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

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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*.

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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...
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 Bacto-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 EcoRV and XbaI 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 lipofect (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 CaCl2, 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 (107 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 antiseraum (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 MgCl2, 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 MgCl2, 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 (Blanchon 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 (Cytoskeleton 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 MgCl2, 1 mM ATP, supplemented with sBV or HA2 alone or in the presence of HA2 and Arp2/3 complex (Cytoskeleton Inc.). Ca2+-actin was converted to Mg2+-actin prior to each polymerization reaction by incubation with 50 μM MgCl2, 200 μM EGTA for 2 min. Pyrene fluorescence was measured using a spectrophotometer (RF-5301 PC Shimadzu) at 25°C, with excitation and emission wavelengths of 365 and 407 nm, respectively.
2.7. Immunofluorescence staining

Sterile coverslips were placed in Petri dishes and seeded at a density of 10^6 cells/dish. After the cells had attached, the culture medium was removed and the cells were infected with vHa-Ha2-egfp at an MOI of 10 PFU/cell. At 72 h p.i., cells were fixed with 2% paraformaldehyde for 15 min and permeabilized in 0.2% Triton X-100 in PBS for 10 min. The cells were then incubated with primary antibodies for 60 min, washed with PBS, and incubated with fluorescently labeled secondary antibodies for 60 min. The fixed cells were viewed using an S2 Leica laser confocal scanning microscope. F-actin was stained with rhodamine-conjugated phalloidin (Molecular Probes).

2.8. Membrane targeting of HA2-CAAX

To express HA2 at the plasma membrane of insect cells, plasmid pIZ/V5-Myc-ha2-CAAX, which allows the expression of N-terminal Myc-tagged and C-terminal CAAX-tagged protein in insect cells (Friederich et al., 1995), was used. To fuse the plasma membrane localization signal CAAX (KKSKTCKVIM) to HA2, the following primers were used for PCR: 5′-CGGAATTCGGATCC (BamHI) A TGGTTCA-3′ and 5′-TTCACTTTGACCTTTGTA-3′. The DNA fragments encoding HA2-CAAX were cloned into the BamHI and EcoRI sites of pIZ/V5-Myc, which contained the Myc tag from pRK5Myc-cortactin (kindly provided by Dr. JT Parsons of UC Berkeley) (Weed et al., 2000). The plasmid constructed was termed pIZ/V5-Myc-ha2-CAAX. Hz-AM1 cells were transfected with this plasmid to express HA2 protein. Plasmid pRK5Myc-ha2-CAAX needed to transfect mammalian cells was constructed by replacing cortactin with ha2-CAAX.

3. Results

3.1. HearNPV nucleocapsid-induced actin polymerization

It has been reported that the nucleocapsids of AcMNPV activate actin polymerization (Lanier and Volkman, 1998). Solubilized BV of HearNPV was mixed with G-actin under polymerization conditions, resulting in increased levels of F-actin as observed by labeling with rhodamine-phalloidin (Fig. 1A, a). The effect of HearNPV sBV on activation of actin polymerization was positive when compared with G-actin incubated with AcMNPV sBV (Fig. 1A, b). Some background spontaneous nucleation of actin polymerization occurred in the absence of virions (Fig. 1A, c), which is normal for this type of assay (Blanchoin et al., 2000b).

The kinetics of actin assembly was monitored using the pyrene–actin assay to analyze the ability of HearNPV sBV to stimulate actin nucleation and accelerate actin polymerization (Cooper et al., 1983). Polymerization of actin alone was characterized by an initial lag phase, indicative of the kinetic barrier
to nucleation, followed by a period of relatively rapid filament elongation (Fig. 1B). Addition of HearNPV sBV resulted in a modest acceleration of polymerization relative to the control, but this did not eliminate the lag phase (Fig. 1B). With increasing amounts of HearNPV sBV, we observed a dose-dependent acceleration of actin polymerization (Fig. 1C). Thus, sBV is sufficient for actin-filament nucleation. This suggests that HearNPV sBV may influence the spatial and temporal distribution of actin polymerization in cells and actin might be involved in the assembly and transport of HearNPV virions.

3.2. HA2 stimulates the nucleation activity of the Arp2/3 complex in vitro

Many baculoviruses encode a nucleocapsid structural protein homologous to HA2 of HearNPV and AcMNPV P78/83, which contains all the conserved domains of WASP-family proteins (Machesky et al., 2001; Nie et al., 2006). It has been reported that P78/83 binds to actin and that HA2 co-localizes with cellular actin in insect cells (Lanier and Volkman, 1998; Nie et al., 2006). Immunoprecipitation experiments with a specific HA2 antibody (αHa2-C) further demonstrated the direct interaction of HA2 with insect cellular actin (Fig. 2A). These data suggest that HA2 and its homologues in baculoviruses bind to and activate the Arp2/3 complex and induce actin assembly, as previously described for WASP-family proteins (Mullins and Machesky, 2000).

To test this hypothesis, we investigated whether purified HA2 and the Arp2/3 complex could accelerate actin polymerization in a synergistic manner, as was observed for the ActA protein of Listeria (Welch et al., 1997). The entire HA2 protein was first expressed in Sf9 using an AcMNPV Bac-to-Bac expression system. Western blot analysis showed that the HA2 protein extracted from vAc-His-Ha2-infected Sf9 cells was about 60 kDa (Fig. 2B), which is larger than both the predicted 49.8 kDa based on its sequence and 50 kDa of wild-type HA2 (Nie et al., 2006). This could be due to different post-translational modifications in Sf9 and Hz-AM1 lines.

We further visualized the polymerization of actin induced by purified HA2 in the presence of Arp2/3 complex and found a dramatic increase in the amount of F-actin stained with rhodamine-phalloidin in vitro (data not shown). These results suggest that HA2 can induce G-actin polymerization into F-actin and activate Arp2/3-mediated actin polymerization.

To obtain a quantitative measure of the activity of HA2, pyrene–actin fluorescence was used to monitor actin assembly in the presence of HA2. The kinetics of assembly with HA2 was not significantly different from that of actin alone, indicating that, on its own, HA2 was unable to stimulate actin polymerization (Fig. 2C). When both Arp2/3 complex and HA2 were present, the slope of the elongation phase was increased and the lag phase was shorter, although not completely eliminated, indicating that Arp2/3 complex and HA2 function together as a highly efficient nucleating site. Moreover, increased amounts of HA2 and a fixed concentration of Arp2/3 complex caused a concentration-dependent acceleration of actin polymerization (Fig. 2C). Thus, HA2 is sufficient to activate Arp2/3 complex and to produce an acceleration of actin polymerization in vitro.

3.3. Branching assay of actin filaments

The Arp2/3 complex binds to the sides of actin filaments and caps the pointed ends, and has been shown to localize at the branch points of dendritic actin arrays in vitro and in vivo (Mullins et al., 1998; Svitkina and Borisy, 1999). Light microscopy provided evidence that stimulation of actin nucleation by WASP-family proteins increased the number of actin branches formed by the Arp2/3 complex (Mullins and Machesky, 2000). The Arp2/3 complex links the pointed ends of actin filaments to the sides of other filaments at an angle of 70° (Mullins et al., 1998).

To define whether HA2 stimulates the actin branching activity of the Arp2/3 complex, we visualized the structures formed in vitro by staining actin filaments with rhodamine-phalloidin and found that 44.8% of the actin filaments generated were associated with branched structures (Fig. 3). In contrast, actin filaments polymerized in the presence of the Arp2/3 complex alone or HA2 alone were infrequently branched (2.0% and 1.0%, respectively) (Fig. 3). When actin alone was tested, a few, relatively long actin filaments were observed. This demonstrated that HA2 stimulated the branching activity of the Arp2/3 complex. It should be noted that HearNPV sBV, without the addition of Arp2/3
complex, could also stimulate the actin branching. This strongly suggests that baculovirus virions might contain some elements that function in a manner similar to the Arp2/3 complex.

3.4. HA2 induces actin rearrangement in vivo

Since known Arp2/3 activators are involved in the formation of plasma membrane protrusions or disruption of the actin cytoskeleton, we were interested in analyzing how HA2 would behave when expressed at the plasma membrane. We therefore constructed a plasmid pIZ/V5-Myc-ha2-CAAX expressing HA2-CAAX, designed to drive expression of the protein at the inner face of the plasma membrane (Fig. 4A). Hz-AM1 cells were transfected with this plasmid as previously performed for RickA (Gouin et al., 2004). HA2 expression resulted in an increase in diffuse phalloidin staining at the cell periphery and a complete loss of cell filopodia (Fig. 4B). In contrast, F-actin labeling was limited to the cell cortex in cells transfected with empty plasmids (Fig. 4C).

3.5. Arp2/3 complex was recruited to the nucleus and co-localized with Ha2 in infected Hz-AM1 cells

It has been reported that F-actin is located in the nucleus in the late stages of baculovirus infection (Ohkawa and Volkman, 1999), while Arp2/3 complex was found mainly in
the lamellipodia of crawling cells and in actin-rich spots of unknown function (Mullins et al., 1997; Welch et al., 1997). It was therefore very interesting to investigate whether Arp2/3 complex was also involved in nuclear actin polymerization during infection with HearNPV. We infected Hz-AM1 cells with vHa-Ha2-egfp and detected Arp2/3 recruitment in the cells. Using anti-Arp3 antibody (Cytoskeleton Inc.), we found that Arp3 was recruited to the nucleus of infected cells and co-localized with HA2 (Fig. 5). It has previously been shown that the Arp2/3 complex subunits Arp3, Arp2, p34-Arc and p21-Arc co-localize in cells (Machesky et al., 1997; Welch et al., 1997).

4. Discussion

Several viruses and intracellular bacterial pathogens exploit the dynamics of the actin cytoskeleton of their host cells during their life cycles, but most of them do so only upon entry into host cells (Cossart and Sansonetti, 2004; Cudmore et al., 1997; Smith et al., 2003). Some also use actin for intracellular locomotion (Dramsi and Cossart, 1998). Over the last few years, studies of actin-based motility of the bacterial pathogens Listeria, Shigella and Rickettsia have yielded insights into the events occurring at the leading edges of motile cells (Carlier et al., 2003; Loisel et al., 1999; Welch et al., 1997; Welch et al., 1998).

The data presented here show that HearNPV sBV has the ability to nucleate actin polymerization in vitro. This finding is consistent with previous research demonstrating that AcMNPV nucleocapsids induce the formation of actin cables shortly after entry into the host-cell cytoplasm and that filamentous actin is required for lepidopteran nucleopolyhedrovirus progeny production (Charlton and Volkman, 1993; Kasman and Volkman, 2000). Therefore, both group I (AcMNPV) and Group II (HearNPV) NPVs are able to induce actin polymerization.

Recently, we showed that HA2 shares the same motifs as the mammalian WASP family (Nie et al., 2006). Supporting a role for HA2 as an activator that directs actin-polymerization-based motility in vitro, we first demonstrated an interaction between HA2 and actin in cells infected with vHa-Ha2-egfp through immunoprecipitation. This finding is in total agreement with the finding that HA2-EGFP co-localized with the cellular actin skeleton (Nie et al., 2006), suggesting that HA2 may shuttle G-actin to the Arp2/3 complex for addition into filaments and may recruit actin to play a critical role in motility. This clearly demonstrates that the replication of group I or II nucleopolyhedroviruses is F-actin-dependent. F-actin-dependent progeny morphogenesis appears to be a characteristic common to viruses in this genus that have lepidopteran hosts (Kasman and Volkman, 2000). We found that HA2, indeed, stimulated the nucleating activity of the Arp2/3 complex based on data obtained by pyrene–actin assay and fluorescence microscopy. Over-expression of HA2 on the cell membrane disrupted actin assembly at the cell periphery and led to an increase in diffuse phalloidin staining (Fig. 4). The disruption of peripheral actin assembly is likely to be a consequence of rearrangement of the actin cytoskeleton by HA2 and the Arp2/3 complex. Recently, it was reported that AcMNPV P78/83 has the capability of substituting for WASP in Arp2/3-dependent actin-polymerization reactions in vitro (Goley et al., 2006).

The Arp2/3 complex binds to and cross-links actin filaments end-to-side at a fixed angle (70°) and organizes them into mechanically rigid networks. Branched assays provided evidence of the ability of HA2 to promote branched-filament formation by the Arp2/3 complex. Since nucleocapsids of HearNPV directly nucleated actin filaments, we next investigated whether nucleocapsids had an effect on actin filament architecture. Our data clearly show that nucleocapsids induced actin filament nucleation and formed branched arrays. We postulate that nucleocapsid-associated actin may bind to HA2, with the total complex acting as a nucleus or a seed for actin polymerization.

Evidence obtained from biochemical data and cellular localization of HA2 indicates that HA2 stimulates actin nucleation by interacting with the Arp2/3 complex and regulating the cellular actin cytoskeleton by de novo formation of actin filaments through a dendritic nucleation mechanism.

It has been reported that nuclear F-actin is required for NPV infection of lepidopteran cells (Ohkawa and Volkman, 1999). Six nuclear localization G-actin (NLA) genes have been identified that mediate nuclear localization of G-actin (Ohkawa et al., 2002). Here we determined recruitment of the Arp2/3 complex in the nucleus of infected cells and its interaction with HA2. The re-localization of Arp2/3 suggests that there might be an organizational role for the Arp2/3 complex in the actin cytoskeleton that affects the assembly of virions. Taken together, both the in vitro and in vivo data demonstrate that HA2 is another baculovirus activator of actin nucleation and requires the Arp2/3 complex to initiate an actin polymerization process similar to ActA in Listeria, which represents a general strategy.
used to regulate the distribution of actin polymerization in cells.

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