Supporting Online Material for

Dynamic Nuclear Actin Assembly by Arp2/3 Complex and a Baculovirus WASP-Like Protein

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Materials and methods

Cell culture and viral infections. TN-368 cells were maintained at 27°C in Hink’s TNM-FH insect medium (JRH Biosciences) with 10% fetal bovine serum (FBS, Invitrogen), 0.1% pluronic F-68 (Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (pen-strep, Invitrogen). Sf9 cells were maintained at 27°C in Ex-Cell 420 insect medium (JRH Biosciences) supplemented with 2% FBS and pen-strep. For infections, viral inoculum was added to cells at 27°C for 1 h.

Recombinant bacmid generation. A bacmid called WOBpos, based on the Volkman lab strain of AcMNPV E2, was generated by introducing a mini-F replicon (from pCRcass1) and kanamycin-resistance cassette just upstream of the polyhedrin gene (Fig. S4). To engineer p78/83 mutations in WOBpos, a plasmid containing p78/83 and its flanking sequences (EcoRI-I 3.3kb) was generated by excision of the 3.3 kb EcoRI/EcoRV fragment from the cloned EcoRI-I fragment of the AcMNPV E2 genome (EcoRI-I/pUC7), blunting, and insertion into a pBluescript backbone. A chloramphenicol acetyltransferase (CAT) cassette was inserted into EcoRI-I 3.3kb between the genes encoding p78/83 and polyhedrin. The following point mutations in p78/83 in this plasmid were generated using the Quikchange Site Directed Mutagenesis Kit (Stratagene): I358A/V361A (labeled p78-I358A), V361A/D364A (labeled p78-D364A), RRR368-370AAA, DE384-5AA, and W387A. The V361A substitution is a naturally occurring variation among AcMNPV strain isolates and has no effect on activity in vitro or on growth in cells. The p78/83 deletion bacmid was generated by first using SnaBI and SalI to excise 84 % of the coding region of p78/83 (amino acid residues 75-328) in plasmid EcoRI-I 3.3 kb, blunting, and ligating in a blunt CAT cassette. To replace the wild type p78/83 gene in WOBpos with mutant variants, linearized, gel-purified plasmid DNA (~30 fmol) was co-electroporated with WOBpos DNA (~0.2 μg) into BW25113/pKD46 E. coli (1). Recombinants were selected on plates containing both kanamycin and chloramphenicol, and their bacmid DNAs verified by gel electrophoresis, PCR, and sequencing. WOBpos virus (WT or mutant) was obtained by transfecting 2 μg bacmid DNA into Sf9 cells using Cellfectin (Invitrogen) and harvesting culture supernatants 3-7 d post transfection (dpt). Rescue transfections were performed with inviable p78/83 mutants by co-transfecting bacmid DNA with 5 μg of EcoRI-I 3.3 kb plasmid into Sf9 cells.

Expression constructs. A vector for expressing mCherry fusion proteins in lepidopteran cells was generated by amplifying the gene encoding mCherry (a gift from Roger Y. Tsien, Howard Hughes Medical Institute, University of California-San Diego) by polymerase chain reaction (PCR) and subcloning it into the EcoRI and BamHI sites in pIZ/V5-His (Invitrogen) to make plZ-mCherry. To express mCherry-actin, the gene encoding Bombyx mori A4 actin was subcloned from pI1actGA (2) into the BamHI and NotI sites in plZ-mCherry to make plZ-mCherry-actin. To express EGFP-actin, plE1actGA was used. A vector for expressing enhanced yellow fluorescent protein (EYFP) fusions in lepidopteran cells was constructed by amplifying and subcloning the EYFP gene (Clontech), as above for mCherry, to make plZ-EYFP. To express a p21-EYFP fusion, the gene encoding Trichoplusia ni ARPC3/p21 (a gift from Michael Roe, North Carolina State University) was amplified by PCR and subcloned into the SacI and KpnI sites in plZ-EYFP to make plZ-p21-EYFP. To express p78/83 alone, the gene encoding p78/83 was amplified by PCR and inserted into plE1/153 (3) to make plE1/153A-p78/83. The plasmid expressing p21-EGFP containing a C-terminal nuclear localization signal (NLS) was generated by amplifying the gene encoding EGFP using primers that incorporate a sequence encoding a spacer and classical NLS (G2PKKKRKVED) at the C-terminus. The PCR product was subcloned into the SacI and NotI sites of plZ-p21-YFP to generate plZ-p21-GFP-NLS. The plasmid expressing p78/83 with a C-terminal NLS was generated by amplifying the p78/83 gene
using primers incorporating a spacer and NLS (G<GPKKKRKVED) at the C-terminus. The PCR product was subcloned into the EcoRI and NotI sites of plIZ/V5-His to generate plZ-p78-NLS.

**Live cell imaging.** To image the timecourse of nuclear actin accumulation and polymerization, TN-368 cells were transfected with plZ-mCherry-actin or plE1actGA using Cellfectin (Invitrogen), and 1 d after transfection cells were infected with WOBpos virus at an MOI of 20. Images were taken using an Olympus IX71 microscope equipped with a Photometrics Coolsnap HQ camera and Metamorph imaging software. For latA treatment during long time-lapse imaging, 4 μM latA was added just after nuclear actin polymerized, and imaging continued. For photobleaching experiments, TN-368 cells were transfected with plE1actGA and infected as above. Images were taken using a Zeiss 510 confocal scanning microscope equipped with a C-Apochromat 63X/1.2 water objective lens. For bleaching, the laser was used at 100% transmission for 100 iterations at 488 nm. Images were corrected for background and loss of fluorescence as described previously (4) using Metamorph software, and recovery half times were determined using Prism software. For drug treatments during FRAP experiments, jasplakinolide (1 μM) was added to culture medium 4 h prior to imaging, or latA (4 μM) was added 1 h prior to imaging.

**Protein expression, purification and characterization.** The truncated genes encoding AcMNPV p78/83 WPWCA (residues 220-543) or WCA (residues 316-543) were amplified by PCR and subcloned into the EcoRI and XhoI sites in pGAT2 (EMBL). Mutations in the gene encoding p78-WCA were made using the Quikchange Site Directed Mutagenesis Kit using the same primers used for mutagenesis of p78/83 for mutant bacmid generation. To express *T. ni* ARPC3/p21 for antibody production, the gene was amplified by PCR and inserted into the EcoRI and SalI sites of pGEX4T-1 (GE Biosciences). GST-p78-WPWCA, GST-p78-WCA (WT and mutants), and GST-p21 were purified by glutathione affinity followed by gel filtration chromatography. Recombinant human Arp2/3 complex (5), rabbit-muscle actin (6), pyrene labelled actin (7), and viral nucleocapsids (8) were prepared as described previously. Pyrene actin polymerization and branching assays were performed as described previously (5).

**Antibodies and immunofluorescence analysis.** Antibodies directed against p78/83 were generated by immunizing chickens with purified GST-p78-WPWCA (Aves Labs). Antibodies were purified first by depleting anti-GST antibodies by passage over a GST Affigel 10 (BioRad) column, and then by affinity purification on a GST-p78-WPWCA Affigel 10 column. Antibodies directed against ARPC3/p21 were generated by immunizing guinea pigs with purified GST-p21 (Pocono Rabbit Farm and Labs). Neither bleeds nor affinity purified anti-p21 antibody gave a signal by immunofluorescence, but bleeds recognized a single band on TN-368 lysates by immunoblotting (Fig. S5). To image Arp2/3 complex and p78/83 localization, TN-368 cells were either transfected with plZ-p21-EYFP and infected as above for mCherry-actin, or infected without transfecting. Cells were fixed in 4% formaldehyde at 22 hours post infection (hpi) and processed for immunofluorescence. Alexa Fluor 488- or rhodamine-phalloidin (Molecular Probes) was used to visualize actin and 4',6-diamidino-2-phenylindole (DAPI) was used to visualize DNA. p78/83 was visualized by indirect immunofluorescence using anti-p78-WPWCA primary antibody with FITC- or TRITC anti-chicken (Jackson Immuno Research) secondary antibody. The signal from p21-EYFP was amplified by indirect immunofluorescence using anti-GFP primary antibody (Roche) with FITC anti-mouse secondary antibody (Jackson Immuno Research). Images were taken as described above for the time lapse experiments. To observe F-actin localization in bacmid-transfected cells, TN-368 cells were transfected with recombinant bacmids encoding WT or mutant p78/83, fixed at 24 hpt and processed for immunofluorescence as above. To identify cells that were transfected with the Δp78/83 bacmid, indirect immunofluorescence was carried out using an anti-pp31 primary antibody (9) (a generous gift
from Linda Guarino, Texas A&M University) with FITC- or TRITC anti-rabbit secondary antibody (Jackson Immuno Research). The ratio of nuclear to cytoplasmic F-actin intensity was quantified by using Metamorph software to calculate average F-actin pixel intensity in the nucleus and in the cytoplasm of cells expressing p78/83. A ratio >1.5 consistently reflected accumulation of nuclear actin filaments that could be confirmed by visual inspection. To image p78/83 when expressed in the absence of infection, TN-368 cells were transfected with pIE1/153A-p78/83, fixed at 24 hpt, and stained for p78/83, F-actin, and DNA as above.

**Replication kinetics and drug treatments.** Viral replication was assessed in triplicate for each experiment, as described previously (10). Sf9 cells in suspension at 4°C were inoculated with WT or mutant WOBpos virus at an MOI of 10. After a 1 h adsorption period, inoculated cells were washed twice with fresh warm media and distributed to 24-well plates at 27°C. For determination of growth in the presence of 1 µM jasplakinolide, drug or DMSO was added at 0 or 15 hpi. Culture supernatant samples were taken at time 0 h and indicated times post infection and stored at –80°C until titration. Viral titers were determined by immunoplaque assay as described previously (11).

**dsRNA-mediated gene silencing.** The genes encoding *T. ni* ARPC3/p21 and EYFP (used as a control) were amplified by PCR using primers to append T7 RNA polymerase promoters at the 5’ ends of both the coding and non-coding strands. 5 µL of each PCR product was used as a template for *in vitro* transcription by T7 RNA polymerase using the Megascript kit (Ambion) following the manufacturer’s protocol. Transcripts were precipitated with LiCl, melted at 95°C, and then annealed by cooling from 65°C to 25°C over 1 h to make dsRNA. 1x10^5 cells per well of a 24-well plate were transfected with 3.75 µg p21 or EYFP dsRNA using Cellfectin (Invitrogen). Cells were harvested at 5, 6, and 7 d post transfection (dpt) and ARPC3/p21 and cofilin (loading control) protein levels were probed by immunoblotting (the anti-cofilin antibody was a generous gift of Michael L. Goldberg, Cornell University). For determination of viral growth in cells with reduced levels of ARPC3/p21, dsRNA-treated cells were infected as above at 5 dpt and culture supernatants were taken at 0, 24, and 48 hpi. Viral titers were determined as above.

**Electron microscopy.** TN-368 cells were plated on sapphire discs suitable for high pressure freezing and transfected with 2 µg wt or DE384-5AA mutant bacmid using Cellfectin. At ~48 hpt, cells were high pressure frozen and processed for transmission electron microscopy (TEM) as previously described (12, 13). Because transfection efficiency was relatively low (~5%), sapphire discs were removed from polymerized blocs and samples were scanned using phase microscopy to identify transfected cells by the presence of viral occlusions. Areas with occlusions were marked, cut out, remounted, and sectioned with a diamond knife. Sections were stained with uranyl acetate and lead citrate (13) and imaged on a JEOL 1200 TEM.
Supporting Figures

Figure S1. Alignment of the connector and acidic regions of p78/83 homologs from diverse lepidopteran NPVs with human WASP. Acidic residues are highlighted in bold. Conserved non-acidic residues are outlined in gray boxes. NPV phylogenetic group (I or II) for each virus is indicated on the right. Abbreviations and accession numbers: NPV, nucleopolyhedrovirus; S, single; M, multiple; Ac, Autographa californica [NCBI accession number L22858]; Adho, Adoxophyes honmai [AP006270]; Bm, Bombyx mori [L33180]; Cf, Choristoneura fumiferana [AF512031]; Chch, Chrysodeixis chalcites [AY864330]; Eppo, Epiphyas postvittana [AY043265]; Hear, Helicoverpa armigera [AF303045]; Hz, Helicoverpa zea [AF334030]; Ld, Lymansia dispar [AF081810]; Maco, Mamestra configurata [U59461]; Op, Orgyia pseudotsugata [U75930]; Ro, Rachiplusia ou [AY145471]; Se, Spodoptera exigua [AF169823]; Splt, Spodoptera litura [AF325155]; Tn, Trichoplusia ni [DQ017380].
**Figure S2.** p78/83 activates Arp2/3 complex to mediate y-branch formation. Branching assays performed with 4 µM actin, 4 µM rhodamine-phalloidin, 60 nM Arp2/3 complex, and 600 nM GST-p78-WCA. Branched structures only form in the presence of Arp2/3 complex and p78/83. Scale bar = 2.5 µm.
Figure S3. p78/83 is specifically recognized by anti-p78/83 antibody and localizes primarily to the cytoplasm in the absence of other viral factors. **A.** Anti-p78 recognizes a band of the expected molecular weight in infected cells (I) and cells expressing p78/83 from a plasmid (T), but not control TN-368 cells (U). 25 µg total TN-368 cell lysate was subjected to SDS-PAGE and immunoblotting with affinity-purified chicken anti-p78-WPWCA antibody (1:1000). **B.** TN-368 cells fixed and stained 24 h after transfection with a construct driving constitutive expression of p78/83. p78/83 was visualized by indirect immunofluorescence using anti-p78/83 antibody, F-actin was stained Alexa Fluor 488-phalloidin, and DNA was visualized using DAPI. The actin staining in cells expressing p78/83 is indistinguishable from cells that do not stain with anti-p78 antibody. Scale bar = 10 µm.
Figure S4. Generation and characterization of WOBpos virus. A. A schematic outline of the procedure for generating WOBpos bacmids containing point mutations in p78/83. Star indicates point mutation in p78/83. B. Replication kinetics of wild-type WOBpos-derived AcMNPV compared with parental AcMNPV E2 strain in cultured Sf9 cells. C. The percent mortality in newly molted fourth instar Heliothis virescens induced by oral inoculation with the indicated number of occlusions of WOBpos virus or parental AcMNPV-E2. Percent mortality in insects was determined as described in (1).

Figure S5. Arp2/3 complex is critical for AcMNPV replication. (A) dsRNA-mediated gene silencing of ARPC3/p21 in TN-368 cells. Cells were transfected with dsRNA targeting ARPC3/p21 or YFP (control), harvested at 5, 6, or 7 days post transfection (dpt) and probed by immunoblotting with anti-p21 and anti-cofilin antibodies. In cells treated with ARPC3/p21 dsRNA, the p21 protein levels were reduced 52±11% compared to YFP dsRNA-treated cells. (B) Viral replication in cells treated with ARPC3/p21 or YFP dsRNA. Cells were transfected with the indicated dsRNA and infected at 5 dpt. The viral titer of the supernatant was determined at 0, 24, and 48 hpi. Viral titer in ARPC3/p21 dsRNA-treated cells was reduced by 65 and 55% at 24 and 48 hpi, respectively, when compared to YFP dsRNA-treated cells. Asterisks indicate statistically significant differences between titer from YFP- and ARPC3/p21 dsRNA-treated samples at the same timepoint (**: P<0.001, *: P<0.01).
Figure S6. p78/83 activation of Arp2/3 complex is required for baculovirus-induced nuclear actin polymerization. TN-368 cells transfected with the indicated p78/83 mutant bacmids were fixed at 24 hpt and processed for immunofluorescence with anti-p78-WPWCA to label p78/83. Because cells transfected with the Δp78/83 mutant showed no p78/83 staining, anti-pp31 antibodies were used to stain and identify transfected cells (pp31 is a viral late transcription factor). Alexa Fluor 488-phalloidin to visualize actin filaments, and DAPI to label DNA. Significant nuclear F-actin was observed in cells transfected with the p78-D364A and -I358A mutants (which are viable), but not in cells transfected with the p78-W387A, -RRR368-70AAA, or Δp78/83 mutants (which are inviable). Scale bar = 10 µm
Figure S7. p78/83 is not necessary for nuclear localization of Arp2/3 complex or sufficient for nuclear polymerization of actin. (A) p78/83 is not necessary for nuclear targeting of Arp2/3 complex. TN-368 cells were co-transfected with bacmid (WT or Δp78/83) and a plasmid that expresses p21-YFP and fixed at 24 hpt. Cells were processed for immunofluorescence using anti-p78-WPWCA (WT) or anti-pp31 (Δp78/83), anti-GFP, and DAPI. Arp2/3 complex localizes to the nucleus in the absence of p78/83 expression (top left panel). (B) Nuclear p78/83 and Arp2/3 complex are not sufficient to induce nuclear actin polymerization in the absence of other viral factors. TN-368 cells were transfected with a construct expressing p78/83 with a C-terminal nuclear localization signal (p78-NLS) (top) or co-transfected with constructs expressing p78-NLS and p21-YFP-NLS, fixed at 24 hpt and processed for immunofluorescence with anti-p78-WPWCA (top and bottom), anti-GFP (bottom), Alexa Fluor 488-(top) or 350-(bottom) phalloidin, and DAPI (top). Neither treatment was sufficient to induce nuclear actin polymerization. Scale bars = 10 µm.
Supporting tables

Table S1. Summary of phenotypes of p78/83 mutants.

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Supporting references

Supplementary Movie Captions

**Movie S1.** Nuclear actin accumulation and polymerization in infected TN-368 cells expressing EGFP-actin (14:30 to 21:42 hpi). 4 µM latA is added immediately after the bottom cell begins to polymerize actin in the nucleus causing reversion to diffuse EGFP-G-actin signal. Images were acquired every 8 min and movie is ~2400X real time.

**Movie S2.** Nuclear actin accumulation and polymerization in infected TN-368 cells expressing EGFP-actin (18:45 to 22:05 hpi). Images were acquired every 8 min and movie is ~2400X real time.

**Movie S3.** Nuclear actin accumulation and polymerization in infected TN-368 cells expressing mCherry-actin (5:44 hpi to 22:40 hpi). Images were acquired every 8 min and movie is ~2400X real time.

**Movie S4.** Fluorescence recovery after photobleaching a small region of the nucleus in a representative infected TN-368 cell expressing EGFP-actin. Imaging was initiated at ~12 hpi and images were acquired at 6 s intervals (~7.75 s between frames including scan time; ~40X real time; bleaching was performed after the second frame of the movie).

**Movie S5.** Fluorescence loss in photobleaching of nuclear GFP-actin in a representative infected TN-368 cell after treatment with latA. Imaging was initiated at ~15 hpi and images were acquired at 6 s intervals (~7.75 s between frames including scan time; ~40X real time; bleaching was performed after the first frame in the movie).