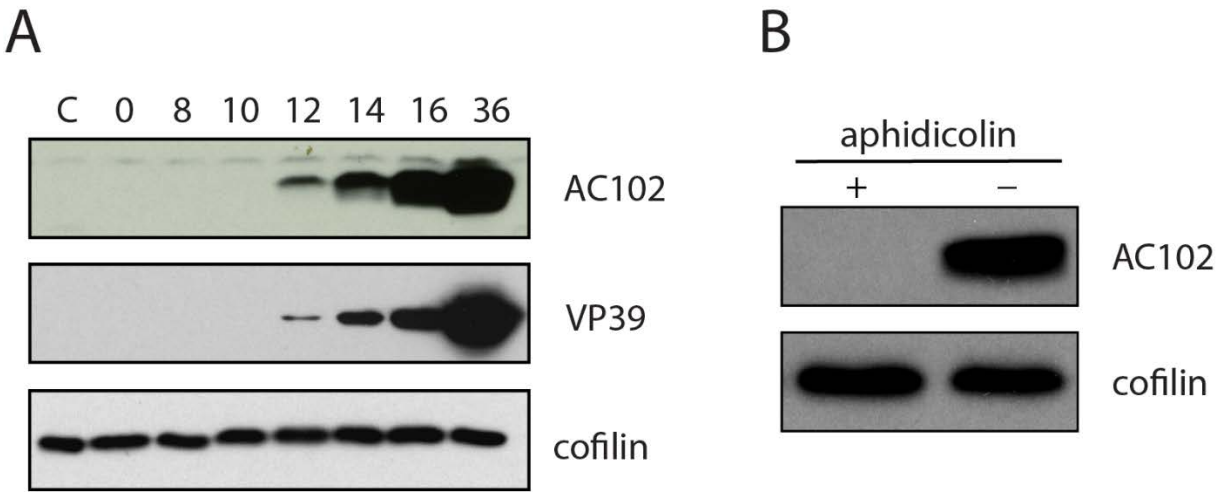


Figure 2.1: AC102 is predominantly expressed late in infection. (A) Western blots of lysates from uninfected Sf9 control cells (labeled 'C') and cells infected with WOBpos at an MOI of 10 at various times post infection, probed for AC102, VP39 (as a late expression control), and cofilin (as a loading control). (B) Western blots of infected cells treated with 5 µg/ml aphidicolin (+) or DMSO control (-) probed for AC102 and cofilin (as a loading control).

The AC102-K66A mutation results in 10-fold reduced viral titers and a small plaque phenotype

Given that AC102 is primarily expressed late in infection, we next sought to investigate the late function(s) of AC102, as its precise role had not yet been investigated. Because *ac102* is an essential gene (Lu et al., 1996; Gandhi et al., 2012), it is not possible to produce virus carrying a null mutation. Therefore, to further investigate the function(s) of AC102, we generated ten mutant viruses, each carrying one of the following point mutations in AC102: N47A, T52A, A55V, D61A, K66A, S77A, A80V, L96A, L105A, or N114A. These amino acid residues were chosen because they are highly conserved between orthologs of AC102 in diverse alpha baculoviruses (**Fig. 2.2**).

Growth of the mutants was initially assessed by infecting cells at MOI of 10 and then measuring viral titer at 18 or 48 hpi (**Table 1**). One virus containing the AC102-D61A mutation did not produce any detectable progeny. Most others exhibited modest 2- to 5-fold reductions in viral titer compared with wild type WOBpos at one or both time points. The AC102-K66A mutant virus, in contrast, produced 10-fold fewer progeny than WOBpos at both time points, and was therefore chosen for further analysis. In a more comprehensive one-step growth curve, the AC102-K66A mutant produced 10-fold fewer progeny at all late time points tested (18, 24, 36, and 48 hpi; **Fig. 2.3A**, left panel). To assess whether the AC102-K66A mutation was the cause of the growth defect and confirm there were no polar effects on other genes, an AC102-K66A-rescue virus was generated by inserting a wild type copy of *ac102* into the bacterial replication cassette within the AC102-K66A bacmid (Goley et al., 2006). The growth kinetics of the AC102-K66A-rescue virus were indistinguishable from those of WOBpos at all time points tested (**Fig. 2.3A**, right panel). Thus, the K66A mutation is the cause of the growth defect in the mutant virus.

In addition to the replication defect, the AC102-K66A mutant virus also produced plaques that were typically only one or a few cells large, considerably smaller than those produced by WOBpos (**Fig. 2.3B**). Quantification of plaque areas showed that AC102-K66A mutant plaques were 6-fold smaller in area than those produced by either WOBpos or AC102-K66A-rescue viruses, which were indistinguishable from one another (**Fig. 2.3C**). Taken together, these results indicate that the K66A mutation in AC102 results in a lower number of infectious viral particles escaping the host cell and a reduced capacity to spread from cell to cell in a plaque assay.

The AC102-K66A mutation results in lower AC102 expression and reveals a role for AC102 in maintaining a condensed virogenic stroma

To further investigate the nature of the defect caused by the AC102-K66A mutation, we compared the relative levels of AC102 and AC102-K66A expression over the course of infection by Western blotting (**Fig. 2.4A**). At 18, 24 and 36 hpi, the levels of AC102-K66A were significantly reduced compared with wild type AC102 at the same time points (**Fig. 2.4B**). These results indicate that the AC102-K66A mutation does not affect the onset of AC102 expression, but results in lower protein levels at all time points, likely by impacting the expression or stability of AC102.

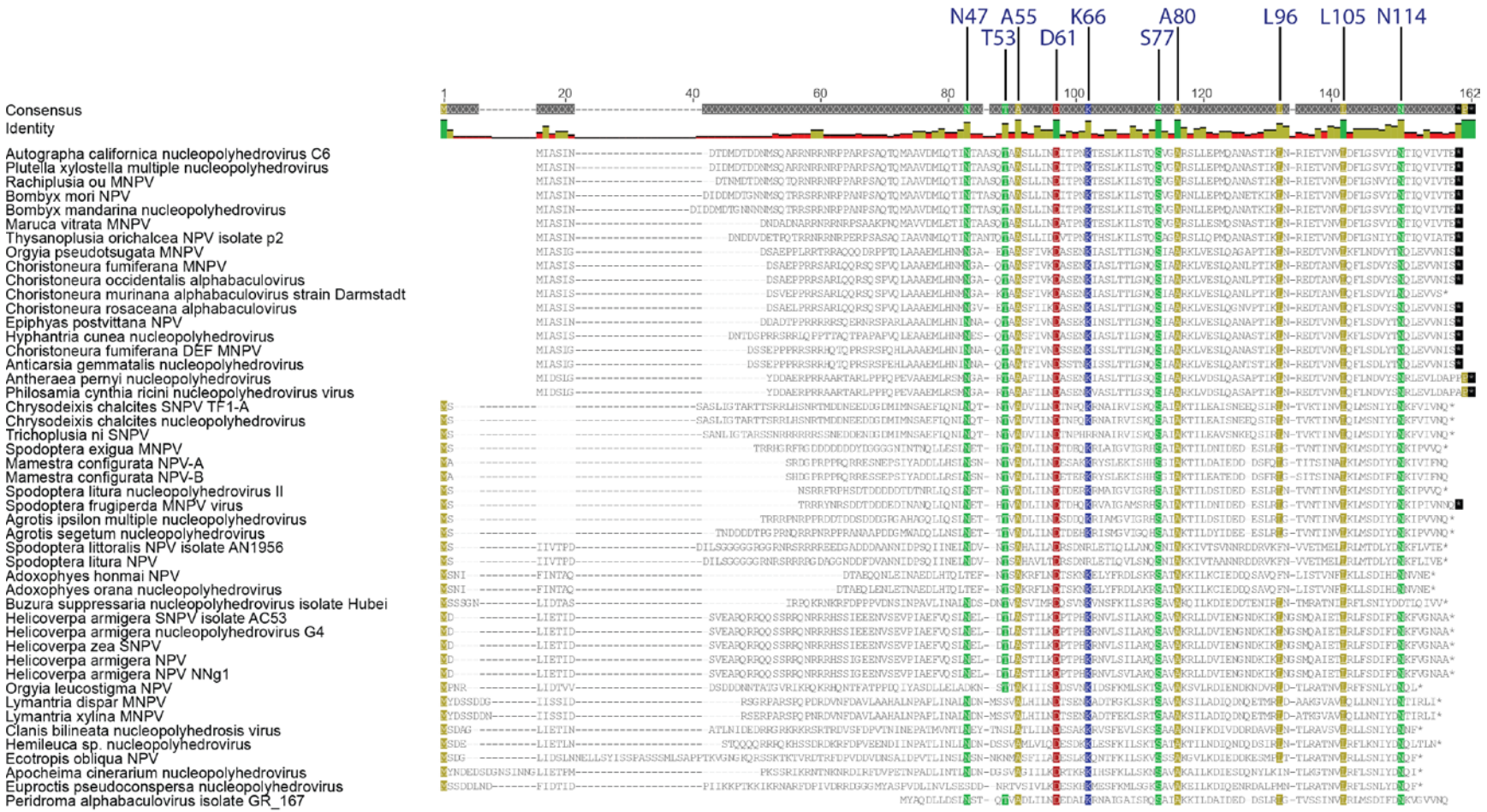


Figure 2.2: Alignment of AC102 protein sequences from alphabaculoviruses. AC102 amino acid sequences were gathered from NCBI, aligned using MAFFT, then manually edited for quality. Residues with at least 85% identity across all alphabaculoviruses are highlighted, according to their polarity as follows: yellow = non-polar, green = polar uncharged, red = polar acidic, and blue = polar basic.

Table 2.1: Titers of AC102 mutant viruses at 18 and 24 hpi

	WOBpos	AC102-N47A	AC102-T53A	AC102-A55V	AC102-D61A	AC102-K66A	AC102-S77A	AC102-A80V	AC102-L96A	AC102-L105A	AC102-N114A
18 hpi	3.49E+05	1.49E+05	3.21E+05	1.51E+05	-	3.61E+04	1.83E+05	4.44E+05	5.46E+04	3.78E+05	1.13E+05
48hpi	7.70E+07	4.80E+07	6.27E+07	3.90E+07	-	7.71E+06	7.70E+07	1.47E+07	6.30E+07	3.60E+07	2.60E+07

*AC102-D61A produced no viral progeny, and could not be amplified for titering.

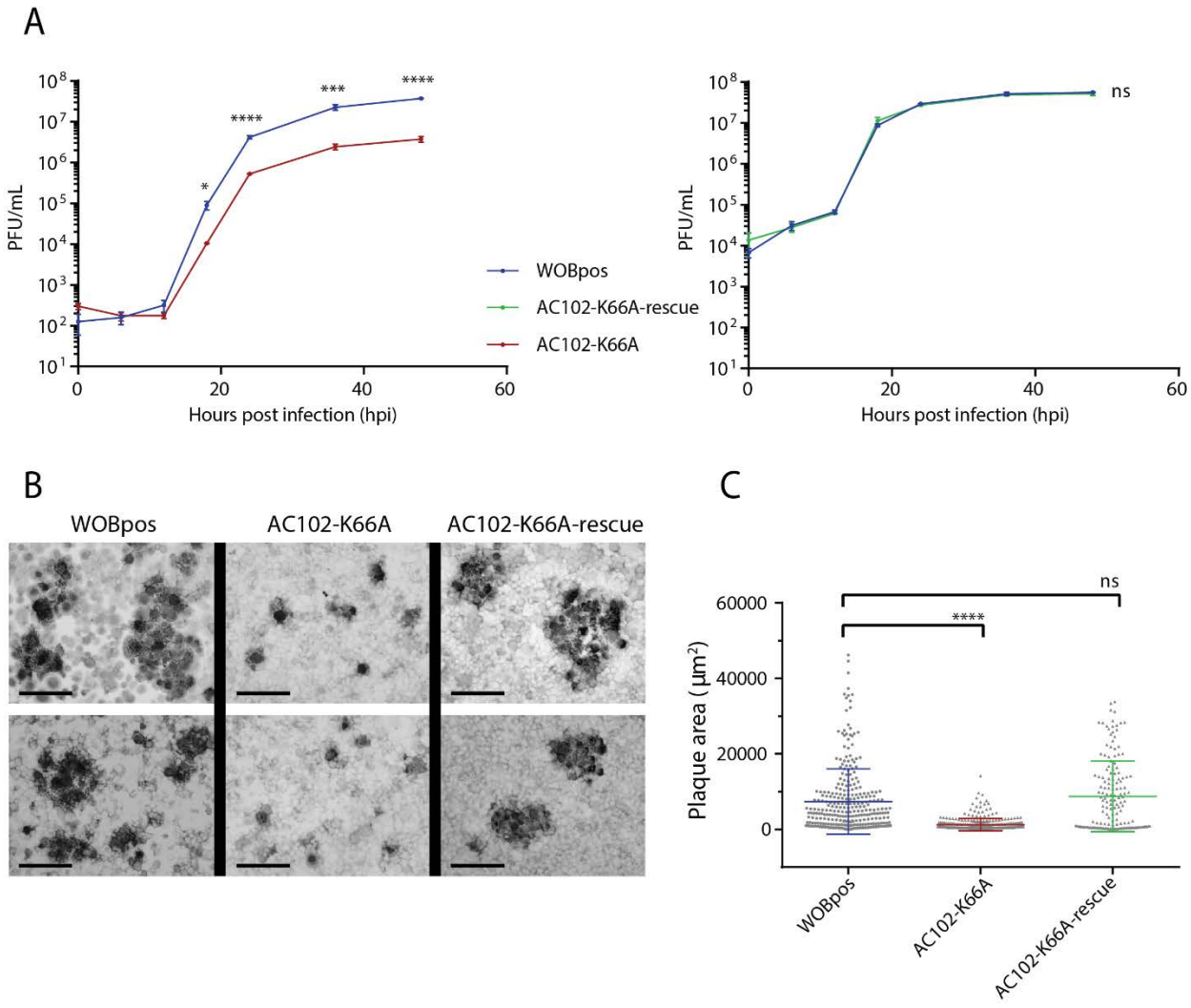


Figure 2.3: AC102-K66A mutation results in 10-fold reduced viral titers and a small plaque phenotype. (A) One-step growth curves of WOBpos and AC102-K66A viruses (left panel) or WOBpos and AC102-K66A-rescue viruses (right panel). Sf9 cells were infected at an MOI of 10 and progeny virus released into the growth media was quantified by titering. Data are the mean from three independent experiments and error bars represent SD. **(B)** Plaques resulting from WOBpos, 102-K66A, or 102-K66A-rescue viruses visualized by immunostaining for viral envelope protein GP64. Scale bars are 100 µm. **(C)** Quantification of plaque area for WOBpos (n=303), AC102-K66A (n=344), AC102-K66A-rescue (n=160). Each point represents one plaque size measurement, pooled from three independent experiments. Center bar represents the mean, and top and bottom bars represent SD. For (A) and (C), P-values are indicated as follows: ns = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$.

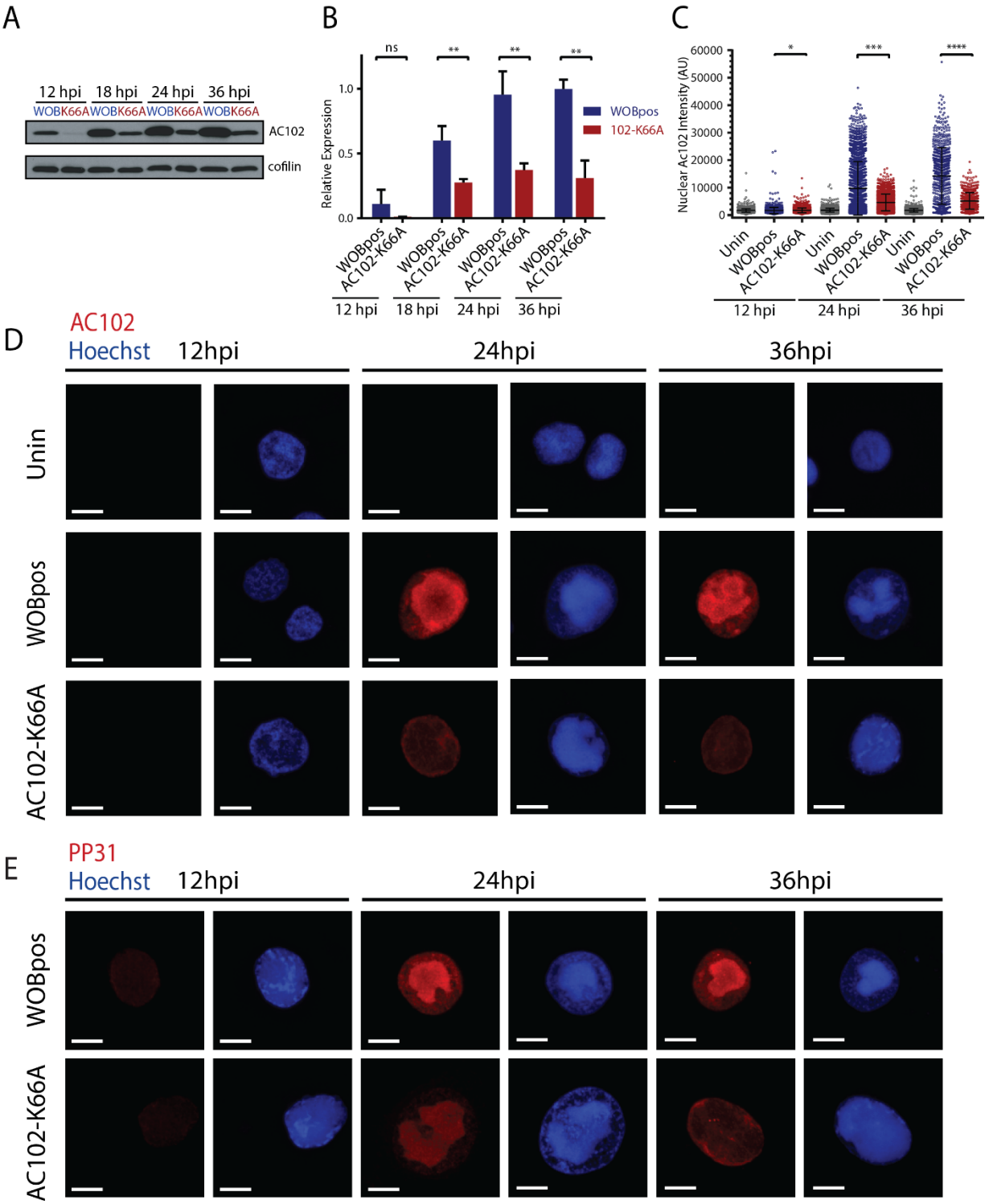

A
12 hpi
18 hpi
24 hpi
36 hpi
WOBK66A
AC102
cofilin
B
Relative Expression
WOBpos
102-K66A
ns
**
12 hpi
18 hpi
24 hpi
36 hpi
C
Nuclear Ac102 Intensity (AU)
*
***
****
Unin
WOBpos
AC102-K66A
D
AC102
Hoechst
12hpi
24hpi
36hpi
Unin
WOBpos
AC102-K66A
E
PP31
Hoechst
12hpi
24hpi
36hpi
WOBpos
AC102-K66A

Figure 2.4: The AC102-K66A mutation results in lower AC102 expression and reveals a role for AC102 in forming a condensed virogenic stroma. (A) Western blot of lysates from Sf9 cells infected with WOBpos or AC102-K66A virus at an MOI of 10, prepared at 12, 18, 24, or 36 hpi, and probed for AC102 and cofilin (as a loading control). **(B)** Quantification of AC102 protein levels from the Westerns in (A) by densitometry. Bars are the mean from three independent replicates, and error bars are SD. **(C)** Nuclear intensity of AC102 immunofluorescence staining in uninfected Sf21 cells, or cells infected with WOBpos or AC102-K66A virus at an MOI of 10, fixed at 12, 24, or 36 hpi. Bars are the mean from three independent replicates, and error bars are SD. **(D)** Sf21 cells stained for AC102 (immunofluorescence; red) and DNA (Hoechst; blue) as in (C), showing the relative localization patterns of AC102 and DNA in the nucleus. **(E)** Sf21 cells stained for PP31 (red) and DNA (blue) as in (D). For (B) and (C), P-values are indicated as follows: ns = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, and **** = $p<0.0001$. For (D) and (E), scale bars are 10 μm.

We also observed the effect of the AC102-K66A mutation on the abundance and localization of AC102 in infected cells by immunofluorescence microscopy. At 12 hpi, AC102 was not detectable in WOBpos or AC102-K66A infected cells (**Fig. 2.4C**; **Fig. 2.4D, left panels**). At 24 and 36 hpi, WOBpos-infected cells showed strong AC102 signal in the nucleus, particularly in and around the VS (as identified by a central nuclear region of intense Hoechst staining of the viral DNA). In contrast, at the same time points most AC102-K66A infected cells show a weaker AC102 signal in the nucleus with reduced staining that was limited to a thin outline of the VS (**Fig. 2.4C**; **Fig. 2.4D**, middle and right panels). In addition, the VS itself in AC102-K66A infected cells appeared to be less condensed than that of WOBpos-infected cells, with the structure taking up almost the entire nucleus (**Fig. 2.4D**, middle and right panels, see Hoechst stain). Thus, the K66A mutation causes a reduction in the amount of AC102 in the nucleus, and an apparent decondensation of the VS.

To further examine the effect of AC102-K66A on the VS, we performed immunofluorescence microscopy using an antibody against PP31, a delayed early protein marker of the VS (Guarino and Smith, 1990; Yamagishi et al., 2007). The timing of PP31 expression was not altered in AC102-K66A versus WOBpos infected cells (**Fig. 2.5A**). At 12 hpi, only dim PP31 signal could be detected in the nuclei of both AC102-K66A and WOBpos infected cells (**Fig. 2.4E, left panel**). At 24 hpi, PP31 was localized to the condensed VS in WOBpos infected cells, and to a less condensed VS in AC102-K66A infected cells (**Fig. 2.4E, middle panel**). At 36 hpi, PP31 remained localized to the VS in WOBpos infected cells, but was less abundant in the VS in AC102-K66A infected cells, instead forming a thin outline around the decondensed structure (**Fig. 2.4E, right panel**). These results confirm that the AC102-K66A mutation results in an aberrant and decondensed VS structure and an altered localization of the VS component PP31.

AC102 is important for proper VP39 assembly into nucleocapsids

We next investigated the effect of AC102-K66A on the expression and localization of the major capsid protein VP39 by immunofluorescence microscopy. As with PP31, the timing of VP39 expression was not affected by the AC102-K66A mutation (**Fig. 2.5B**). At 12 hpi, VP39 showed diffuse or punctate localization to the VS both in WOBpos and AC102-K66A infected cells (**Fig. 2.5A, left panel**). At 24 hpi, however, when WOBpos infected cells continued to show punctate or diffuse VP39 localization in the VS, the majority of AC102-K66A infected cells had VP39 in the RZ, where it often assembled into long filaments (**Fig. 2.6A, middle panel**). At 36 hpi, VP39 redistributed to a diffuse or punctate localization in the RZ in WOBpos infected cells, whereas it remained as filaments in the ring zone of AC102-K66A infected cells (**Fig. 2.6A, right panel**).

We further quantified aberrant VP39 distributions in WOBpos and AC102-K66A infected cells by dividing cells at 24 and 36 hpi into three different VP39 phenotypic classes: (1) wild type with punctate or diffuse distribution in the VS or RZ, (2) “filamentous” with long thin filaments localized in the RZ, and (3) “cable-like” with thick VP39 cables localized in the RZ. At both time points, the majority of WOBpos infected cells had wild type VP39 distribution, whereas the majority of AC102-K66A cells had a filamentous phenotype (**Fig. 2.6B-E**). Moreover, only AC102-K66A infected cells contained pronounced cable-like VP39 structures

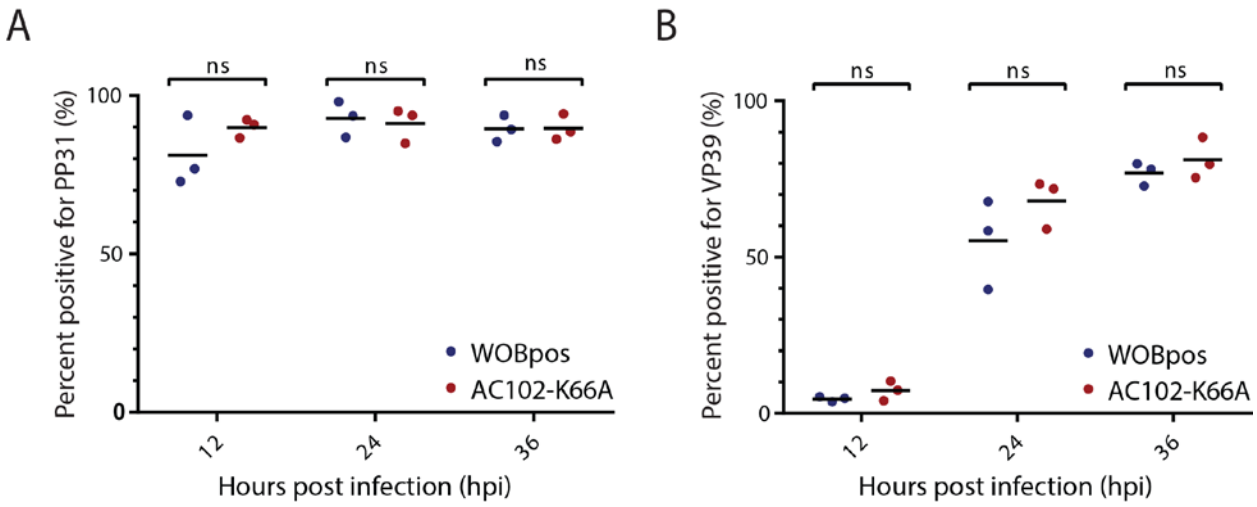


Figure 2.5: The AC102-K66A mutation does not delay the onset of early or late gene expression. (A) Quantification of the percent cells expressing the delayed early marker PP31 or **(B)** the late marker VP39. Sf21 cells were infected with WOBpos or AC102-K66A virus at an MOI of 10, fixed at 12, 24, or 36 hpi, then stained for (A) PP31 or (B) VP39. Lines indicate the mean for three independent experiments. P-values were non-significant at all time points.

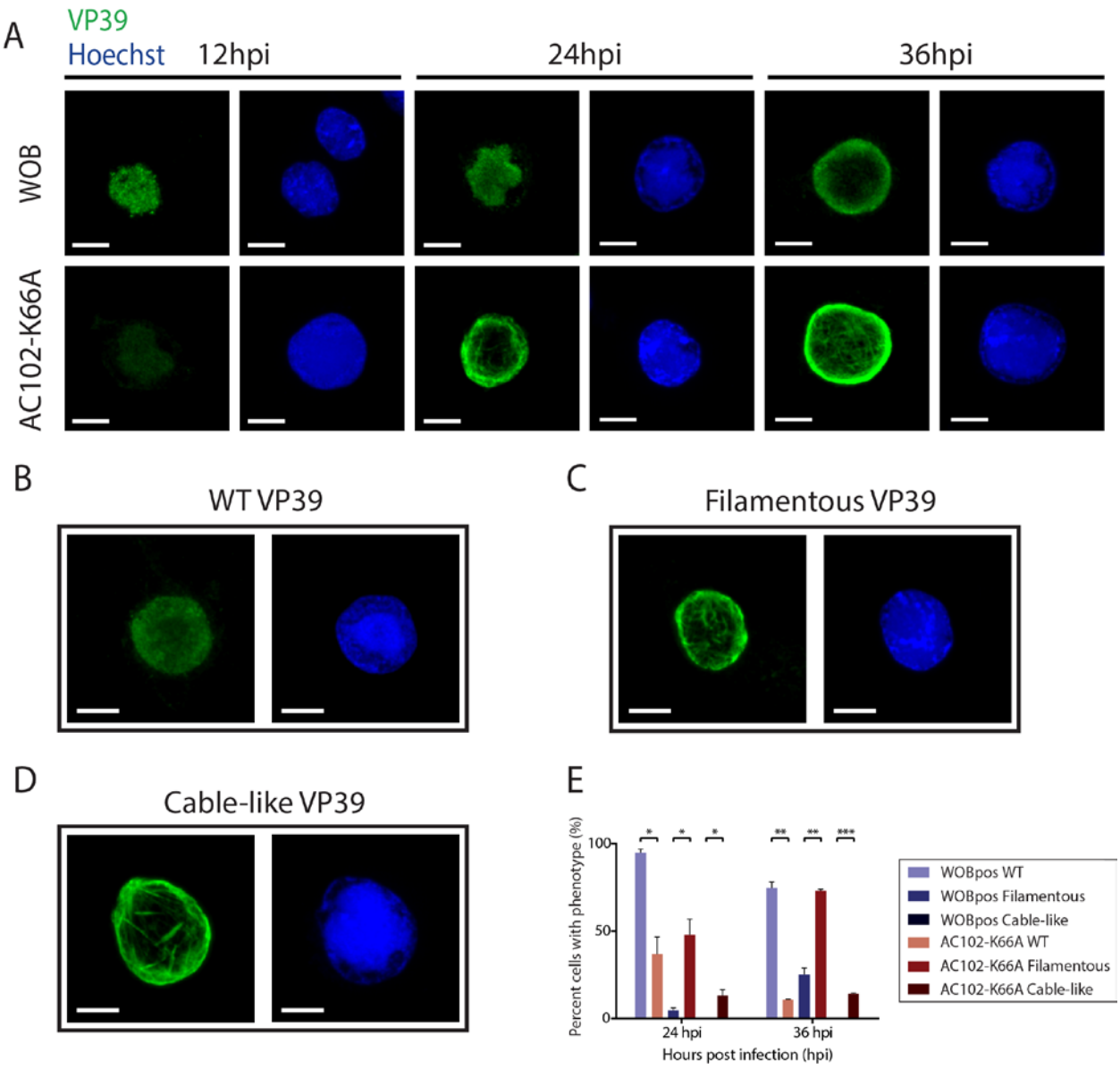


Figure 2.6: AC102 is important for proper VP39 localization in the nucleus of infected cells. **(A)** Sf21 cells stained for VP39 (immunofluorescence; green) and DNA (Hoechst; blue) following infection with WOBpos or AC102-K66A virus at an MOI of 10, and fixed at 12, 24, or 36 hpi. **(B-D)** Examples of VP39 localization patterns: **(B)** wild-type, with punctate or diffuse VP39 distribution in the VS or RZ; **(C)** "filamentous" with long and thin VP39 filaments localized in the RZ; and **(D)** "cable-like" with thick VP39 cables localized in the RZ. **(E)** Quantification of the percentage of cells with each VP39 phenotype at 24 and 36 hpi for WOBpos or AC102-K66A infected cells in (A). Data are the mean from three independent experiments. Error bars represent SD. P-values are indicated as follows: ns = non-significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. All scale bars are 10 μm.

(**Fig. 2.6B-E**). These results indicate that the AC102-K66A mutation causes aberrant assembly of VP39 into long filamentous and cable-like structures late in infection.

Electron microscopy confirms roles for AC102 in forming a condensed virogenic stroma and in proper nucleocapsid morphogenesis

We used transmission electron microscopy (TEM) to further compare intranuclear structure in WOBpos and AC102-K66A infected cells. At 24 hpi, WOBpos infected cells showed a condensed VS, composed of electron-dense lobes, and a peripheral RZ, both of which contained nucleocapsids (**Fig. 2.7A**). In contrast, AC102-K66A infected cells lacked a well-defined VS, instead containing a more amorphous and decondensed region that occupied most of the nucleus and did not contain electron-dense lobes or visible nucleocapsids (**Fig. 2.7D**). Moreover, in AC102-K66A infected cells, the peripheral RZ was densely packed with long tubular structures that were variable in length and were often bundled or clustered (**Fig. 2.7D-H**). The tubules also varied in electron density, with some being nearly electron-lucent and others having electron-dense areas indicating the packaging of viral DNA (**Fig. 2.7D-F**). Cross sections of the tubular structures showed that most regions are electron-lucent (**Fig. 2.7I**). Taken together with the VP39 localization studies, these results suggest that the tubular structures are aberrant assemblies of VP39 that are not properly formed into unit-length nucleocapsids, and are instead assembled into long tubular structures that sometimes contain viral DNA.

AC102 is crucial for nuclear actin polymerization in the ring zone during late infection.

A previous study suggested that AC102 is required for nuclear actin polymerization, as transfection of cells with a WOBpos bacmid containing a deletion of *ac102* (*AcΔ102*) caused a failure in nuclear F-actin accumulation (Gandhi et al., 2012). However, the *AcΔ102* virus does not complete a replication cycle, and thus does not necessarily capture the roles of AC102 during a viral infection. To further investigate the role of AC102 in nuclear actin polymerization, we tested the ability of the AC102-K66A virus to cause nuclear accumulation of F-actin late in infection by fluorescence microscopy. A substantial percentage of WOBpos infected cells contained nuclear F-actin (defined as a nuclear/cytoplasmic actin intensity ratio of 2 or greater) at 24 and 36 hpi (**Fig. 2.8A, top**; **Fig. 2.8B**). In comparison, substantially fewer AC102-K66A infected cells exhibited nuclear F-actin accumulation at these time points (**Fig. 2.8A, bottom**; **Fig. 2.8B**). These results confirm that AC102 is important for nuclear F-actin polymerization late in infection.

AC102 is important for polyhedrin expression and polyhedra formation very late in infection.

To complete our investigation of the roles of AC102, we sought to investigate a possible function very late in infection. We therefore assessed the timing and expression of the very late protein polyhedrin in WOBpos and AC102-K66A infected cells by Western blotting (**Fig. 2.9A**). At 18 hpi there was no detectable expression of polyhedrin in any infected cells, as expected for a very late protein (**Fig. 2.9A**). At 24 hpi, WOBpos infected cells showed strong expression of polyhedrin, whereas AC102-K66A infected cells showed low but detectable expression. At 36 hpi, polyhedrin expression was significantly higher in WOBpos infected cells than in

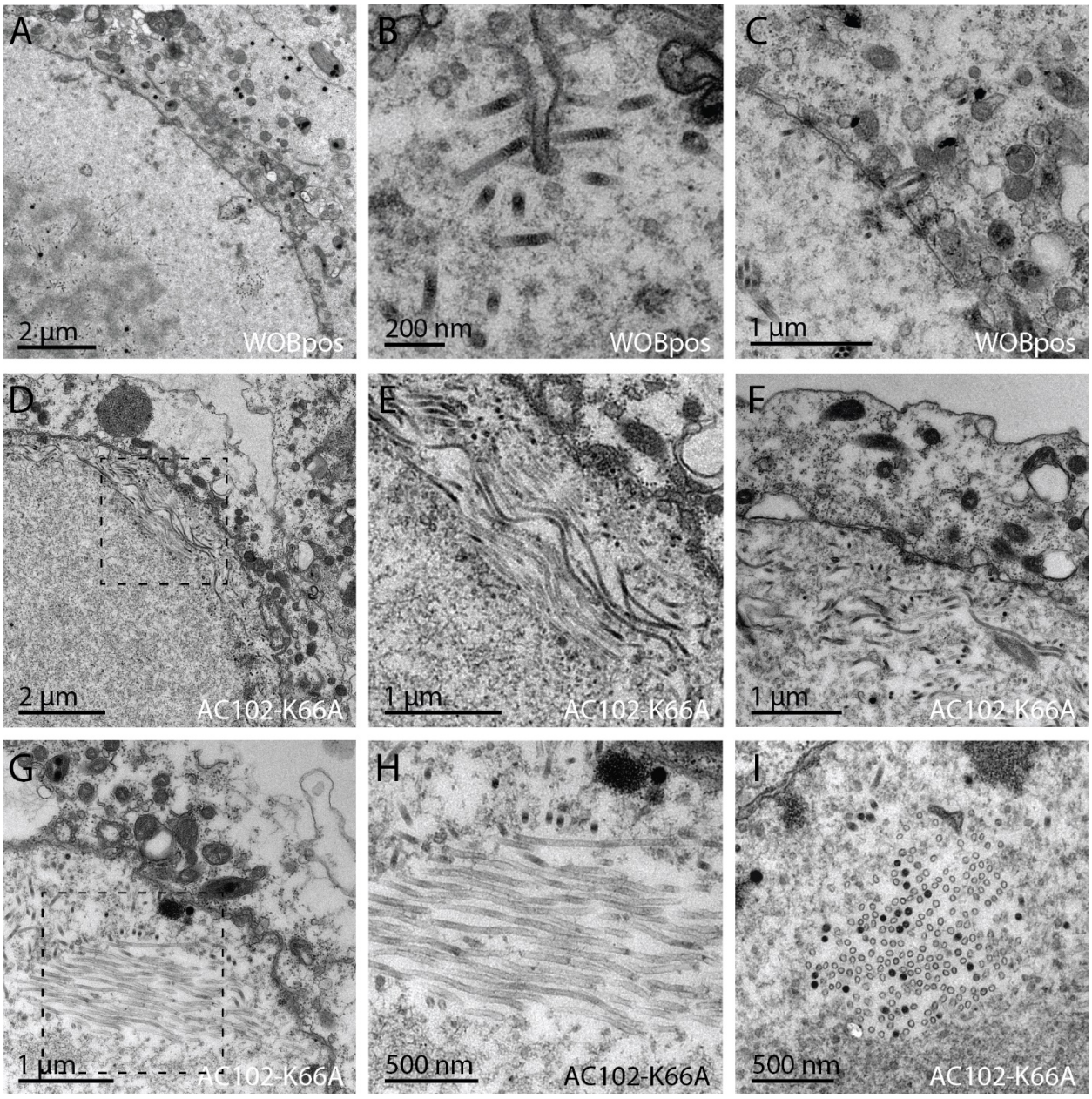

A
2 µm
WOBpos
B
200 nm
WOBpos
C
1 µm
WOBpos
D
2 µm
AC102-K66A
E
1 µm
AC102-K66A
F
1 µm
AC102-K66A
G
1 µm
AC102-K66A
H
500 nm
AC102-K66A
I
500 nm
AC102-K66A

Figure 2.7: Electron microscopy confirms roles for AC102 in proper nucleocapsid morphogenesis and virogenic stroma formation. (A-C) Electron micrographs of WOBpos infected cells and **(D-I)** AC102-K66A infected cells at 24 hpi. Sf9 cells were infected at an MOI of 10, fixed, and prepared for TEM. **(A)** A typical RZ of WOBpos infected cells, with some free and enveloped virions present, and typical VS containing virions between the well-defined electron-dense lobes. **(B)** Typical nucleocapsids, either free or in the process of nuclear envelopment during ODV formation. **(C)** Virions inside the nucleus, budding out of the nuclear membrane, and in the cytoplasm. **(D)** An AC102-K66A infected cell, showing a decondensed VS that lacks electron-dense lobes and a RZ that contains a dense packing of tubular capsid-like structures, some electron-dense and some electron-lucent. **(E)** Magnified view of the boxed region in (D). **(F)** An AC102-K66A infected cell, showing virions and tubular structures inside the nucleus, with no virions budding out of the nuclear membrane or in the cytoplasm. **(G)** Aggregates of electron-lucent tubular structures. **(H)** Magnified view of the boxed region in (G). **(I)** Cross-section of a tubular structure aggregate showing electron-lucent structures and a few electron-dense structures.

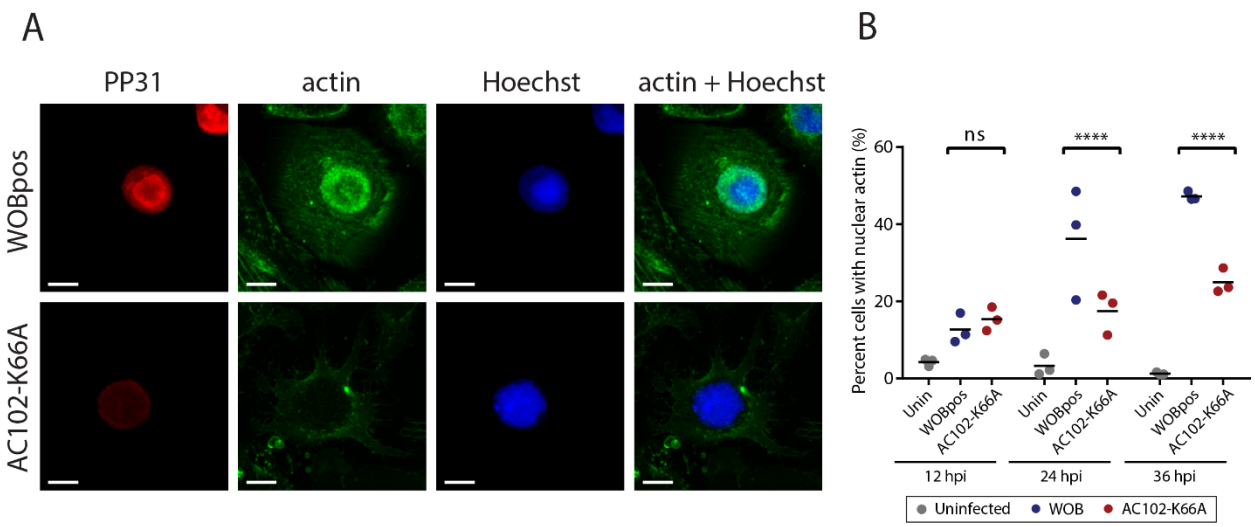


Figure 2.8: AC102 is crucial for nuclear actin polymerization in the ring zone during late infection. (A) Sf21 cells stained for PP31 (immunofluorescence; red), F-actin (Alexa Fluor-phalloidin; green) and DNA (Hoechst; blue). Sf21 cells were infected with WOBpos or AC102-K66A virus at an MOI of 10 and fixed at 36 hpi. Scale bars are 10 μm. **(B)** Quantification of the percent cells with nuclear F-actin at 12, 24, and 36 hpi, defined as cells with a nuclear to cytoplasmic F-actin intensity ratio of 2 or greater, as quantified from Alexa 488-phalloidin staining. Each point represents the average percentage of cells containing nuclear actin for one independent experiment. Lines indicate the means for three independent experiments. P-values are indicated as follows: ns = non-significant and **** = p<0.0001.

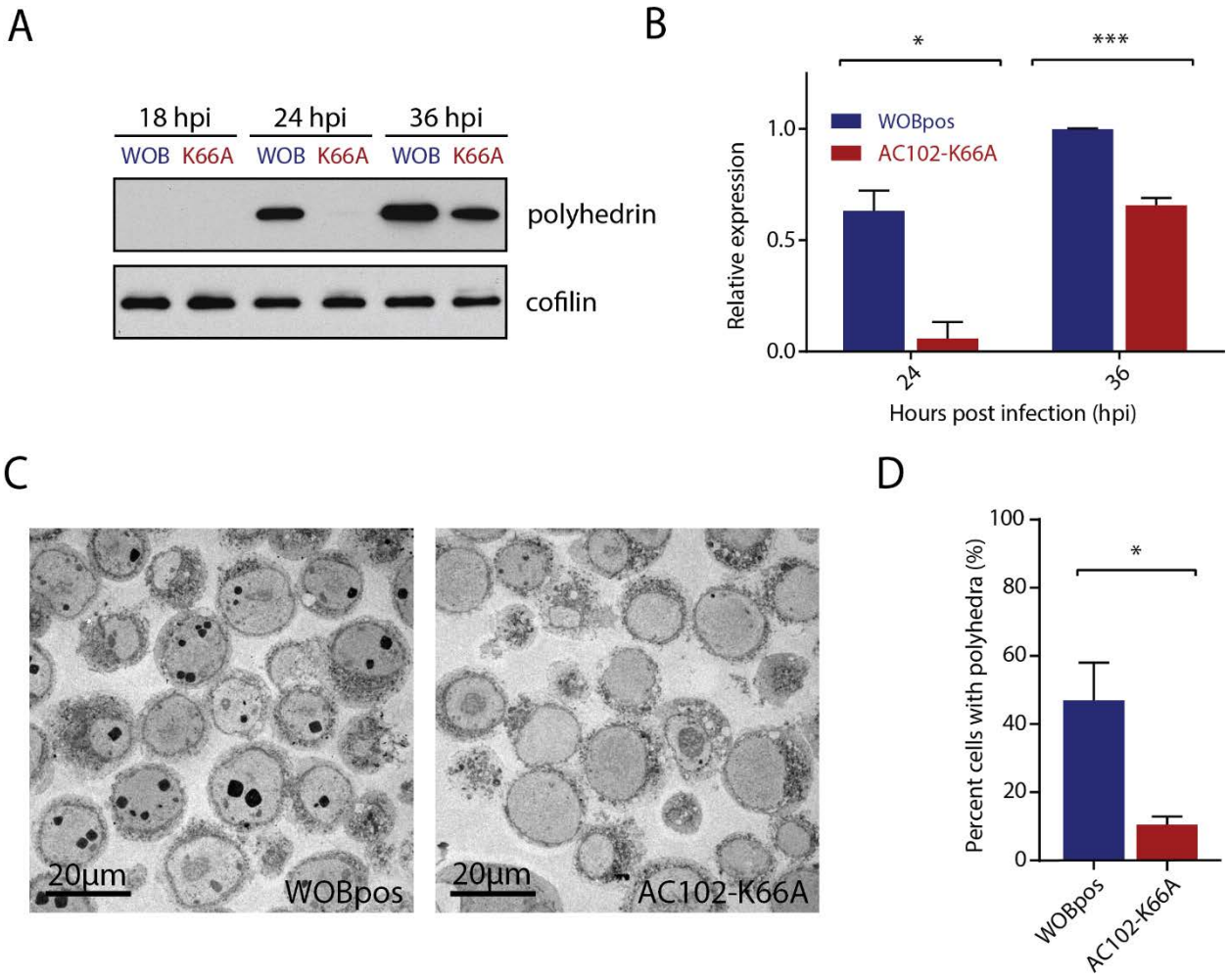


Figure 2.9: AC102 is important for polyhedrin expression and polyhedra formation very late in infection. (A) Western blots of lysates from Sf9 cells infected with WOBpos at an MOI of 10 and probed at 18, 24, or 36 hpi for polyhedrin (top) or cofilin (bottom, loading control). **(B)** Quantification of AC102 protein levels from Western blots as in (A). Bars are the mean from three independent replicates, and error bars are SD. **(C)** TEM micrographs of WOBpos or AC102-K66A infected cells at 36 hpi showing the presence or absence of electron-dense polyhedra. **(D)** Percentage of cells with at least one polyhedra for WOBpos or AC102-K66A infected cells as in (C). Bars are the mean from three independent replicates, and error bars are SD. In (B) and (D), P-values are indicated as follows: *=$p<0.05$ and ***=$p<0.001$.

AC102-K66A infected cells (**Fig. 2.9B**). This suggests that very late gene expression is delayed and/or reduced by the AC102-K66A mutation.

To determine whether lower polyhedrin expression also correlated with fewer cells containing polyhedra, we imaged infected cells at 36 hpi using TEM and counted the fraction of cells with at least one polyhedron. Compared with WOBpos infected cells (**Fig. 2.9C, left**), fewer AC102-K66A infected cells contained polyhedra (**Fig. 2.9C, right; Fig. 2.9D**). Together, these data indicate that the AC102-K66A mutation delays the timing of polyhedrin expression and impairs the formation of polyhedra during the transition to very late stage infection.

AC102 is a nucleocapsid protein that interacts with P78/83, C42, and EC27

The timing of AC102 expression as a late gene and the effect of the AC102-K66A mutation on nucleocapsid morphogenesis suggested that AC102 may be a structural component of the nucleocapsid. To test whether AC102 is a budded virion-associated protein, budded virus particles were isolated via ultracentrifugation over a sucrose cushion, then fractionated into their envelope and nucleocapsid components. Western blotting revealed that AC102 is associated with the nucleocapsid fraction, but not with the envelope fraction (**Fig. 2.10A**). These results indicate that AC102 is a structural component of nucleocapsids.

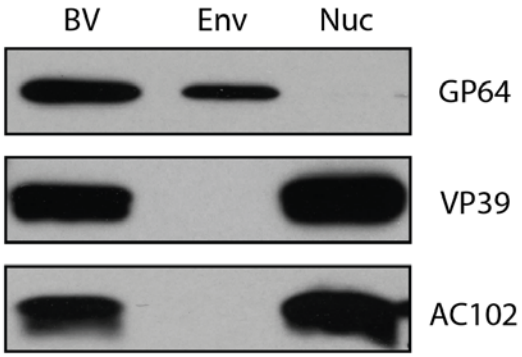
As a nucleocapsid structural component, AC102 would be predicted to interact with other capsid proteins. To identify the specific viral proteins with which AC102 interacts, a virus was constructed that expresses AC102 fused to a twin Strep-tag (Schmidt et al., 2013) in the native *ac102* locus of the viral genome (AC102-Strep). The AC102-Strep virus grows at a rate that is indistinguishable from WOBpos (**Fig. 2.11**), indicating that the AC102-Strep protein is fully functional. To isolate AC102 with its potential interacting proteins, Sf9 cells were infected with AC102-Strep (or WOBpos as a control), and at 24 hpi cells were lysed and AC102-Strep protein was isolated by Streptactin affinity chromatography (IBA Lifesciences). Interestingly, AC102-Strep co-purified with three other viral proteins as identified by mass spectrometry of isolated protein bands following SDS-PAGE. These proteins were EC27, C42 and P78/83 (**Fig. 2.10B**). Furthermore, mass spectrometry of whole elution samples revealed that these three proteins were the most abundant viral proteins in the pulldown (**Fig. 2.10C**). Other less abundant proteins identified in the pulldown have also been characterized as virion-associated proteins. Taken together, these results indicate that AC102 is a nucleocapsid protein that specifically interacts in a complex with the other nucleocapsid proteins P78/83, C42, and EC27.

DISCUSSION

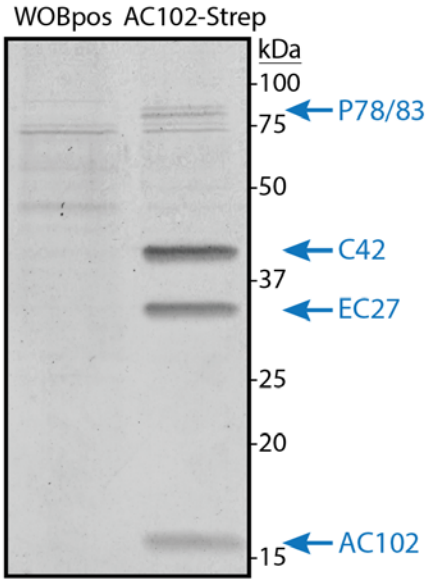
AcMNPV drastically increases nuclear actin levels in host cells during early infection through a process called the nuclear localization of actin (NLA), but little is known about the role that this phenomenon plays in viral infection. Viral protein AC102 is essential in viral replication and was previously shown to play a role in NLA, leading to the presumption that AC102 is an early protein (Ohkawa, Volkman, and Welch, 2010; Gandhi et al., 2012). Here we reveal a role for AC102 as a late-expressed nucleocapsid protein that is part of the P78/83-C42-EC27 complex and affects nuclear F-actin assembly during late infection.

In this study, we show that AC102 is expressed predominantly late in infection, with detectable protein at 12 hpi that continued to accumulate throughout late infection to 36 hpi. The

A



B



C

Accession	Protein name	Normalized abundance	Normalized abundance in control	p-value	HGSCore	Protein spectral counts/ total spectral counts			
						Replicate 1	Replicate 2	Control 1	Control 2
P25695	C42	3.64	0.03	0.0002	7.417	2010/26093	3058/30523	21/6511	26/6785
P41702	EC27	1.94	0.03	0.0002	7.722	1019/26093	1152/30523	15/6511	18/6785
Q03209	P78/83	1.10	0.03	0.0001	7.868	1227/26093	1074/30523	28/6511	33/6785
P41482	AC102	1.00	0.02	*	N/A	227/26093	244/30523	6/6511	4/6785
P41483	E25	0.98	0.52	0.0008	5.583	345/26093	514/30523	237/6511	225/6785
P41701	E18	0.90	0.17	0.0015	4.631	78/26093	233/30523	34/6511	24/6785
P17499	VP39	0.55	0.09	0.0057	1.888	309/26093	434/30523	65/6511	56/6785
Q00733	VP80	0.27	0.02	0.0201	0.354	334/26093	398/30523	18/6511	26/6785

* p-value not calculated for bait

Figure 2.10: AC102 is a nucleocapsid protein that interacts with P78/83, C42, and EC27. **(A)** Western blots of total budded virus (BV), envelope (Env), and nucleocapsid fractions (Nuc) probed for GP64 (envelope control), VP39 (nucleocapsid control), and AC102. **(B)** Streptactin-affinity chromatography eluates from WOBpos and AC102-Strep infected Sf9 cell lysates at 24 hpi. Equal amounts of sample were subjected to SDS-PAGE, the gel was stained with Safestain, and bands that were unique to the AC102-Strep sample were excised and analyzed by mass spectrometry. The protein identity of each band is indicated. **(C)** Total Streptactin affinity chromatography eluates from either WOBpos or AC102-Strep infected Sf9 cells were subjected to mass spectrometry. Statistically significant protein hits were determined using Spotlite (Goldfarb 2014), a web-based platform designed to identify significant protein hits from affinity purification/mass spectrometry data. Protein abundance was calculated by dividing total spectra per protein by protein length. Normalized abundance was calculated by dividing protein abundance by the abundance of AC102, in order to set AC102 abundance to 1. Positive protein hits are ranked by normalized abundance. A higher HGScore means a higher probability of bait-prey protein interaction. A higher HGScore means a higher probability of bait-prey protein interaction.

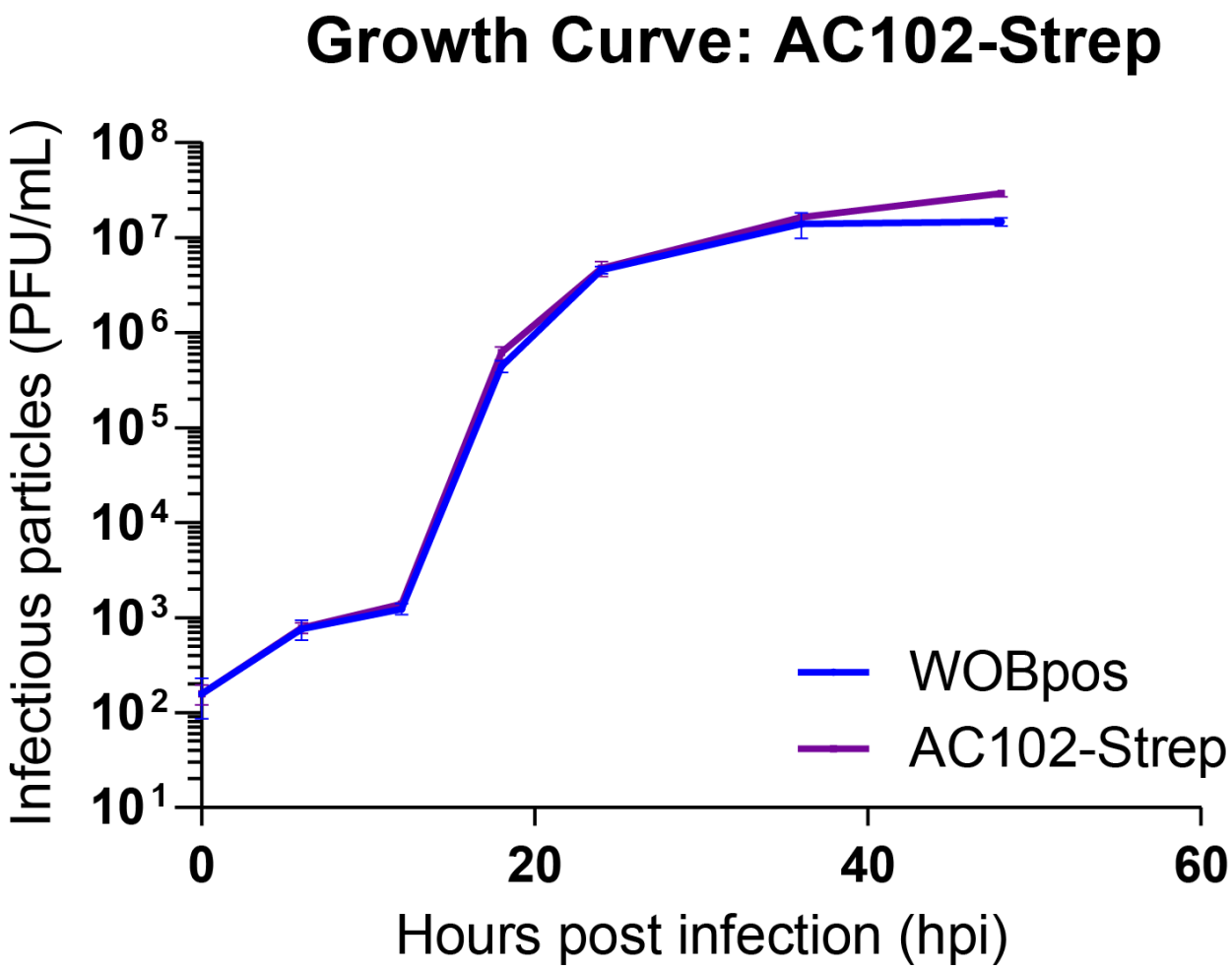


Figure 2.11: AC102-Strep virus has similar growth kinetics as WOBpos.

One-step growth curves of WOBpos and AC102-Strep viruses. Sf9 cells were infected at an MOI of 10 and progeny virus released into the growth media was quantified by titering. Data are the mean from three independent experiments and error bars represent SD.

onset of AC102 expression is consistent with the onset of *ac102* mRNA accumulation in *T. ni* cells (Chen et al., 2013), which peaks at 18 hpi. AC102 protein levels continued to accumulate throughout late infection, suggesting that mRNA and protein levels are not directly correlated at later time points. The onset of AC102 expression is similar to that of VP39 expression, which is strictly expressed during late infection (Friesen and Miller, 1985; Thiem and Miller, 1989; Todd et al., 1996; Passarelli and Guarino, 2007). Furthermore, AC102 expression is undetectable after treating cells with aphidicolin, which prevents late gene expression. Thus, AC102 is predominantly expressed late in infection. AC102 has, however, been shown to function during early infection and can be transactivated by early viral protein AC152 in the absence of late gene expression (Ohkawa, Rowe, and Volkman, 2002), suggesting that there may be low levels of AC102 expressed early in infection.

Many late expressed viral proteins are structural components of virions, which led us to test whether AC102 has a structural function in addition to its known function in NLA. Our data indicates that AC102 associates with nucleocapsids and not envelopes, confirming that it is indeed a structural nucleocapsid-associated protein. Furthermore, we found that AC102 specifically interacts with the nucleocapsid proteins P78/83, C42, and EC27, which were previously shown to interact with each other and form a complex at the basal end of the nucleocapsid (Braunagel et al., 2001; Russell, Funk, and Rohrmann, 1997; Vialard and Richardson, 1993). AC102 had not, until now, been recognized as a member of this complex. The interactions between P78/83, C42, and EC27 were discovered through yeast-two-hybrid (Y2H) studies (Braunagel et al., 2001), which can miss protein interactions for a variety of reasons, including protein non-functionality or steric hindrance of protein-protein interactions due to fusion with a Y2H transcription factor fragment. Given that AC102 is a small protein, it is reasonable to believe that it could be rendered non-functional or sterically hindered when fused to a larger Y2H transcription factor fragment. In addition, it is possible that AC102 requires multiple members of its complex to form a stable interaction, which cannot be determined using a Y2H screen.

Analysis of the phenotype of the AC102-K66A mutant virus suggests that AC102 performs an important function in nucleocapsid morphogenesis. Using TEM, we observed capsid-like tubular structures in the RZ of AC102-K66A infected cells at 36 hpi. This has been observed for many other viruses with deleted or mutant versions of nucleocapsid proteins, including viruses with deleted or mutant versions of C42, EC27, 49K, 38K, VP1054, AC53, PK-1, and VLF-1 (Guan et al., 2016; Liang et al., 2013; Liu et al., 2008; Marek et al., 2013; Vanarsdall et al., 2006; Vanarsdall, Peason, and Rohrmann, 2007; Wu et al., 2008). Many of these studies have also used immuno-EM to show that the capsid-like tubular structures contain VP39. Our immunofluorescence microscopy data also shows the presence of VP39 filaments and cables in the RZ of AC102-K66A infected cells at 24 and 36 hpi. Taken together, these data confirm that AC102 is important for proper nucleocapsid morphogenesis, most likely due to its role as a structural component of nucleocapsids.

AC102 also affects the transition to very late-stage infection. Western blots revealed that polyhedrin expression is reduced, and TEM revealed that fewer AC102-K66A infected cells contain polyhedra as compared to WOBpos infected cells, both of which are hallmarks of a defect in very late-stage infection (Partington et al., 1990). Several viruses with deleted or mutated structural proteins including PK-1, VLF-1, AC53, and 38K also show reduced polyhedra formation, suggesting that a link between nucleocapsid morphogenesis and very late infection exists (Liang et al., 2013; Liu et al., 2008; McLachlin and Miller, 1994; Wu et al.,

2006). AC102's involvement in the transition to very late infection is therefore likely due to its importance in nucleocapsid morphogenesis.

AC102's specific interaction with the P78/83-containing complex also suggests a role in F-actin polymerization. P78/83 is a viral NPF which recruits and activates the Arp2/3 complex to promote actin polymerization, and is required for both actin-based motility during early infection and nuclear F-actin polymerization during late infection (Ohkawa, Volkman, and Welch, 2010; Goley et al., 2006; Machesky and Insall, 2001). TN-368 cells transfected with an *ac102* deletion virus (*AcΔ102*) lack nuclear F-actin (Gandhi et al., 2012), however, it was previously unclear whether this was due to a direct effect of AC102 on nuclear F-actin assembly, or due to an inability for infected cells to progress to late stage infection. We observed decreased levels of nuclear F-actin in late-stage AC102-K66A infected cells, confirming that AC102 is crucial for F-actin assembly. The defect in nuclear actin polymerization seen for our AC102-K66A mutant virus could therefore be attributed to an inability of the P78/83-C42-EC27-AC102 complex to correctly localize to the nucleocapsid in the absence of functional AC102, or a defect in the actin polymerization of P78/83 in the absence of AC102. Furthermore, defects in actin assembly may influence nucleocapsid morphogenesis. F-actin is known to be required for BV formation, as treatment of AcMNPV infected cells with actin depolymerizing or actin stabilizing drugs results in no BV formation (Kasman and Volkman, 2000; Hess, Goldsmith, and Volkman, 1989; Goley and Welch, 2006). In addition, cytochalasin D treatment of infected cells, which inhibits the polymerization of actin, results in the same capsid-like tubular structures as reported in our study, giving credence to the idea that F-actin is involved in nucleocapsid morphogenesis (Volkman, 1988; Hess, Goldsmith, and Volkman, 1989). Whether a lack of F-actin causes the aberrant nucleocapsid morphogenesis seen in our study, or the aberrant nucleocapsid morphogenesis in our study is independent of F-actin polymerization and relates to a structural role for AC102 in nucleocapsids, has yet to be investigated.

Components of the P78-C42-EC27-AC102 complex are also thought to have early functions. For example, C42 and EC27 have been implicated in cell cycle regulation, an important process for early cellular reprogramming and viral DNA replication (Braunagel et al., 2001; Li et al., 2010; Belyavskyi, Braunagel, and Summers, 1998). AC102 is involved in the NLA phenotype seen in early infection (Ohkawa, Rowe, and Volkman, 2002). How the early functions of these proteins are carried out is unclear, as P78/83, C42, and EC27 are known to be expressed during late infection, after any early functions would be carried out. One explanation could be that, because these proteins are associated with the nucleocapsid, they are imported into the nucleus upon viral nuclear entry, where they can carry out their early functions. An alternative explanation is that these proteins could be translated at low levels early in infection, from either low levels of early de novo transcription, or potentially through mRNA incorporation into virions and subsequent carry over into new cells, as is the case with herpes simplex virus-1 and human cytomegalovirus (Bresnahan, 2000; Greijer, Dekkers, and Middeldorp, 2000; Sciortino et al., 2001). Transcriptome data supports the idea that low levels of early transcripts exist, as significant transcript levels of *c42*, *ec27*, and *ac102* are seen in *T. ni* infected cells at 0 hpi, only 60 min after viral inoculum is added to cells (Chen et al., 2013, supplementary material). Additionally, RT-PCR experiments reveal low levels of *ac102* mRNA in infected Sf9 cells as early as 2 hpi, supporting the theory that early transcripts of at least *ac102* exist during early infection (data not shown).

Our studies of AC102 reveal that this protein is a central player in linking the early NLA phenomenon with later nuclear F-actin assembly and nucleocapsid morphogenesis. Future

studies into the specific relationship between P78/83 and AC102, as well as further investigation into the role of AC102 in early NLA will reveal how AC102 mechanistically contributes to the drastic nuclear actin transformations seen during baculovirus infection and will contribute to the growing field of nuclear actin research.

MATERIALS AND METHODS

Cell Lines and Viruses

Sf9 cells were maintained in ESF921 media (Expression Systems) at 28°C in shaker flasks. Sf21 cells were maintained in Grace's insect media (Gemini Bio-Products) with 10% FBS and 0.1% Pluronic F-68 (Invitrogen) at 28°C in shaker flasks. AcMNPV WOBpos (Goley et al., 2006) was used as the wild-type virus in this study.

Generation of Recombinant Viruses

To generate viruses with point mutations in *ac102*, we first generated transfer vectors carrying the AcMNPV viral fragment KpnI-E (2.0-kb KpnI fragment of PstI-C containing *ac102*; Ohkawa, Rowe, and Volkman, 2002) and a downstream chloramphenicol resistance (*cat*) cassette for later use in recombinant bacmid selection. These were cloned into the KpnI site of pBSKS+. The QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used according to the manufacturer's protocol to produce ten p102_mutant transfer vectors encoding AC102 with one of the following point mutations: N47A, T52A, A55V, D61A, K66A, S77A, A80V, L96A, L105A, and N114A.

To generate mutant viruses (including WOBpos-AC102-K66A, referred to as 'AC102-K66A' in this study), *E. coli* strain GS1783 (provided by Laurent Coscoy; Tischer, Smith, and Osterrieder, 2010) was transformed with WOBpos viral DNA carrying a deletion of *ac102* (*Ac*Δ*102*; Gandhi et al., 2012), induced at 42°C to express recombinase, then subsequently transformed with linearized p102_mutant transfer vectors. Recombinant bacmids were selected by plating recovered cells on LB containing chloramphenicol. Bacmid DNA was isolated then transfected into Sf9 cells using TransIT-Insect Transfection Reagent (Mirus Bio LLC), and virus was recovered from transfected cell supernatants.

To generate the WOBpos-AC102-K66A-rescue (referred to in this study as 'AC102-K66A-rescue') virus, the transfer vector pAC102_rescue was engineered by amplifying *ac102* and its native promoter from viral DNA using PCR, and then subcloning it into pWOBGent3 (Ohkawa, Volkman, and Welch, 2010). *E. coli* strain GS1783 containing the AC102-K66A bacmid was induced as described above, then transformed with linearized pAC102_rescue DNA. This resulted in one copy of *ac102* under the control of its native promoter being recombined into the AC102-K66A bacmid, just upstream of the bacmid's kanamycin resistance cassette. Recombinant bacmids were selected for by plating transformed *E. coli* on LB containing gentamicin. Bacmid DNA was isolated then transfected into Sf9 cells, and virus was recovered as described above.

To generate the WOBpos-AC102-StrepII virus (referred to in this study as 'AC102-Strep'), the transfer vector pAC102_StrepII was engineered by amplifying a gene encoding AC102 tagged with twin Strep tags (Schmidt et al., 2013). *E. coli* strain GS1783 containing the

*Ac*Δ*102* bacmid (Gandhi et al., 2012) was induced as described above, then transformed with linearized pAC102_StrepII DNA. This results in the insertion of the *ac102-Strep* gene into the bacmid's native *ac102* locus. Recombinant bacmids were selected for by plating transformed *E. coli* on LB containing chloramphenicol. Bacmid DNA was isolated then transfected into Sf9 cells, and virus was recovered as described above.

For all recombinant viruses, to verify proper homologous recombination and confirm the presence of *ac102* point mutations, viral DNA was subjected to verification by restriction endonuclease analysis, as well as PCR and DNA sequencing (data not shown).

Viral Growth Curves and Plaque Size Assays

To compare the progeny budded virus production kinetics of WOBpos, AC102-K66A, and AC102-K66A-rescue viruses, one-step growth curves were performed in triplicate. An immunoplaque assay adapted from (Volkman and Phyllis, 1982) to assess viral production for each time point post-infection. Briefly, Sf9 cells were seeded onto 12-well HTC supercured slides (Cel-Line) and infected with viral supernatant samples from each growth curve time point. Infected cells were overlaid with 0.6% methylcellulose, incubated for 3 d at 28°C, fixed with formyl buffered acetone (25% formalin, 45% acetone, 30% phosphate buffer), and immunostained with mouse-anti-GP64 primary antibody (B12D5; kindly provided by Loy Volkman; Keddie, Aponte, and Volkman, 1989) and goat-anti-mouse-HRP secondary antibody (Santa Cruz Biotechnology). Slides were mounted with D.P.X. (Sigma) and plaques were counted to obtain viral titers for each time point.

To measure plaque size, immunoplaque assays were performed in triplicate for WOBpos, AC102-K66A, and AC102-K66A-rescue viruses as described above. Images of plaques were captured on an Olympus IX71 microscope with a CoolSNAP HQ camera (Photometrics). The Fiji distribution of ImageJ (Schneider, Rasband, and Eliceiri, 2012; Schindelin et al., 2012) was used to measure the area of individual plaques.

Budded Virus Purification and Fractionation

To obtain purified viral particles, Sf9 cells were infected with WOBpos virus at an MOI of 10 and supernatant containing budded viruses was collected at 2 dpi. Virus-containing cell supernatant was then overlaid onto a 40% sucrose cushion and ultracentrifuged at 100,000 × *g* for 1 hour at 15°C (Beckman SW-28 rotor and Beckman L8-M Ultracentrifuge) to purify budded viral particles. To fractionate budded virus into envelope and nucleocapsid components, a final concentration of 1% NP-40 was added to 100 μg of budded virus for 1 h at 4°C with rotation. Ultracentrifugation was then carried out at 80,000 × *g* for 1 hour at 15°C (Beckman TLA-100 rotor and Beckman TL-100 Ultracentrifuge) to separate the supernatant fraction containing viral envelope proteins from the pellet fraction containing nucleocapsid associated proteins. The nucleocapsid fraction was then washed and ultracentrifuged a second time (80,000 × *g*, 1 h, 15°C, Beckman TLA-100 rotor, Beckman TL-100 Ultracentrifuge) to ensure that there was no contamination from the envelope fraction.

Purification and Identification of AC102 Interacting Proteins

To purify AC102-Strep proteins together with interacting proteins, Sf9 cells were infected with WOBpos-AC102-StrepII or control WOBpos virus at an MOI of 10, and infected cells were harvested 24 h post infection. Cells were lysed on ice for 10 min in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1μg/ml LPC, 1μg/ml aprotinin, and 1mM PMSF) and centrifuged at 16,000 × *g* for 2 min at room temperature to separate nuclei and cellular debris from the cytoplasmic supernatant. The clarified cell lysate was incubated with 50 μL (packed volume) of Streptactin Sepharose resin (IBA Lifesciences) in microcentrifuge tubes for 2 h at 4°C on a rotator. Beads were washed then eluted with lysis buffer containing 6 μM D-desthiobiotin (IBA Lifesciences). Protein concentration was assessed via Bradford assay (Bradford, 1976). Equal amounts of protein samples were separated by SDS-PAGE and gels were stained with SimplyBlue SafeStain (Thermo Fisher Scientific) according to the manufacturer's maximum sensitivity protocol.

Bands that were unique to the AC102-Strep pulldown (*i.e.*, not in the WOBpos control) were cut out, in-gel digested with trypsin (Rosenfeld et al., 1992), and subjected to mass spectrometry to identify high affinity and/or high abundance candidate proteins that interact with AC102 (see below). In addition, whole samples from three independent affinity chromatography isolates of both AC102-Strep and WOBpos were trypsin digested and subjected to mass spectrometry to identify other candidate proteins that may be less abundant and/or indirect binding partners of AC102.

Mass spectrometry was performed by the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley. For whole samples, a nano LC column was packed in a 100 μm inner diameter glass capillary with an emitter tip. The column consisted of 10 cm of Polaris c18 5 μm packing material (Varian), followed by 4 cm of Partisphere 5 SCX (Whatman). The column was loaded by use of a pressure bomb and washed extensively with buffer A (5% acetonitrile/ 0.02% heptaflurobutyric acid (HBFA)). The column was then directly coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL linear ion trap mass spectrometer. An Agilent 1200 HPLC equipped with a split line so as to deliver a flow rate of 300 nl/min was used for chromatography. Peptides were eluted using an 8-step MudPIT procedure (Washburn, Wolters, and Yates, 2001). Buffer A was 5% acetonitrile/ 0.02% heptaflurobutyric acid (HBFA); buffer B was 80% acetonitrile/ 0.02% HBFA. Buffer C was 250 mM ammonium acetate/ 5% acetonitrile/ 0.02% HBFA; buffer D was same as buffer C, but with 500 mM ammonium acetate. This protocol was also followed for proteins originating from cut out gel bands, except that the nano LC column was only packed with 10 cm of Polaris c18 5 μm packing material (Varian), and not with Partisphere 5 SCX (Whatman), and the chromatography consisted of a simple gradient from 100% buffer A to 40% buffer A/ 60% buffer B.

Protein identification and quantification were done with IntegratedProteomics Pipeline (IP2, Integrated Proteomics Applications, Inc. San Diego, CA) using ProLuCID/Sequest, DTASelect2 and Census (Xu et al., 2006; Cociorva, Tabb, and Yates, 2007; Tabb, McDonald, and Yates, 2002; Park et al., 2008). Tandem mass spectra were extracted into ms1 and ms2 files from raw files using RawExtractor (McDonald et al., 2004). Data was searched against the *Autographa californica* nucleopolyhedrovirus (NC_001623.1) protein database supplemented with sequences of common contaminants, concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). LTQ data was searched with 3000.0 milli-amu precursor tolerance and the fragment ions were restricted to a

600.0 ppm tolerance. All searches were parallelized and searched on the VJC proteomics cluster. Search space included all fully tryptic peptide candidates with no missed cleavage restrictions. Carbamidomethylation (+57.02146) of cysteine was considered a static modification. We required 1 peptide per protein and both trypitic termini for each peptide identification. The ProLuCID search results were assembled and filtered using the DTASelect program (Cociorva, Tabb, and Yates, 2007; Tabb, McDonald, and Yates, 2002) with a peptide false discovery rate (FDR) of 0.001 for single peptides and a peptide FDR of 0.005 for additional peptides for the same protein. Under such filtering conditions, the estimated false discovery rate was about 1% for the datasets used.

Statistically significant protein hits were determined using Spotlite (Goldfarb et al., 2014), a web-based platform designed to identify significant protein hits from AP-MS data (affinity purification followed by mass spectrometry). The HGScore (Guruharsha et al., 2011) scoring algorithm was used to determine the probability of true protein interactions with AC102-Strep. This method uses a hypergeometric distribution error model that factors in negative controls and incorporates total spectral counts into the final score. A higher score means a higher probability of protein interaction.

AC102 Purification, Anti-AC102 Antibody Generation

To express recombinant AC102 protein, *ac102* was amplified by PCR and subcloned into the SspI site of pET-1M (Addgene plasmid # 29656; kindly provided by Scott Gradia at the UC Berkeley QB3 Macrolab) to create an inducible vector that codes for a fusion protein of a histidine tag, maltose-binding protein, a tobacco-etch virus protease cleavage site, and AC102 (His-MBP-TEV-AC102). pET-1M-AC102 *E. coli* strain BL21(DE3) (New England Biolabs) was transformed with pET-1M-AC102, grown in 1L shaker cultures at 37°C to an OD of 0.6-0.8, induced with 250 µM ITPG for 2 h, then harvested. *E. coli* were lysed by sonication, lysates were centrifuged (Sorval RC5C plus centrifuge, SS34 rotor, 4˚C, 20 min at 20,000 × *g*), and clarified lysate was used for amylose affinity chromatography. Briefly, lysates were incubated with 10 ml (packed volume) of amylose resin (New England Biolabs) in a 50 ml column for 2 h at 4°C on a rotator, washed with 10 volumes of column buffer (100 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, pH 8.0), then eluted in 1 ml fractions with column buffer containing 10 mM maltose. Protein containing fractions (as determined by Bradford assay), were pooled and MBP was cleaved from AC102 using 1 mg/ml TEV Protease. Released His-MBP and uncleaved His-MBP-TEV-AC102 protein were removed by binding to HisPur™ Ni-NTA Resin (Thermo Fisher Scientific). The resulting purified AC102 was concentrated to 1 mg/ml with a 3 kDa molecular weight cut-off protein concentrator (Thermo Fisher Scientific).

To generate custom antibodies that recognize AC102, rabbits were immunized with the purified AC102 protein by Pocono Rabbit Farm and Laboratory using their standard 91-day protocol. For antibody affinity purification, purified and concentrated AC102 was further purified via ion exchange chromatography (Akta Explorer 10 system (GE Healthcare), HiTrap SP HP 1ml column (GE Healthcare)) and coupled to NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare). Anti-AC102 serum was passed over the AC102-coupled resin, and antibodies were eluted with 100 mM Glycine pH 2.5. Antibodies were immediately neutralized to pH 7.5 by adding ~70µL 1M Tris pH 8.8 per 1ml fraction and stored at -20°C or -80°C in 50% glycerol.

Analysis of AC102 and AC102-K66A Expression by Western Blotting

To determine AC102 protein expression levels during the course of infection, Sf9 cells were infected in triplicate with WOBpos virus at an MOI of 10 and infected cells were harvested at 0, 8, 10, 12, 14, 16, and 36 hpi. Cells were lysed on ice for 10 min in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1μg/ml LPC, 1μg/ml aprotinin, and 1mM PMSF) and centrifuged at 16,000 × *g* for 2 min at room temperature to separate nuclei and cellular debris from the cytoplasmic supernatant. Cell lysates were subjected to SDS-PAGE, with equal loading of total protein in all lanes (determined via Bradford assay). Proteins were transferred to nitrocellulose membranes, and probed by Western blotting using our rabbit anti-AC102 antibody (1:5000 dilution, 5% milk in TBST). As a control for the onset of late infection, Western blotting was also performed using mouse-anti-VP39 antibody (P10C6; kindly provided by JaRue Manning; Whitt and Manning, 1988). Rabbit-anti-cofilin (GA15; kindly provided by Michael Goldberg and Kris Gunslaus) was used as a loading control. To confirm early expression of AC102, cells were infected with WOBpos at an MOI of 10 and were treated with either 5 μg/ml aphidicolin or DMSO (as a control) added to the media. At 24 hpi, cells were lysed and subjected to SDS-PAGE and Western blotting as described above using rabbit-anti-AC102 and rabbit-anti-cofilin (loading control) antibodies.

To compare protein expression levels of AC102 in WOBpos and AC102-K66A infected cells, Sf9 cells were infected in triplicate as described above, and lysed at 0, 6, 12, 18, 24, and 36 hpi. Cell lysate was prepared and Western blots were carried out as described above to probe for AC102, polyhedrin (anti-polyhedrin antibody was generously provided by Loy Volkman; Volkman, 1983), and cofilin (the loading control).

Immunofluorescence Microscopy

For immunofluorescence microscopy, Sf21 cells were seeded onto Greiner CELLSTAR 96-well plates (black-walled plates with micro-clear bottoms) to ~75% confluency, infected in triplicate with WOBpos or AC102-K66A virus at an MOI of 10, and fixed with 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH6.9) at 12, 24, or 36 hpi. Fixed cells were permeabilized with 0.15% Triton X-100 in PHEM, blocked with 5% normal goat serum (MP Biomedicals) + 1% BSA, and immunostained (Gandhi et al., 2012). Primary antibodies used were anti-AC102 (this study), anti-VP39 (P10C6), anti-PP31 (kindly provided by Linda Guarino; Guarino et al., 1992), or anti-P78 (kindly provided by Chris Richardson; Vialard, 1994; Iorioa et al., 1998). Phalloidin conjugated to Alexa Fluor 488 or 568 (Molecular Probes) was used to visualize F-actin. Cells were stained with Hoechst to visualize DNA within the nucleus.

Cells were imaged using an Opera Phenix High Content Screening confocal spinning disk system (PerkinElmer) using either the Opera Phenix 20x water immersion objective (PerkinElmer, NA 1.0, WD 1.7 mm) or the Opera Phenix 63x water immersion objective (PerkinElmer, NA 1.15, WD 0.6 mm) and the system's two sCMOS cameras (4.4-megapixel 2100x2100, 16-bit resolution, 6.5μm pixel size). Image analysis was carried out using Harmony High-Content Imaging and Analysis Software (PerkinElmer) on 16-bit images taken at 20x or 63x magnification. All analysis was done using maximum intensity projections except for nuclear and cytoplasmic actin quantification, which was done on single z-plane images through the center of cell nuclei.

Electron Microscopy

Sf9 cells were infected in duplicate with WOBpos or AC102-K66A virus at an MOI of 10 and infected cells were fixed at 18 and 36 hpi for 45 min with 1.5% paraformaldehyde and 2.0% glutaraldehyde, in a final concentration of 0.05 M cacodylate buffer, pH 7.3. Cell pellets were embedded in 2% agarose, rinsed three times in 0.05 M sodium cacodylate buffer, pH 7.3, and postfixed in a solution of 1% osmium tetroxide, 1.6% potassium ferricyanide, and 0.1 M cacodylate buffer, pH 7.2. Postfixed samples were then embedded in resin, sectioned, and stained with 2% methanolic uranyl acetate and lead citrate (Bozzola and Russell, 1998). Samples were imaged with a FEI Tecnai 12 transmission electron microscope equipped with a Rio 9 CMOS camera (3072 x 3072 pixels, Gatan).

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CHAPTER 3

Future directions

My work has focused on the previously unknown functions of the viral protein AC102 during late infection. I discovered that AC102 is an important member of the P78/83-C42-EC27-AC102 complex, which is critical for nucleocapsid formation. In addition, I found that AC102 plays a role in nuclear F-actin polymerization. These findings raise many new and intriguing questions for future research. First, the mechanism by which AC102 contributes to the functions of the P78/83-C42-EC27-AC102 complex is still unknown. Evidence suggests that P78/83, C42, and EC27 are regulated through phosphorylation, and our preliminary data suggests that AC102 may also be phosphorylated in this complex. We speculate that phosphorylation of AC102 may regulate the assembly or functions of the P78/83-C42-EC27-AC102 complex. AC102 phosphorylation may therefore affect cell cycle regulation or F-actin assembly through its association with the P78/83-C42-EC27-AC102 complex. Second, it is unknown how AC102 facilitates NLA early in infection. Do the previously identified NLA genes function in concert with AC102, or do other yet unidentified players work together with AC102 to facilitate NLA? Answering these questions will shed light on how nuclear actin is regulated in early and late infection, and may reveal a mechanistic link between early and late nuclear actin events through an AC102-facilitated process.

Does AC102 phosphorylation contribute to the functions and/or regulation of the P78/83-C42-EC27-AC102 complex?

My work shows that the P78/83-C42-EC27-AC102 complex is critical for nucleocapsid morphogenesis during late infection and also plays key roles in early infection processes (Chapter 2). The function or formation of this complex may be regulated through phosphorylation, as P78/83, C42, and EC27 are all phosphoproteins (Ohkawa, Volkman, and Welch, 2010; Belyavskyi, Braunagel, and Summers, 1998; Li et al., 2010; Hou et al., 2012; Vialard and Richardson, 1993). Analysis of the mechanisms of action of P78/83, C42, and EC27 provides insight into the possible functions of AC102 and a basis for future research.

As mentioned previously, P78/83 is required for early actin-based motility and for late nuclear F-actin polymerization through the recruitment and activation of the Arp2/3 actin-nucleating complex (Goley et al., 2006; Ohkawa, Volkman, and Welch, 2010). P78/83 is a phosphoprotein and a homolog of the eukaryotic Wiskott-Aldrich Syndrome Protein (WASP) (Vialard, 1994; Machesky and Insall, 2001). WASP activity is boosted through tyrosine phosphorylation by Abl kinases (Burton, Oliver, and Pendergast, 2005; Fogerty et al., 1999; Welch and Wang, 1993) and through serine phosphorylation by casein kinase 2 (CK2) (Cory et al., 2003), and it is therefore presumed that P78/83 is also activated through phosphorylation. Interestingly, AC102 contains three residues that are predicted to be phosphorylated by CK2 (as predicted by NetPhos 3.1; Blom, Gammeltoft, and Brunak, 1999), suggesting that AC102 and P78/83 could be co-regulated. Investigating the interactions of host and viral kinases with the P78/83-C42-EC27-AC102 complex will be important in understanding the complex's functional regulation.

Viral proteins C42 and EC27 act by recruiting kinases to phosphorylate other protein targets. Viral protein C42 contains a putative pocket protein binding site, which is thought to bind the retinoblastoma protein (pRB) (Li et al., 2010). pRB is a multifunctional protein that is regulated via phosphorylation and is central to cellular signaling and cell cycle regulation (Braunagel et al., 2001; Korenjak and Brehm, 2006; Cobrinik, 2005). Many DNA viruses phosphorylate pRB to inactivate it, which contributes to cell cycle progression to S phase. This is

advantageous for DNA viruses because cellular DNA replication machinery is amplified more than threefold during S phase, and most DNA viruses require at least a few components of the host DNA replication machinery to replicate their own genomes (Zeng et al., 1994; Rohrmann, 2013). While AcMNPV encodes its own DNA polymerase, it does not encode all necessary factors for DNA replication and therefore benefits from the ability to control cellular progression into an S-like phase (Tomalski, Wu, and Miller, 1988; McDougal and Guarino, 1999; Rohrmann, 2013). pRB has also been reported to bind to c-Abl tyrosine kinase (among other kinases), which can phosphorylate the nucleation-promoting factor N-WASP to boost the actin polymerization activity of the Arp2/3 complex (Welch and Wang, 1993). It is hypothesized that C42 acts as a scaffold protein to bring kinases into close proximity to P78/83 in order to phosphorylate P78/83 and promote F-actin assembly (Li et al., 2010). However, no experimental evidence yet exists to support the theory that kinases recruited by EC27 and/or pRB phosphorylate P78/83. Further research on the interaction between the P78/83-C42-EC27-AC102 complex and kinases may reveal a phosphorylation-based regulatory mechanism that ties together AcMNPV's control of the cell cycle with F-actin polymerization and sheds light on a role for AC102 as a phosphoprotein.

EC27 is a multifunctional viral cyclin that has also been shown to be important for viral regulation of the cell cycle (Belyavskyi, Braunagel, and Summers, 1998). As described above, AcMNPV drives cells into an S-like phase in order to replicate its own viral genome (Monteiro et al., 2012; Ikeda and Kobayashi, 1999). EC27 recruits a variety of host cyclin-dependent kinases to control the progression of the cell cycle during infection. First, EC27 displays cyclin D-like activity, which controls cell cycle progression into S phase. EC27 recruits cyclin-dependent kinase 6 (CDK6) to phosphorylate pRB, leading to transcription of E2F-controlled genes that act to push the cell into S phase (Belyavskyi, Braunagel, and Summers, 1998). The interaction between EC27 and pRB is likely facilitated by C42, which binds both proteins (Braunagel et al., 2001). EC27 also displays cyclin B-like activity, which controls cell cycle progression into mitosis (M phase) (Belyavskyi, Braunagel, and Summers, 1998). Normally, the degradation of the host cyclin B-CDC2 complex allows for the cell cycle to progress from G_2 phase to M phase. However, during AcMNPV infection, EC27 forms a complex with CDC2 after cyclin B-CDC2 is degraded, resulting in the arrest of host cells at the G_2/M phase of infection (Belyavskyi, Braunagel, and Summers, 1998; Braunagel et al., 1998). Together, EC27's cyclin B- and cyclin D-like activity allow viral DNA to replicate while the host cell is technically arrested at the G_2/M phase, creating a viral S-like phase.

The role of C42 and EC27 as phosphoproteins that interact with a number of kinases suggests that phosphorylation plays a key role in their respective cell cycle regulatory functions. As discussed above, the recruitment of these kinases may also play a role in activating P78/83 for the promotion of nuclear F-actin assembly. Investigating the phosphorylation states of AC102 through the course of infection may reveal how this protein contributes to the regulation of nuclear F-actin polymerization and cell cycle regulation.

It is interesting that the members of the P78/83-C42-EC27-AC102 complex are intimately dependent on phosphorylation and/or the recruitment of several kinases. Protein phosphorylation is a common post-translational modification for AcMNPV virion-associated proteins (Hou et al., 2012). While AC102 is not known to be a phosphoprotein, preliminary data from our affinity purification/ mass spectrometry experiments (see **Fig. 2.10B, C**) show that at least serine residue 4 (S4) of AC102 can be phosphorylated in the P78/83-C42-EC27-AC102 complex (data not shown). AC102 orthologs from most alphabaculoviruses contain serine or

threonine at position 4 in their amino acid sequence (see **Fig. 2.2**), suggesting that a serine/threonine kinase may be responsible for the phosphorylation of AC102. Several lines of reasoning support this theory. First, all phosphorylated residues identified so far in P78/83, C42, and EC27 proteins are serine (Hou et al., 2012), which suggests that the entire P78/83-C42-EC27-AC102 complex may be regulated through the action of serine/threonine kinases. Indeed, CDC2 and CDK6 are both serine/threonine kinases and specifically interact with EC27 (Belyavskyi, Braunagel, and Summers, 1998). Second, in Hou et al. (2012), serine/threonine phosphorylation accounts for 95% of phosphorylated residues in virion-associated AcMNPV proteins, implicating serine/threonine kinase activity in the global post-translational modifications of virion-associated proteins. Third, AC102 contains eleven serines, thirteen threonines, and one tyrosine. Of these, six serines and eight threonines are predicted to be possible kinase substrates (as predicted by NetPhos 3.1; Blom, Gammeltoft, and Brunak, 1999). Lastly, AcMNPV encodes its own serine/threonine kinase, PK-1, which is known to be associated with one end of the nucleocapsid (Braunagel et al., 2003; Vanarsdall et al., 2006; Wang et al., 2010; Yang and Miller, 1998), making PK-1 a tempting candidate kinase for P78/83-C42-EC27-AC102 phosphorylation.

Curiously, deleting PK-1 from the viral genome leads to no BV production and the formation of electron-lucent tubular structures in the nucleus (Liang et al., 2013) that are similar to those seen in cells infected with the AC102-K66A virus. One explanation could be that PK-1 facilitates the formation of the P78/83-C42-EC27-AC102 complex by phosphorylating AC102 or another member of the complex. If true, this would facilitate the formation of the basal structure of nucleocapsids and regulate nucleocapsid morphogenesis. Alternatively, PK-1 phosphorylation could activate P78/83's activity in promoting actin polymerization, which would also control nucleocapsid formation, as F-actin is required for the formation of nucleocapsids (Volkman, 1988; Ohkawa and Volkman, 1999). Investigating the phosphorylation of AC102 in particular would shed light on how it may regulate the activation of complex formation or function.

AC102 phosphorylation may be involved in the regulation of viral F-actin assembly and/or the manipulation of the cell cycle through its membership in the P78/83-C42-EC27-AC102 complex. Only S4 phosphorylation in AC102 was seen in all of the affinity chromatography replicates that we analyzed (data not shown). However, other phosphorylated serine or threonine residues in AC102 were seen in single replicates (data not shown). Phosphorylation inconsistencies between replicates in our study could be explained by the fact that lysis and affinity chromatography of AC102-Strep-infected cells were performed in the presence of protease inhibitors, but not phosphatase inhibitors. Affinity chromatography/mass spectrometry experiments using AC102-Strep-infected cell lysates could therefore be repeated in the presence of phosphatase inhibitors to identify additional phosphorylated residues of AC102. Once these residues are identified, viruses with either phosphomimetic (serine/threonine to glutamate/aspartate) or phosphoablative (serine/threonine to alanine) mutations could be created and screened for defects in viral growth, cell cycle regulation, and F-actin assembly. Viral growth and F-actin assembly could be assessed using one-step growth curves and phalloidin staining, respectively (see methods in Chapter 2). Defects in cell cycle regulation could be assessed by measuring the DNA content of infected Sf9 cells as performed in Braunagel et al. (1998).

A defect in F-actin assembly caused by one of these mutant viruses would be particularly interesting. If Western blots showed that AC102 expression and stability was not affected by the AC102 phosphorylation mutations, and affinity chromatography experiments showed that all

members of the P78/83-C42-EC27-AC102 complex are present, that would suggest that the P78/83-C42-EC27-AC102 complex is able to form correctly, but that a defect in P78/83 activation exists. This would suggest that AC102 phosphorylation is involved in regulating P78/83 promotion of F-actin assembly.

How does AC102 facilitate NLA?

AC102 is the only identified viral protein that is essential for the early nuclear localization of actin (NLA) during early AcMNPV infection (Gandhi et al., 2012) and as such, it provides a tool for studying the cellular functions of nuclear actin. While the existence of nuclear actin was once a hotly debated topic, nuclear actin is now known to play critical roles in gene regulation and cellular reprogramming during processes such as cellular development, senescence, dedifferentiation, and stress responses (Ho and Crabtree, 2010; Kwak et al., 2004; Miyamoto et al., 2011; Miralles et al., 2003; Iida, Matsumoto, and Yahara, 1992; Belin and Mullins, 2013; Misu, Takebayashi, and Miyamoto, 2017). G-actin brought into the nucleus through NLA is necessary for nuclear F-actin polymerization in late infection, which in turn is important for proper BV formation (Charlton and Volkman, 1991; Goley et al., 2006; Kasman and Volkman, 2000). However, it is not known why G-actin accumulates in the nucleus during early infection, or if and how the NLA phenotype affects the progression of viral infection (Ohkawa, Rowe, and Volkman, 2002; Hess, Goldsmith, and Volkman, 1989).

One possibility is that NLA acts to reprogram the host cell through gene expression regulation (Volkman, 2015). Host cell reprogramming is critical to successful viral infection, and is frequently controlled through manipulating host gene expression (Paschos and Allday, 2010; Adhya and Basu, 2010; White et al., 2010). Nuclear G-actin has been shown to affect gene expression through its interactions with all three RNA polymerases, RNPs, chromatin remodeling complexes, and stress response regulating factors such as MRTF (Belin and Mullins, 2013; Kapoor and Shen, 2014; Kwak et al., 2004; Misu, Takebayashi, and Miyamoto, 2017; Miyamoto et al., 2011; Serebryannyy, Cruz, and DeLanerolle, 2016; Visa and Percipalle, 2010; Weissbach et al., 2016). It is therefore possible that NLA and the buildup of G-actin in the nucleus plays a role in reprogramming the host cell to support viral replication.

The role of AC102 in the NLA phenotype is of special interest. As the only NLA gene that is absolutely necessary for G-actin accumulation in the nucleus (Gandhi et al., 2012), AC102 likely interacts with actin, actin binding proteins, and/or proteins that indirectly augment the levels of actin in the nucleus. For example, AC102 could interact with proteins such as the actin-specific karyopherins exportin-6 and importin-9 (Stüven, Hartmann, and Görlich, 2003; Dopie et al., 2012), cofilin (Bamburg, Harris, and Weeds, 1980), or actin itself to increase the nuclear levels of actin in early infection. AC102 and its interacting proteins could also globally affect the levels of nuclear import by targeting Ran, for example. Finally, it would be interesting to see if AC102 physically interacts with the other NLA proteins identified by Ohkawa and colleagues (Ohkawa, Rowe, and Volkman, 2002), or if NLA proteins act separately to stimulate NLA.

To identify AC102-interacting proteins during early infection, we performed Streptactin affinity chromatography using lysates of aphidicolin-treated Sf9 cells infected with AC102-Strep virus (see methods in Chapter 2). Aphidicolin prevents DNA replication and therefore arrests infected cells in early infection. Unfortunately, these experiments failed to capture any significant amounts of protein (data not shown). After establishing that AC102 was predominantly expressed late in infection, this was not surprising, as there evidently was not

enough AC102-Strep in the lysates to capture AC102 or any AC102-interacting proteins. Future experiments could address this problem by expressing and purifying AC102, conjugating it to NHS Sepharose to create an affinity chromatography column (see methods in Chapter 2), then performing affinity chromatography on large-scale lysates of aphidicolin-treated Sf9 cells infected with AC102-Strep virus. The contribution of candidate AC102-interacting proteins to the NLA phenotype could then be assessed through RNAi experiments, screening for low levels or delayed accumulation of nuclear G-actin. RNA-seq could also be used to identify differences in gene expression that result from defective NLA. The AC102-K66A viral mutant has not yet been assessed for NLA defects and could therefore also be investigated using these methods. Future research on AC102's role in NLA will be an excellent tool for studying how nuclear actin affects gene expression and cellular reprogramming.

It is possible that the phosphorylation state of AC102 and/or identified AC102-interacting proteins may be important for NLA as well. For example, only cofilin-bound actin can be imported into the nucleus by importin-9 (Dopie et al., 2012), and cofilin must be dephosphorylated to bind actin. An RNAi screen in *Drosophila melanogaster* S2R+ cells for proteins that augment nuclear actin levels revealed that several Twinstar (the cofilin ortholog in *Drosophila*) regulators either maintain nuclear actin levels by blocking Twinstar phosphorylation, or decrease nuclear actin levels by phosphorylating Twinstar (Dopie et al., 2015). AC102 and its interacting proteins could potentially affect the phosphorylation state of proteins that are involved in nuclear actin import or export in a similar way. Phosphorylation is also important for the nuclear import of cargo in general, and can either stimulate or repress nuclear import (Nardozzi, Lott, and Cingolani, 2010). If AC102 or AC102-interacting proteins directly bind G-actin for import into the nucleus, protein phosphorylation state could indeed be an important regulator of the rate of nuclear import. These scenarios are purely demonstrative, but are used to highlight the value of assessing both protein interactions and phosphorylation modifications when studying AC102 in the context of NLA. If a phosphorylation-based regulatory mechanism of AC102 is discovered for both nuclear F-actin polymerization and NLA, this would uncover a common function of AC102 as a regulatory phosphoprotein of nuclear actin.

Summary

The work in this dissertation sets the stage for identifying regulatory links between early and late nuclear actin processes during AcMNPV infection. In addition, our discovery that AC102 is a previously unknown member of the P78/83-C42-EC27-AC102 complex provides new opportunities to study how AcMNPV regulates nuclear accumulation of G-actin, nuclear F-actin polymerization, and cell cycle regulation. AcMNPV provides an ideal system to study the regulation and functions of nuclear actin, and continued investigation of AC102's role in nuclear actin-related processes will not only shed light on AcMNPV's control of the viral replication cycle, but will also illuminate general principles of cell biology.

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