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HA2 from the *Helicoverpa armigera* nucleopolyhedrovirus: a WASP-related protein that activates Arp2/3-induced actin filament formation

Qian Wang [a,b], Changyong Liang [a], Jianhua Song [a], Xinwen Chen [a,*]

[a] *State Key Lab of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100039, People's Republic of China*

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Abstract

Filamentous actin is required for the productive replication of lepidopteran nucleopolyhedroviruses. We have demonstrated that nucleocapsids of the *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) are capable of nucleating actin polymerization *in vitro* in a dose-dependent manner. Actin polymerization is the main mechanism used in cell locomotion and is also utilized by the *Listeria* bacteria and by vaccinia virus for intracellular and intercellular movements. The WASP family of proteins has been shown to stimulate the assembly of branched actin filaments by the Arp2/3 complex. The process is conserved in eukaryotic cells. HearNPV ORF 2 (HA2), a WASP homologue, could nucleate branched actin filaments in the presence of Arp2/3 complex *in vitro*. We also demonstrate that HA2 co-localizes with Arp2/3 complex in the nucleus of infected cells, suggesting that HA2 and Arp2/3 complex are involved in nuclear actin polymerization. In summary, HA2 activates Arp2/3-induced actin filament network formation *in vitro* and *in vivo*.

Keywords: Baculovirus; *Helicoverpa armigera* nucleopolyhedrovirus; HearNPV; HaSNPV; WASP; Arp2/3 complex; Actin

1. Introduction

The actin cytoskeleton is a dynamic filament network essential for cell movement, morphogenesis, polarization and cell division (Drubin and Nelson, 1996; Mitchison and Cramer, 1996). These processes rely on the rapid and localized assembly and disassembly of actin filaments. Cellular signals, such as the activated Rho-family G proteins, direct construction of new actin filaments *de novo* by localizing and activating the nucleation machinery. The seven-protein Arp2/3 complex is one of the prime candidates that can generate new barbed ends by stimulating nucleation (Machesky et al., 1999). Two actin-related proteins (Arp2 and Arp3) of the complex serve as the nucleation site for a filament with a free barbed end (Robinson et al., 2001). Except for cortactin, CARMIL, myosin I, etc., most of nucleation-promoting factors identified to date are members of the Wiskott-Aldrich syndrome protein (WASP) family, which includes isoforms of WASP, N-WASP, and Scar. These proteins contain a number of domains known to interact with both the cytoskeleton and various signaling complexes that regulate cell and pathogen movements (Higgs and Pollard, 1999; Rohatgi et al., 1999).

A number of unrelated intracellular pathogens such as *Listeria monocytogenes*, *Shigella* spp., *Rickettsia* spp. and vaccinia virus have developed strategies to manipulate the cytoskeletal machinery to invade, move within and spread between host cells (Goldberg, 2001). These strategies combine structural and functional mimicry of host proteins and activities.

It has been demonstrated that filamentous actin is essential for nucleocapsid morphogenesis and progeny production of *Autographa californica* multi nucleopolyhedrovirus (AcMNPV) and others in the *Nucleopolyhedrovirus* genus (Kasman and Volkman, 2000). It is also shown that nucleocapsids of AcMNPV are capable of nucleating actin polymerization *in vitro* and that the structural protein P78/83 locate at the basal end of the nucleocapsid binds directly to actin (Lanier and Volkman, 1998; Russell et al., 1997). P78/83 shares proline-rich WH2 and acidic motifs with mammalian WASP family (Machesky

* Corresponding author. Tel.: +86 27 87199106; fax: +86 27 87199106.
E-mail address: chenxw@pentium.whiov.ac.cn (X. Chen).

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et al., 2001; Nie et al., 2006). Recently, Goley et al. (2006) demonstrated that P78/83 was a viral nucleation-promoting factor for the Arp2/3 complex and nuclear actin assembly by P78/83 and Arp2/3 complex was essential for viral progeny production.

The open reading frame *ha2* of the *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV), a homologue of P78/83, was shown to interact with host cell actin (Chen et al., 2001; Nie et al., 2006). In this paper, we demonstrate that HearNPV nucleocapsids induce actin polymerization *in vitro*, and demonstrate the mechanism of actin nucleation induced by HA2 and the interaction with the Arp2/3 complex.

2. Materials and methods

2.1. Cells and viruses

The *H. zea* cell line (BCIRL-Hz-AM1, Hz-AM1) and *Spodoptera frugiperda* (Sf9) cells were maintained at 28 °C in Grace's medium supplemented with 10% fetal bovine serum. HearNPV G4, the genome of which has been entirely sequenced (Chen et al., 2001), and vHa-Ha2-egfp (Nie et al., 2006) was propagated in Hz-AM1 cells. An AcMNPV bacmid (bMON14272; Invitrogen) was also used.

2.2. Purification of budded virus (BV) and solubilized BV (sBV)

Medium from infected cells was clarified by low-speed centrifugation and BV was then pelleted using a 25% sucrose cushion at 100,000 × *g* for 45 min. The virus pellet was resuspended in 10 mM Tris, pH 8.5, and an aliquot was removed for protein analysis using BCA reagent (Beyotime). Aliquots of BV (100 μg) were solubilized with 1% Nonidet P-40 to prepare sBV (Lanier and Volkman, 1998).

2.3. Protein expression and purification

Full-length HA2 was expressed in Sf9 cells using the Bac-to-Bac baculovirus expression system (Gibco BRL) with an N-terminal 6 × His-tag. To obtain the recombinant virus, the entire *ha2* gene was cloned into the vector pFactbacHTb utilizing the *Eco*RV and *Xba*I sites to produce the donor plasmid pFactHTb-His-ha2. This plasmid was used to transform competent DH10B cells containing helper and AcMNPV bacmid (Invitrogen). The recombinant bacmid was used to transfect Sf9 cells with the aid of lipofectin (Invitrogen) to generate the recombinant virus, vAc-His-Ha2. Sf9 cells infected with vAc-His-Ha2 were lysed and clarified, and the protein was purified on affinity columns containing Ni-NTA resin (Novagen). The purified Ha2 was run on SDS-PAGE to detect its size and gel was stained with Commassie Blue. Western blot analysis was performed using the HA2-specific antibody αHa2-C (Nie et al., 2006) as the primary antibody. The signal was detected using a BCIP/NBT kit (Sino-American).

Actin was purified from rabbit skeletal muscle as described by Pardee and Spudich (1982) and stored at −80 °C in a modified G buffer (5 mM Tris–HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 0.2 mM ATP).

2.4. Immunoprecipitations

Immunoprecipitations were carried out using Seize Protein A (Ptglab, USA). Briefly, 20-ml cultures of Hz-AM1 cells (10^7 cells) were infected with vHa-Ha2-egfp virus at a multiplicity of 10 PFU/cell. The cells were harvested at 72 h post-infection (p.i.) by centrifugation and lysed using buffer NTP (50 mM Tris–HCl, pH 7.9, 150 mM NaCl, and 0.5% (v/v) Nonidet P-40). Resin slurry (0.1 ml) containing Protein A was equilibrated and washed with the lysis buffer. Then 20 μg of antibody αHa2-C (Nie et al., 2006) was immobilized on the precleared resin and the protein extracts were added to the column and incubated at 4 °C overnight in a rotary mixer. The mixture was sedimented in the column and the crude material was washed with the lysis buffer. The remaining antigen was dissociated from the antibody with elution buffer. The elution process was repeated three more times and the fractions were examined by SDS-PAGE and Western blot analysis using a polyclonal rabbit anti actin antiserum (Cytoskeleton Inc.) as the primary antibody. The signal was detected using a BCIP/NBT kit (Sino-American).

2.5. Visualization of polymerization

Virus or protein samples were mixed with 3 μM G-actin (unlabeled), then 20 μl of rhodamine phalloidin in AP buffer (25 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 mM EGTA) was added, mixed well and incubated overnight on ice in the dark. Fluorescent filaments were examined by microscopy (Lanier and Volkman, 1998). After polymerization in the presence of rhodamine-phalloidin, actin was diluted to a final concentration of 10 nM in fluorescence buffer containing 50 mM KCl, 1 mM $MgCl_2$, 100 mM DTT, 10 mM imidazole, pH 7.0, 0.5% methylcellulose, 20 mg/ml catalase, 100 mg/ml glucose oxidase, 3 mg/ml glucose. Samples of 2 μl were applied to coverslips coated in 0.1% nitrocellulose and the fluorescence was viewed using a Leica DC 300F microscope with a mercury illumination source (Blanchoin et al., 2000a). Actin branches were visualized by fluorescence microscopy and images were analyzed using Image-Pro Plus 5.0 software.

2.6. Pyrene–actin polymerization studies

Pyrene–actin (Cytosleleton Inc.) and unlabeled actin were mixed in G-buffer to generate a 3-μM monomeric actin solution with 10–15% pyrene–actin. Polymerization reactions contained 50 mM KCl, 10 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM ATP, supplemented with sBV or HA2 alone or in the presence of HA2 and Arp2/3 complex (Cytoskeleton Inc.). Ca^{2+}-actin was converted to Mg^{2+}-actin prior to each polymerization reaction by incubation with 50 μM $MgCl_2$, 200 μM EGTA for 2 min. Pyrene fluorescence was measured using a spectrofluorimeter (RF-5301 PC Shimadzu) at 25 °C, with excitation and emission wavelengths of 365 and 407 nm, respectively.