

Initial characterization of Vaccinia Virus B4 suggests a role in virus spread

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ABSTRACT

Currently, little is known about the ankyrin/F-box protein B4. Here, we report that B4R-null viruses exhibited reduced plaque size in tissue culture, and decreased ability to spread, as assessed by multiple-step growth analysis. Electron microscopy indicated that B4R-null viruses still formed mature and extracellular virions; however, there was a slight decrease of virions released into the media following deletion of B4R. Deletion of B4R did not affect the ability of the virus to rearrange actin; however, VACV811, a large vaccinia virus deletion mutant missing 55 open reading frames, had decreased ability to produce actin tails. Using ectromelia virus, a natural mouse pathogen, we demonstrated that virus devoid of EVM154, the B4R homolog, showed decreased spread to organs and was attenuated during infection. This initial characterization suggests that B4 may play a role in virus spread, and that other unidentified mediators of actin tail formation may exist in vaccinia virus.

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Introduction

The *Poxviridae* comprise a unique family of viruses distinguished by a single linear double-stranded DNA (dsDNA) genome, a large brick-shaped virion, and the ability to replicate exclusively in the cytoplasm of an infected cell (Moss, 2007). The most well-known member of the *Poxviridae* family, variola virus, is the causative agent of smallpox, a disease that killed more humans in recorded history than all other infectious diseases combined (Fenner et al., 1988). Although smallpox was eradicated in 1977 through a dedicated effort by the World Health Organization (Fenner et al., 1988), other poxviruses cause clinically relevant diseases in animals and humans, and recent outbreaks of monkeypox virus underline the importance of studying these viruses (Reed et al., 2004). The use of poxviruses for gene-delivery vectors and oncolytic therapies has also generated interest in studying poxvirus biology (Seet et al., 2003; Thorne, 2011).

Poxviruses form two types of infectious particles, mature virions (MV; also known as intracellular mature virus, IMV), and extracellular virions (EV; also known as extracellular enveloped virus, EEV) (Condit et al., 2006; Moss, 2007). Virion production has

been well studied in vaccinia virus (VACV). The process begins in the virus factories with the formation of crescent-shaped structures, comprised of lipids and virion core proteins, which surround the newly replicated DNA genome (Dales and Mosbach, 1968; Moss, 2007). Poxviruses encode a scaffold protein that provides a stable structure in order for the lipid bilayer to form (Heuser, 2005; Szajner et al., 2005), and proteins synthesized in the endoplasmic reticulum are transported to the growing lipid bilayer (Husain et al., 2006, 2007b). An immature virion (IV) is formed once the lipid bilayer fully encloses the core (Condit et al., 2006; Moss, 2007). Transition to infectious MV occurs following genome packaging, proteolytic cleavage, loss of the D13 scaffold protein, association of additional membrane proteins, and core rearrangement (Condit et al., 2006). While most virus is thought to be released upon cell lysis, some MVs can undergo additional maturation steps, which are thought to aid in their exit prior to lysis. In this process, MVs are transported away from virus factories and acquire two additional lipid membranes from either endosomes or the trans-Golgi network to become wrapped virions (WV) (see Table 1 for a list of genes involved in this process). WV are then transported along microtubules to the cell surface, where they fuse with the plasma membrane and lose one set of membranes to become EVs (Geda et al., 2001; Hollinshead et al., 2001; Rieddorf et al., 2001; Ward and Moss, 2001a). EVs can be immediately released, or remain associated with the

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Table 1

Summary of VACV genes important for virus spread and infectivity.

Gene class	Gene	Activity	References
Virion release and spread	A27L	Transport MV away from virus factory; also implicated in wrapping	(Blasco et al., 1991; Howard et al., 2010; Ward, 2005)
	A33R	Important for wrapping; anchors A36 to viral membrane, thus aiding actin projectile formation	(Breiman et al., 2013; Chan et al., 2010; Chan and Ward, 2010, 2012a, b; Katz et al., 2003; Roper et al., 1996; Roper et al., 1998; Ward et al., 2003; Wolffe et al., 2001)
	A34R	Regulate WV formation and rate of EV release; may be involved in actin projectile formation	(Blasco et al., 1993; Breiman et al., 2013; Breiman and Smith, 2010; Earley et al., 2008; Husain et al., 2007a; McIntosh and Smith, 1996; Perdigero et al., 2008; Vanderplasschen et al., 1998; Wolffe et al., 1997)
	A36R	Anchors F12 to WV for transport to cell surface along microtubules; essential for actin projectile formation	(Frischknecht et al., 1999; Herrero-Martinez et al., 2005; Horsington et al., 2013; Humphries et al., 2012; Johnston and Ward, 2009; Newsome et al., 2006; Rieddorf et al., 2001; Rottger et al., 1999; Sanderson et al., 1998; van Eijl et al., 2000; Ward and Moss, 2004; Wolffe et al., 1998, 2001)
	B5R	Important for wrapping; stimulates actin projectile formation	(Chan and Ward, 2010; Earley et al., 2008; Engelstad et al., 1992; Engelstad and Smith, 1993; Herrera et al., 1998; Isaacs et al., 1992; Katz et al., 2003; Katz et al., 2002; Katz et al., 1997; Mathew et al., 1998; Mathew et al., 1999; Mathew et al., 2001; Newsome et al., 2004; Rottger et al., 1999; Ward and Moss, 2001b; Wolffe et al., 1993)
	E2L	Transport WV to cell surface; also implicated in wrapping	(Dodding et al., 2009; Domi et al., 2008; Morgan et al., 2010; Rottger et al., 1999)
	F11L	Transport WV to cell surface; important for disruption of actin and transition from WV to EV	(Arakawa et al., 2007a; Arakawa et al., 2007b; Cordeiro et al., 2009; Handa et al., 2013; Morgan et al., 2010)
	F12L	Transport WV to cell surface	(Dodding et al., 2009; Morgan et al., 2010; van Eijl et al., 2002; Zhang et al., 2000)
	F13L	Important for wrapping; has phospholipase activity	(Blasco and Moss, 1991; Borrego et al., 1999; Honeychurch et al., 2007; Husain and Moss, 2001, 2002, 2003; Husain et al., 2003; Roper and Moss, 1999; Schmelz et al., 1994)
	A25L/A26L	Mediates binding/fusion; probably determines preference for endocytic or direct fusion pathway	(Chiu et al., 2007)
Binding to cell surface	A27L	Mediates binding to heparan sulfate	(Chung et al., 1998; Hsiao et al., 1998)
	D8L	Mediates binding to chondroitin sulfate	(Hsiao et al., 1999)
	H3L	Mediates binding to heparan sulfate	(Lin et al., 2000)
	A16L	Transmembrane protein; mediates internalization	(Ojeda et al., 2006b)
	A21L	Transmembrane protein; mediates internalization	(Townsley et al., 2005b)
	A28L	Transmembrane protein; mediates internalization	(Senkevich et al. (2004a, b))
	G3L	Transmembrane protein; mediates internalization	(Izmailyan et al., 2006; Senkevich et al., 2005; Turner et al., 2007)
	G9R	Transmembrane protein; mediates internalization	(Ojeda et al., 2006a)
	H2R	Transmembrane protein; mediates internalization	(Nelson et al., 2008; Senkevich and Moss, 2005)
	J5L	Transmembrane protein; mediates internalization	(Wolfe et al., 2012)
Entry-fusion complex (EFC)	L5R	Transmembrane protein; mediates internalization	(Townsley et al., 2005a)
	O3L	Membrane-associated; mediates fusion	(Satheshkumar and Moss, 2009, 2012)
	F9L	Membrane-associated; mediates fusion	(Brown et al., 2006)
Associated with EFC	L1R	Membrane-associated; mediates fusion	(Bisht et al., 2008)
May be associated with EFC	I2L	Membrane-associated; mediates fusion	(Nichols et al., 2008)

outside of the plasma membrane and catalyze the formation of actin tails to infect adjacent cells (Blasco and Moss, 1992; Cudmore et al., 1995). MVs mediate host-to-host transmission, while EVs, the less abundant of the two, mediate spread within an infected host (Smith et al., 2003). Generally, disruption of the genes involved in virion formation and transport confers a small-plaque phenotype in cell culture, and results in reduced virulence *in vivo*; this has been extensively reviewed (Roberts and Smith, 2008a; Smith and Law, 2004).

Within the B *HindIII* region of the VACV genome, a number of open reading frames (ORFs) have been characterized; however, the role of B4R during poxvirus infection remains unknown. B4 contains multiple Ankyrin (Ank) domains in conjunction with a C-terminal F-box (Mercer et al., 2005; Sonnberg et al., 2008); a combination that was unique to poxviruses until recently, when Ank/F-box proteins were identified in the parasitoid wasp, *Nasonia* (Werren et al., 2010). The F-box domain is necessary for interaction with Skp1 in the SCF ubiquitin ligase, an important mediator of protein degradation (Bai et al., 1996; Nakayama and Nakayama, 2006). Cellular F-box proteins recruit substrates to the SCF to be ubiquitinated (Kipreos and Pagano, 2000). Ank repeats are present in a number of cellular proteins, and mediate distinctive protein–protein interactions (Li et al., 2006).

Ank repeats have been implicated in a number signaling events that regulate cell cycle control, cell differentiation, apoptosis, and vesicular trafficking (Li et al., 2006).

Here, we sought characterize the role of B4 during poxvirus infection. We report that deletion of B4R resulted in a reduction in plaque size. We demonstrate that viruses lacking B4R progressed through the stages of crescent, IV, MV, WV, and EV formation, but show decreased levels of virions released in the media. Furthermore, using an ectromelia (ECTV) mouse model, we demonstrate that deletion of the homolog of B4R, EVM154, resulted in reduced spread of the virus to the spleen, liver, lungs, and kidneys, and attenuation of the virus. This initial characterization suggests that B4 might contribute to virion spread.

In addition, we used the large deletion virus, vaccinia virus strain 811 (VACV811) to further probe the question of virus spread. This virus was strategically engineered by progressive deletions and lacks a total of 55 ORFs, when compared to the parental VACV Copenhagen. This includes 38 ORFs from the left end of the genome (C23L to F4L) and 17 from the right end (B13R to B29R) (Perkus et al., 1991; Fagan-Garcia and Barry, 2011). Despite the large number of genes missing from this virus, VACV811 encodes all genes known to contribute to efficient virus formation and spread (Table 1). Here,

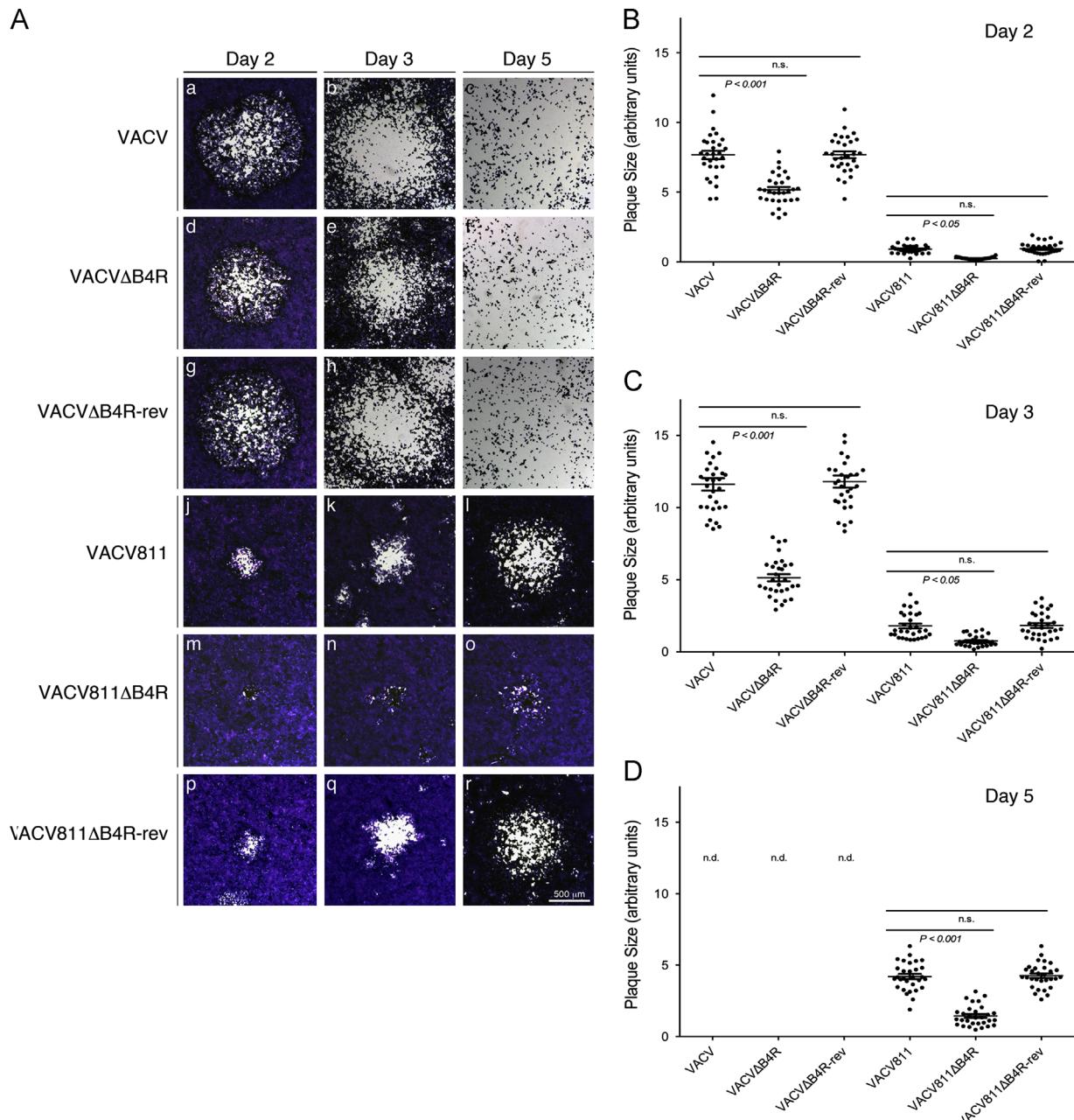


Fig. 1. Effect of deleting B4R on plaque size in tissue culture: (A) plaque sizes. BGMK cells were infected with VACV (a–c), VACVΔB4R (d–f), VACVΔB4R-rev (g–i), VACV811 (j–l), VACV811ΔB4R (m–o), or VACV811ΔB4R-rev (p–r). Cells were stained with crystal violet 2 days (a, d, g, j, m, p), 3 days (b, e, h, k, n, q), or 5 days post-infection (c, f, i, l, o, r). (B, C, and D) Comparison of plaque sizes. 45 plaques were measured over three independent experiments using ImageJ. GraphPad was used to determine significant differences in plaque size between viruses. n.s., not significant; n.d., no data.

we demonstrate that VACV811 had decreased ability to produce actin tail formation. Furthermore, we demonstrate that deletion of B4R from VACV811 produced a decrease in virus spread compared to deletion from VACV, suggesting that B4 might work in cooperation with another protein missing from VACV811.

Results

Deletion of B4R seems to reduce the ability of VACV to spread in tissue culture

The purpose of this study was to perform initial characterization studies on the Ank/F-box protein B4. Using homologous

recombination, we generated a deletion virus from VACV strain Copenhagen, VACVΔB4R, which contains a YFP/gpt cassette in place of the B4R ORF (Rintoul et al., 2011). A revertant virus, VACVΔB4R-rev, was also generated. To determine if the B4 protein was important for virus propagation in tissue culture, a plaque assay was performed (Fig. 1). VACV, VACVΔB4R, or VACVΔB4R-rev, were serially diluted and titrated on BGMK cells and plaques were allowed to form for a period of up to five days. At two days post infection, VACV and VACVΔB4R-rev produced large, round plaques, and secondary plaque formation was visible three days post-infection (Fig. 1A a–c and g–i). At five days post-infection, all of the cells infected with VACV and VACVΔB4R-rev were lysed, and plaques were no longer visible (Fig. 1A). Interestingly, plaques formed by VACVΔB4R were smaller when compared to VACV or VACVΔB4R-rev, indicating that the B4

protein might contribute to virus spread in tissue culture (Fig. 1A d–f). Since the large deletion virus VACV811 also had a reduction in plaque size compared to VACV (Fig. 1A j–o), we generated the deletion virus VACV811ΔB4R, also containing a YFP/gpt cassette in place of the B4R, to determine if the reduction in plaque size was affected by further loss of B4R (Fig. 1A m–o). The revertant virus, VACV811ΔB4R-rev, was also generated (Fig. 1A p–r). Strikingly, plaque formation by VACV811

was significantly reduced compared to VACV (Fig. 1A j and k). Plaques formed by VACV811ΔB4R were barely visible following five days of infection, indicating that deletion of B4R again resulted in a reduction in plaque size (Fig. 1A m–o). VACV811ΔB4R-rev and VACV811 formed the same sized plaques, indicating that the method of deletion did not affect the virus elsewhere in the genome (Fig. 1A j and p). Statistical analysis further confirmed our results (Fig. 1B–D).

To determine the replicative capacity of viruses devoid of the B4 protein, we used single-step growth analysis (Fig. 2A). BGMK cells were infected at a MOI of 10 with VACV, VACVΔB4R, VACVΔB4R-rev, VACV811, VACV811ΔB4R, or VACV811ΔB4R-rev. One hour post-infection, inoculum was removed and incubated with fresh medium. At 0, 4, 8, 12, 24 and 48 h post-infection, media and cells were harvested and virus was released by three freeze-thaw cycles followed by sonication. Similar levels of virus replication occurred, suggesting no defect in viral replication (Fig. 2A). Typically, a reduction in plaque size is indicative of decreased virion morphogenesis, release, or spread. For instance, deletion of the VACV genes A33R, A34R, A36R, B5R, F12L, or F13L results in a small plaque phenotype (Blasco and Moss, 1991; Domi et al., 2008; Duncan and Smith, 1992; Parkinson and Smith, 1994; Roper et al., 1996; Wolffe et al., 1993; Zhang et al., 2000) (Table 1). We used a multi-step growth curve to examine EV formation and virus spread (Fig. 2B). BGMK cells were infected with the indicated viruses and samples were harvested at 0, 12, 24, 48, and 72 h post-infection. The titers of VACVΔB4R and VACVΔB4R-rev were not significantly lower compared to VACV. However, in all three experimental replicates, VACV811 grew to titers two logs lower than VACV, while the titers of VACV811ΔB4R were approximately half to one full log less than VACV811 (Fig. 2B). The revertant VACV811ΔB4R-rev grew as well as its parental virus, again indicating that the method of deletion did not affect the virus elsewhere in the genome. Together, these data confirmed that VACV811 is defective in spread compared to VACV, and suggested that the loss of B4R from VACV811 decreases the ability of VACV811 to spread in tissue culture.

Late genes are still synthesized in viruses devoid of B4R

Since late gene synthesis is required for virion production (Moss, 2007), we wanted to determine if loss of B4 altered late gene synthesis. For this, BGMK cells were infected with VACV, VACVΔB4R, VACV811, or VACV811ΔB4R and whole cell lysates were collected at the indicated times post-infection. Samples were immunoblotted for E3, a VACV protein that is expressed early during infection, and B5, A34, and I5, which are expressed late during infection (Assarsson et al., 2008; Broyles, 2003) (Fig. 3). In cells infected with VACV and VACV811, expression of E3 occurred almost immediately and protein levels increased during

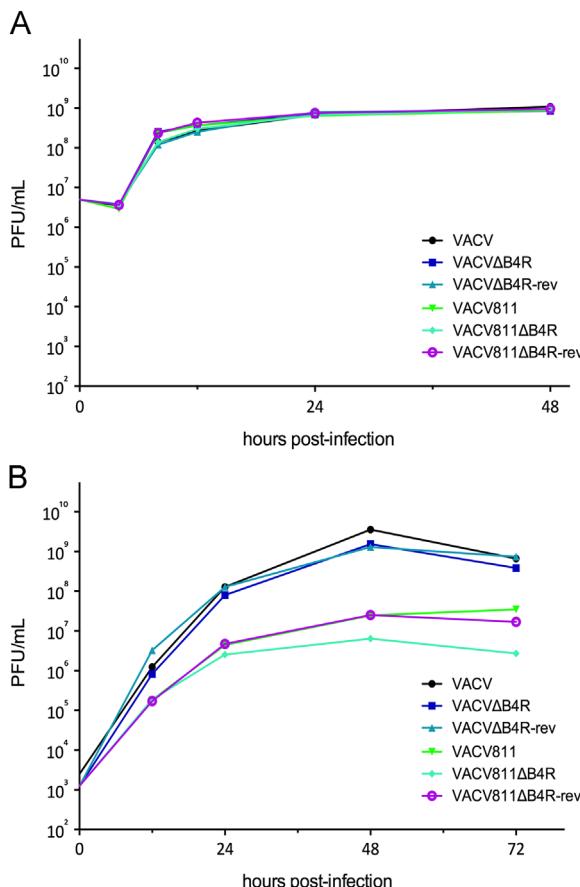


Fig. 2. Analysis of virus growth following deletion of B4R. BGMK cells were infected with VACV, VACVΔB4R, VACVΔB4R-rev, VACV811, VACV811ΔB4R, or VACV811ΔB4R-rev at a MOI of 10 for single-step growth analysis (A) and at a MOI of 0.01 for multiple-step growth analysis (B). Infected cells were harvested and lysed to release infectious virus. Serial dilutions of infectious virus were plated on BGMK cells. Infected monolayers were fixed and stained with crystal violet and plaques were counted to generate growth curves. Representative of three independent experiments, each titered in duplicate.

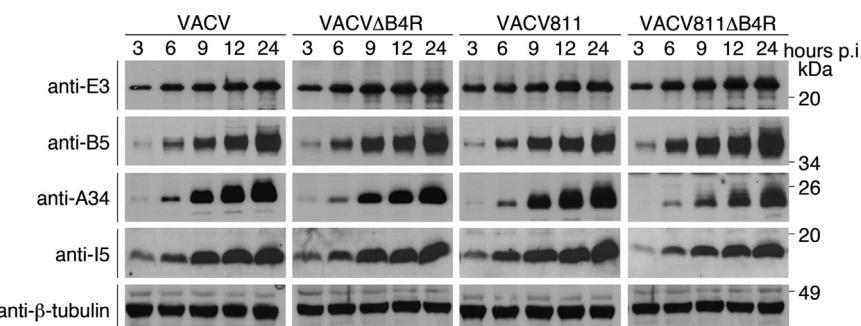


Fig. 3. Effect on late gene synthesis in viruses devoid of B4R. BGMK cells were infected with VACV, VACVΔB4R, VACV811, or VACV811ΔB4R at a MOI of 10. Whole cell lysates were harvested at the indicated times post infection and immunoblotted for the early protein E3, and the late proteins B5, A34, and I5. β -tubulin was used as a loading control.

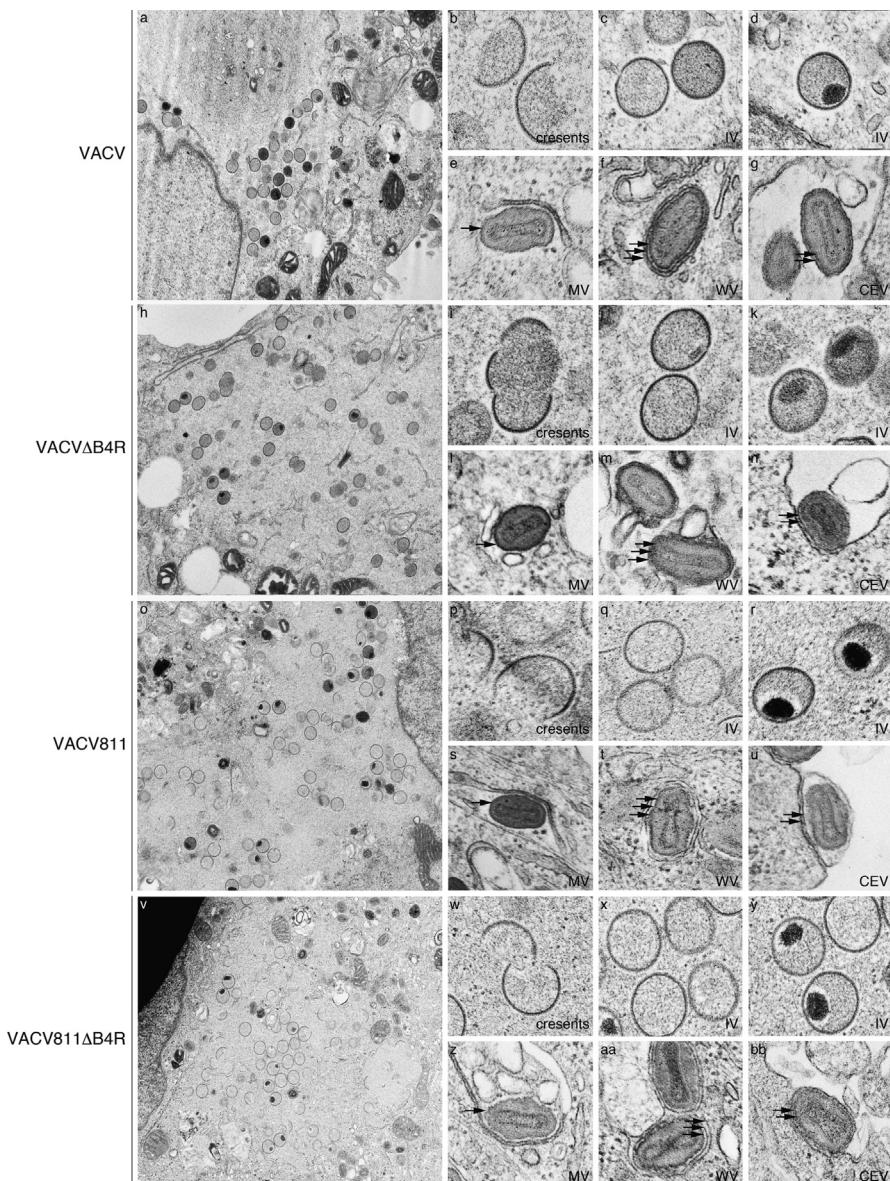


Fig. 4. Transmission electron microscopy of infected cells. BGMK cells were infected at a MOI of 10 with VACV (a–g), VACV Δ B4R (h–n), VACV811 (o–u), or VACV811 Δ B4R (v–bb). Twenty-four hours post-infection, cells were fixed, sectioned, and visualized using transmission electron microscopy. Micrographs display crescent formation (b, c, i, j, p, l, w, x), IV (d, k, r, y), MV (e, l, s, z), WV (f, m, t, aa), and CEV (g, n, u, bb). Arrows indicate membranes.

the course of infection (Fig. 3). Expression of the late proteins B5, A34, and I5 began at six hours post-infection and continued to increase throughout infection (Fig. 3). In all cases, the mutant viruses expressed the indicated proteins to levels similar to their respective parental strain, suggesting that deletion of B4R does not affect late protein expression (Fig. 3).

Viruses devoid of B4R form virus particles, but exhibit decreased EV release into the media

Since late genes were being produced in viruses devoid of B4R, we sought to determine whether EV formation was prevented at some step, using transmission electron microscopy (Fig. 4). BGMK cells were infected at a MOI of 10 with VACV (Fig. 4a–g), VACV Δ B4R (Fig. 4h–n), VACV811 (Fig. 4o–u), or VACV811 Δ B4R (Fig. 4v–bb). Fixed cells were visualized by transmission electron microscopy to observe the stages of virion assembly (Duncan and Smith, 1992). In cells infected with VACV and VACV Δ B4R, crescents (Fig. 4b and i), immature virions (Fig. 4d and k), MVs (Fig. 4e

and l), WV (Fig. 4f and m), and EVs (Fig. 4g and n) were visualized; indicating that loss of the B4 protein did not prevent EV production. In addition, cells infected with VACV811 (Fig. 4o–u) or VACV811 Δ B4R (Fig. 4v–bb) displayed similar results, indicating that virion formation is not hindered. To determine if the same numbers of virions are released from cells infected with the various viruses, we performed a media release assay (Fig. 5). BGMK cells were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Supernatant and cellular fractions were harvested 24 h post-infection, and samples were titrated to determine the percentage of virions in the media compared to the total number of virions. Interestingly, we observed a lower number of virions being released from cells infected with VACV811 Δ B4R compared to VACV811 (Fig. 5). There was no significant difference in the number of virions released from cells infected with VACV Δ B4R compared to VACV (Fig. 5). It is possible that the effect is more pronounced since more genes are missing from VACV811, and one or more gene products may work cooperatively with B4.

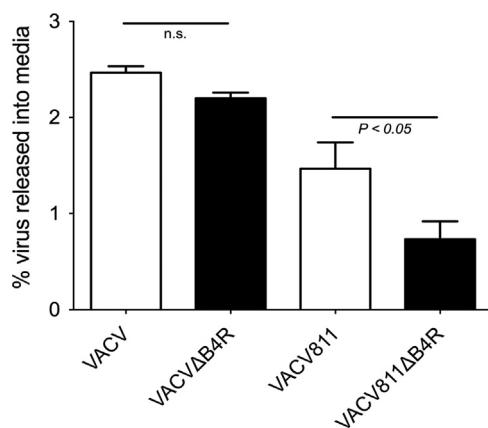


Fig. 5. Effect on virion release following deletion of B4R. BGMK cells were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Twenty-four hours post-infection, media were harvested and cellular debris was removed by centrifugation. Cell monolayers were harvested in PBS and freeze-thawed three times to release virus particles. The media and cell fractions were titrated and the values were used to determine the number of virions that were released from the cell. The amount of released virus, as a percent of total virus (released+cell-associated) was then calculated. The experiment was performed in triplicate, and standard error of the mean (SEM) was determined. n.s., not significant.

Deletion of B4R does not affect actin tails

Following fusion of WV with the cell membrane, the virion remains associated with the membrane until it initiates a rearrangement of actin that projects it away from the cell (Roberts and Smith, 2008b; Smith et al., 2002; Welch and Way, 2013). Since VACV811 devoid of B4R had reduced numbers of virions released from the cell, we sought to determine if formation of actin tails was impaired by loss of B4R (Fig. 6). BSC-1 cells on coverslips were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Twenty hours post-infection, cells were stained with phalloidin to visualize actin tails, and to the viral EV protein B5, to observe the location of WV and EVs in the cell (Fig. 6A). Mean number of projectiles per cell was quantified by counting the actin tails in 50 cells in each of three separate trials (Fig. 6B). Cells infected with viruses devoid of B4R formed actin tails as well as their respective parental strain; however, we noticed a decrease in the number of actin tails formed in cells infected with VACV811 compared to cells infected with VACV (Fig. 6B). Notably, we observed that the localization of B5, a WV/EV specific protein, was not drastically altered by the presence of B4 (Fig. 6A).

The ECTV-mouse model indicates that loss of B4R results in virus attenuation

Since our data suggested that B4 might be important for the virus infection cycle, we wanted to determine whether B4 contributed to virus virulence *in vivo*. Mouse models are useful for studying poxvirus infection; however, VACV is not a natural pathogen to mice. Infection with ECTV, the natural pathogen of mice, is a well-established model to study poxvirus-host interactions (Esteban et al., 2012; Esteban and Buller, 2005). ECTV contains a homolog to B4R, EVM154. The amino acid sequences of EVM154 and B4R are 94% identical, and both ORFs contain the same domains: an F-box domain at the C-terminus which has been demonstrated to be functional, and six Ank domains dispersed throughout the N-terminus (van Buuren et al., 2008). To study the contribution of B4R *in vivo*, we generated an ECTV devoid of EVM154. A revertant was also generated. To assess the role of EVM154 in spread, we performed a plaque assay similar to the one described above (Fig. 2), where ECTV, ECTV Δ 154, or ECTV Δ 154-rev were serially diluted and titrated on BGMK cells

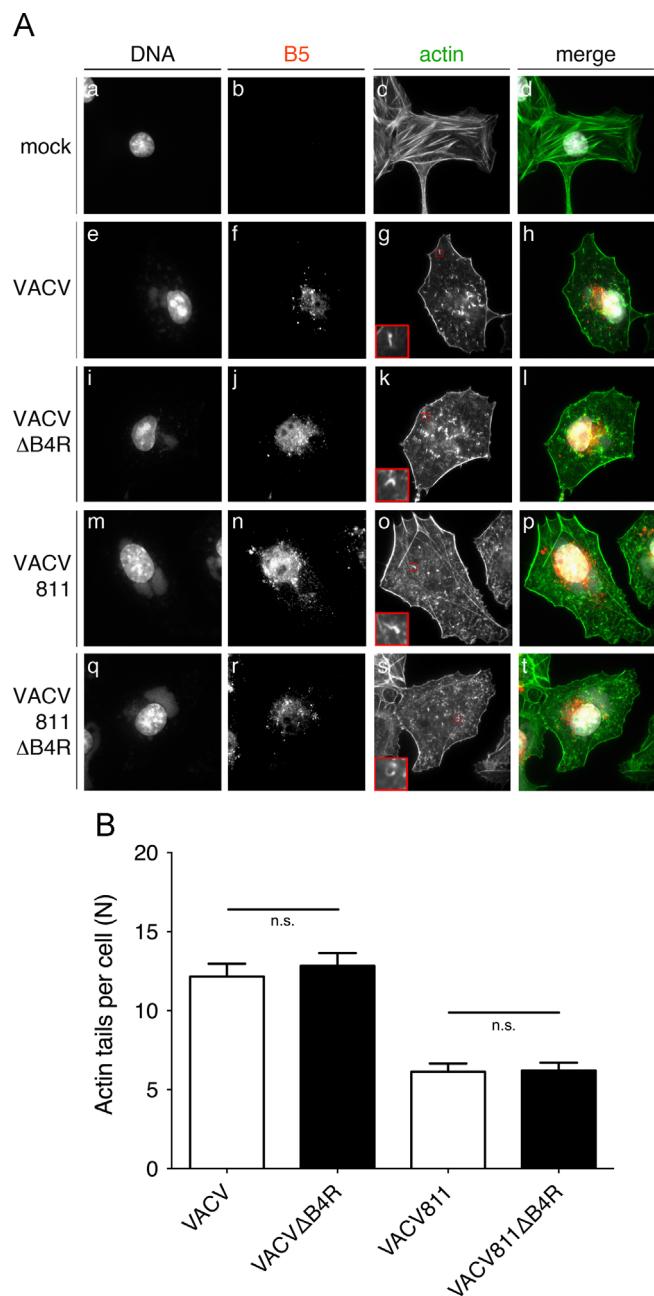
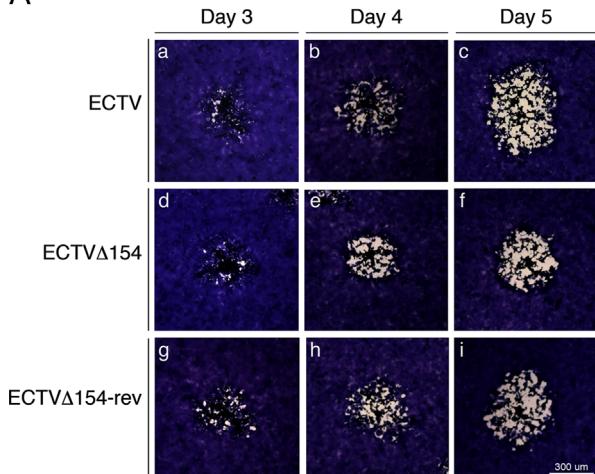


Fig. 6. Effect on actin tail formation following deletion of B4R: (A) BGMK cells were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Twenty hours post-infection, cells were stained for B5 to visualize virion distribution, actin to visualize tails, and DAPI to visualize the nucleus. (B) Quantification of actin tails. Data were quantified by counting 150 cells over three separate experiments. n.s., not significant.

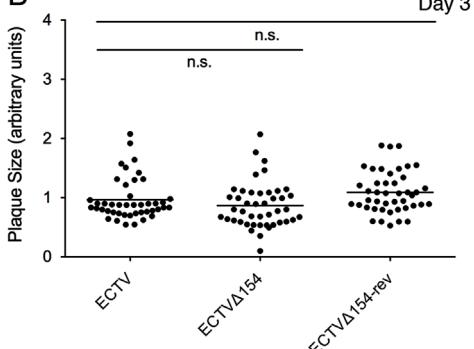
and plaque formation was analyzed after 3, 4, and 5 days post-infection (Fig. 7). In agreement with our observations in VACV, ECTV devoid of EVM154 formed smaller plaques than its parental virus, ECTV, or the revertant virus, ECTV Δ 154-rev (Fig. 7).

Since deletion of EVM154 demonstrated similar results to deletion of B4R, we turned to our mouse model to determine if B4R/EVM154 played a role in virus virulence (Fig. 8A-D). C57BL/6 mice were infected intranasally with ECTV or ECTV Δ 154. Mice were sacrificed at the indicated days post-infection, and tissue from the liver, spleen, lungs, and kidneys was harvested. The amount of virus present was determined by titration. While wild type ECTV exhibited high titers in all organs after seven days of infection, an increase in ECTV Δ 154 viral titers was not detected in

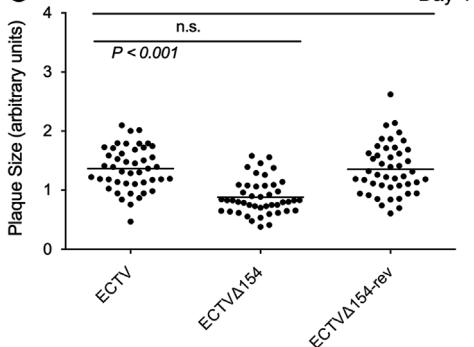
A



B



C



D

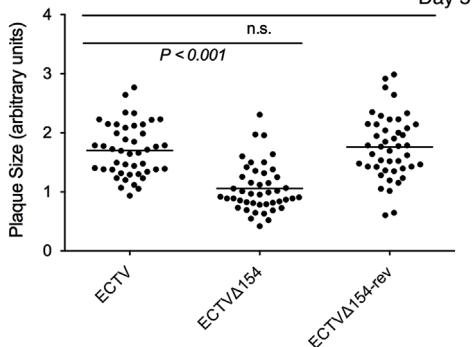


Fig. 7. Effect of deleting EVM154 on plaque size in tissue culture: (A) plaque sizes. BGMK cells were infected with ECTV (a–c), ECTVΔ154 (d–f), or ECTVΔ154-rev (g–i). Cells were stained with crystal violet 3 days (a, d, g), 4 days (b, e, h), or 5 days (c, f, i, l, o, r) post-infection. (B, C, and D) Comparison of mean plaque sizes. 45 photographed plaques were measured over three experiments using ImageJ and analyzed using GraphPad to determine significant differences in plaque size between viruses. n.s., not significant.

any of the organs, indicating a significant defect in the ability of ECTVΔ154 to spread within the mouse. Survival of the mice was also monitored at the indicated days post-infection (Fig. 8E). Mice infected ECTV succumbed to infection 8 days post-infection. In contrast, mice infected with ECTVΔ154 survived infection, further confirming that EVM154 was important for virulence.

Discussion

The purpose of this study was to perform initial characterization of B4. We found that deletion of B4R from VACV resulted in a reduction in plaque size in tissue culture (Fig. 1), and a slight decrease in virion spread, as assessed using a multi-step growth curve (Fig. 2B). Deletion of the homolog of B4R, EVM154, from ECTV also results in a reduction in plaque size (Fig. 7), lending further support to the idea that this gene may play a role in *Orthopoxvirus* spread.

For our experiments, we also utilized the large deletion virus VACV811, which lacks 55 ORFs, to further investigate the role of B4 in spread. Interestingly, VACV811 contains all of the currently known ORFs that are implicated in release and spread (Table 1), yet compared to VACV, VACV811 produced plaques that were reduced in size (Fig. 1) and showed decreased growth in a multiple-step growth curve (Fig. 2B). Following deletion of B4R, we observed that VACV811ΔB4R produces smaller plaques than VACV811 (Fig. 1), and multiple-step growth analysis suggested that VACV811ΔB4R is defective in release or spread (Fig. 2B).

While the exact mechanism as to how B4 contributes virus spread remains elusive, we ruled out the possibility that B4 might be important for MV morphogenesis since these deletion viruses underwent all stages of viral morphogenesis (Fig. 4) and grew to similar levels under high MOI, single-step growth analysis (Fig. 2A). Furthermore these viruses appeared to support similar levels of late gene synthesis (Fig. 3). The disruption of the B4R gene did not affect the production, or localization, of the adjacent B5R gene (a gene known to be important for EV/actin tail formation) suggesting that these observations were not the result of an unintended disruption of B5R (Figs. 3 and 6).

We did observe that cells infected with VACV811ΔB4R released fewer viruses into the medium of infected cells than the parental VACV811 strain (Fig. 5). Bioinformatics predicts that B4 encodes six Ank repeats, and these domains have been shown to be involved in a number of processes, such as maintenance of the actin cytoskeleton, cytoskeleton rearrangement, and linking membrane proteins to the cytoskeletons (Cunha and Mohler, 2009). Given that the rearrangement of actin plays an important role in viral release and spread (Arakawa et al., 2007a, 2007b; Frischknecht et al., 1999; Hiller et al., 1979; Horsington et al., 2013; Reeves et al., 2005) we examined whether B4 affected the formation of actin tails. The presence of B4R did not seem to affect the frequency of actin tails formed by VACV-infected cells; however, VACV811 formed significantly fewer actin tails than VACV (Fig. 6). Since VACV811 is thought to encode all known genes in EV/actin tail formation it suggests that there may be additional, yet-to-be identified, viral genes involved in these processes that are absent in VACV811.

Another question that remains is why there was a significant reduction in plaque size following deletion of B4R from VACV (Fig. 1), yet there was no significant difference in the number of virions released from cells infected with each virus (Fig. 5). One possibility is that B4 plays a role in cell motility during infection. It has been established that actin dynamics affect cell motility; however, there was no reduction in actin tails in cells infected with VACVΔB4R compared to VACV (Fig. 6). Interestingly, reintroduction of F11L, an important mediator of actin formation, into

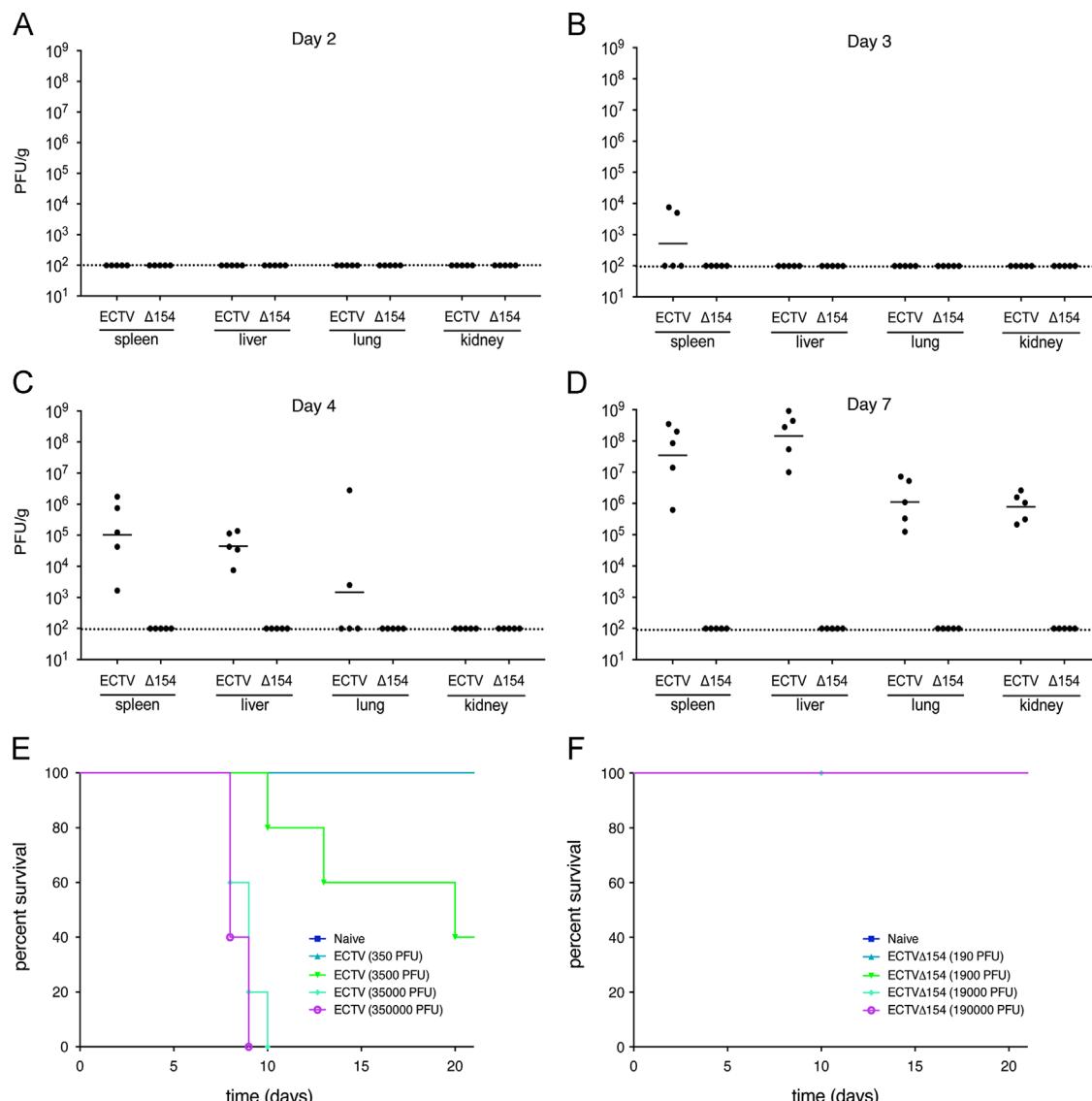


Fig. 8. (A–D) Organ titers. C57BL/6 mice were infected intranasally with ECTV and ECTVΔ154. Spleen, liver, lung, and kidney tissue were harvested at days 2, 3, 4, and 7 post-infection and viral titers were determined on BSC-40 cells ($n=5$). (E and F) Survival of mice. C57BL/6 mice were infected intranasally with ECTV and ECTVΔ154 and survival was monitored up to 21 days post-infection.

myxoma or modified vaccinia Ankara (MVA), did not restore cell motility to the same degree as motility seen in wild-type VACV, suggesting that an additional gene maybe involved in VACV-mediated cell motility (Irwin and Evans, 2012; Valderrama et al., 2006; Zwilling et al., 2010). Whether B4 plays a role in this phenomenon will be explored in the future.

Many poxviral proteins that are important for release and spread are packaged into the virion; however, B4 has not been identified in the VACV virion (Moss, 2007). No work has been done to characterize the ECTV virion, so whether EVM154 is packaged is unknown; however, given the similarities in genes encoded by VACV and ECTV, one would predict EVM154 is also absent from virions. Moreover, transfection experiments have indicated that B4R/EVM154 are expressed diffuse throughout the cytoplasm (data not shown), further suggesting that this protein is not incorporated into the virion.

B4R/EVM154 contain Ank/F-box domains that are important for modulating the SCF ubiquitin ligase. Some poxviral proteins that mediate release and spread require post-translational modifications in order to function. For instance, F13 is palmitoylated, and A34 is glycosylated (Breiman and Smith, 2010; Grosenbach et al., 1997). It is

possible that some poxviral proteins that are important for virion release and spread might require processing by the proteasome, an event that could be mediated by B4R/EVM154. Promoter analysis and semi-quantitative RT-PCR indicate that B4R/EVM154 is a late gene (data not shown), so this speculation is not unreasonable. Unfortunately, with the exception of the GRR signal that mediates processing of the NF κ B precursors p100 and p105, proteasomal processing motifs are not well characterized (Lin and Ghosh, 1996). Consequently, it is difficult at this point to screen the poxviral proteins to determine if any of them contain proteasomal-processing signals.

Our results with VACV811 present a number of interesting observations and indicate that there may be additional unidentified ORFs that contribute to virion release, spread, or infectivity. Importantly, our data also suggest a possible role for B4, a protein that has until now remained uncharacterized. The importance of B4 was underlined when we infected mice with ECTV devoid of EVM154, the homolog of B4R, and saw decreased virus spread to the liver, lung, kidneys, and spleen (Fig. 8). Additionally, survival curves indicated that a virus devoid of EVM154 was attenuated. An important consideration is the MOI that was used during our experiments. Though a reduction in plaque size and restriction in a

multi-step growth curve is often indicative of a defect in spread, these conclusions are often drawn with additional data that supports a defect in spread following deletion of the gene in question. Since we have not determined the exact mechanism of B4, we draw our conclusions with caution. We also recognize that using a high MOI in many of our experiments may have obscured the effects of deleting B4R. Future experiments will include assessing gene synthesis, virion formation, and actin tails at a low MOI, to ensure that a high MOI did not partially overcome defects that we observed during a single growth cycle. Moving forward, additional studies will be necessary to confirm and elucidate the mechanism of B4; however, this study suggests that B4 might contribute to virion release and spread. Overall, our study provides new insights into the poxvirus lifecycle and raises the question of whether we have identified all the ORFs involved in virion release and spread.

Materials and methods

Cell lines and virus strains

BSC-1 and BSC-40 cells were obtained from the American Type Culture Collection (ATCC) and Buffalo green monkey kidney (BGMK) from Diagnostic Hybrids. U2OS cells stably expressing a cytoplasmic mutant of the Cre recombinase (U2OS-Cre) were kindly provided by Dr. J. Bell, University of Ottawa. Cells were grown at 37 °C in 5% CO₂ in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (NCS) (Invitrogen), 50 U/mL of penicillin (Invitrogen), 50 U/mL of streptomycin (Invitrogen), and 200 µM of L-glutamine (Invitrogen). Vaccinia virus strain Copenhagen (VACV) was provided by Dr. G. McFadden (University of Florida, USA). Vaccinia virus strain 811 (VACV811), which is missing 55 ORFs from the variable regions of the VACV genome, was provided by E. Paolelli (Perkus et al., 1991). Ectromelia virus strain Moscow (ECTV) was provided by Dr. M. Buller (St. Louis University, USA). All viruses were propagated in BGMK cells as previously described (Stuart et al., 1991).

Plasmid constructs

pDGloXP was described previously (Rintoul et al., 2011). pDGloXP contains a yellow fluorescent protein (YFP)/guanine phosphoribosyltransferase (*gpt*) fusion open reading frame under the control of a poxvirus early/late promoter. The promoter and open reading frame are flanked by loxP sites. pDGloXP-ΔB4R was constructed by PCR amplification of 400-nucleotide sequences located directly upstream and downstream of B4R in the VACV genome. The upstream region of homology was amplified using the following primers: forward 5'-*(Spel)*-ACT AGT CAA GGT ATA TAA ACC TGG-3' and reverse 5'-*(HindIII)*-AAG CTT ATA TAA TTT ATA TTC TGT AAC ATG TTA-3'. The downstream region of homology was amplified using the following primers: forward 5'-*(NotI)*-GC GGC CGC ACA CTA TTA AAA TAT AAA-3' and reverse 5'-*(BglII)*-AGA TCT TAG TGT CAT GGT GGA AAT-3'. The 400 base pair amplicons were cloned into pGEM-T using the *Spel* and *HindIII* restriction sites for the upstream region, and *NotI* and *BglII* for the downstream region. The sequences were subcloned into the pDGloXP vector to create pDGloXP-DB4R. pDGloXP-Δ154 was constructed by amplification of the 200-nucleotide sequences located directly upstream and downstream of EVM154 in the ECTV genome, using Taq polymerase (Invitrogen Corporation). The upstream region of homology was amplified using the following primers: forward 5'-*(XbaI)*-CTC GAG ATC ATA TAG ACA ATA ACT-3' and reverse 5'-*(HindIII)*-AAG CTT GAC ATA TAA TTT ATA TTC TGT-3'. The downstream region of homology was

amplified using the following primers: forward 5'-*(BamHI)*-GGA TCC AAT CTA AGT AGG ATA AAA-3' and reverse 5'-*(NotI)*-GC GGC CGC AAA CGA TGT TTC GGT AGA-3'. The 400 base pair sequences were cloned into pGEM-T using the *XbaI* and *BamHI* and *NotI* for the downstream region. Next, the sequences were digested and subcloned into the pDGloXP vector to create pDGloXP-Δ154.

Generation of recombinant viruses

Recombinant viruses were generated as described previously (Rintoul et al., 2011). Recombinant viruses containing the YFP/*gpt* marker, VACVΔB4R and VACV811ΔB4R, were generated by infecting BGMK cells with VACV or VACV811 at a MOI of 0.01 and transfecting with 10 µg of linearized pDGloXP-DB4R using Lipofectamine 2000 (Invitrogen Corporation) (Gammon et al., 2010; Rintoul et al., 2011) for one hour post-infection. Recombinant virus ECTVΔ154 was generated by infecting BGMK cells with ECTV at a MOI of 0.01 and transfecting with 10 µg of linearized pDGloXP-Δ154 using Lipofectamine 2000 one hour post-infection. Recombinant viruses were selected using media containing 25 µg/mL mycophenolic acid (MPA) (Sigma-Aldrich), 250 µg/mL xanthine (Sigma-Aldrich), and 15 µg/mL hypoxanthine (Sigma-Aldrich). YFP⁺ recombinants were screened visually using an inverted fluorescent microscope (Leica). Marker-free ECTVΔ154, lacking drug resistance and fluorescent markers, was generated in order to infect mice. The YFP/*gpt*-containing virus was passaged through U2OS-Cre cells and plaques were screened using the absence of YFP as an indicator that excision by the Cre recombinase had occurred. To generate the revertant viruses, VACVΔB4R-rev and VACV811ΔB4R-rev, primers to the 5' end of the upstream flanking region and the 3' end of the downstream flanking region of the gene were used to generate a PCR product using VACV or VACV811 as a template. BGMK cells were infected with VACVΔB4R and VACV811ΔB4R at a MOI of 0.01 followed by transfection with the PCR product. Absence of YFP indicated that recombination had occurred, and the gene was re-inserted. PCR analysis of viral genomes verified deletion and reinsertion of the B4R and EVM154 ORFs.

Plaque assays

Viruses were serially diluted and titred on BGMK cells (1 × 10⁶). Plaques were allowed to form over 2–5 days and cells were stained with crystal violet. Fifteen randomly isolated plaques were photographed for each sample using an Axioscope 2 plus microscope (Zeiss) at a magnification of 2.5× and measured in ImageJ v1.44i (National Institutes of Health). The edge of the plaque (defined by the edge of cells that had piled) was traced using the shape tool in ImageJ. The experiment was performed in triplicate. Plaque sizes were compared using GraphPad Prism version 5 and statistical significance was determined using a one-way ANOVA with a Tukey post-test (GraphPad Software, La Jolla California USA, www.graphpad.com).

Growth curves

Single-step growth curves were performed by infecting six-well dishes of BGMK cells (1 × 10⁶) with the indicated viruses at a MOI of 10. One hour post-infection, cells were washed with warm PBS and 2 mL of supplemented DMEM were added back to each well. Media and cells were harvested at the indicated times post-infection and released from cells by freeze thaw. Titers were determined in duplicate on BGMK cells. The experiment was performed in triplicate. Alternatively, multi-step growth curves were performed by infecting BGMK cells at a MOI of 0.01 and

harvesting samples at the indicated times post-infection. For multi-step growth curves, no wash step was performed 1 h post-infection. Titers were determined in duplicate on BGMK cells, and the experiment was performed in triplicate.

Immunoblot analysis

Six-well dishes of BGMK cells (1×10^6) were mock-infected or infected with the indicated virus at a MOI of 10. Whole cell lysates were collected at the indicated times post-infection in 150 μ L of sodium dodecyl sulfate (SDS)-containing sample buffer and samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane (Fisher Scientific) for 2 h at 420 mA using a semi-dry transfer apparatus (TYLER Research Instruments) and transfer buffer containing 192 mM glycine, 25 mM Tris, and 20% v/v methanol. Membranes were blocked for 3 h at room temperature in 5% w/v skimmed milk in Tris-buffered saline with Tween 20 (TBS-T), containing 200 mM Tris (pH 7.5), 15 mM NaCl, and 0.1% v/v Tween-20 (Fisher Scientific). Samples were immunoblotted overnight with mouse anti-E3L (Weaver et al., 2007), mouse anti-B5R (Izmailyan et al., 2006), rabbit anti-A34R (Irwin and Evans, 2012), rabbit anti-I5L (Wilton et al., 2008), and mouse anti- β -tubulin (ECM Biosciences) antibodies, followed by the appropriate secondary antibody, either Peroxidase-conjugated AffiniPure goat anti-rabbit (Jackson Laboratories) or Peroxidase-conjugated AffiniPure goat anti-mouse (Jackson Laboratories). Proteins were visualized using enhanced chemiluminescence (ECL) (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). To quantify relative levels of protein, membranes were instead blocked for one hour at room temperature with commercial blocking buffer (Licor), and immunoblotted overnight with mouse anti-B5R (Izmailyan et al., 2006), rabbit anti-A34R (Irwin and Evans, 2012), rabbit anti-I5L (Wilton et al., 2008), or mouse anti-I3L (Lin et al., 2008) followed by incubation with IR-680- or IR-800-conjugated secondary antibodies (Licor). Protein levels were visualized using an Odyssey Imager (Licor) and relative levels were determined compared to I3L.

Electron microscopy

BGMK cells (1×10^5) grown on 13 mm poly-L-lysine-coated coverslips (Thermanox) were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Twenty-four hours post-infection, cells were fixed with 4% glutaraldehyde/2% paraformaldehyde, 0.2 M sucrose and 4 mM CaCl₂ in 0.16 M sodium cacodylate buffer (pH 7.4) at 37 °C for one hour. Following fixation, coverslips were washed with 0.05 M sodium cacodylate buffer to remove aldehyde from the fixative, and the lipids were then fixed with 1% ice-cold osmium tetroxide (OsO₄) in 0.05 M sodium cacodylate buffer. Following lipid fixation, coverslips were again washed with 0.05 M sodium cacodylate buffer. To increase contrast, cells were blocked in en bloc stain with 1% uranyl acetate in 0.1 M sodium acetate buffer (pH 5.2) for 15 min. Coverslips were washed with 0.1 M sodium acetate buffer followed by Milli-Q filtered water and then dehydrated with increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95% and 100%) in propylene oxide. Cells were infiltrated with a mixture of embed 812 and araldite 502 resins and embedded on gelatin capsules before being thermally polymerized at 60 °C for 48 h. Ultra-thin sections with a thickness of 60 nm were generated using a Leica UC7 ultramicrotome (Leica Microsystems, Inc.) and contrasted with 2% uranyl acetate and Reinolds' lead citrate. Sections were imaged using a Hitachi H-7650 transmission electron microscope (Hitachi-High Technologies) at 80 kV and a 16 mega pixel TEM camera (XR111, Advanced Microscopy Techniques, MA, USA).

Media release experiments

Six-well dishes of BGMK cells (1×10^6) were infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. One hour post-infection, cells were washed twice with PBS to remove any unbound virus, and fresh media was added. Twenty-four hours post-infection, media was removed and centrifuged at 1000g for 10 min. The supernatant was then transferred to a new tube and titered to determine the number of virions released into the media. The cell layer was harvested with PBS, freeze-thawed three times, and then titered to determine the number of virions associated with the cell. The amount of released virus, as a percent of total virus (released+cell-associated) was then calculated. The experiment was performed in triplicate, and standard error of the mean (SEM) was determined.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as previously described (Irwin and Evans, 2012). BSC-40 cells (2×10^5) were seeded on coverslips and mock-infected or infected at a MOI of 10 with VACV, VACV Δ B4R, VACV811, or VACV811 Δ B4R. Twenty hours post-infection, cells were fixed with 4% w/v paraformaldehyde (Sigma-Aldrich) in PBS for 30 min on ice. Cells were permeabilized with 100 mM glycine in PBS-T, washed three times, and blocked for 30 min with 3% BSA in TBS-T. Cells were incubated for one hour with mouse anti-B5, washed with PBS-T, and incubated with Cy5-conjugated secondary antibody (Molecular Probes). Following a one hour incubation with secondary antibody, cells were counterstained with rhodamine phalloidin (0.3 U/mL) and 5 ng/mL DAPI for 20 min, and washed six times with PBS-T. Coverslips were mounted using Mowiol mounting medium containing 0.1 mg/mL Mowiol, 0.1 M PBS (pH 7.4), 25% glycerol, and 2.4% triethylenediamine (DABCO). Cells were visualized using the 60 \times oil immersion objective of a personal Delta-vision microscope. Number of actin tails per cell were quantified by counting 150 infected cells over three independent trials.

Six to 11 weeks old female C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Five female mice with similar body mass were infected intranasally with 10-fold escalating doses of ECTV or ECTV Δ 154. Body weights were monitored daily. Mice were sacrificed at 2, 3, 4, and 7 days post-infection, and tissue from the spleen, liver, lungs, and kidney were harvested. Tissue was homogenized using a tissue homogenizer (Next Advance), followed by dilution in PBS (10% w/v). Viral titers were determined on BSC-1 cells. To prevent avoidable suffering, mice demonstrating a drop in body weight to 70% of their original mass, or signs of severe morbidity, were euthanized. These experiments were performed in accordance with mouse ethics outlined by the Canadian Council on Animal Care and the University of Alberta.

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