

The Host Phosphoinositide 5-Phosphatase SHIP2 Regulates Dissemination of Vaccinia Virus^{∇†}

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After fusing with the plasma membrane, enveloped poxvirus virions form actin-filled membranous protrusions, called tails, beneath themselves and move toward adjacent uninfected cells. While much is known about the host and viral proteins that mediate formation of actin tails, much less is known about the factors controlling release. We found that the phosphoinositide 5-phosphatase SHIP2 localizes to actin tails. Localization requires phosphotyrosine, Abl and Src family tyrosine kinases, and neural Wiskott-Aldrich syndrome protein (N-WASP) but not the Arp2/Arp3 complex or actin. Cells lacking SHIP2 have normal actin tails but release more virus. Moreover, cells infected with viral strains with mutations in the release inhibitor A34 release more virus but recruit less SHIP2 to tails. Thus, the inhibitory effects of A34 on virus release are mediated by SHIP2. Together, these data suggest that SHIP2 and A34 may act as gatekeepers to regulate dissemination of poxviruses when environmental conditions are conducive.

Orthopoxviruses, including vaccinia virus (VACV), monkeypox virus (MPXV), and variola virus (VARV), are large double-stranded DNA (dsDNA) viruses that cause characteristic umbilicated vesiculopustular skin lesions (pox) (12). VARV is the causative agent of smallpox, and VACV is used for vaccination against smallpox (12). Although smallpox has been eradicated, naturally occurring poxviruses are still of concern to humans. In particular, MPXV is endemic in Africa (57) and has the potential for spread to humans from bushmeat and squirrels (28, 29, 53, 57, 58), and recent outbreaks in the Democratic Republic of Congo have raised the possibility of human-to-human transmission (58). Efforts to understand the capacity for human-to-human transmission among poxviruses have focused on how the virus spreads from cell to cell.

Infection by poxviruses is initiated upon entry of either of two different forms of the virus. The first, called the intracellular mature virus (IMV; also called mature virion [MV]), consists of a viral core surrounded by one or two lipid bilayers derived from an endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) (21, 59, 61, 71). A second infectious form of the virus, called the extracellular enveloped virus (EEV; also called enveloped virus [EV]) (67), consists of an IMV enveloped in additional membranes derived from the host cell. IMV is released following lysis of host cells (60), whereas the precursors of EEV traffic along microtubules to the cell periphery (15, 20, 56, 75). Upon fusion with the plasma

membrane, the doubly enveloped virion stimulates formation of actin-filled membranous protrusions called tails and then disengages from the host cell (67).

Formation of actin tails occurs by a mechanism conserved among VACV, MPXV, and VARV (55). EEV recruits host Abl and Src family tyrosine kinases (39, 40, 54), which phosphorylate viral protein A36 at residues 112 and 132 (40), thereby facilitating recruitment of Nck, Grb2, Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP), and neural WASP (N-WASP) (13, 14, 37, 63, 76). Interactions with phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] in the plasma membrane induce conformational changes in N-WASP that allow the protein to bind to and activate Arp2/Arp3 (Arp2/3) complex, a nucleator for actin polymerization (37, 62). The rate of actin-mediated propulsion and actin tail length appear to be a function of the turnover rate and interactions among viral factors and recruited host proteins (8, 76).

While extensive information is available about the viral and host factors that initiate actin polymerization, much less is known about the factors that contribute to virion release. Based on mutation experiments, several viral factors (including F12, F13, A33, A34, B5, and A36) have been implicated in viral release (67) although in many cases, such mutations also affect actin tail formation or specific infectivity, thereby precluding unequivocal determination of the role these proteins play in release. That virus release also depends on cell type (36, 44) indicates that host factors also participate. Reeves et al. separated actin motility from release by demonstrating that redundant Src and Abl family tyrosine kinases mediate tail formation, whereas only Abl family kinases mediate release (54).

Previous work from our lab and from others has implicated phosphatidylinositol-3 (PI3)-kinase activities at several distinct steps of viral maturation though not in formation of actin tails or in release (35, 70, 81). Nevertheless, the observation that host proteins involved with vesicular trafficking, such as Alix,

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Tsg101 and Eps15, also affect poxviral spread (22) suggests that lipid signaling may also regulate viral dissemination. In this regard, we considered the possibility that other lipid and phosphoinositide (PI) signaling molecules, including lipid phosphatases, might also participate in virion release.

SHIP2 and its related isoform SHIP1 are SH2 domain-containing inositol polyphosphate 5-phosphatases (7, 27, 31, 32, 47). Whereas SHIP1 is expressed in hematopoietic cells, SHIP2 is expressed ubiquitously (18, 65). Both isoforms exhibit PI 5-phosphatase activity with phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P₃] and PI(4,5)P₂ as substrates (5, 16, 18, 38, 49, 72, 77). SHIP2 was originally identified as a negative regulator of insulin signaling (6), and mutations in SHIP2 have also been linked to metabolic disorders, including type II diabetes (23–25, 33, 66). In addition, SHIP2 has also been implicated in regulating cytoskeletal organization and endocytosis (38, 51, 68, 80). Smith et al. recently demonstrated that SHIP2 localizes to actin protrusions, called pedestals, which form beneath enteropathogenic *Escherichia coli* (EPEC) cells, and that reduction of SHIP2 levels causes an aberrant pedestal structure (68).

Here, we demonstrate that SHIP2 localizes beneath VACV during actin tail formation in a manner that depends on both tyrosine kinases and N-WASP but not actin. We also show that SHIP2 negatively regulates release of virions and may act as a gatekeeper molecule that limits poxvirus dissemination.

MATERIALS AND METHODS

Cells, viruses, and reagents. BSC40, 3T3, Abl1^{-/-} Abl2^{-/-}, N-WASP^{fllox/lox}, and N-WASP^{-/-} cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Cellgro, MediaTech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and 10 IU/ml penicillin and 10 µg/ml streptomycin (P-S) (Cellgro, MediaTech, Inc., Manassas, VA). Src^{-/-} Fyn^{-/-} Yes^{-/-} cells were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS and P-S. SHIP2^{+/+} and SHIP2^{-/-} cells were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS, P-S, and 1% of 50 mM β-mercaptoethanol in Dulbecco's phosphate-buffered saline (DPBS). All cells were grown at 37°C in a 5% CO₂ incubator. Mouse embryonic fibroblasts were isolated from embryos that were homozygous for a knockout of the SHIP2 (inositol polyphosphate phosphatase-like protein 1 [INPPL1]) gene (6) and were prepared as reported in Zhang et al. (82). Viral strains were grown and propagated as previously described (54). Viral strain WI was provided by Bernard Moss and consisted of the WR strain containing A34 derived from strain IHD-J (3). VACV strain IHD-J expressing luciferase was made by Reeves et al. and consisted of the firefly luciferase gene under the control of a synthetic early/late promoter (55). Titers of all viral strains were determined on BSC40 cells.

Microscopy. Various cell lines used for microscopy were plated onto poly-D-lysine (PDL)-collagen-coated glass coverslips and infected with ~10⁶ PFU of WR or virus expressing an F13-green fluorescent protein (F13-GFP) fusion for 16 h. After 16 h, cells were fixed as previously described (54) and processed for microscopy. To detect DNA, cells were stained with 17 µg/ml 4',6'-diamidino-2-phenylindole (DAPI). Actin was visualized using phalloidin-564 (Molecular Probes/Invitrogen, Carlsbad, CA). The following antibodies for microscopy were used: anti-SHIP1 (Cell Signaling Technology, Beverly, MA), anti-SHIP2 (anti-INPPL1; Novus Biologicals, Littleton, CO), anti-lamellipodium (LPD) (anti-RAPH; Sigma, St. Louis, MO), and anti-SHC (BD Transduction Labs) were used at a 1:50 dilution; anti-Xpress (Invitrogen, Carlsbad, CA) was used at 1:200; and anti-phosphotyrosine (PY) (4G10; Millipore, Billerica, MA) and anti-Myc (9E10; Millipore, Billerica, MA) were used at 1:250. Anti-B5 was a gift from Jay Hooper, USAMRIID, and was isolated from rabbits vaccinated with a B5 DNA vaccine. Anti-B5 was added to cells after permeabilization and used at a 1:5,000 dilution. Tyrosine kinase inhibitors, imatinib mesylate (Gleevec) or dasatinib (Sprycel), were added to cells at 10 µM for 30 min after a 15.5-h infection. For transfections, cells were transfected with various SHIP2 constructs (49, 82) or Myc-SHIP1 using FuGene 6 (Roche, Indianapolis, IN) for 48 h. We did not

observe a difference in the numbers of actin tails formed on infected transfected cells.

Comet assays. Experiments with vaccinia virus were conducted under biosafety level 2 (BSL-2) conditions. One hundred or 10 PFU of vaccinia virus strain WR, IHD-J, vRB12 (WR ΔF13), or WI was diluted in 500 µl of 2% FBS-DMEM and added to monolayers of naïve BSC40, SHIP2^{+/+}, or SHIP2^{-/-} cells in six-well dishes. Virus was allowed to adsorb to and enter the cells for 1 h at 37°C in 5% CO₂. Unbound virus was then removed by washing monolayers with 1 ml of PBS. Medium was then replaced with 10% FBS-DMEM. Two days after infection, monolayers were fixed and stained with crystal violet solution (0.1% crystal violet and 20% ethanol).

EEV and CAV measurements. To quantify the amount of EEV and cell-associated virus (CAV) produced, SHIP2^{+/+} and SHIP2^{-/-} cells were grown in six-well dishes in triplicate wells. Cell numbers were quantified, and cells were infected at a multiplicity of infection (MOI) of 5 or 0.01 with strain IHD-J. Virus was diluted in 500 µl of 2% FBS in DMEM and allowed to adsorb to cells for 1 h. Unbound virus was then removed by washing cells three times with PBS and then adding 1.5 ml of 10% FBS in DMEM. To quantify EEV, supernatant was removed 24 or 48 h later and spun at 400 × g for 10 min to remove cells. IMV was neutralized with 1:1,000 10F5 (anti-L1) antibody (a gift from Jay Hooper, USAMRIID) for 1 h at 37°C; the supernatant was then diluted and added to naïve BSC40 cell monolayers, and plaques were enumerated after 2 days. To quantify CAV, monolayers were scraped into 1 ml of 2% FBS in DMEM, and virus was released through three freeze-thaw cycles. CAV was diluted and added to naïve BSC40 monolayers.

RNA interference (RNAi). To knock down SHIP2 protein, BSC40 cells in duplicate six-well plates were transfected with 50 nM RNA complementary to SHIP2 (INPPL1 On-Targetplus small interfering RNA [siRNA] human sequences 1, 2, 3, and 4; Dharmacon) using RNAiMAX Lipofectamine reagent (Invitrogen, Carlsbad, CA). After 3 days cells were infected with 100 PFU of VACV strain IHD-J for 48 h. After this time plaques were fixed and stained with crystal violet. To confirm protein knockdown, BSC40 cells in triplicate six-well plates were transfected with 50 nM RNA complementary to SHIP2 for 3 days, and protein knockdown was confirmed by Western blotting with anti-SHIP2 (anti-INPPL1 at 1:500; Novus Biologicals, Littleton, CO) and anti-glyceraldehyde-3-phosphate dehydrogenase ([GAPDH] 1:1,000; Sigma-Aldrich, St. Louis, MO).

Luciferase assay. To quantify viral entry, SHIP2^{+/+} and SHIP2^{-/-} cells were grown in six-well dishes in triplicate wells. Cell numbers were quantified, and cells were infected at a multiplicity of infection of 5 with strain IHD-J expressing luciferase (55). Virus was diluted in 500 µl of cold 2% FBS in DMEM and allowed to adsorb to cells for 1 h at 4°C. Unbound virus was then removed by washing cells three times with cold PBS and adding 1.5 ml of prewarmed 10% FBS in DMEM. After a 2-h infection cells were lysed, and protein concentration was determined using a Bio-Rad DC (detergent-compatible) Protein Assay kit (Bio-Rad, Hercules, CA). Luciferase expression was measured using 20 µg of protein with a Bright-Glo luciferase assay system (Promega, Madison, WI) and a Synergy HT BioTek plate reader.

Specific infectivity. Specific infectivity was calculated based on a method using the optical density at 260 nm (OD₂₆₀) as described by Parrish and Moss (42). Briefly, SHIP2^{+/+} and SHIP2^{-/-} cells were grown in three 150-cm² tissue culture dishes and infected with ~10⁸ PFU/ml IHD-J for 3 days. To liberate virus, flasks were subjected to three freeze-thaw cycles. Medium and cell debris were purified from medium by spinning samples at 10,000 rpm in a JA-10 rotor for 1.25 h. Cell debris and virus were resuspended in 2 ml of 2% FBS in DMEM. To remove cell debris, samples were spun at 400 × g for 10 min. Virus was further purified through a 36% sucrose cushion, followed by two, 25 to 40% sucrose gradients. Viral particles were quantified by the OD₂₆₀, where 1 OD₂₆₀ unit is equivalent to 1.2 × 10¹⁰ particles. To calculate the number of infectious particles, purified virus was diluted in 2% FBS in DMEM and added to naïve monolayers, and the plaques were enumerated 2 days later. Specific infectivity was calculated as 136.3 ± 46.5 particles/PFU for SHIP2^{+/+} cells and 80.0 ± 18.0 particles/PFU for SHIP2^{-/-} cells.

Western analysis. BSC40 or CRL2019 cells were grown in 10-cm tissue culture dishes. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, Beverly, MA), and protein concentration was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA). One hundred micrograms of protein was separated by SDS-PAGE; proteins were then transferred to nitrocellulose membranes. Those were blocked with 3% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h. Membranes were probed with anti-SHIP1 (1:1,000), anti-SHIP2 (1:500), or anti-tubulin (1:5,000; Abcam, Cambridge, MA) in blocking solution for an additional hour. Bands were detected using anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase

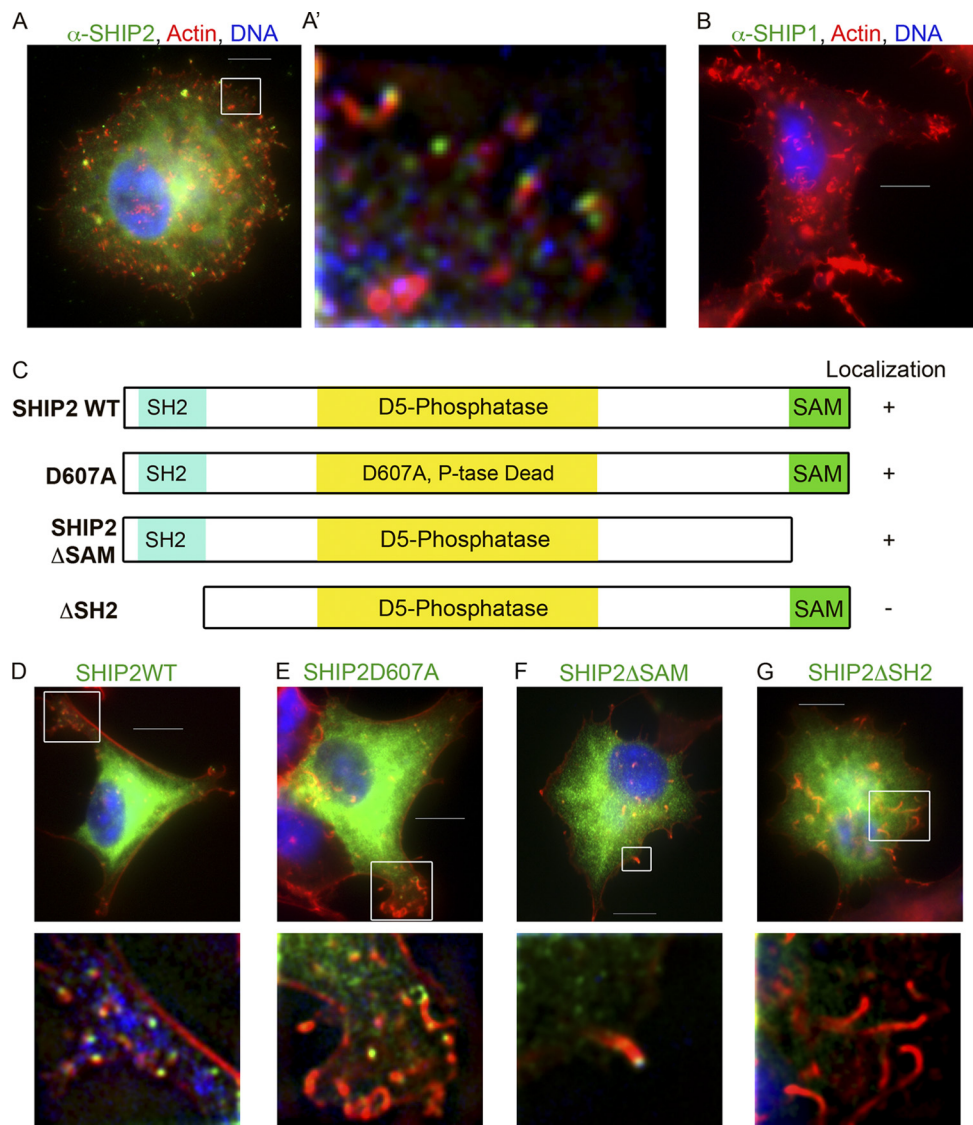


FIG. 1. SHIP2 is recruited to VACV actin tails via its SH2 domain. (A) Images of BSC40 cells infected with VACV strain WR and stained with antibodies recognizing endogenous SHIP2. Panel A' shows a magnification of the boxed region in panel A. (B) Images of BSC40 cells infected with VACV strain WR and stained with antibodies recognizing endogenous SHIP1. See also Fig. S1 in the supplemental material. (C) Domain organization of SHIP2 and SHIP2 mutants. (D to G) Images of BSC40 cells expressing Xpress-SHIP2-WT, or SHIP2 mutants Xpress-SHIP2-D607A, Xpress-SHIP2- Δ SAM, or Xpress-SHIP2- Δ SH2. Note that deletion of the SH2 domain prevented localization to actin tails. Images in the lower panels are magnifications of the respective boxed regions. Scale bar, 10 μ m. P-tase, phosphatase; α , anti.

([HRP] GE Healthcare, United Kingdom), and blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA).

RESULTS

The inositol phosphatase SHIP2 localizes to VACV actin tails. To explore a role for PI signaling in actin tail formation and virion release, we first assessed whether PI kinases or phosphatases localized beneath virions. We could not detect localization of type I PI3-kinases with several antisera (data not shown). However, an endogenous protein recognized by an antibody to the phosphoinositide phosphatase SHIP2 was enriched relative to the cytoplasm at the tips of actin tails and directly apposed to the virion (Fig. 1A and A'). Localization of SHIP2 was apparent in the majority of actin tails in BSC40

cells. The localization appeared specific as antiserum that detects SHIP1 but not SHIP2 did not recognize epitopes in actin tails (Fig. 1B). Western analysis confirmed that SHIP1 was not expressed at detectable levels in BSC40 cells, in contrast to SHIP2 (see Fig. S1A and B in the supplemental material). However, exogenously expressed SHIP1 did localize to tails in BSC40 cells (see Fig. S1C). To identify the domains of SHIP2 necessary for recruitment to actin tails, we transiently transfected vectors expressing wild type (WT) SHIP2 (SHIP2-WT) or SHIP2 variants containing point mutations or deletions of particular domains into BSC40 cells (Fig. 1C). Overexpressed SHIP2-WT (Fig. 1D) localized to the tops of tails, as did a phosphatase dead mutant (SHIP-D607A) (Fig. 1E), and a sterile alpha motif (SAM) domain

deletion mutant (SHIP2- Δ SAM) (Fig. 1F). The localization of SHIP2- Δ SAM to actin tails appeared consistent with the localization of exogenous SHIP1, which lacks a SAM domain (see Fig. S1C in the supplemental material). In contrast, SHIP2 lacking the SH2 domain (SHIP2- Δ SH2) did not localize to actin tails (Fig. 1G). Together, these data suggest that the SH2 domain is required for localization of SHIP2 to actin tails; however, neither phosphatase activity nor the SAM domain appeared required for localization.

Localization of SHIP2 requires Abl and Src family tyrosine kinases and N-WASP but not actin. Observations with SHIP2- Δ SH2 indicated that tyrosine phosphorylation might be required for localization of SHIP2 to actin tails. We next assessed whether localization of SHIP2 was evident in cell lines lacking particular tyrosine kinases or in cells treated with tyrosine kinase inhibitors. SHIP2 remained localized to actin tails formed on Abl1^{-/-} Abl2^{-/-} cells, Src^{-/-} Fyn^{-/-} Yes^{-/-} cells, and on cells treated with 10 μ M imatinib mesylate (STI-571), an inhibitor of Abl family kinases (Fig. 2A) (64). However, in cells treated with dasatinib (BMS-354825), an inhibitor of both Abl and Src family kinases and actin tails (55), no localization of SHIP2 was evident apposed to virions (Fig. 2B).

To determine whether localization of SHIP2 requires other components of VACV actin tails, we next assessed SHIP2 localization in fibroblast cell lines derived from N-WASP^{flox/flox} or N-WASP^{-/-} mice and infected with VACV. Cell lines deficient in N-WASP fail to form tails (69); therefore, we measured the colocalization of SHIP2 with phosphotyrosine and anti-B5, components of cell-associated virions. In N-WASP^{flox/flox} cells, 25% of virions colocalized with both SHIP2 and phosphotyrosine (Fig. 2C and D; see also Fig. S2 in the supplemental material). In contrast, only 1.9% of virions lacking a phosphotyrosine signal colocalized with SHIP2. In N-WASP^{-/-} cells, the percentage of virions colocalizing with SHIP2 was similar whether phosphotyrosine was present or not (1.8 to 2.7%) and similar to that observed in N-WASP^{flox/flox} cells for virions lacking phosphotyrosine. These data suggest that N-WASP and phosphotyrosine together are required for localization of SHIP2. To rule out the possibility that localization of SHIP2 required the Arp2/3 complex or actin, we assessed localization of SHIP2 in BSC40 cells expressing N-WASP with a deletion of the cofilin and acidic domains (N-WASP- Δ CA), which fails to recruit the Arp2/3 complex and thereby precludes formation of actin tails (26). As shown in Fig. 2E, SHIP2, N-WASP- Δ CA, and the virion (detected with anti-B5 monoclonal antibody [MAB]) colocalized. Collectively, these data suggest that activity of Abl or Src family kinases, perhaps acting redundantly (54), is required for localization of SHIP2 and that localization depends on N-WASP but not on the Arp2/3 complex or actin. The interaction between SHIP2 and N-WASP appears to be indirect as we were unable to detect a direct association of SHIP2 with N-WASP in immunoprecipitation experiments (data not shown).

SHIP2 does not regulate formation of actin tails. Localization of SHIP2 beneath virions on actin tails raised the possibility that the protein might regulate either actin tails or virion release or both. To test these possibilities, we first assessed actin tails in embryonic fibroblasts derived from SHIP2^{+/+} and SHIP2^{-/-} mice. As shown in Fig. 3A, the numbers and sizes of

actin tails appeared similar in the two cell types, as did the velocities of virions on tails (data not shown). We also did not observe a difference in the numbers or localization of B5-positive virions outside these cells (data not shown). We confirmed that SHIP1 was not expressed in either SHIP2^{+/+} or SHIP2^{-/-} cells, SHIP2 was not expressed in the SHIP2^{-/-} cells, and SHIP2 was evident only on the tops of actin tails found in SHIP2^{+/+} but not in SHIP2^{-/-} cells (see Fig. S3 in the supplemental material). Previous work by Smith et al. identified two interacting partners of SHIP2, SHC and LPD, which localize to EPEC pedestals (68), and Krause et al. identified LPD as localizing to VACV actin tails (30). We confirmed that LPD as well as SHC localized to the tips of VACV tails (See Fig. S4A and B). However, both SHC and LPD were recruited to tails in both SHIP2^{+/+} cells and SHIP2^{-/-} cells. Together, these data suggest that SHIP2 is required for neither the formation of actin tails nor the recruitment of LPD or SHC.

SHIP2 regulates virion dissemination by inhibiting release of EEV. We next investigated the effects of SHIP2 on plaque formation. To do this, SHIP2^{+/+} or SHIP2^{-/-} cells were infected with VACV strain IHD-J, which releases large numbers of EEV particles (3). At 32 h postinfection, plaques formed by IHD-J are similar in size to those seen with other strains (e.g., WR) (Fig. 3B, upper panel). However, unlike WR plaques, IHD-J plaques are associated with an archipelago of smaller plaques, termed “comets,” which are evident 48 h after infection and which are indicative of enhanced EEV release in this strain (1, 19). Characteristic plaques formed by IHD-J were evident on SHIP2^{+/+} and SHIP2^{-/-} cells by 32 h (Fig. 3B, upper panel) though plaques were slightly larger on SHIP2^{-/-} cells. Comets visualized at 48 h postinfection were significantly larger in SHIP2^{-/-} cells than those in SHIP2^{+/+} cells, often merging with adjacent comets and extending across the plate (Fig. 3B, lower panel). We next measured the amount of EEV released by SHIP2^{+/+} or SHIP2^{-/-} cells into the supernatant. In accordance with the plaque assays, ~3-fold more EEV and CAV grew in SHIP2^{-/-} cells than in SHIP2^{+/+} cells at a low MOI (0.01) (Fig. 3C), consistent with increased viral spread in the monolayer. We did not observe a difference in the ratio of EEV to CAV between the two cell types, and viral replication was similar at an MOI of 5, suggesting that SHIP2 does not affect viral replication (data not shown). To corroborate data from SHIP2^{-/-} cells, we next knocked down SHIP2 in BSC40 cells. BSC40 cells treated with either of three siRNAs specific to SHIP2 (sequences 2, 3, and 4) exhibited larger comets than those seen with negative siRNA, the transfection reagent (RNAiMAX), or untreated cells (Fig. 3D). Knockdown of SHIP2 was confirmed by Western analysis (Fig. 3E).

To confirm that the large comets formed on the SHIP2^{-/-} cells were specifically due to increased release of EEV, we carried out two additional experiments. Reeves et al. found that release of EEV required activity of Abl family tyrosine kinases (54). In accordance with the idea that more EEV is released from SHIP2^{-/-} cells than from SHIP2^{+/+} cells, we found that the Abl family tyrosine kinase inhibitor imatinib mesylate blocked comets in SHIP2^{+/+} cells and reduced the size and extent of comets in SHIP2^{-/-} cells (Fig. 4A). Second, we infected both cell types with WR vRB12, a virus lacking F13L (2), a gene required to form EEV and comets. As shown

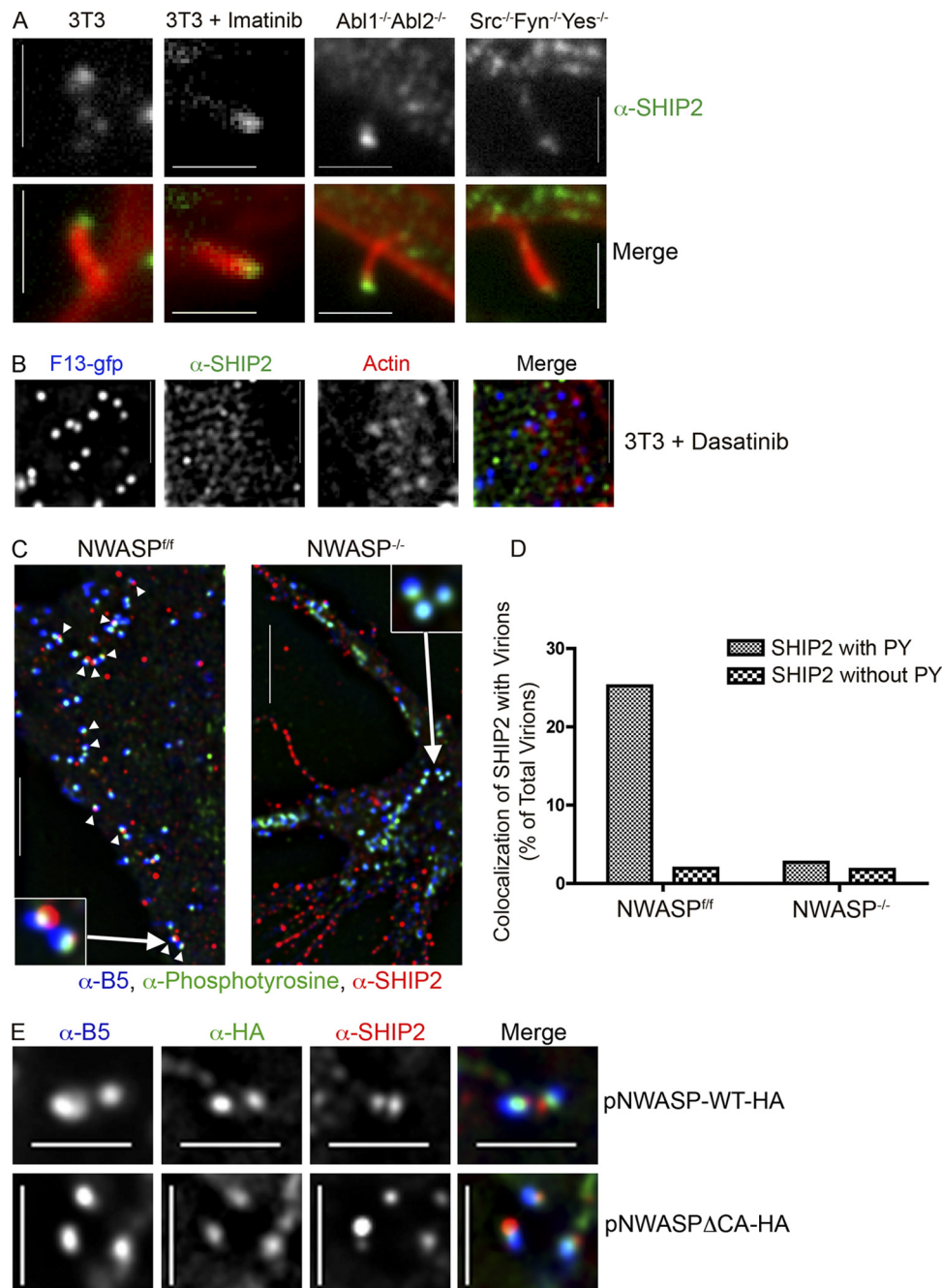


FIG. 2. Localization of SHIP2 requires Abl and Src family tyrosine kinases and N-WASP but not actin. (A) Images of endogenous SHIP2 in actin tails formed by WR on 3T3 cells, 3T3 cells treated with 10 μ M imatinib mesylate (inhibits Abl1 and Abl2), Abl1^{-/-} Abl2^{-/-} cells, or Src^{-/-} Fyn^{-/-} Yes^{-/-} cells. Scale bar, 2 μ m. (B) Endogenous SHIP2 does not appear localized with F13-GFP virions following dasatinib treatment, which inhibits both Src and Abl family kinases and blocks actin tails. Scale bar, 2 μ m. (C) Images of colocalization of SHIP2 with vaccinia virus protein B5 and PY in N-WASP^{flox/flox} or N-WASP^{-/-} cells. Arrows indicate colocalization of all three proteins, and insets are magnifications of the indicated WR virions. Scale bar, 5 μ m. See also Fig. S2 in the supplemental material. (D) Quantification of colocalization of B5 fluorescence, SHIP2, and PY in images such as those in panel C. Data are expressed as a percentage of total virions on which SHIP2 was colocalized with B5 with or without PY staining in N-WASP^{flox/flox} cells ($n = 1,244$ virions) and N-WASP^{-/-} cells ($n = 1,133$ virions). (E) Localization of SHIP2 to WR virions does not require actin. BSC40 cells were transfected with pNWASP-WT-HA (where HA is the hemagglutinin A epitope tag) or pNWASP Δ CA-HA and infected with VACV. Colocalization of SHIP2 with B5 was evident in cells transfected with either N-WASP-WT-HA or N-WASP Δ CA-HA. Scale bar, 2 μ m.

in Fig. 4B, vRB12 formed similarly sized plaques on SHIP2^{+/+} and SHIP2^{-/-} cells but did not form comets on either cell type. We could find no evidence to support the possibility that differences in infectivity or cellular migration rates could account

for the apparent increase in the size of comets in SHIP2^{-/-} cells. Furthermore, luciferase under the control of an early/late promoter was similarly expressed in both cell types at 2 h postinfection, indicating that increased viral entry did not ac-

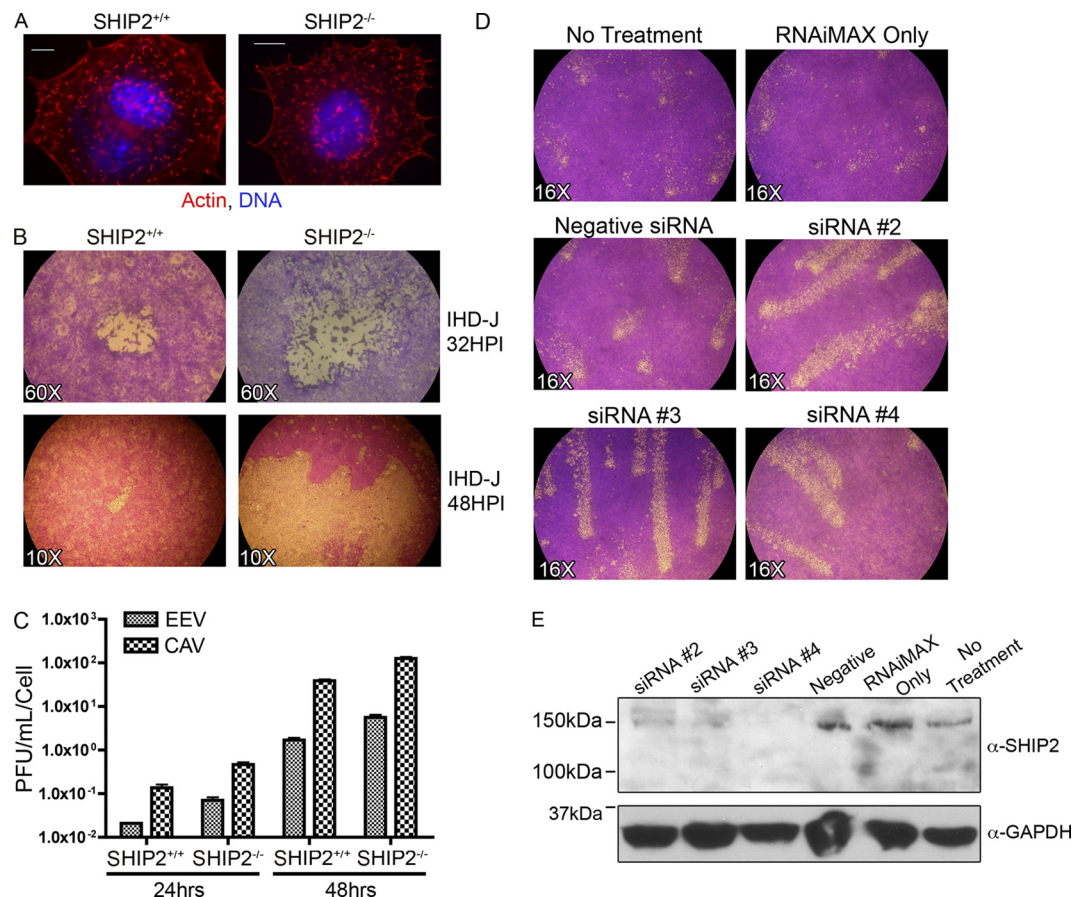


FIG. 3. SHIP2 regulates virion dissemination independent of actin tail formation. (A) VACV strain WR forms actin tails on SHIP2^{+/+} cells and SHIP2^{-/-} cells. Cells were stained for actin (red) and DNA (blue). Scale bar, 10 μ m. See also Fig. S3 and S4 in the supplemental material. (B) VACV strain IHD-J forms larger comet tails in SHIP2^{-/-} cells than in SHIP2^{+/+} cells. Cells were infected for 32 h (upper panels; magnification, $\times 60$) or 48 h (lower panels; magnification, $\times 10$) and stained with crystal violet to visualize plaques. (C) Quantification of EEV and CAV of strain IHD-J from SHIP2^{+/+} and SHIP2^{-/-} cells at 24 and 48 h postinfection. (D) SHIP2 knockdown with siRNA enhances virion release relative to control cells. BSC40 cells were left untreated or were treated with Lipofectamine reagent (RNAiMAX), negative siRNA, or either of three siRNAs that target SHIP2 (2, 3, or 4) for 3 days; cells were then infected with VACV strain IHD-J for 2 days and stained and visualized at a magnification of $\times 16$. (E) The upper panel shows Western analysis with SHIP2 antiserum of lysates of BSC40 cells left untreated or treated as described for panel D. The lower panel shows Western analysis of lysates with GAPDH antiserum to confirm equivalent loading of samples.

count for enhanced comet size (see Fig. S5 in the supplemental material). In addition, specific infectivity levels of VACV produced in SHIP2^{-/-} and SHIP2^{+/+} cells were similar, as were the numbers of plaques formed on both cell types, and no differences in rates of movement of SHIP2^{+/+} and SHIP2^{-/-} cells were evident (data not shown). Collectively, these data suggest that SHIP2 inhibits release of EEV.

VACV protein A34 mediates the effects of SHIP2 on inhibition of EEV release. The observation that deletion of A34 in VACV enhances release of virus (34) suggests that A34, a component of EEV, acts as an inhibitor of release. In accordance with this idea, the VACV strain IHD-J, which contains a mutation in A34, releases more EEV and forms larger comets than strain WR, and a WR strain containing A34 derived from IHD-J (called WI) forms larger comets than WR (3). The large comets evident in SHIP2^{-/-} cells compared to those in SHIP2^{+/+} cells led us to hypothesize that A34 may act via SHIP2. To test this possibility, we assessed the effects of WR and WI comets formed on SHIP2^{-/-} and SHIP2^{+/+} cells. As

shown in Fig. 4C, WR was unable to form comets on SHIP2^{+/+} cells but did form comets on SHIP2^{-/-} cells (Fig. 4C). WI produced small comets on SHIP2^{+/+} cells but large comets on SHIP2^{-/-} cells (Fig. 4D), reminiscent of those seen with IHD-J in these cells (Fig. 3B). We hypothesized that the capacity of IHD-J and WI to form larger comets than WR was due to differences in the recruitment of SHIP2 to actin tails in these strains. As shown in Fig. 4E, an inverse correlation exists between EEV release and the efficacy of SHIP2 recruitment to actin tails. Thus, whereas strain WR recruited SHIP2 to 69% of actin tails, strains IHD-J and WI recruited SHIP2 to 54% and 49% of tails, respectively, a statistically significant difference compared to WR ($P < 0.0003$ and $P < 0.0001$, respectively) (Fig. 4E). Collectively, these data suggest the following: (i) that recruitment of SHIP2, via its SH2 domain, requires N-WASP and the Abl and Src family tyrosine kinases but that these proteins alone are not sufficient; (ii) that viral protein A34 recruits SHIP2 to actin tails; and (iii) that SHIP2 at least in part mediates inhibition of release by A34.

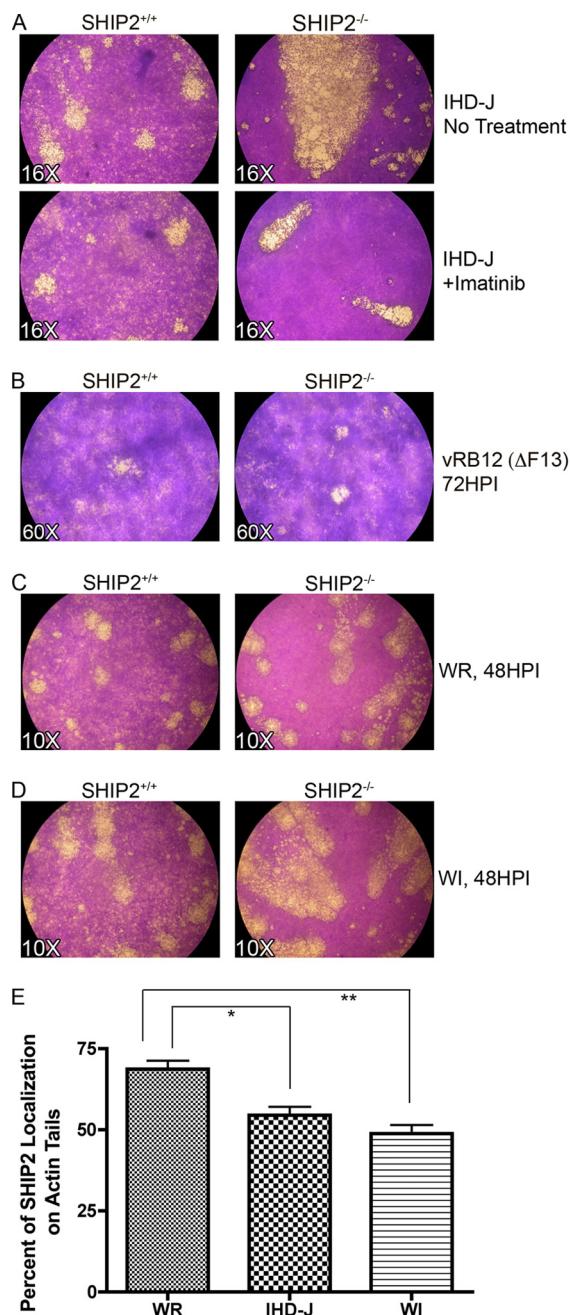


FIG. 4. A34 and SHIP2 negatively regulate virion dissemination. (A) Release of virions from SHIP2^{+/+} and SHIP2^{-/-} cells is sensitive to imatinib mesylate. Cells were infected with VACV IHD-J for 48 h postinfection and stained with crystal violet. Plaques were visualized by microscopy at a magnification of $\times 16$. Imatinib mesylate (10 μ M) was added to cells at 1 h postinfection. (B) Comets formed on SHIP2^{+/+} and SHIP2^{-/-} cells are specifically due to EEV. Cells were infected for 72 h with WR vRB12, a virus lacking the F13L gene and unable to form EEV. Although plaques formed on the two cell types were of similar sizes, no comets were evident. Plaques were visualized at a magnification of $\times 60$. (C) Plaques formed by VACV strain WR on SHIP2^{+/+} and SHIP2^{-/-} cells 48 h postinfection (magnification, $\times 10$). (D) Plaques formed by VACV strain WI on SHIP2^{+/+} and SHIP2^{-/-} cells at 48 h postinfection (magnification, $\times 10$). (E) Quantification of SHIP2 recruitment to actin tails in cells infected with VACV strains WR, IHD-J, or WI. WR ($n = 2597$ tails, 45 cells) recruits more SHIP2 to tails than IHD-J ($n = 2,046$ tails, 58 cells) ($P = 0.0003$) or WI ($n = 2,763$ tails, 32 cells) ($P = 0.0001$). HPI, hours postinfection.



FIG. 5. Overview of SHIP2 recruitment and regulation of virion release.

DISCUSSION

Here, we explore the role of the phosphoinositide 5-phosphatase SHIP2 in release of VACV from infected cells. We found that SHIP2 localizes to actin tails in an SH2-dependent manner, suggesting that phosphorylated tyrosines on cellular or viral proteins within the tail mediate recruitment. Host Src and Abl family kinases localize to and redundantly form tails (14, 54), and we find that these kinases are also required for recruitment of SHIP2 (Fig. 2). Abl but not Src family kinases also appear to play a role in enhancing release of EEV (54). In this regard, comets formed on SHIP2^{+/+} and SHIP2^{-/-} cells are blocked by imatinib mesylate, a specific inhibitor of Abl family kinases (54). Thus, Abl family kinases play dual but antagonistic roles in EEV release, by both facilitating recruitment of a release inhibitor (SHIP2) and promoting virion release (Fig. 5).

Src and Abl family tyrosine kinases phosphorylate the viral protein A36 at two sites, suggesting that phosphorylated A36 may directly recruit SHIP2 (40). However, this seems unlikely because phospho-A36 recruits N-WASP via Nck and WIP (14, 37), and SHIP2 recruitment also depends on N-WASP. We cannot rule out the possibility that factors distal to N-WASP may recruit SHIP2. Whereas Grb2 is one such candidate (63), the Arp2/3 complex and actin do not appear to be involved as SHIP2 does not affect actin tails and as N-WASP- Δ CA, which does not recruit the Arp2/3 complex, still recruits SHIP2. In this regard, Smith et al. found that SHIP2 appears to regulate host lipids and actin polymerization in EPEC pedestals (68), which resemble actin tails formed by vaccinia virus (17). However, our data do not recapitulate the EPEC phenotype, suggesting that VACV utilizes SHIP2 in a manner distinct from EPEC.

Still unresolved is whether SHIP2 inhibits virion release via its catalytic activity or, alternatively, serves as a scaffold to recruit effectors. Two known SHIP2 binding partners, SHC and LPD, are recruited to VACV actin tails, though by a mechanism that appears to be independent of SHIP2 (see Fig. S3 in the supplemental material). Nevertheless, other SHIP2 effectors have been described, including epidermal growth factor receptor (EGFR), filamin, p130^{Cas}, Cbl, vinexin, Arap3, APS, JIP-1, and intersectin (11, 38, 41, 43, 48, 50, 52, 73, 78, 79).

Another possibility is that SHIP2 phosphatase activity is required to inhibit release of EEV. In this regard, we have attempted to localize PH domains that specifically recognize various phosphatidylinositol phosphates (PIPs) on actin tails. Although some PH domains do appear to localize, point mutants that abolish binding to lipid moieties *in vitro* also localize, suggesting that recruitment is nonspecific (S. McNulty and D. Kalman, unpublished data). Furthermore, we have been un-

able to detect Akt-PH, Akt, or Akt-(P)S473 at the tops of tails (McNulty and Kalman, unpublished). Notably, we cannot rule out the possibility that expression of the PH domain alone does not compete effectively with intact proteins that utilize multiple binding sites, a phenomenon we observed previously with localization of proline-rich regions and SH2 domains from tyrosine kinases in EPEC pedestals (4).

Do viral proteins participate with SHIP2 to regulate release of EEV? The observation that deletion of A34 or point mutations within A34 (K151E) enhance release of EEV (3) raises the possibility that the normal function of A34 is to suppress EEV release. Our data suggest that A34 mediates recruitment of SHIP2 and that SHIP2 at least in part mediates inhibition of release by A34 (Fig. 4). A34 exists in a complex with viral proteins A33, A36, B5, and F13 (45, 46); therefore, it remains possible that SHIP2 tethers a complex of host and viral proteins that regulate viral release. Other intrinsic host defense molecules have been recently described, including tetherin and viperin, which are induced in an interferon-dependent manner. Tetherin is antagonized by the HIV protein Vpu, and viperin is antagonized by a cellular protein, farnesyl diphosphate synthase (FPPS), which facilitates virion release (9, 74). In contrast, VACV regulation of release through SHIP2 appears distinct. Rather than antagonizing release, A34 cooperates with SHIP2 to inhibit release. A34 is a transmembrane glycoprotein with homology to C-type lectins (10) and may detect the presence of leukocytes, cell adhesion molecules, or endocytic receptors. In so doing, A34 may play a gatekeeper role so as to limit release except when environmental conditions are conducive to dissemination.

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