Identification of a Novel Regulatory Sequence of Actin Nucleation Promoting Factor Encoded by Autographa californica Multiple Nucleopolyhedrovirus*

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Yun Wang‡, Yongli Zhang†¶, Shili Han†, Xue Hu†, Yuan Zhou‡, Jingfang Mu‡†§, Rongjuan Pei‡, Chunchen Wu‡, and Xinwen Chen‡

From the ‡State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, 430071, the †University of Chinese Academy of Sciences, Beijing, China, 100049, and the ¶Central China Normal University, Wuhan, China, 430000

Background: The regulatory mechanism of baculoviral actin nucleation promoting factor (NPF) remains elusive.
Results: Baculoviral NPF harbors a multifunctional regulatory sequence (MRS) at its N terminus.
Conclusion: Baculoviral NPF is modulated by the host proteasome and viral nucleocapsid protein C42 through its N-terminal MRS.
Significance: Identification of a novel regulatory mechanism of viral NPF.

Actin polymerization induced by nucleation promoting factors (NPFs) is one of the most fundamental biological processes in eukaryotic cells. NPFs contain a conserved output domain (VCA domain) near the C terminus, which interacts with and activates the cellular actin-related protein 2/3 complex (Arp2/3) to induce actin polymerization and a diverse regulatory domain near the N terminus. Autographa californica multiple nucleopolyhedrovirus (AcMNPV) nucleocapsid protein P78/83 is a virus-encoded NPF that contains a C-terminal VCA domain and induces actin polymerization in virus-infected cells. However, there is no similarity between the N terminus of P78/83 and that of other identified NPFs, suggesting that P78/83 may possess a unique regulatory mechanism. In this study, we identified a multifunctional regulatory sequence (MRS) located near the N terminus of P78/83 and determined that one of its functions is to serve as a degron to mediate P78/83 degradation in a proteasome-dependent manner. In AcMNPV-infected cells, the MRS also binds to another nucleocapsid protein, BV/ODV-C42, which stabilizes P78/83 and modulates the P78/83-Arp2/3 interaction to orchestrate actin polymerization. In addition, the MRS is also essential for the incorporation of P78/83 into the nucleocapsid, ensuring virion mobility powered by P78/83-induced actin polymerization. The triple functions of the MRS enable P78/83 to serve as an essential viral protein in the AcMNPV replication cycle, and the possible roles of the MRS in orchestrating the virus-induced actin polymerization and viral genome decapsidation are discussed.

Actin is one of the most abundant and evolutionarily conserved molecules in eukaryotic cells. Morphologically, globular actin (G-actin) can nucleate and polymerize to filamentous actin (F-actin) to form a functional actin cytoskeleton. Actin polymerization is mediated by actin nucleators, and the actin-related protein 2/3 complex (Arp2/3), which consists of seven subunits, is one of the most important nucleators that nucleates G-actin to Y-branched F-actin (reviewed in Ref. 1). Arp2/3 activity in nucleating G-actin heavily depends on nucleation promoting factors (NPFs)‡ (reviewed in Ref. 2). The conserved output region of NPFs is the C-terminal VCA domain, which includes a verprolin homology motif (V), an amphipathic connector region (C), and an acidic peptide (A) that interact with Arp2/3 through the P40 subunit (reviewed in Ref. 2; see also Ref. 3). In addition to the conserved VCA domain, NPFs harbor diverse N-terminal regulatory sequences that confer a variety of regulation mechanisms and functions. Therefore, an understanding of NPF regulation, especially the identification and characterization of their N-terminal regulatory sequences, is central to determining the mechanism and function of actin polymerization. Currently, at least eight NPFs (Class I) have been identified in mammalian cells based on different N-terminal sequences (reviewed in Ref. 4). One of the most studied NPFs is Wiskott-Aldrich syndrome protein (WASP) (5). The N terminus of WASP contains a GTPase-binding domain that binds to the VCA domain via intramolecular interaction and keeps WASP in an inactive state (6). Upon stimulation, the small GTPase Cdc42 can competitively bind to the GTPase-binding domain and subsequently abolish the intramolecular interaction to release the VCA domain, thus activating WASP to direct Arp2/3 to initiate actin polymerization (6).

Pathogens also encode NPFs to promote host actin polymerization to assist in their replication. Pathogen-derived NPFs

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† These authors contributed equally to this work.
‡ To whom correspondence may be addressed: State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Tel.: 86-27-87197575; Fax: 86-27-87199106; E-mail: wangyun@wh.iov.cn.
¶ To whom correspondence may be addressed: State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China. Tel.: 86-27-87199106; Fax: 86-27-87199106; E-mail: chenxw@wh.iov.cn.
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such as ActA encoded by *Listeria monocytogenes* (7), RickA encoded by *Rickettsia* sp. (8), and BimA encoded by *Burkholderia thailandensis* contain conserved VCA domains and different N-terminal regulatory sequences that enable the NPFs to precisely control actin polymerization in pathogen multiplication (9). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most studied baculovirus. After AcMNPV entry into the host cell, the virus induces host actin polymerization to aid in its replication: during the early infection phase, the viral nucleocapsid induces cytoplasmic actin polymerization to propel nucleocapsid migration toward the nucleus for replication (10, 11); after the nucleocapsid reaches the nuclear membrane, the cytoplasmic F-actin tail depolymerizes and detaches from the nucleocapsid, allowing the nucleocapsid alone to enter the nucleus (11); during the late infection phase, the virus promotes nuclear actin polymerization to assist in nucleocapsid assembly (12, 13). P78/83, a viral nucleocapsid protein encoded by AcMNPV ac9, is the NPF responsible for virus-induced actin polymerization (12). Sequence comparison with other well-characterized NPFs has revealed a classic VCA domain located near the C terminus of P78/83 (14). In contrast, its N terminus exhibits no similarity with any identified NPFs, suggesting that P78/83 may possess a unique regulation mechanism.

Our previous work revealed that another viral protein, BV/ODV-C42 (C42), is functionally related to P78/83. In addition to its role in mediating the nuclear relocation of P78/83 in virus-infected cells (15), P78/83 fails to initiate actin polymerization in the absence of C42 (16), suggesting that C42 plays a key role in regulating the NPF activity of P78/83. In the present study, we identified a multifunctional regulatory sequence (MRS) located at the N terminus of P78/83. This MRS serves as a degron to mediate P78/83 degradation by the host proteasome. In virus-infected cells, the MRS is masked by C42, and P78/83 is thus exempt from proteasomal degradation, ensuring its NPF activity in mediating actin polymerization. In addition to modulating P78/83 stability, the MRS is also required for P78/83 incorporation into the viral nucleocapsid, which ensures the nucleocapsid integrity and viral mobility powered by P78/83-induced actin polymerization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Virus Manipulation**—Sf9 cells were maintained at 27 °C in Grace’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). The proteasome inhibitor PS-341 (LC Laboratories) was added to the medium 4 h prior to cell lysis for Western blot or immunoprecipitation (IP) assays. Viral supernatants were filtered through a 0.45-μm syringe filter (Millipore), followed by incubation with fresh Sf9 cells to initiate secondary infection for virus titration or ultracentrifugation at 120,000 × g for 150 min at 4 °C to collect purified virions. Virus titration and infectivity assays were performed as described previously (15).

**Preparation of an ac9 Knock-out Bacmid**—To remove ac9 from the bacmid (bMON14272; Invitrogen), the ρ-red recombination system was employed, as described previously (15). The recombinant bacmid (vAc<ac9ko>) was verified by PCR and sequencing (data not shown).

**Construction of Plasmids and Recombinant Bacmids**—A standard molecular cloning protocol was used to prepare the indicated plasmid constructs. Genes were cloned into pLZ-V5/flag/myc (Invitrogen) for transient expression, and a Bac-to-Bac protocol was employed to prepare recombinant bacmids. In brief, gene expression cassettes were cloned into pFastBac dual (Invitrogen), and the resulting shuttle vectors were transformed to DH10B *Escherichia coli* cells harboring bMON14272, vAc<ac9ko> (15), or vAc<ac9ko> to generate transposed bacmids.

**Western Blot and IP Assay**—Cells were washed with PBS and lysed in radioimmune precipitation assay buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris, pH 8.0) with complete protease inhibitor mixture (Roche). Total proteins were quantified by Quickstart Bradford (Bio-Rad), and lysates containing 100 μg of total proteins were mixed with 2× Laemmli buffer before performing SDS-PAGE. The proteins were transferred to nitrocellulose membranes, which were blocked in 0.5% nonfat dry milk and incubated with the indicated antibodies (anti-V5 was purchased from Invitrogen; anti-flag (M2) was purchased from Sigma; anti-tubulin and anti-ubiquitin were purchased from Cell Signaling; anti-EGFP and anti-actin were purchased from Santa Cruz; anti-myc and anti-P78/83 were purchased from Abmart) overnight at 4 °C. After incubation with HRP-conjugated secondary antibodies (Jackson Labs), the membranes were developed using enhanced chemiluminescence (Pierce). For the IP assay, 2000 μg of total protein was incubated with 2 μg of the indicated antibodies and 50 μl of protein G-agarose (Millipore) overnight at 4 °C. The immunoprecipitated agarose was extensively washed with radioimmune precipitation assay buffer, mixed with 2× Laemmli buffer, and subjected to the Western blot assay.

**F-actin Staining**—Cells grown on coverslips were washed with PBS, fixed in 3.7% formaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 5 min. The cells were then incubated with a 1:40 dilution of rhodamine-phalloidin (Invitrogen) at room temperature inside a covered container for 20 min. After being extensively washed with PBS, the samples were subjected to fluorescence microscopy observation using a BX53 microscope.

**RESULTS**

**Baculoviral NPFs Exhibit Different Protein Abundance when Transiently Expressed in Insect Cells**—Viral genes encoding P78/83 and its baculoviral homologs are essential genes that exist in all sequenced Lepidoptera nuclear polyhedrosis virus (NPV) genomes (17). To compare the expression pattern of different baculoviral NPFs, we transiently expressed three P78/83 baculoviral homologs using the same vector in Sf9 cells, AcMNPV ORF9 (P78/83), *Bombyx mori* NPV (BmNPV) ORF2 (Bm2), and *Helicoverpa armigera* NPV (HearNPV) ORF2 (Ha2), which represent NPFs encoded by group I NPV (AcMNPV and BmNPV) and group II NPV (HearNPV). All three baculoviral NPFs were tagged with the FLAG epitope, and Western blotting using an anti-flag antibody demonstrated a strong band at ~45 kDa and a weak band at ~60 kDa only for Ha2, as we reported previously (18). P78/83 and Bm2 were not detected.
Amino Acid Region 40–143 Is a Key Determinant of P78/83 Protein Abundance—To determine the possible reasons that contribute to the different protein abundance of baculoviral NPFs, we compared the amino acid (aa) sequences of P78/83, Bm2, and Ha2. The sequence comparison assay showed that all three NPFs contain the well characterized VCA domain at the C terminus and the proline-rich domain in the middle region (Fig. 1B). However, the similarity of the N-terminal sequences (aa 1–143 of AcMNPV) was complicated, because P78/83 and Bm2 appeared to contain an extra sequence distributed at the N-terminal sequences influence P78/83 protein abundance (Fig. 1C). For all tested truncations, P78/83 with a deletion of aa 1–143 (P78/83Δ1–143) or 1–225 (P78/83Δ1–225) showed similar strong bands when probed with an anti-P78/83 antibody; P78/83Δ1–39 or P78/83Δ1–59 showed similar weaker bands, and P78/83Δ1–20 or P78/83 showed no bands (Fig. 1C). These results indicate that aa 40–143 play a key role in determining P78/83 protein abundance. To further dissect the role of aa 40–143, aa segments 40–59, 60–143, and 40–143 were individually deleted from P78/83 (Fig. 1D), and the constructs were transiently expressed in Sf9 cells. P78/83Δ40–59, P78/83Δ60–143, and P78/83Δ40–143 exhibited increased protein abundance (Fig. 1D), confirming that the region comprising aa 40–143 is a key determinant of P78/83 protein abundance.

To reveal how aa 40–143 modulate P78/83 protein abundance, aa 40–143 were fused to enhanced GFP (EGFP) at either the N terminus (40–143-GFP) or C terminus (GFP-40–143) (Fig. 1E). The subsequent expression cassettes were transiently expressed in Sf9 cells. Fluorescence microscopy indicated that both fusion proteins generated an extremely low level of EGFP fluorescence, in contrast to cells expressing native EGFP (GFP) or GFP-1–40 (aa 1–40 of P78/83 fused with EGFP) (Fig. 1E). A Western blot assay also provided consistent evidence that both fusion proteins were barely detectable in Sf9 cells (Fig. 1E). A degron is generally defined as a minimal element within a protein that is sufficient for recognition and degradation by proteolytic machinery (19). One of the key properties of degrons is that they are transferable, which means that genetically engineered fusions of such sequences confer instability on otherwise long-lived proteins (reviewed in Refs. 20 and 21). The phenotype of the fusion of aa 40–143 to EGFP resulted in fusion protein degradation, demonstrating that aa 40–143 serve as a degron that directly mediates protein degradation in insect cells as well as in mammalian cells (data not shown).

P78/83 Degradation Is Proteasome-dependent—The proteasomal degradation pathway is the most frequently used pathway for protein degradation. We next determined whether a chemical blocker of the proteasome could influence P78/83 protein abundance. Western blot assays demonstrated that the proteasome blocker PS-341, but not the lysosome inhibitor chloroquine (data not shown), could effectively increase P78/83 protein abundance in a concentration-dependent manner (Fig. 2A). Similar to P78/83, Bm2 protein abundance also appeared to be increased by PS-341, in contrast to Ha2, which exhibited...
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no significant change in protein abundance in response to PS-341 (Fig. 2B).

Because aa 40–143 serve as a degron to mediate protein degradation, to assess whether PS-341 can inhibit the degron-mediated protein degradation, an EGFP fusion protein (GFP-40–143) and P78/83340–143 were transiently expressed in Sf9 cells in the presence or absence of PS-341. A Western blot assay demonstrated that PS-341 significantly increased the protein abundance of GFP-40–143 but did not exert a significant influence on P78/83340–143 (Fig. 2C). This finding suggested that aa 40–143 modulate protein abundance by promoting protein degradation via a proteasome-dependent pathway, which can be inhibited by PS-341.

Because most substrates of proteasomal degradation are ubiquitinated proteins, to determine whether aa 40–143 contain important ubiquitination residues responsible for P78/83 degradation, all potential lysine ubiquitination sites within the region were mutated. The resulting mutants were expressed in Sf9 cells and compared with P78/83 and P78/83340–143. Western blotting demonstrated that none of these ubiquitination site mutants exhibited significantly increased stability in comparison with P78/83 (Fig. 2D). Accordingly, the levels of ubiquitination of P78/83 in the presence or absence of aa 40–143 were the same, implying that aa 40–143 contains no important ubiquitination sites (Fig. 2, E and F).

C42 Modulates P78/83 Protein Abundance—We next investigated the P78/83 expression pattern in AcMNPV-infected cells. Unlike the previous result that P78/83 is highly unstable in virus-free cells, P78/83 appeared to be a stable protein in AcMNPV-infected cells (Fig. 3A), suggesting that certain unidentified viral products can protect P78/83 from degradation during AcMNPV infection.

C42 is a viral late gene product that is not only expressed simultaneously with P78/83 in the AcMNPV replication cycle but also physically binds to P78/83 (22, 23). We then characterized the C42-P78/83 interaction and assessed whether C42 contributes to modulating P78/83 protein abundance. A series of V5 epitope-tagged C42 truncations were prepared and co-expressed in Sf9 cells with P78/83 (Fig. 3B). A co-immunoprecipitation (co-IP) assay demonstrated that only full-length C42 exhibited a strong interaction with P78/83 (Fig. 3B). Removal of aa 1–5 of C42 greatly weakened the C42-P78/83 interaction, and extending the deletion to aa 1–10 completely abrogated the interaction (Fig. 3B), indicating that aa 1–10 of C42 is essential for the C42-P78/83 interaction. A closer examination of the P78/83 content in whole cell lysates revealed that P78/83 protein abundance is strictly correlated to the C42-P78/83 interaction: when the C42-P78/83 interaction occurred (C42-V5, C4231–5-V5), P78/83 was stable; when the C42-P78/83 interaction was disrupted, P78/83 was degraded (Fig. 3B). This phenotype suggested that C42 is involved in the modulation of P78/83 protein abundance through the C42-P78/83 interaction. To confirm that the C42 N terminus is essential to P78/83 protein abundance, C42-V5 and C4231–10-V5 were co-expressed with P78/83 in Sf9 cells. Western blotting demonstrated that the full-length C42 protected P78/83 from degradation, whereas the removal of aa 1–10 of C42 greatly compromised P78/83 protein abundance (Fig. 3C).

We continued to explore whether C42 is responsible for P78/83 protein abundance in virus-infected cells. Sf9 cells were transfected with either AcMNPV (bMON14272) or c42 knockout bacmids expressing transposoned EGFP (vAc242ko), C42 (vAc242ko-c42), or C4231–10 (vAc242ko-c4231–10) (15). P78/83 exhibited high protein abundance in the cells transfected with bMON14272 or vAc242ko-c42, whereas vAc242ko-c4231–10 and vAc242ko resulted in very low levels of P78/83 (Fig. 3D). This phenotype in bacmid-transfected cells is in accordance with the phenotype in plasmid-transfected cells, indicating that C42 modulates P78/83 protein abundance through interaction with P78/83 during AcMNPV infection.

C42 protects P78/83 from degradation by binding to aa 40–143 of P78/83—Previous data demonstrated that C42 harbors the nuclear localization sequence (NLS) KRKK at its C terminus, the removal of which results in the loss of the capacity of C42 to relocate P78/83 to the nucleus by co-transportation.
C42 Modulates P78/83-Arp2/3 Interaction—Actin polymerization induced by AcMNPV infection is dependent on the interaction of P78/83 with host Arp2/3 (12). Because C42 was demonstrated to modulate P78/83 protein abundance, we next investigated whether C42 interferes with the P78/83-Arp2/3 interaction. The Spodoptera frugiperda P40 subunit (GenBank™ accession no. AFD50557.1) was chosen as a representative of the Arp2/3 (24).

A co-IP assay demonstrated that P78/83 failed to interact with P40 in the absence of C42; in contrast, PS-341 partially

(15, 23). We therefore speculated that C42 could possibly modulate P78/83 protein abundance by changing its subcellular distribution. When P78/83 was co-expressed with C42 (C42-V5) or a C42 NLS mutant (C42nls-V5) in Sf9 cells, both generated abundant P78/83, as indicated by equally strong bands on Western blots (Fig. 3E). This finding is in sharp contrast to the co-expression of P78/83 and EGFP (Fig. 3E), indicating that the mechanism by which C42 modulates P78/83 protein abundance is independent of the change in its subcellular localization.

Because P78/83 degradation is attributed to aa 40–143, another possible scenario is that C42 binding to P78/83 either directly or indirectly influences aa 40–143, which subsequently suppresses protein degradation. To test our hypothesis, we characterized how P78/83 interacts with C42. The series of P78/83 truncations described above were co-expressed with C42 in Sf9 cells. Co-IP assays demonstrated a relatively strong interaction between C42 and P78/83 with the removal of aa 1–20 or 1–39 (P78/831–20-flag, P78/831–39-flag); the extended removal of aa 1–59 (P78/831–59-flag) significantly compromised P78/83-C42 interaction, and the removal of aa 1–143 (P78/831–143-flag) completely abrogated the interaction (Fig. 3F). In accordance with these phenotypes, the removal of aa 40–59, 60–143, or 40–143 of P78/83 also abrogated the P78/83-C42 interaction (Fig. 3G), demonstrating that aa 40–143 of P78/83 play an essential role in P78/83 binding to C42 and suggesting that C42 protects P78/83 from proteasome-mediated degradation by either direct or indirect masking its degron sequence. Note that all three nonoverlapping P78/83 deletions shown in Fig. 3G completely blocked the P78/83-C42 interaction, which raises the possibility that deletions in aa 40–143 alter the overall P78/83 protein conformation.

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FIGURE 3. C42 increases P78/83 protein abundance by binding to the MRS. A, the expression pattern of P78/83 in mock- and AcMNPV-infected cells. P78/83-flag was transiently expressed in Sf9 cells. At 24 hpt, the cells were either mock-infected or AcMNPV-infected (multiplicity of infection = 5). At 48 hpt, the cells were harvested, and the proteins were subjected to a Western blot assay using an anti-flag antibody. B, characterization of the C42-P78/83 interaction. A series of C42 truncations tagged with the V5 epitope were generated as described by the diagram. The resulting C42 truncations were co-expressed with P78/83 in Sf9 cells. At 48 hpt, the cells were harvested and subjected to a co-IP assay using an anti-V5 antibody. The immunoprecipitated proteins and whole cell lysates (WCL) were probed with the indicated antibodies. C, C42-P78/83 interaction is essential for P78/83 stability. EGFP, C42-V5, and C42nls-V5 were co-expressed with P78/83 in Sf9 cells, respectively. At 48 hpt, cells were harvested, and the proteins were subjected to a Western blot assay using an anti-P78/83 antibody. The viral major capsid protein VP39 served as the exogenous reference to indicate bacmid transfection efficiency.

Because P78/83 degradation is attributed to aa 40–143, another possible scenario is that C42 binding to P78/83 either directly or indirectly influences aa 40–143, which subsequently suppresses protein degradation. To test our hypothesis, we characterized how P78/83 interacts with C42. The series of P78/83 truncations described above were co-expressed with C42 in Sf9 cells. Co-IP assays demonstrated a relatively strong interaction between C42 and P78/83 with the removal of aa 1–20 or 1–39 (P78/831–20-flag, P78/831–39-flag); the extended removal of aa 1–59 (P78/831–59-flag) significantly compromised P78/83-C42 interaction, and the removal of aa 1–143 (P78/831–143-flag) completely abrogated the interaction (Fig. 3F). In accordance with these phenotypes, the removal of aa 40–59, 60–143, or 40–143 of P78/83 also abrogated the P78/83-C42 interaction (Fig. 3G), demonstrating that aa 40–143 of P78/83 play an essential role in P78/83 binding to C42 and suggesting that C42 protects P78/83 from proteasome-mediated degradation by either direct or indirect masking its degron sequence. Note that all three nonoverlapping P78/83 deletions shown in Fig. 3G completely blocked the P78/83-C42 interaction, which raises the possibility that deletions in aa 40–143 alter the overall P78/83 protein conformation.

C42 Modulates P78/83-Arp2/3 Interaction—Actin polymerization induced by AcMNPV infection is dependent on the interaction of P78/83 with host Arp2/3 (12). Because C42 was demonstrated to modulate P78/83 protein abundance, we next investigated whether C42 interferes with the P78/83-Arp2/3 interaction. The Spodoptera frugiperda P40 subunit (GenBank™ accession no. AFD50557.1) was chosen as a representative of the Arp2/3 (24).

A co-IP assay demonstrated that P78/83 failed to interact with P40 in the absence of C42; in contrast, PS-341 partially
restored the P78/83-P40 interaction, and C42 maximized the P78/83-P40 interaction (Fig. 4A). These results could easily be attributed to the fact that C42 is essential for P78/83 to be at a measurable level to interact with P40 (Fig. 4A).

P78/83-C42 Interaction Is Pivotal for Nuclear Actin Polymerization and AcMNPV Replication—Previous reports have shown that both P78/83 and C42 are essential for nuclear actin polymerization and AcMNPV replication (12, 16). In this study, C42 was demonstrated to stabilize P78/83 and subsequently enhance the P78/83-Arp2/3 interaction level. We next explored how C42 influences nuclear actin polymerization and AcMNPV replication by disrupting the C42-P78/83 interaction.

Transposed bacmids were generated based on vAcC42ko, a c42 knock-out bacmid previously described (15). To ensure that P78/83 could self-localize to the nucleus in the absence of C42, an NLS-tagged P78/83 (P78/83-KRK) was transposed to vAcC42ko with EGFP (vAcC42ko-ac9nls) (16), full-length C42 (vAcC42ko-c42-ac9nls), or C42 without the C42-P78/83 interaction domain (vAcC42ko-c42Δ1–10-ac9nls) (Fig. 4B). The recombinant bacmids were transfected into Sf9 cells, and the nuclear actin polymerization status was characterized by phallolidin staining at 24 h post-transfection (hpt). In vAcC42ko-c42-ac9nls transfected cells, F-actin was observed to accumulate inside the nuclear domain (vAcC42ko-c42-ac9nls) (16). The recombinant bacmids were transfected into Sf9 cells at 24 hpt, the cells were stained by rhodamine-phalloidin (F-actin staining) and Hoechst 33258 (nucleus staining). The images were captured with a BX53 fluorescence microscope. D, C42-P78/83 interaction is required for AcMNPV replication. The indicated bacmids were transfected into Sf9 cells. At 120 hpt, viral supernatants were collected and filtered before being added to fresh Sf9 cells to initiate secondary infection. At 120 hpi, virus infectivity was determined by fluorescent microscopy.

We continued to determine the infectivity of these bacmids. After transfection of these bacmids into Sf9 cells, viral supernatants were collected at 120 hpt. Viral infectivity assays showed that the supernatants from vAcC42ko-ac9nls and vAcC42ko-c42Δ1–10-ac9nls transfected cells failed to infect fresh Sf9 cells at 120 h postinfection (hpi) (Fig. 4D), indicating that the P78/83-C42 interaction is also essential for AcMNPV replication. The replication deficiency of vAcC42ko-ac9nls and vAcC42ko-c42Δ1–10-ac9nls is due, at least in part, to the instability of P78/83 and the resulting failure of nuclear actin polymerization, which is a key step for viral nucleocapsid assembly in the AcMNPV replication cycle (12, 13).

AA 40–143 Are Required for P78/83 Incorporation into the Viral Nucleocapsid—Because P78/83 is a viral structural protein distributed at the basal region of the nucleocapsid (25), one possible function of P78/83 degradation is that it may generate a leak in the enclosed nucleocapsid and that the viral genome may escape through that leak (decapsidation), resulting in viral DNA repication. An easy way to verify this hypothesis is to check whether P78/8340–143 (the nondegradable version of P78/83), as a nucleocapsid protein, can block viral genome decapsidation.

To incorporate P78/8340–143 into the nucleocapsid, we introduced exogenous P78/83 expression cassettes into an ac9 knock-out bacmid (vAcAc9ko), expressing either wild-type P78/83 (vAcAc9ko-ac9nls-gfp) or P78/8340–143 (vAcAc9ko-ac9Δ40–143nls-gfp), and both versions of P78/83 were tagged with an NLS to ensure their self-nuclear localization (Fig. 5A) (16). The recombinant bacmids were co-transfected into Sf9 cells in the presence or absence of PS-341 at 44 hpt. At 48 hpt, the cells were harvested, and the proteins were subjected to a co-IP assay using an anti-V5 antibody. The immunoprecipitated proteins and whole cell lysates (WCL) were probed with the indicated antibodies. B, diagram of bacmid constructs. Expression cassettes expressing EGFP and NLS-tagged wild-type P78/83 (ac9-nls) with either wild-type C42 (c42) or C42 with aa 1–10 deleted (c42Δ1–10), both controlled by the c42 promoter (Pc42), were transposed into vAc42ko by the Bac-to-Bac system. C, C42-P78/83 interaction is required for virus-induced nuclear actin polymerization. The indicated bacmids were transfected into Sf9 cells. At 24 hpt, the cells were stained by rhodamine-phalloidin (F-actin staining) and Hoechst 33258 (nucleus staining). The images were captured with a BX53 fluorescence microscope. D, C42-P78/83 interaction is required for AcMNPV replication. The indicated bacmids were transfected into Sf9 cells. At 120 hpt, viral supernatants were collected and filtered before being added to fresh Sf9 cells to initiate secondary infection. At 120 hpi, virus infectivity was determined by fluorescent microscopy.
mids were transfected into Sf9 cells, and the resulting viral supernatants were collected to initiate secondary infection. Fluorescence microscopy showed that vAcac9ko-ac9nls-gfp could generate infectious viral particles (Fig. 5A), indicating that the NLS-tagged P78/83 could successfully restore the infectivity of vAcac9ko. In contrast to vAcac9ko-ac9nls-gfp, vAcac9ko-ac940–143nls-gfp could not produce infectious viral particles (Fig. 5B), indicating that the NLS-tagged P78/83340–143 (P78/83340–143nls-gfp) failed to restore the infectivity of vAcac9ko and that aa 40–143 are essential for P78/83 functionality. At least two possibilities could result in the loss of vAcac9ko-ac9340–143nls-gfp infectivity. First, P78/83340–143nls cannot be packaged into the nucleocapsid, which leads to nucleocapsid assembly failure; second, P78/83340–143nls can be packaged into the nucleocapsid but cannot be degraded, which leads to viral genome decapsidation failure.

To distinguish between these two possibilities, two recombinant bacmids, vAcac9nls-gfp and vAcac9340–143nls-gfp, were prepared by introducing the same P78/83 expression cassettes described in Fig. 6A into bMON14272 (Fig. 5C). Viral stocks of vAcac9nls-gfp and vAcac9340–143nls-gfp were used to infect Sf9 cells. When we compared the intracellular P78/83 in virus-infected cells and the extracellular P78/83 in purified virions, we found that P78/83340–143nls (~70 kDa, matching the predicted molecular weight of P78/83340–143nls) was only present intracellularly in vAcac9340–143nls-gfp-infected cells but was absent in the lysates of purified virions (Fig. 5D). This result indicates that the nucleocapsid of vAcac9340–143nls-gfp contains no P78/83340–143nls and that aa 40–143 are required for P78/83 incorporation into the nucleocapsid. Therefore, aa 40–143 serve as a MRS of P78/83 in the virus replication cycle. However, we could not yet determine whether MRS-mediated P78/83 proteasomal degradation is involved in viral genome decapsidation because of the failure of P78/83340–143nls to be incorporated into the viral nucleocapsid.

**DISCUSSION**

Most NPFs contain a conserved VCA domain near their C terminus that plays a similar role in binding and activation of the Arp2/3 to initiate actin polymerization. However, the diverse N-terminal regulatory domains confer different regulatory mechanisms to NPFs and subsequently enable the precise control of actin polymerization to exert different functions in eukaryotic cells. Therefore, the identification of NPF N-terminal regulatory sequences and investigation of their regulation mechanisms are key to understanding the control of actin polymerization. In this study, we identified an MRS of the AcMNPV-encoded NPF that is novel not only because it presents a completely different regulatory mechanism from the well-defined intramolecular conformational switch model employed by WASP and other NPFs but also because of its multiple roles in assisting in virus replication. Actin polymerization plays a crucial role in regulating a variety of biological functions in eukaryotic cells. The modulation of NPF stability is one of the major ways of orchestrating actin polymerization. Reicher et al. (26) reported that WASP could be ubiquitinated and degraded upon T-cell antigen receptor

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**FIGURE 5. The MRS is essential for P78/83 incorporation into the viral nucleocapsid.** A and C, diagram of bacmids. Two expression cassettes expressing EGFP and either NLS-tagged wild-type P78/83 (ac9nls) or P78/83 with aa 40–143 deleted (ac9340–143nls), both controlled by the ac9 promoter (Pac9), were transposed into vAcac9ko (A) or bMON14272 (C), respectively. B, virus infectivity assay. vAcac9ko-ac9nls-gfp and vAcac9ko-ac9340–143nls-gfp were transcribed into Sf9 cells. At 120 hpt, viral supernatants were collected and used to infect fresh Sf9 cells. Cells expressing EGFP were captured at 24 hpt and 24 hpi using an IX-51 fluorescence microscope. D, the impact of the MRS on the P78/83 nucleocapsid incorporation. Viral stocks of vAcac9nls-gfp and vAcac9340–143nls-gfp were used to infect fresh Sf9 cells at a multiplicity of infection of 5. At 24 hpi, viral supernatants were collected and subjected to ultracentrifugation at 120,000 × g for 150 min at 4 °C to harvest the purified virions before Western blotting; the infected cells were lysed, and 100 μg of cell lysate was subjected to Western blotting with the indicated antibodies. WCL, whole cell lysates.
A Novel Regulatory Sequence of AcMNPV NPF

FIGURE 6. The possible roles of the MRS in regulating the virus-induced actin polymerization and viral genome decapsidation. AcMNPV-encoded NPF P78/83 contains an MRS located near its N terminus that promotes P78/83 degradation via the host proteasome. After AcMNPV entry into the cytoplasm, the MRS of P78/83 is proposed to be buried in the nucleocapsid, avoiding host proteasome degradation; in contrast, the VCA domain of P78/83 is proposed to be exposed on the nucleocapsid surface to induce cytoplasmic actin polymerization, providing viral mobility that propels the nucleocapsid to migrate to the nucleus (step 1). When the nucleocapsid is ready to transport across the nuclear membrane, the MRS may be exposed to the host proteasome, leading to P78/83 degradation, which subsequently ends P78/83-induced actin polymerization and detaches the nucleocapsid from the F-actin tail (step 2). After the nucleocapsid enters the nucleus, the viral genome may be released through the nucleocapsid leak generated by P78/83 degradation, resulting in viral genome replication and gene transcription (step 3). In the cytoplasm, the viral late gene product C42 is expressed simultaneously with P78/83, stabilizes P78/83 by binding to the MRS, and relocates P78/83 to the nucleus (step 4), where nuclear actin polymerization occurs and the nucleocapsid is assembled (step 5).

activation, which subsequently enhances T cell activation. King et al. (27) also showed that β1-integrin can facilitate platelet-derived growth factor receptor trafficking and fibroblast chemotaxis by modulating actin polymerization through stabilizing Neural-WASP. As a virus-encoded NPF, P78/83 has been fully proven to be responsible for AcMNPV-induced actin polymerization (11, 12). However, the molecular mechanism responsible for the regulation of virus-induced actin polymerization remains obscure. In this study, the identified MRS of P78/83 indicates that the modulation of P78/83 stability by the host proteasome and viral protein C42 is a major mechanism for orchestrating AcMNPV-induced actin polymerization.

It has been reported that P78/83 is located at the end of the nucleocapsid, which contains the basal structure (25). The requirement of the MRS for P78/83 integration into the nucleocapsid suggests that the MRS is buried in the nucleocapsid, preventing the cellular proteolytic machinery from interacting with and degrading P78/83. Conversely, the VCA domain of P78/83 is proposed to be exposed on the nucleocapsid surface to induce cytoplasmic actin polymerization, providing the viral mobility that propels the nucleocapsid to migrate to the nucleus after AcMNPV entry into the host cytoplasm (10, 11). When the nucleocapsid transports across the nuclear membrane, the F-actin tail detaches from the nucleocapsid, allowing the nucleocapsid to enter the nucleus through the tight nuclear pore (11). It is possible that the MRS may be exposed to the cellular proteolytic machinery at this phase because of unidentified reasons, leading to P78/83 degradation, which subsequently ends P78/83-induced actin polymerization and detaches the nucleocapsid from the F-actin tail. After the nucleocapsid enters the nucleus, the viral genome may be released from the F-actin tail (end 1). It is possible that the MRS may be exposed to the decapsidation machinery of the viral nucleocapsid.

In summary, we identified a novel regulatory sequence of baculovirus-encoded NPF. The interplay between the proteolytic machinery and viral protein C42 through the MRS presents a novel regulatory model for NPF and helps to shed light on the decapsidation mechanism of the viral nucleocapsid.

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