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# Functional Analysis of Vaccinia Virus B5R Protein: Role of the Cytoplasmic Tail

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Vaccinia extracellular enveloped virus (EEV) is important for cell-to-cell and long-range virus spread both *in vitro* and *in vivo*. Six genes have been identified that encode protein constituents of the EEV outer membrane, and some of these proteins are critical for EEV formation. The B5R gene encodes an EEV-specific type I membrane protein, and deletion of this gene markedly decreases EEV formation and results in a small plaque phenotype. Data suggest that the transmembrane domain, cytoplasmic tail, or both contain the EEV localization signals that are required for targeting of the B5R protein to EEV and for EEV formation. Here, we report the construction of mutant vaccinia viruses in which the wild-type B5R gene was replaced with a mutated one that encodes a protein with the putative cytoplasmic tail deleted. The mutated protein showed normal intracellular distribution and was properly incorporated into EEV. Vaccinia viruses expressing the B5R protein lacking the cytoplasmic tail formed plaques that were similar in type and size to those formed by wild-type viruses and produced equivalent amounts of infectious EEV. These results indicate that the B5R cytoplasmic tail is not necessary for EEV formation and points to the transmembrane domain as the major determinant for targeting the B5R protein to the outer membrane of EEV and for supporting EEV formation. 

## INTRODUCTION

Vaccinia virus has two infectious virus forms: the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) (Moss, 1996). The majority of progeny virus remains within the cell as IMV, and it is speculated that this form of virus is ultimately important in spread of the virus from animal to animal. In contrast, EEV is critical for the cell-to-cell and distant spread of the virus in tissue culture and within the host (Payne, 1980) .

EEV is derived from IMV that has been enwrapped with membranes from the *trans*-Golgi network containing additional viral proteins and forms the outer envelope of EEV (Schmelz *et al.,* 1994; Tooze *et al.,* 1993). These enwrapped virions are propelled out of the cell by actin tails in a manner similar to the movement of certain intracellular prokaryotic organisms (Cossart, 1995; Cossart and Kocks, 1994; Cudmore *et al.,* 1995, 1996; Tilney and Tilney, 1993). The intracellular enveloped virus also is transported as a vesicle to the cell periphery, where it fuses with the plasma membrane to gain access to the extracellular compartment. In the case of vaccinia virus strain WR, the majority of this enveloped virus remains attached to the cell surface (Blasco and Moss, 1992). It is believed that this virus can mediate direct spread between neighboring cells, resulting in the production of a large, round plaque phenotype in tissue culture. In contrast, vaccinia virus strain IHD-J releases most of the enveloped virus to the extracellular space as EEV, presumably generating the characteristic comet plaque phenotype.

To date, six vaccinia virus genes have been identified that encode proteins specifically incorporated into the EEV outer membrane, and some of these proteins are critical for EEV formation (Blasco and Moss, 1991; Duncan and Smith, 1992; Engelstad *et al.,* 1992; Hirt *et al.,* 1986; Isaacs *et al.,* 1992; Parkinson and Smith, 1994; Payne and Norrby, 1976; Roper *et al.,* 1996; Shida, 1986). The B5R open reading frame (ORF) encodes one of these EEV-specific proteins (Engelstad *et al.,* 1992; Isaacs *et al.,* 1992; Takahashi-Nishimaki *et al.,* 1991). Deletion of the B5R gene markedly decreases EEV formation and results in a small plaque phenotype (Engelstad and Smith, 1993; Martinez-Pomares *et al.,* 1993; Wolffe *et al.,* 1993). Based on the hydrophobicity plot and mutational analysis, the B5R gene encodes a 317-amino-acid polypeptide that contains an N-terminal signal peptide (Isaacs *et al.,* 1992), large extracellular domain, transmembrane region, and short cytoplasmic tail (Engelstad *et al.,* 1992; Isaacs *et al.,* 1992). Recently, we and others showed that the majority of the extracellular domain of the B5R protein is not required for EEV formation or for B5R protein incorporation into EEV (Herrera *et al.,* 1998; Mathew *et al.,* 1998). This indicates that B5R outer-envelope targeting and its ability to support EEV formation are

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likely encoded by the transmembrane or cytoplasmic domains, or both. This is further supported by the finding that when expressed along with the wild-type B5R protein, a chimeric protein containing only the B5R transmembrane and cytoplasmic domains fused to a foreign protein extracellular portion was targeted to EEV (Katz *et al.,* 1997).

To address B5R structure–function relationships, we tested the role of the predicted cytoplasmic tail in EEV formation. The cytoplasmic tail seemed a likely candidate to contain the targeting signal that directs the B5R protein to a specific cellular location that contributes to the formation of EEV. Alternatively, the 14-amino-acid cytoplasmic tail may aid in EEV formation by interacting directly with viral proteins on IMV to support the enwrapping of IMV with Golgi-derived membranes to form EEV. Possibly, the cytoplasmic tail contribution to EEV formation may be because it is involved in the oligomerization of the B5R protein and the complex formed is critical for EEV formation. Here, we detail the investigations to determine the contribution of the cytoplasmic tail domain of the B5R protein in targeting of the B5R protein to EEV and in EEV formation.

## RESULTS

### Construction of recombinant vaccinia viruses expressing a mutated B5R protein lacking the cytoplasmic tail

We constructed a transfer plasmid in which the wild-type B5R gene from vaccinia virus strain WR was replaced with a mutated one that lacked the coding sequence for the final 12 amino acids of the predicted 14-amino-acid B5R protein cytoplasmic tail. To facilitate identification of plaques that we anticipated might be aberrant in size, a color marker was incorporated in the transfer plasmid so that after recombination, viral plaques could be detected by color staining. We used the *Escherichia coli* β-glucuronidase gene under control of the vaccinia virus P7.5 promoter (GUS) so the inclusion of X-glu in the media would turn cells infected with the mutant virus blue (Carroll and Moss, 1995) and because X-glu is not a substrate for β-galactosidase (which is present in the parental B5R-deletion viruses, W-B5R$^-$ and I-B5R$^-$).

Mutant viruses were isolated and plaque purified using reverse gpt selection (Isaacs *et al.*, 1990) and X-glu staining (Carroll and Moss, 1995). Proper genomic positioning of the mutated gene was confirmed by Southern blotting (data not shown). We also verified by PCR that the mutant viruses did not contain sequence corresponding to the predicted cytoplasmic tail by using primers that amplified a PCR product only in wild-type viruses (data not shown).

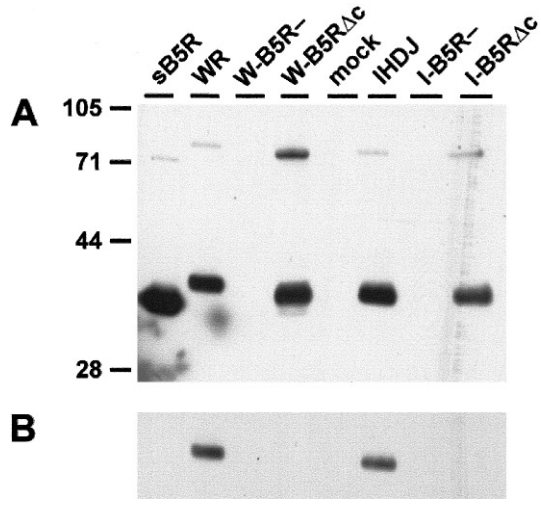


FIG. 1. Western blot analysis of cells infected with the panel of vaccinia viruses. BSC-1 cells were infected with wild-type viruses (WR, IHD-J), B5R-deletion viruses (W-B5R$^-$, I-B5R$^-$), or mutant viruses lacking the cytoplasmic tail (W-B5RΔc, I-B5RΔc). At 48 h, cells were harvested in lysis buffer, loaded, and separated by SDS–PAGE, and proteins were transferred to nitrocellulose. The blot was probed with rabbit polyclonal antibody (C′-B5R) raised to an extracellular domain of B5R (A). Molecular masses in kilodaltons are indicated on the left based on color protein markers (LTI). The blot was then stripped and probed with CT-B5R, a rabbit polyclonal antibody raised against the cytoplasmic tail peptide (B). CT-B5R detected only the full-length ~42-kDa B5R protein. sB5R refers to a previously described secreted form of the B5R protein that lacks both the transmembrane and cytoplasmic domains (Isaacs *et al.*, 1992).

### Expression of mutated B5R protein lacking the cytoplasmic tail

Western blots of lysates from cells infected with wild-type virus WR or IHD-J probed with the rabbit polyclonal anti-B5R antibody showed the expected 42-kDa band, which was absent in the B5R-deletion viruses W-B5R$^-$ and I-B5R$^-$ (Fig. 1A). As described previously (Herrera *et al.*, 1998), the wild-type B5R protein produced by IHD-J migrated slightly faster compared with the WR protein. A smaller B5R protein was made by the WR-based virus expressing the B5R protein lacking the putative cytoplasmic tail (W-B5RΔc), consistent with the 12-residue deletion. I-B5RΔc produced a mutated protein of the same size as W-B5RΔc because it contained strain WR B5R sequence lacking the cytoplasmic tail. In addition, rabbit antibody raised against a peptide corresponding to the cytoplasmic tail that recognized full-length B5R proteins did not detect the mutated B5R protein produced by W-B5RΔc amd I-B5RΔc, further confirming the absence of the cytoplasmic tail (Fig. 1B). Of note, the higher-molecular-weight bands in Fig. 1A likely represent B5R homodimers or heterodimers (Engelstad *et al.,* 1992; Herrera *et al.,* 1998; Isaacs *et al.,* 1992) and are seen in both the wild-type and tail-deleted B5R proteins, indicating that the cytoplasmic region is not required for oligomerization.

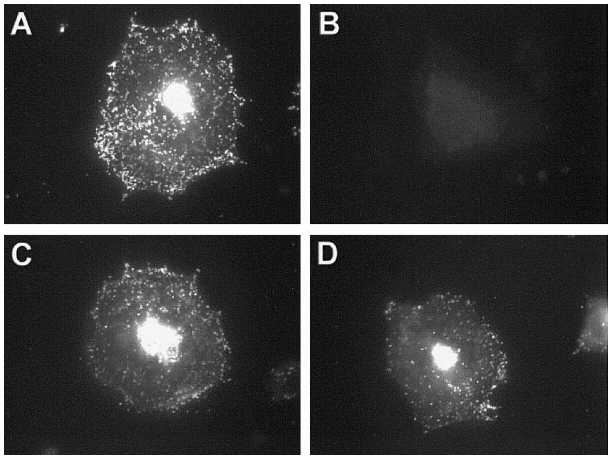


FIG. 2. Localization of B5R in infected cells. BHK-21 cells were infected with wild-type virus (WR) (A), B5R-deletion virus (W-B5R$^-$) (B), B5R-rescued virus (W-B5R$_{rescue}$) (C), or mutant virus lacking the cytoplasmic tail (W-B5RΔc) (D). At 7 h after infection, cells were fixed, permeabilized, and incubated with a monoclonal anti-B5R antibody. Secondary antibody coupled with FITC was used to visualize the primary antibody. Representative cells are shown. Note the intense juxtanuclear staining and dispersed punctate staining in all except W-B5R$^-$ (B).

## B5RΔc protein distribution in infected cells

To determine whether deletion of the predicted cytoplasmic tail disrupted normal targeting of the B5R protein in infected cells, we carried out immunofluorescent staining of infected BHK 21 cells using the anti-B5R monoclonal antibody (Fig. 2). Cells infected with wild-type virus (WR) showed a pattern of intense juxtanuclear staining, suggesting a Golgi distribution, as well as a dispersed punctate pattern that corresponds to enveloped virions. The B5R-deletion virus showed no staining, whereas rescue of the B5R deletion resulted in a pattern similar to that of the wild-type virus. In cells infected with W-B5RΔc, B5R staining showed a pattern indistinguishable from wild-type and B5R-rescue viruses, indicating that the intracellular distribution of the B5R protein in the context of a vaccinia virus infection is not determined by the cytoplasmic tail.

## Plaque phenotype of mutant viruses W-B5RΔc and I-B5RΔc

We previously showed that deletion of the B5R gene produced a small plaque phenotype (Wolffe *et al.*, 1993), indicating a critical role for the B5R protein in cell-to-cell vaccinia virus spread. Here, we determined the role of the B5R cytoplasmic tail in plaque formation (Fig. 3). Deletion of the cytoplasmic tail of the B5R protein resulted in a virus that formed plaques identical to the virus carrying the wild-type B5R gene. This demonstrates that the B5R protein cytoplasmic tail is not necessary for efficient cell-to-cell spread.

## EEV formation by mutant I-B5RΔc

The similarity in plaque phenotypes between W-B5RΔc and I-B5RΔc to their respective wild-type viruses suggested that EEV formation was not likely to be disrupted by deletion of the cytoplasmic tail. To confirm this, EEV formation was analyzed directly using cesium chloride gradients of supernatants from metabolically labeled infected RK-13 cells (Fig. 4). As expected, the B5R-deletion virus (I-B5R$^-$) produced minimal amounts

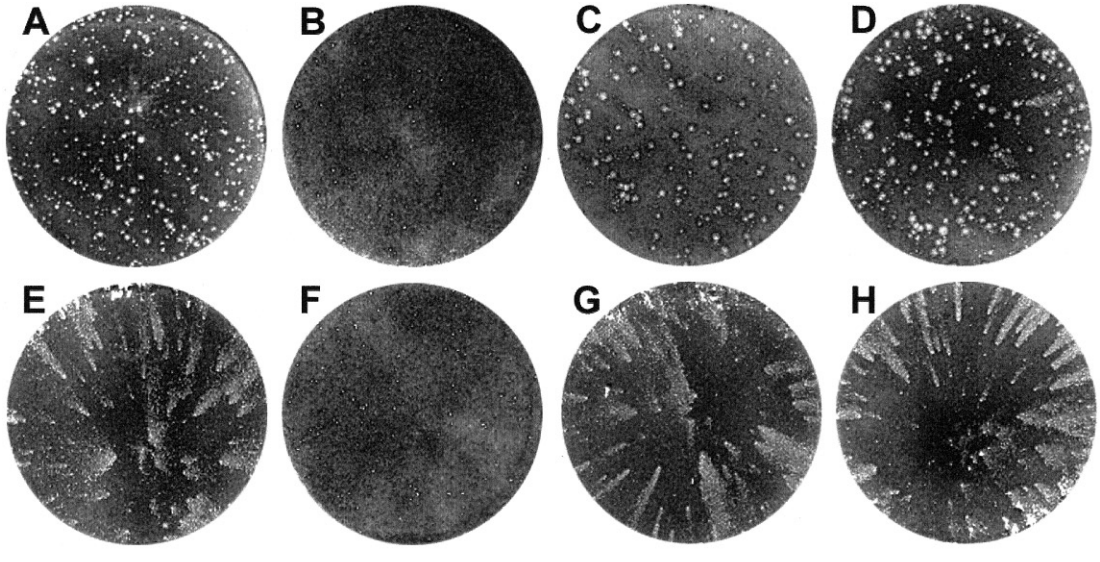


FIG. 3. Plaque phenotype under liquid overlay. BSC-1 cells were infected with either WR-based viruses (A–D) or IHDJ-based viruses (E–H) and incubated under liquid media. At 48 h, cell monolayers were stained with 0.1% crystal violet in 20% ethanol, and wells were photographed. Viruses are WR (A), W-B5R$^-$ (B), W-B5R$_{rescue}$ (C), W-B5RΔc (D), IHD-J (E), I-B5R$^-$ (F), I-B5R$_{rescue}$ (G), and I-B5RΔc (H). Note that the mutant viruses lacking the B5R cytoplasmic tail (D and H) form plaques similar to their respective wild-type (A and E) and rescued (C and G) viruses.

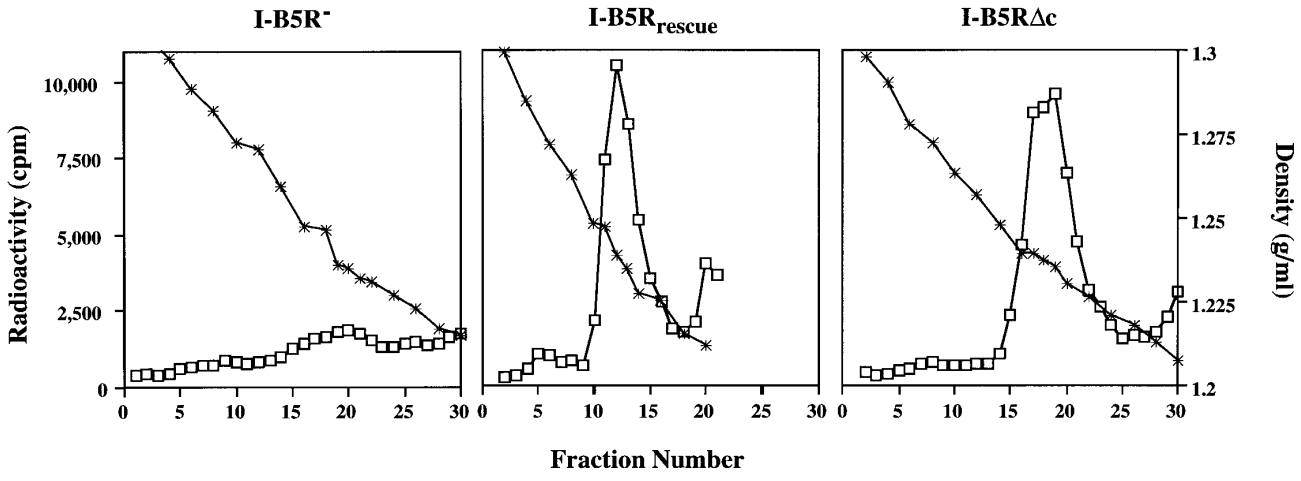


FIG. 4. CsCl gradients of metabolically labeled virus released into the media of infected cells. RK-13 cells were infected with the B5R-deletion virus (I-B5R$^{-}$), the B5R-rescued virus (I-B5R$_{rescue}$), or the mutant virus lacking the B5R cytoplasmic domain (I-B5RΔc) and incubated in the presence of [$^{35}$S]methionine and [$^{35}$S]cysteine as described under Materials and Methods. After 48 h, media were centrifuged through a CsCl density gradient. Fractions were collected from the bottom of the tube, and the radioactivity of an aliquot from each fraction was measured in a liquid scintillation spectrometer (open symbols). The density of every other fraction was determined based on the refractive index (star symbols).

of EEV, which was restored to normal levels in the rescue virus (I-B5R$_{rescue}$). The amount of virus at a buoyant density corresponding to EEV found in the media of cells infected with the cytoplasmic deletion virus (I-B5RΔc) was indistinguishable from that with the wild-type or rescue viruses. Furthermore, titration of this virus revealed that I-B5RΔc had the same titer as the wild-type and I-B5R$_{rescue}$ viruses, indicating that the absence of the cytoplasmic tail did not affect the infectivity of the mutant virus.

We determined whether the mutant B5R protein was incorporated into EEV at wild-type levels. This was examined by Western blotting of unlabeled EEV released into the media of RK-13 cells (Fig. 5). This revealed that the mutant virus lacking the B5R cytoplasmic tail (I-B5RΔc) incorporated protein at levels similar to that of wild-type (IHD-J) or the rescued virus (I-B5R$_{rescue}$) when compared with the amount of p37 found in EEV. As described previously (Herrera *et al.*, 1998), because I-B5R$_{rescue}$ was rescued with B5R sequence derived from strain WR, the B5R protein produced by IHD-J migrates slightly faster compared with the rescued virus.

## Actin tail formation by mutant W-B5RΔc

In addition to its role in EEV formation, the B5R protein is important for the production of intracellular enveloped forms of virus that are required for the formation of actin tails in cells infected with vaccinia virus. The association between intracellular enveloped virus and actin tails is believed to play an important role in cell-to-cell spread (Roper *et al.*, 1998; Wolffe *et al.,* 1997, 1998). Because the B5R putative cytoplasmic tail might be in a position to interact with cellular proteins involved in actin tail formation on the intracellular enveloped virus, we used phalloidin staining to determine whether actin tails were formed in cells infected with our panel of viruses (Fig. 6). As shown previously, wild-type and the rescue of the B5R-deletion viruses formed actin tails, whereas the B5R-deletion virus did not. Cells infected with W-B5RΔc also formed actin tails, indicating that the cytoplasmic tail was not essential for actin tail formation.

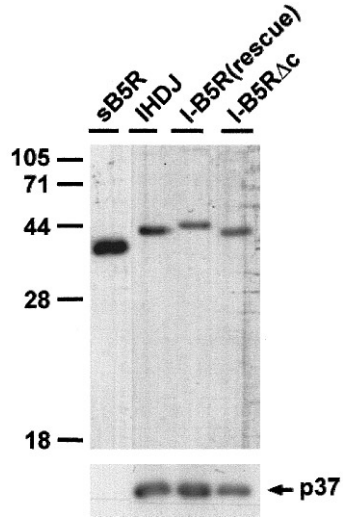


FIG. 5. Western blot of EEV-specific proteins. EEV was isolated from the media from RK-13 cells infected with wild-type virus (IHDJ), the rescued B5R-deletion virus (I-B5R$_{rescue}$), and the mutant virus lacking the B5R cytoplasmic domain (I-B5RΔc). Proteins were subjected to SDS–PAGE, followed by transfer to nitrocellulose. The blot then was probed with rabbit polyclonal antibody C′-B5R. Molecular masses in kilodaltons are indicated on the left based on color protein markers (LTI). sB5R refers to a previously described secreted form of the B5R protein that serves as a positive control (Isaacs *et al.*, 1992). The blot was stripped and reprobed with anti-p37 antibody, and the resulting autoradiogram is shown below the main blot.

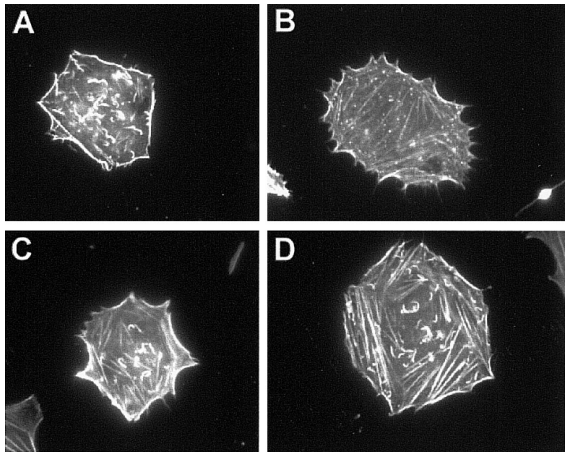


FIG. 6. Actin tail formation in cells infected with vaccinia virus. BHK-21 cells were infected with wild-type virus (WR) (A), B5R-deletion virus (W-B5R$^-$) (B), B5R-rescued virus (W-B5R$_{rescue}$) (C), or mutant virus lacking the cytoplasmic tail (W-B5RΔc) (D). At 7 h after infection, cells were fixed, permeabilized, and incubated with tetramethyl rhodamine isothiocyanate–phalloidin. Representative cells were photographed. Note the thick actin tails in the cells infected with the mutant virus lacking the cytoplasmic tail (W-B5RΔc) (D), as well as cells infected with the wild-type virus (WR) (A) and B5R-rescued virus (W-B5R$_{rescue}$) (C).

## DISCUSSION

Defining the structure–function relationships underlying vaccinia virus EEV-specific proteins is critical for elucidating the complex process of vaccinia virus morphogenesis, which remains poorly understood at the molecular level. Furthermore, characterizing these steps will also provide critical information regarding orthopoxvirus pathogenesis and for the development of safe and effective recombinant poxvirus-based vaccines.

We have studied the effect of specific mutations in the function of the B5R protein. Previously, we showed that the deletion of the B5R gene results in viruses that are severely impaired in IMV wrapping, EEV formation, and actin tail induction (Herrera *et al.,* 1998; Wolffe *et al.,* 1993). To define the domain or domains of the protein responsible for these functions, here we constructed viruses lacking most of the putative cytoplasmic tail in the B5R protein. The cytoplasmic tail seemed a likely candidate to contain the targeting signal that directs the protein to a specific cellular location. Although the predicted B5R protein 14-amino-acid cytoplasmic tail (DKNNDQYKFHKLLP) has no classic targeting motif, the sequence of HKLLP is similar to the KKXX or KXKXX localization signals that result in targeting to the endoplasmic reticulum or *cis*-Golgi compartments (Teasdale and Jackson, 1996). In addition, the sequence KFHKLL potentiality represents a di-leucine motif involved in endosomal trafficking (Pond *et al.,* 1995). We also anticipated that the cytoplasmic tail might have been involved in several other aspects of B5R protein function, including wrapping of IMV to form EEV, targeting of B5R to EEV outer envelope, induction of actin tails, and mediating interaction of the B5R protein with other proteins. Surprisingly, however, the cytoplasmic tail is not required for any of these functions. Because we previously showed that the majority of the extracellular domain of the B5R protein was dispensable for EEV formation (Herrera *et al.,* 1998), this indicates that the transmembrane or extracellular juxtamembrane spacer region is responsible for these functions. Given that the transmembrane and cytoplasmic domain of B5R can direct incorporation of a fusion protein to the EEV envelope (Katz *et al.,* 1997) or target a fusion protein to the Golgi region in the absence of infection (M. M. Lorenzo and R. Blasco, unpublished observations), it seems likely that the transmembrane domain plays a crucial role in both intracellular targeting and EEV incorporation. It has yet to be determined whether the B5R transmembrane domain, in the absence of other B5R sequences, carries the information required for EEV targeting and EEV formation.

Curiously, the deletion of the four putative short consensus repeat (SCR) domains that compose the majority of the extracellular domain of the B5R protein produced a WR-derived virus that formed comet-like plaques and did not produce actin tails (Herrera *et al.,* 1998). The formation of comet-like plaques is likely due to enhanced release of cell-associated enveloped virus, suggesting a role of the extracellular domain in tethering EEV to the cell surface. In contrast, disruption of actin tail formation in viruses lacking the B5R protein extracellular SCRs remains more difficult to explain. In contrast, the B5R cytoplasmic tail is topologically more likely to be interacting with the cellular machinery required for actin polymerization and formation of the actin tails on the intracellular enveloped virus, yet the deletion of the cytoplasmic domain does not curtail actin tail formation.

Because the B5R cytoplasmic tail is highly conserved in orthopoxviruses (Engelstad and Smith, 1993), it is likely to possess some crucial function not yet evident, which may be revealed by *in vivo* studies of these mutant viruses. Furthermore, in addition to defining the structural determinants of B5R protein function, our data suggest that the cytoplasmic tail might be used for the delivery of antigens to EEV, which could be useful in vaccinia virus-based vaccine development. For example, utilization of B5R as the basis for fusion proteins would allow efficient incorporation of immunogens into EEV, which might be advantageous for the generation of certain immune responses (Katz and Moss, 1997). Although incorporation of antigens as fusion proteins in place of the extracellular domain of B5R would allow expression on the surface of EEV (Katz *et al.*, 1997), certain cytosolic proteins might be more effectively incorporated as fusion proteins attached to the inner membrane surface or might be more effective immunogens if not exposed to antibodies or other constituents of the extracellular environment.

## MATERIALS AND METHODS

### Cells

BSC-1, CV-1, and RK-13 cells were grown in MEM (LTI/GIBCO) containing Antibiotic-Antimycotic (LTI/GIBCO) and 10% FBS (LTI/GIBCO). STO cells were maintained in Dulbecco's modified Eagle's medium (LTI/GIBCO) containing the identical additives. BHK-21 cells were maintained in Glasgow MEM (GIBCO) containing 10% tryptose phosphate broth, 20 mM HEPES, 2 mM glutamine, and 5% FBS. Vaccinia virus infections were carried out in media containing 2.5% FBS and incubated at 37°C in a 5% $CO_2$ atmosphere.

### Deletion of the cytoplasmic tail of the B5R protein

Deletion of the C-terminal 12 amino acids of the B5R protein was carried out on the previously described plasmid pSI-80, which contains the vaccinia virus (strain WR) B5R gene and flanking sequences (Wolffe *et al.*, 1993). Initially, the majority of the 3′-end of the B5R ORF and the entire right flanking sequence were removed by digesting pSI-80 with *Bcl*I and *Hin*dIII. The right flanking sequence was regenerated by PCR using pSI-80 as a template and primers oISI-66 (5′-GGCCTGATCA*GATCT*CCGTAAATATAAATCCG-3′, where the *Bcl*I site is underlined and a new *Bgl*II site is in italics) and oISI-65 (5′-CCCAAGCTTGCATCAACAGATAATAAC-3′, where the *Hin*dIII site is underlined). After 20 cycles of amplification at 94°C for 2 min, 55°C for 2 min, and 72°C for 1 min 30 s, the ~620-bp PCR product was cut with *Bcl*I and *Hin*dIII, gel purified, and ligated with the *Bcl*I/*Hin*dIII-digested pSI-80 to produce pSIEH-115e. A mutated B5R ORF lacking the predicted cytoplasmic tail was generated in a similar manner using PCR primers oISI-68 (5′-TTTACATGTGATCAGGG-3′) and oISI-67 (5′-GGCCTGATCATTTGTCACAGGAACAAAC-3′), where *Bcl*I sites are underlined and a newly introduced in-frame stop codon is in bold. After amplification using the identical PCR conditions outlined above, the resulting ~780-bp PCR fragment was digested with *Bcl*I, gel purified, and then cloned into *Bcl*I-digested pSIEH-115e to create pSIEH-117o. The correct orientation and proper sequence were confirmed by sequencing of the plasmid. Finally, pSIEH-117o was digested with *Bgl*II to allow introduction of the bacterial $\beta$-glucuronidase gene under control of a vaccinia virus promoter (GUS) (Carroll and Moss, 1995). The ~2.1-kbp GUS cassette was excised from p7.5GUS (kindly provided by Bernie Moss and Miles Carroll, National Institutes of Health) by cutting with *Sal*I and *Sac*I. The cassette was blunted with T4 DNA polymerase and ligated into pSIEH-117o that had been digested with *Bgl*II and blunted. The resulting plasmid, pSIEH-118i, had the entire B5R gene, under its intrinsic promoter, with a new stop codon at amino acid 305, resulting in the deletion of 12 amino acids from the predicted 14-amino-acid cytoplasmic tail, as well as a color marker so that if small plaques were produced by the mutant viruses, they could be identified by X-glu staining.

### Isolation of mutant viruses

The viruses W-B5R$^-$ and I-B5R$^-$, which represent the deletion of the B5R gene in WR and IHD-J, respectively, have been described previously (Wolffe *et al.*, 1993) and served as the parental viruses for the generation of cytoplasmic tail mutants (B5RΔc). The mutated gene was introduced by transfection of infected cells with pSIEH-118i using standard protocols (Earl and Moss, 1991b). Mutant viruses were isolated using reverse gpt selection by growth on STO cells in the presence of 6-thioguanine as described previously (Isaacs *et al.,* 1990) except here we included the substrate X-glu (Clonetech Labs, Palo Alto, CA) at a concentration of 0.2 mg/ml during the overlay step along with neutral red (Carroll and Moss, 1995). Blue plaques were isolated and viruses underwent three plaque purifications before expansion. The WR-based virus expressing the B5R protein lacking the putative cytoplasmic tail (vSIEH-29) is hereafter referred to as W-B5RΔc. The corresponding IHDJ-based virus (vSIEH-30) containing the mutated B5R gene (strain WR) lacking the cytoplasmic tail is referred to as I-B5RΔc. Rescue of the B5R-deletion viruses (W-B5R$^-$ and I-B5R$^-$) in WR and IHD-J with the wild-type B5R gene from strain WR, resulting in W-B5R$_{rescue}$ and I-B5R$_{rescue}$, has been described previously (Herrera *et al.*, 1998) .

### Southern blot and PCR confirmation of mutant viruses

Viral DNA was isolated from infected BSC-1 cells, digested with *Sna*BI, separated on 1% agarose, transferred to Immobilon-S membrane (Millipore), and probed using a B5R-specific probe as described previously (Earl and Moss, 1991a; Herrera *et al.*, 1998). We also confirmed the deletion of the DNA corresponding to the predicted cytoplasmic tail by PCR using a mixture of two pairs of PCR primers. One primer pair, oISI-64 (5′-GCACGGGTCGACCAACATGTACTGTACCCAC-3′) and oISI-71 (5′-GCTCTAGATTACGGTAGCAATTTATGG-3′), creates a ~900-bp product in wild-type viruses that retain the full cytoplasmic tail. The second primer pair (previously described oISI-8 and oISI-9; Isaacs *et al.,* 1990) was used to confirm that viral DNA was present in the B5RΔc viruses that did not produce a product using oISI-64 and oISI-71. Products were amplified (30 cycles at 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min) and separated on a 1.5% agarose gel, and bands were visualized by ethidium bromide staining.

### Western blot

Western blots of infected cell lysates were carried out using BSC-1 cells grown in 24-well plates and harvested

48 h after infection. The cells were pelleted and lysed in 50 μl of lysis buffer as described previously (Herrera *et al.,* 1998). Western blots of EEV proteins were carried out on virus released into the media by infected RK-13 cells in T150 flasks and processed as described previously (Herrera *et al.,* 1998). Samples (14 μl) were boiled in Laemmli's loading buffer and 2-mercaptoethanol, separated by 10% PAGE–0.1% SDS, and transferred to nitrocellulose. Blots were probed with rabbit polyclonal antibody C′-B5R at a dilution of 1:5000. C′-B5R was raised against a peptide corresponding to a portion of the extracellular domain proximal to the transmembrane domain (Isaacs *et al.,* 1992). After incubation with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Boehringer-Mannheim) diluted to 1:10000, bands were visualized by Renaissance Chemiluminescence Reagent (Dupont NEN). To confirm the presence or absence of the putative cytoplasmic tail, blots were stripped according to the manufacturer's instructions and reprobed with another polyclonal rabbit antibody, CT-B5R, at a dilution of 1:7500. CT-B5R polyclonal antibody was raised to a peptide corresponding to the predicted cytoplasmic tail (Katz *et al.,* 1997) and was the kind gift of B. Moss (National Institutes of Health). Stripped blots were also probed with an anti-P37 polyclonal antibody raised to a peptide corresponding to the predicted N-terminus of the p37 protein (the kind gift of B. Moss) (Katz *et al.,* 1997). It was used at a dilution of 1:3000.

### Analysis of EEV by CsCl gradient centrifugation

Virus was metabolically labeled by infecting confluent RK-13 cells in six-well plates incubated overnight in methionine- and cysteine-free media containing 100 μCi of Tran$^{35}$S-Label (ICN) as described previously (Herrera *et al.*, 1998) and then incubated for an additional 24 h in complete media. The medium was removed, clarified, and placed on a CsCl step gradient as described previously (Payne and Norrby, 1976). The gradient then was spun in a SW-41 rotor (Beckman) at 30,000 rpm for 2 h 45 min at 15°C. Gradient fractions were collected from the bottom of the tube, and the radioactive counts of aliquots were measured, as well as the refractive index, as described previously (Herrera *et al.,* 1998).

### Immunofluorescence and phalloidin staining of infected cells

Subconfluent BHK-21 cells grown on coverslips were infected for 2 h at a multiplicity of infection of 5 pfu/cell. After removal of the virus inoculum, complete BHK-21 medium was added, and the cells were incubated for 7 h at 37°C. Cells then were fixed for 10 min at room temperature with cold 4% paraformaldehyde. The coverslips were washed with PBS and permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After washing with PBS, the monolayers were treated for 5 min with PBS–0.1 M glycine and then incubated for 30 min with anti-B5R hybridoma supernatant, 19C2, diluted 1:100 in PBS containing 20% FBS. Rat monoclonal antibody, 19C2 (Schmelz *et al.,* 1994), was kindly made available by G. Griffiths (EMBL, Germany) and used as hybridoma supernatant. Cells were washed and incubated with fluorescein isothiocyanate-conjugated rabbit anti-rat antibody (DAKO) used at a 1:50 dilution in PBS–20% FBS. For phalloidin staining of filamentous actin structures, cells fixed and permeabilized as above were incubated for 30 min with PBS-20% FBS containing 0.33 ng/ml tetramethyl rhodamine isothiocyanate–phalloidin (Sigma). Finally, the monolayers were washed extensively in PBS and mounted with Fluorsave (Calbiochem).

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