

The Effects of Targeting the Vaccinia Virus B5R Protein to the Endoplasmic Reticulum on Virus Morphogenesis and Dissemination

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The consequence of redirecting the vaccinia virus (VV) B5R protein to the endoplasmic reticulum (ER) has been investigated by the addition of an ER retrieval signal KKSL (K_2X_2) to the B5R C-terminus. This mutant B5R gene and a version of the gene with the inactive ER retrieval sequence KKSLAL (K_2X_4) were inserted into the thymidine kinase locus of a VV mutant lacking the B5R gene, $\Delta B5R$. Similar levels of B5R protein were made by each virus, but the B5R- K_2X_2 protein remained sensitive to endoglycosidase H and colocalised with protein disulphide isomerase in the ER. In contrast, the B5R- K_2X_4 protein colocalised with 1,4-galactosyltransferase in the *trans*-Golgi network. Electron microscopy revealed that even when the B5R protein was redirected to the ER, intracellular mature virus particles were wrapped by cellular membranes to form intracellular enveloped virus particles, although more incompletely wrapped particles were evident compared with wild type. These intracellular enveloped virus particles were, however, unable to efficiently induce the polymerisation of actin and the plaque size formed by $\Delta B5R$ - K_2X_2 was small. Nevertheless, the amount and specific infectivity of EEV produced by $\Delta B5R$ - K_2X_2 were similar to those of wild type, despite the dramatic reduction in the amount of B5R protein present in $\Delta B5R$ - K_2X_2 EEV. © 1999 Academic Press

Key Words: vaccinia virus; B5R protein; endoplasmic reticulum; actin tails; extracellular enveloped virus; electron microscopy; plaque size.

INTRODUCTION

Vaccinia virus (VV) is a complex DNA virus that replicates in the cell cytoplasm and has a large double-stranded DNA genome encoding approximately 200 genes (Moss, 1996). VV particles are large and complex: the particles are 250 nm × 350 nm, sufficiently large to be seen easily with fluorescent microscopy (Cudmore *et al.*, 1995) and contain more than 100 proteins (Essani and Dales, 1979).

Virus morphogenesis commences in cytoplasmic foci or factories, and two forms of infectious virus particle, called intracellular mature virus (IMV) and extracellular enveloped virus (EEV), are produced (Appleyard *et al.*, 1971). These virions have different biological properties and different roles in the virus life cycle (Boulter and Appleyard, 1973; Payne and Kristensson, 1985; Vanderplasschen *et al.*, 1998). Morphogenesis begins with the appearance of virus crescents that are composed of lipid

and protein. Early studies suggested that these structures contained a single lipid bilayer and were synthesised *de novo* within the cytoplasm (Joklik and Becker, 1964; Dales and Mosbach, 1968). Later, these structures were proposed to contain two lipid bilayers that were derived from and continuous with host cell membranes of the intermediate compartment between the endoplasmic reticulum (ER) and the Golgi complex (Sodeik *et al.*, 1993). Recently, further electron microscopy has shown that these crescents, immature virus (IV) particles, and IMV particles contain only a single lipid bilayer (Hollinshead *et al.*, 1999). Regardless of the origin and nature of these crescents, it is clear that once formed, they extend to form noninfectious IV particles. After condensation of the core and proteolytic processing of several capsid proteins (Moss and Rosenblum, 1973), infectious IMV particles are formed.

IMV particles represent the majority of infectious progeny, and most remain inside the cell until cell lysis. However, some IMV particles are wrapped by a double layer of membrane (Ichihashi *et al.*, 1971) derived from the *trans*-Golgi network (TGN) (Hiller and Weber, 1985; Schmelz *et al.*, 1994) or tubular endosomes (Tooze *et al.*, 1993) to form intracellular enveloped virus (IEV). IEV particles promote the polymerisation of actin tails on one side of the virion to create a growing tail that gives the particle motility (Cudmore *et al.*, 1995). When an IEV

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reaches the cell periphery, its outer membrane fuses with the plasma membrane to produce an enveloped virus on the cell surface. This virion may remain attached to the cell surface as cell-associated enveloped virus (CEV) (Blasco and Moss, 1991) or be released as EEV. The proportion of enveloped virus that is retained (CEV) or released (EEV) varies with different strains of VV (Payne, 1980) and is affected by several EEV-specific proteins (see later).

Six VV genes have been found to encode EEV-specific proteins: F13L (p37) (Hirt *et al.*, 1986), A33R (gp22-28) (Roper *et al.*, 1996), A34R (gp22-24) (Duncan and Smith, 1992), A36R (p45-50) (Parkinson and Smith, 1994), A56R (gp86, the virus haemagglutinin) (Shida, 1986), and B5R (gp42) (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992). The function of these proteins has been studied using virus mutants in which the virus gene is either mutated, deleted, or repressed. These studies have shown that these proteins have little effect on the formation of IMV. However, the proteins have different roles in the subsequent stages of morphogenesis. In the absence of B5R (Engelstad and Smith, 1993; Wolffe *et al.*, 1993), F13L (Blasco and Moss, 1991), or A34R (Duncan and Smith, 1992; McIntosh and Smith, 1996; Wolffe *et al.*, 1997), the wrapping of IMV particles to form IEV is reduced or abolished, and in the absence of A33R, there is an increased proportion of virions in which the wrapping is incomplete (Roper *et al.*, 1998). Consequently, the formation of actin tails by all of these viruses is reduced or inhibited (Cudmore *et al.*, 1995; Wolffe *et al.*, 1997, 1998; Herrera *et al.*, 1998; Mathew *et al.*, 1998; Roper *et al.*, 1998; Sanderson *et al.*, 1998; Röttger *et al.*, 1999). The A36R (Parkinson and Smith, 1994) and A56R proteins (G. L. Smith, unpublished data) are not required for IEV formation, but A36R is needed for the polymerisation of actin tails (Sanderson *et al.*, 1998; Wolffe *et al.*, 1998; Röttger *et al.*, 1999). Thus the A36R deletion mutant is the only EEV mutant that makes IEV normally but no actin tails, and this, together with the fact that the majority of its amino acids are in the cytosol (Röttger *et al.*, 1999), makes A36R the prime candidate for the initiation of actin tail polymerisation. Many of the virus mutants lacking EEV proteins make small plaques (Blasco and Moss, 1991; Duncan and Smith, 1992; Engelstad and Smith, 1993; Wolffe *et al.*, 1993; Parkinson and Smith, 1994; Roper *et al.*, 1998), and there is a correlation between the inability to make actin tails and a small plaque phenotype. One exception is the virus lacking the four short consensus repeat (SCR) domains of B5R, which makes a normal-size plaque but does not form actin tails (Herrera *et al.*, 1998).

The roles of EEV proteins in EEV formation are variable. Loss of the F13L and B5R genes reduce EEV formation approximately 10-fold (Blasco and Moss, 1991; Engelstad and Smith, 1993; Wolffe *et al.*, 1993), whereas mutation (Blasco *et al.*, 1993) or deletion (McIntosh and

Smith, 1996; Wolffe *et al.*, 1997) of the A34R gene increases EEV formation. In the latter case, the EEV levels increased 25-fold but the EEV had a 5-fold reduced specific infectivity (McIntosh and Smith, 1996). Similarly, removal of all (Herrera *et al.*, 1998) or parts (Mathew *et al.*, 1998) of the B5R extracellular domain increased EEV formation by 10- or more than 50-fold- respectively, but these virions have normal infectivity. Deletion of the A33R or A36R genes has more modest effects on EEV formation, with a twofold to fourfold increase after the loss of A33R (Roper *et al.*, 1998) and a threefold to fivefold decrease after the loss of A36R (Parkinson and Smith, 1994).

The B5R gene encodes a 42-kDa glycoprotein that is expressed throughout infection (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992). In addition, there is a soluble, 35-kDa B5R protein of unknown function (Martinez-Pomares *et al.*, 1993). When expressed independently of vaccinia virus infection, the B5R protein localises in the Golgi complex (Katz *et al.*, 1997). The B5R protein affects virus plaque size, host range, and virulence (Takahashi-Nishimaki *et al.*, 1991; Engelstad and Smith, 1993; Martinez-Pomares *et al.*, 1993; Wolffe *et al.*, 1993; Stern *et al.*, 1997). Its extracellular domain shares amino acid similarity with complement control proteins (Goebel *et al.*, 1990; Smith *et al.*, 1991; Takahashi-Nishimaki *et al.*, 1991) and has four SCRs that are typical of the regulators of complement activation superfamily. Despite this similarity, no role for the B5R protein in inhibiting complement activity has been found (Vanderplasmchen *et al.*, 1998; M. Law and G. L. Smith, unpublished data). Mutagenesis of the B5R protein showed that deletion of one, two, or three SCR domains resulted in a small plaque phenotype but the release of 50-fold more EEV of normal infectivity (Mathew *et al.*, 1998). Deletion of all four SCRs produced a normal plaque size (Herrera *et al.*, 1998). The transmembrane anchor sequence and short cytoplasmic domain of B5R were sufficient to target a chimeric human immunodeficiency virus type 1 glycoprotein to the EEV outer envelope (Katz *et al.*, 1997), and loss of the short cytoplasmic tail did not affect the function of the B5R protein (Lorenzo *et al.*, 1999). Evidence for interactions between the B5R and A34R proteins has been presented (Röttger *et al.*, 1999). These observations taken together with the failure of the B5R deletion mutant to wrap IMV particles to form IEV suggested that B5R is a critical protein for initiation of the wrapping process and perhaps the accumulation of other EEV proteins in the TGN.

In this report, we redirected the B5R protein to the ER by the addition of an ER retrieval sequence (KKSL, K₂X₂) (Jackson *et al.*, 1990, 1993) at the C-terminus. Similar approaches have been used to investigate the effect of ER localisation of herpes simplex virus glycoprotein H (Browne *et al.*, 1996) and simian immunodeficiency virus envelope protein (Vincent *et al.*, 1998). It was anticipated that either the relocation of B5R to the ER might prevent

the formation of EEV due to a failure to wrap IMV particles by membranes lacking the B5R protein (as for the B5R deletion mutant) or B5R in the ER might induce wrapping of IMV particles by ER membranes. In fact, the retention of B5R in the ER did not prevent wrapping of IMV particles by the usual cellular membranes. Instead, it resulted in a considerable reduction in actin tail formation and plaque size and the production of EEV particles at normal levels and infectivity despite the absence of the B5R protein from these virions. The implications of these findings are discussed.

RESULTS

Construction of recombinant VVs expressing B5R-K₂X₂ or B5R-K₂X₄

To evaluate the effect of redirecting the B5R protein to the ER, two recombinant VVs were constructed. One of these had the ER retrieval sequence K₂X₂ (Jackson *et al.*, 1990; 1993) attached to the extreme C-terminus of the B5R protein, whereas the other had an inactive version of the ER retrieval sequence (K₂X₄) (Jackson *et al.*, 1990; 1993) at this position. Each of these B5R genes, under the control of the B5R promoter, was inserted into the TK locus of Δ B5R, a recombinant VV lacking the B5R gene (Engelstad and Smith, 1993), as described in Materials and Methods. As a control, a virus, vB5R/TK (Mathew *et al.*, 1998), that contained the wild-type (WT) B5R gene inserted into the TK locus of Δ B5R was analysed in parallel.

The genomes of the vB5R-K₂X₂ and vB5R-K₂X₄ viruses were analysed by PCR and Southern blotting and found to have the anticipated genome structures: namely, each virus had the B5R ORF plus the anticipated C-terminal motif inserted into the TK gene and retained a deletion in the B5R gene at its natural locus (data not shown).

Analysis of B5R gene expression

To determine whether the mutant B5R proteins were expressed and at what level, cells were either mock-infected or infected with Δ B5R, vB5R/TK, vB5R-K₂X₂, or vB5R-K₂X₄, and extracts from infected cells were analysed by immunoblotting using a polyclonal anti-B5R antiserum (Fig. 1). A protein of 42 kDa was detected at similar levels in cells infected with vB5R/TK, vB5R-K₂X₂, or vB5R-K₂X₄ but was absent in Δ B5R- and mock-infected cells. In the presence of tunicamycin, each protein was reduced in size, as reported previously for the WT B5R protein (Mathew *et al.*, 1998), but again the abundance of the protein was equivalent in each case. As a control to show that the cells had been infected to a similar degree, the nitrocellulose membrane was stripped and then reprobbed with monoclonal antibody (MAb) AB1.1 directed against the D8L gene product (Parkinson and Smith, 1994). Similar amounts of this protein

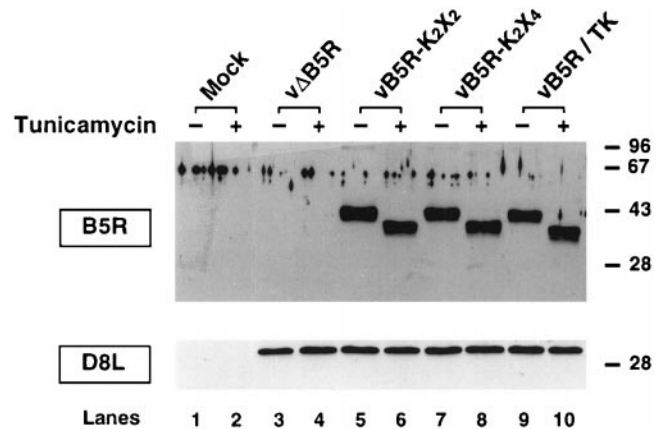


FIG. 1. Immunoblot showing the B5R protein synthesised by mutant viruses. Extracts from mock-infected cells or cells infected with the indicated virus were prepared and immunoblotted as described in Materials and Methods. In the upper panel, the B5R protein was detected with rabbit anti-B5R antiserum. In the lower panel, the membrane was reprobbed with MAb AB1.1 to the VV D8L protein. The sizes of molecular weight markers are shown in kDa.

were detected in all infected cells (lanes 3–10). Evidently, the presence of the C-terminal short amino acid tags did not affect the size or steady-state level of the B5R protein.

The B5R-K₂X₂ protein is present in the ER

Endoglycosidase H (endoH) digests the high mannose carbohydrate side chains of glycoproteins that have been synthesized in the ER but that have not been processed within the Golgi complex (Maley *et al.*, 1989). However, after trimming of the mannose side chains and addition of complex sugars in the TGN, glycoproteins become resistant to endoH digestion (Maley *et al.*, 1989). Thus the sensitivity of a glycoprotein to endoH can be used to determine whether it has been transported through the Golgi complex or is retained in the ER. If the K₂X₂ motif was functioning as anticipated, the B5R-K₂X₂ protein would be expected to be retained in the ER and would remain sensitive to endoH digestion. To test this, cells were infected with WT, vB5R/TK, vB5R-K₂X₂, or vB5R-K₂X₄ viruses and pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine for 5 min at 6.5 h postinfection (p.i.). Cells were then chased with cold methionine and cysteine for different lengths of time, immunoprecipitated with anti-B5R antiserum, incubated with or without endoH, and analysed by SDS-PAGE. For the WT and vB5R/TK viruses, the B5R protein was sensitive to digestion by endoH immediately after pulse, but became resistant by 80 min of chase (Fig. 2). In contrast, the B5R-K₂X₂ protein remained sensitive to endoH digestion throughout this pulse-chase period, indicating it was retained in the ER (Fig. 2). A much longer chase (6 h) showed that a small proportion of the B5R-K₂X₂ protein did become endoH resistant (data not shown). The B5R-K₂X₄ protein had an intermediate phenotype showing

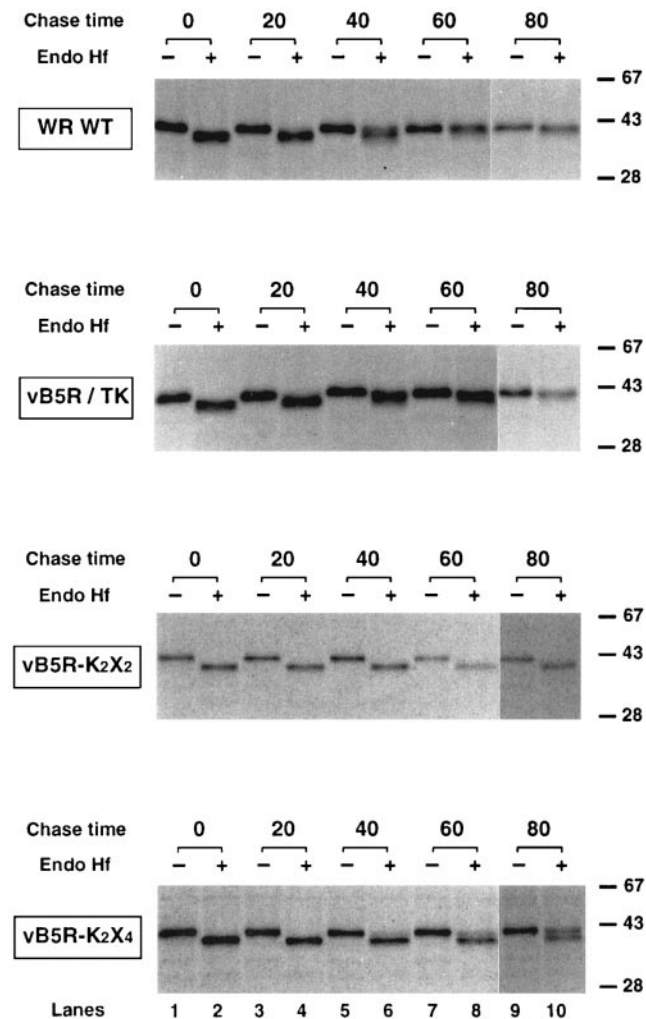


FIG. 2. Pulse-chase analysis. BS-C-1 cells were infected with the indicated viruses and pulse-labeled for 5 min at 6.5 h p.i. as described in Materials and Methods. After immunoprecipitation with antibody to the B5R protein and incubation with (+) or without (–) EndoH, the samples were resolved by SDS-PAGE (12% gels). Autoradiographs of the dried gels are shown. The positions of molecular weight markers are shown.

that the rate of transport from the ER was delayed compared with WT. However, after a 6-h chase, all of this protein was endoH resistant (data not shown) and so had been exported from the ER.

The subcellular location of the B5R-K₂X₂ and K₂X₄ proteins was analysed independently through indirect immunofluorescence using human protein disulphide isomerase (PDI) and 1,4-galactosyltransferase (Gal trans) as markers of the ER and TGN, respectively (Fig. 3). Cells were infected with either vB5R-K₂X₂ or vB5R-K₂X₄ and then stained with antibodies to B5R and PDI or to B5R

and Gal trans. In vB5R-K₂X₄-infected cells, the B5R protein was concentrated within small punctate foci resembling disseminated VV particles (Figs. 3C and 3G) and perinuclear structures that also contained the TGN marker gal Trans (Figs. 3G and 3H). In contrast, in cells infected with vB5R-K₂X₂, the B5R antigen showed a strikingly different pattern that had an extensive reticular distribution similar to that of PDI (Figs. 3A and 3B) and was predominantly segregated from Gal trans-positive membranes (compare Figs. 3E and 3F). Note also the pronounced staining of the nuclear envelope (Figs. 3A and 3E). The distribution of B5R protein in vB5R-K₂X₄ and vB5R/TK-infected cells were indistinguishable (data not shown). Thus the K₂X₂ motif functioned as anticipated, and the B5R-K₂X₂ protein was relocated to the ER.

Previous studies have shown that some B5R protein is present on the cell surface as well as on IEV, CEV, and EEV particles (Schmelz *et al.*, 1994). The level of B5R protein on the surface of cells infected with vB5R/TK, vB5R-K₂X₂, and vB5R-K₂X₄ was assessed by flow cytometry after staining with MAb 19C2 (Fig. 4A). The levels of B5R protein detected on cells infected with vB5R/TK and vB5R-K₂X₄ were similar, but there was a significant reduction in B5R surface expression in vB5R-K₂X₂-infected cells. Control experiments using an isotype-matched rat MAb and vΔB5R-infected cells confirmed the specificity of staining (Figs. 4B and 4C).

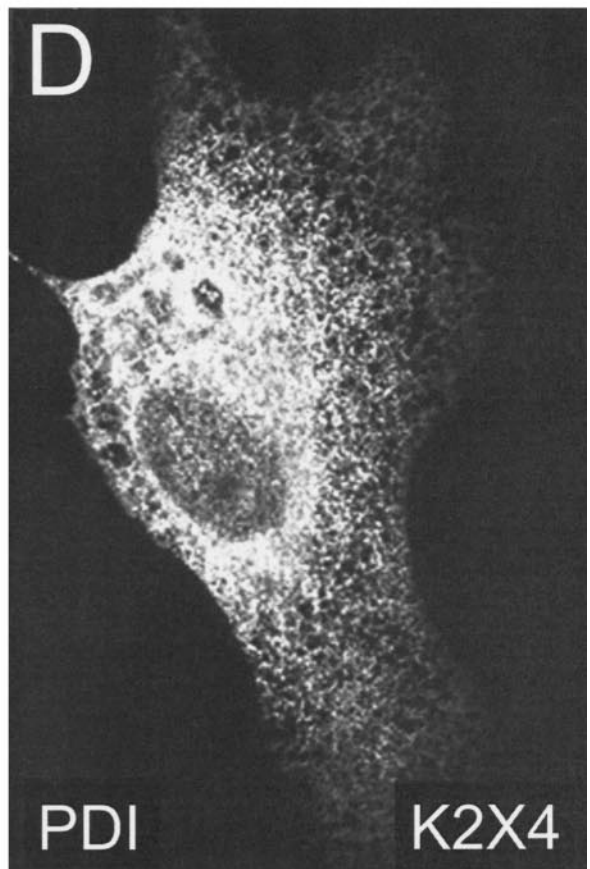
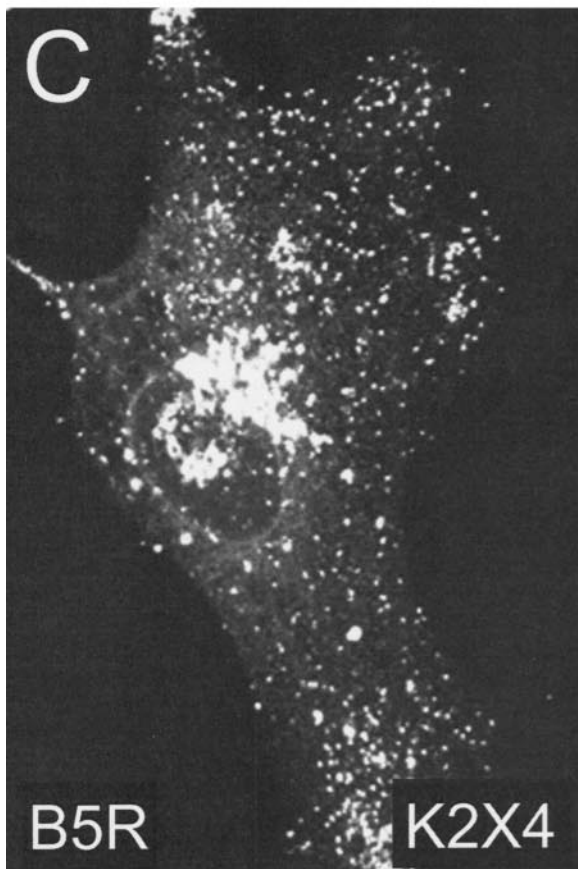
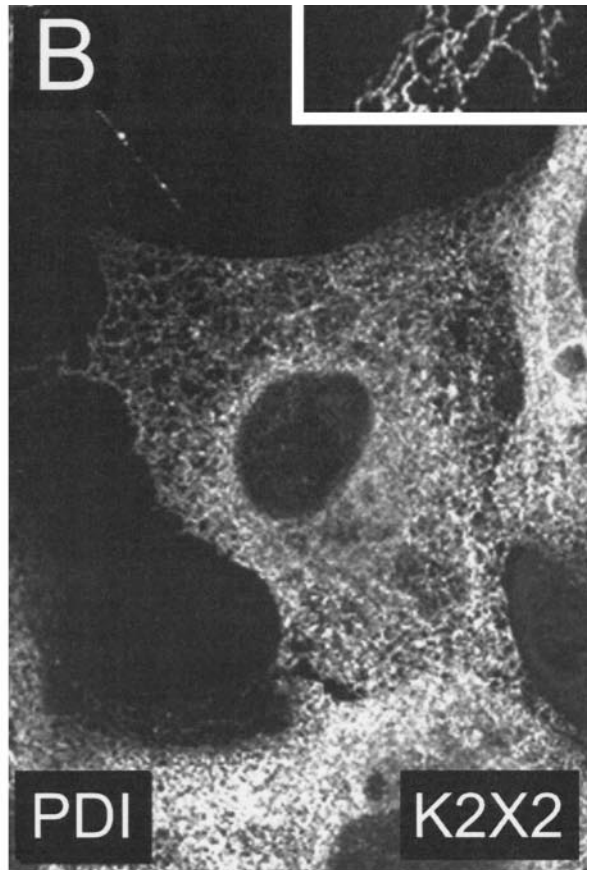
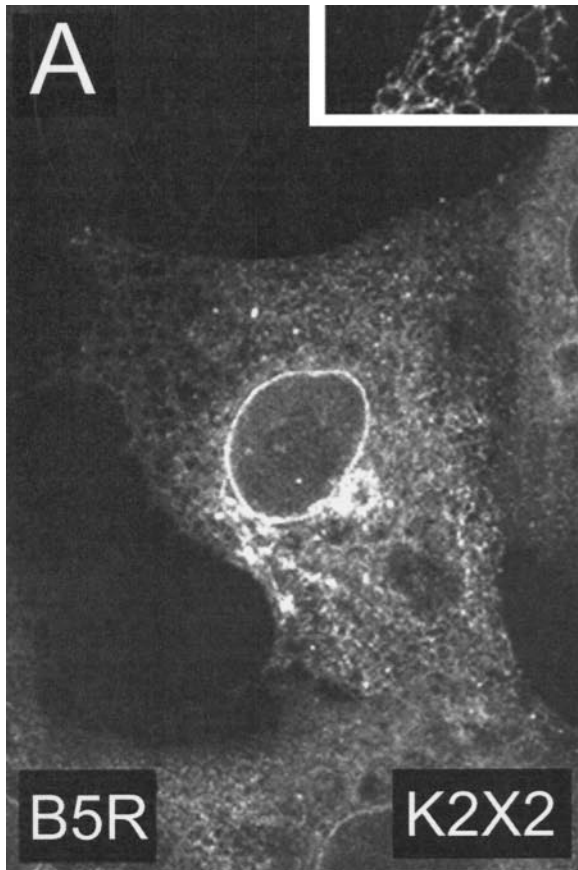
Wrapping of IMV to form IEV

The formation of virus particles in vB5R-K₂X₂-infected cells was examined next. Figure 3 shows that the number of B5R-positive virions in cells infected with vB5R-K₂X₂ was reduced greatly compared with vB5R-K₂X₄ (compare Figs. 3A and 3E with 3C and 3G). This suggested that either IEV particles were not formed when the B5R protein was retained in the ER or IEV particles were formed but these did not contain detectable levels of B5R. These possibilities were addressed by confocal and electron microscopy.

Electron microscopy

To determine whether cells infected with vB5R-K₂X₂ produced IEV particles, cells were fixed at 12 h p.i. and processed for thin section transmission electron microscopy. In these cells, all stages of VV morphogenesis were evident, and IEV particles were formed (Figs. 5A and 5B). To determine whether the frequency of IEV particle formation was different in cells infected with vB5R-K₂X₂, vB5R-K₂X₄, and vB5R/TK, the number of virus particles that were partially or completely enveloped was

FIG. 3. Immunofluorescence showing the B5R-K₂X₂ protein in the ER. BS-C-1 cells were infected with the indicated viruses and processed for indirect immunofluorescence as described in Materials and Methods. Cells shown in panels A, B, E, and F were infected with vB5R-K₂X₂, and cells in panels C, D, G and H were infected with vB5R-K₂X₄. The insets in A and B show the reticular staining pattern at a higher magnification.



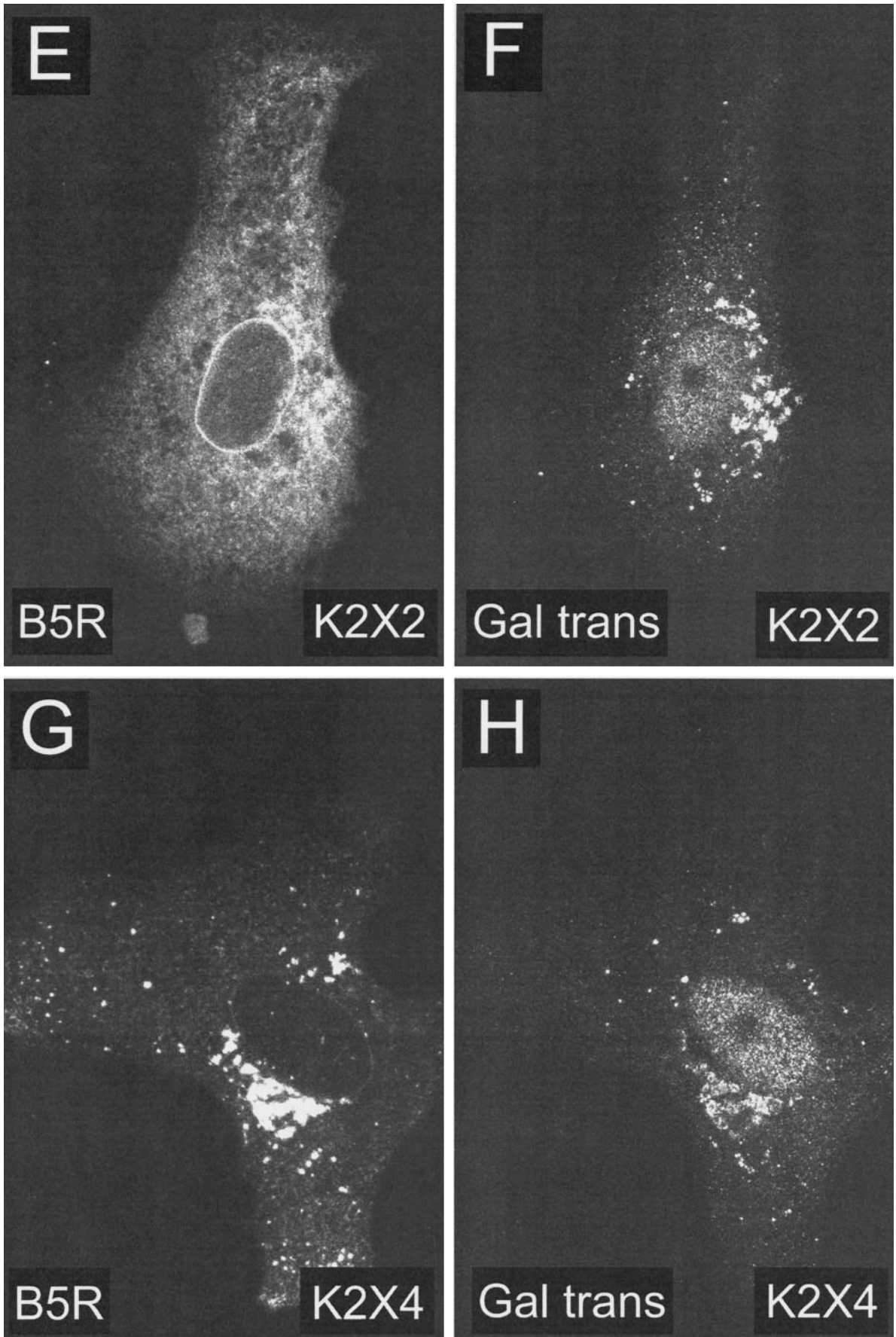


FIG. 3—Continued

expressed as a percentage of the total number of particles counted. The percentage of IMV particles that showed any wrapping (partial or complete) for vB5R-K₂X₂ (22%, 59 of 273) and vB5R-K₂X₄ (34%, 106 of 305) was slightly reduced or similar, respectively, to that for vB5R/TK (31%, 48 of 154) but much higher than that for vΔB5R (1%, 2 of 157) (Mathew *et al.*, 1998). However, the percentage of completely wrapped IEV particles was lower for both vB5R-K₂X₂ (8%, 22 of 273) and vB5R-K₂X₄ (13%, 41 of 305) compared with that for vB5R/TK (29%, 44 of 154). Therefore, either the relocation of the B5R protein to the ER or the addition of extra amino acids at its C-terminus reduced the efficiency of IEV formation. Nevertheless, significant numbers of IEV were formed by vB5R-K₂X₂ and vB5R-K₂X₄. However, an interesting property of vB5R-K₂X₂ morphogenesis was the higher-than-usual frequency of clusters of IEV and CEV particles that were contained within a single outer envelope (Figs. 5C and 5D). For vB5R-K₂X₂, there were 21 examples of this (inside or outside the cell), whereas for vB5R-K₂X₄ and vB5R/TK, only 2 examples were seen.

Do IEV particles made by vB5R-K₂X₂ contain B5R?

To address this question, infected cells were stained with MAbs against the D8L and B5R proteins or against the D8L and F13L proteins and were analysed by immunofluorescence and confocal microscopy. The D8L protein is present on all virions (IMV, IEV, CEV, and EEV), and so its detection served as a measure of the total number of virus particles. On the other hand, the B5R and F13L proteins, being specific for IEV, CEV, and EEV, would detect only particles that had been wrapped by intracellular membranes that contained these antigens. Figure 6 shows that the number of D8L-positive virions was similar in cells infected with either vB5R-K₂X₂ or vB5R/TK, but the number of B5R- or F13L-positive structures differed. In vB5R/TK-infected cells, the percentage of total particles that were B5R positive ($46.7 \pm 3.2\%$) or F13L positive ($49.8 \pm 4.2\%$) were similar, but in vB5R-K₂X₂-infected cells, the proportion of B5R-positive particles was much lower ($4.3 \pm 1.3\%$) than F13L-positive virions ($31.6 \pm 3.7\%$). This result, taken together with the reduced number of B5R-positive structures seen in vB5R-K₂X₂-infected cells compared with vB5R-K₂X₄-infected cells (Fig. 3), and the detection of IEV particles by electron microscopy (Fig. 4), indicated that IEV particles were formed when the majority of B5R was present in the ER, rather than TGN.

Formation of actin tails

To determine whether actin tails were being made in vB5R-K₂X₂-infected cells, the cells were stained with tetramethylrhodamine B isothiocyanate-phalloidin to detect polymerised actin and costained with a MAb to B5R (Fig. 7). No actin tails are evident in the vB5R-K₂X₂-infected

cell shown in Fig. 7B, although occasionally one or more actin tail was seen in some other vB5R-K₂X₂-infected cells, particularly late during infection (not shown). In contrast, actin tails were detected easily after infection with vB5R-K₂X₄ (Fig. 7D). It appears, therefore, that although the loss of B5R from IEV virions does not prevent their formation, it does restrict their ability to induce actin tail formation.

Plaque size

The size of plaques formed by vB5R-K₂X₂ and vB5R-K₂X₄ on BS-C-1 cells is shown in Fig. 8 compared with WT, vΔB5R, and vB5R/TK. Plaques formed by vB5R-K₂X₄ were indistinguishable from WT or vB5R/TK, but those formed by vB5R-K₂X₂ were intermediate between WT and vΔB5R. The reduced plaque size of vB5R-K₂X₂ is consistent with the reduced number of actin tails produced by this virus. The fact that plaques are larger than those made by vΔB5R is consistent with a few actin tails still being made. Plaques formed by all viruses under liquid overlay did not form comets (data not shown), suggesting that enhanced levels of EEV were not produced.

Production of intracellular and extracellular virus

The amount of infectious intracellular and extracellular virus produced by vB5R-K₂X₂ and vB5R-K₂X₄ was determined after infection of RK₁₃ cells at 1 PFU/cell (Fig. 9A). The infectious titres at each location were very similar for all viruses, and as usual with the WR strain of VV, the great majority of infectious progeny was intracellular. To confirm that the extracellular virus was EEV and not IMV that had been released from infected cells on lysis, the sensitivity of the extracellular virus to neutralisation by an IMV-specific MAb was investigated (Fig. 9B). In all cases the majority of the extracellular virus was resistant to neutralisation, whereas the same concentration of this antibody neutralised 86–92% of purified IMV (data not shown). Interestingly, the EEV released from vB5R-K₂X₂-infected cells showed the greatest resistance to neutralisation (95%), comparable to the resistance of the EEV made by vΔB5R (Mathew *et al.*, 1998) (and data not shown).

As an independent method to determine whether the extracellular virus detected was IMV or EEV, virus released from [³⁵S]methionine- and [³⁵S]cysteine-labeled infected cells was analysed by CsCl density gradient centrifugation (Parkinson and Smith, 1994). Analysis of the virus released from 1×10^7 RK₁₃ cells infected with vB5R-K₂X₂ or vB5R-K₂X₄ showed that virtually all (>95%) of the virus present in the culture supernatant had EEV density (data not shown). This observation is not inconsistent with the fact that up to one third of the extracellular virus could be neutralised by the IMV antibody, because EEV with a damaged outer envelope can retain EEV density and be neutralised by IMV antibody (Vanderplasschen and Smith, 1997).

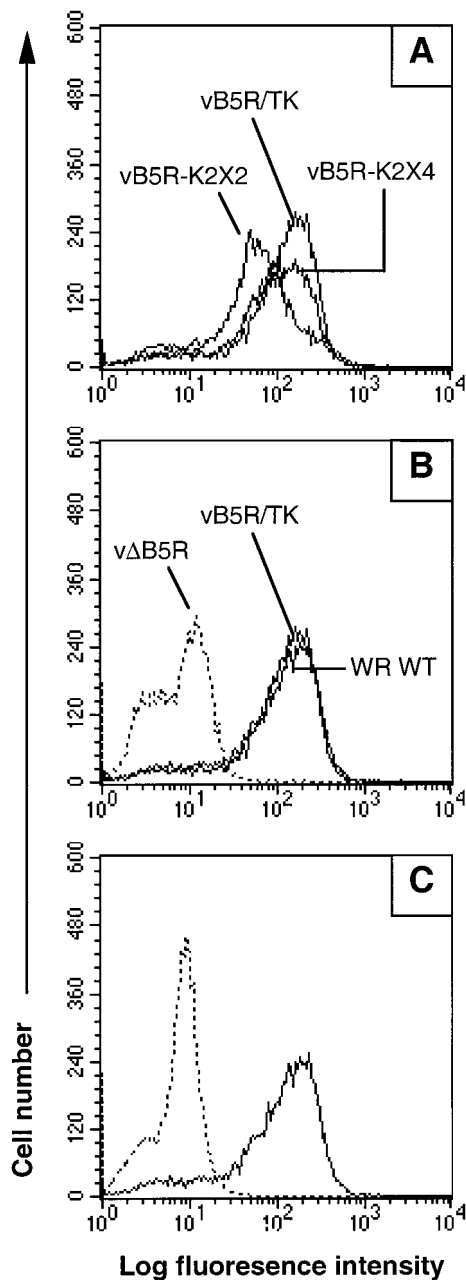


FIG. 4. Flow cytometry. TK⁻143B cells were infected with the indicated viruses at 5 PFU/cell. At 12 h p.i. the level of B5R protein on the cell surface was determined as described in Materials and Methods. (A) Cells were infected with vB5R-K₂X₂, vB5R-K₂X₄, or vB5R/TK and then stained with rat MAb 19C2 against B5R. (B) Cells were infected with vΔB5R, WT, or vB5R/TK and then stained with rat MAb 19C2 against B5R. (C) Cells were infected with WT virus and stained with MAb 19C2 (continuous line) or an isotype-matched rat MAb OX11 (dotted line). The number of cells is shown on the y axis, and the log₁₀ fluorescent intensity is shown on the x axis.

EEV from vB5R-K₂X₂-infected cells lacks B5R

To determine whether EEV released from vB5R-K₂X₂-infected cells contained B5R, virus was concentrated from the supernatant of infected cells and analysed by immunoblotting using polyclonal antibody against B5R

(Fig. 10). The amount of soluble, 35-kDa B5R that was present in the culture supernatant and the relative amount of B5R in infected cells were also determined. As a control, MAb AB1.1 against the IMV protein D8L was used to reprobe the nitrocellulose membrane. Cells infected with vB5R/TK and vB5R-K₂X₄ produced similar amounts of B5R in infected cells (C), extracellular virions (V), and culture supernatant (S). In contrast, vB5R-K₂X₂-infected cells produced no detectable B5R in extracellular virions or the culture supernatant but had similar amounts of cell-associated B5R. The lack of B5R in the extracellular virions was not due to a reduction in the number of virus particles because when duplicate membranes were probed with MAb directed against the D8L protein, similar amounts of D8L antigen were detected in virus (V) released after infection with vB5R-K₂X₂, vB5R-K₂X₄, or vB5R/TK (lower panel). The similar amounts of D8L antigen (Fig. 10), infectious virus (Fig. 9), and [³⁵S]methionine- and [³⁵S]cysteine-labeled virus (data not shown) made by these viruses, but the absence of B5R from the vB5R-K₂X₂ virions indicates that virions lacking B5R have normal infectivity.

Last, the absence of the 35-kDa form of B5R in the supernatant of vB5R-K₂X₂-infected cells indicates that cleavage of the 42-kDa precursor into the 35-kDa form occurs in a post-ER compartment.

DISCUSSION

A recombinant VV that expresses a B5R protein with an ER retrieval sequence (vB5R-K₂X₂) at its C-terminus was constructed and analysed. The B5R protein made by vB5R-K₂X₂ was shown to be located predominantly in the ER, rather than in the TGN as for the B5R protein made by WT virus and the virus with an inactive ER retrieval sequence (vB5R-K₂X₄) at the C-terminus of B5R. vB5R-K₂X₂ formed normal amounts of IMV and EEV, and slightly lower amounts of IEV, but the B5R-K₂X₂ protein was reduced greatly in IEV and not detected in EEV. Despite this, the EEV had normal infectivity. Few actin tails were induced on IEV particles, and consistent with this, the plaque size of the vB5R-K₂X₂ was reduced compared with WT but slightly larger than vΔB5R.

At the outset of this work, we considered that the consequence of redirecting the B5R protein to the ER might be that either IMV particles would be wrapped from that location to form IEV or wrapping would not take place. However, it was found that wrapping took place from the TGN, but the B5R protein was not detected on most wrapped particles (immunofluorescence) and was not detected at all on EEV by immunoblotting. This result was surprising because two groups had shown independently that a mutant virus lacking the B5R protein made normal IMV, but the wrapping of IMV to IEV did not take place (Engelstad and Smith, 1993; Wolffe *et al.*, 1993).

So how is the wrapping observed here explained? One

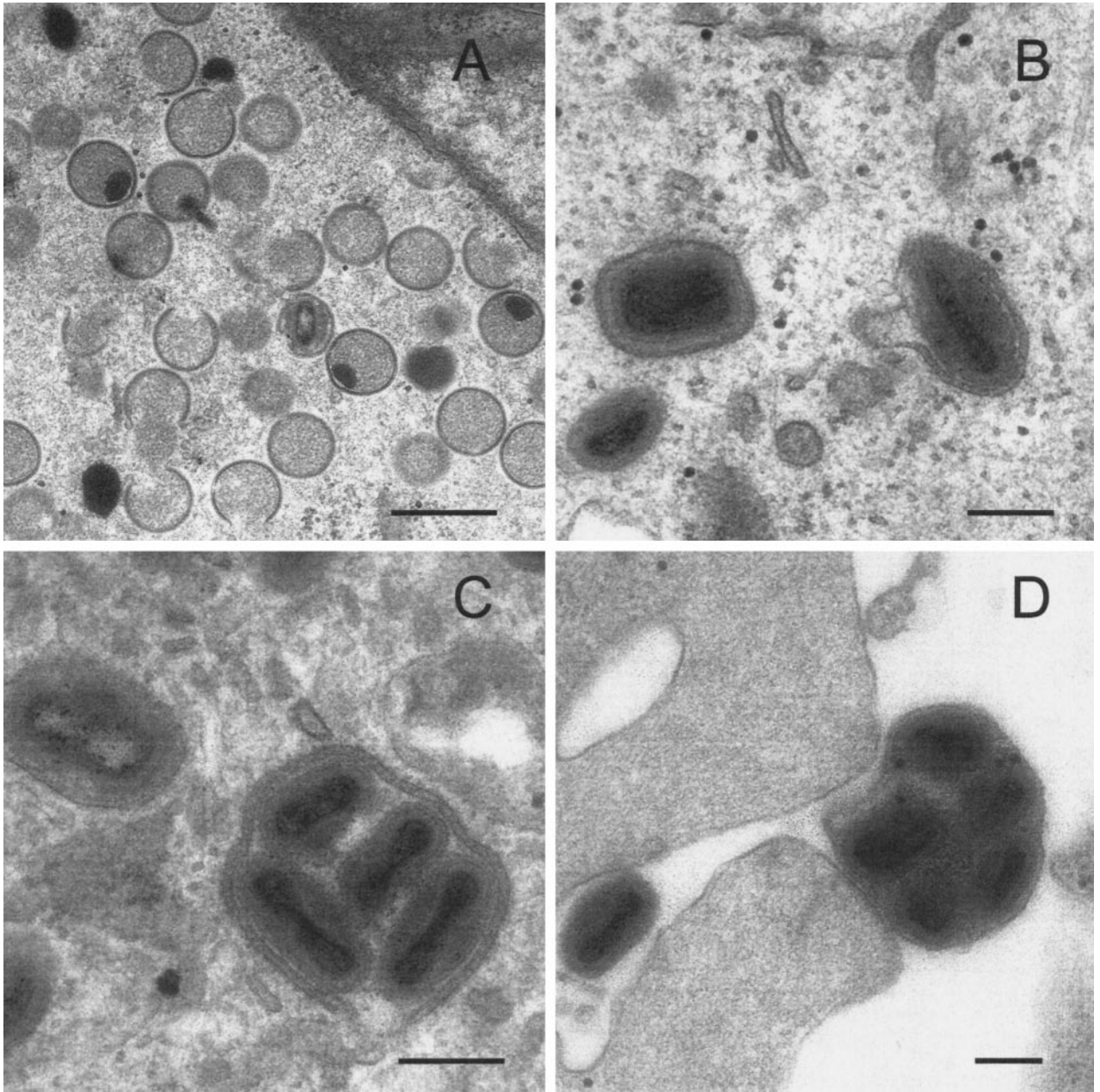


FIG. 5. Electron microscopy. HeLa cells were infected with vB5R-K₂X₂ and processed for electron microscopy as described in Materials and Methods. Panel A shows a typical virus factory, panel B shows two IEV particles, panel C shows the presence of several IMV particles wrapped by the same outer membranes, and panel D shows a cluster of CEV particles enclosed within the same outer envelope and associated with an actin tail. Bar in panel A = 500 nm, bar in panels B–D = 200 nm.

possibility is that a small amount of B5R is present in the TGN and that this is sufficient to initiate the wrapping process. In support of this possibility, the K₂X₂ motif used is a retrieval sequence rather than a retention sequence (Jackson *et al.*, 1990; 1993), so some B5R might be present in the TGN transiently before being retrieved to the ER or incorporated into IEV. In fact a small fraction of the IEV particles did contain B5R (as detected by immunofluorescence), although it is unclear from this analysis how much B5R was incorporated compared with WT. Perhaps IEV containing only a small fraction of the amount of B5R found

in WT IEV might still be revealed by this technique. Certainly the level was low because B5R was not detected in EEV by immunoblotting. The detection of B5R on some IEV particles by immunofluorescence but not on EEV by immunoblotting might represent different sensitivities of the techniques. Alternatively, enveloped virus containing B5R might be retained on the cell surface, whereas virions lacking B5R might be released; in this regard, it is noteworthy that viruses with one or more of the B5R SCR domains deleted released enhanced levels of EEV (Herrera *et al.*, 1998; Mathew *et al.*, 1998).

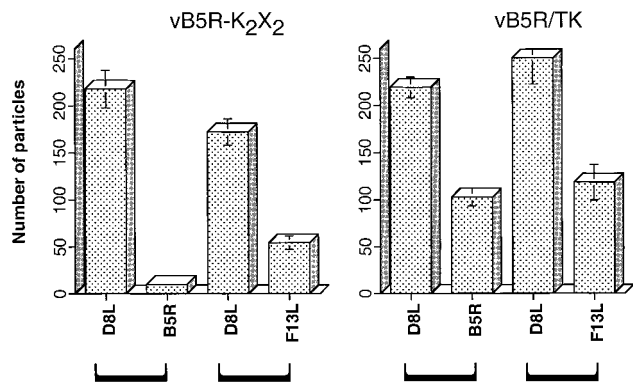


FIG. 6. Bar graphs showing the percentage of intracellular virus particles that were positive for the B5R or F13L proteins determined by double immunofluorescent staining and confocal microscopy. BS-C-1 cells were grown on glass coverslips and infected with vB5R-K₂X₂ or vB5R/TK. At 9 h p.i. two coverslips were removed from the same culture dish and processed for fluorescent microscopy as described in Materials and Methods. One coverslip was stained with MAb AB1.1 against D8L and MAb 19C2 against B5R, whereas the second coverslip was stained with MAb AB1.1 and MAb 15B6 against F13L. The numbers of virions stained with D8L, B5R, and F13L were recorded from confocal images of cells. Bars show the mean number of particles stained by each antibody $\pm$ S.E.M. ($n = 7$). Lines underneath the graph show data derived from cells on the same coverslip.

An alternative possibility to explain the IMV wrapping is that the normal interactions that exist between different EEV proteins (Röttger *et al.*, 1999) are established in the ER and that B5R is important for initiating the formation of these complexes. However, once these complexes are transported to the Golgi complex, the B5R-K₂X₂ protein is stripped from them by the ER retrieval motif and returned to the ER. Ultimately, the complexes are deficient in B5R, but B5R had an important role in their formation. This would contrast with the situation in v Δ B5R-infected cells, in which the complexes might never become established and hence wrapping is inhibited.

In vB5R-K₂X₂-infected cells, the EEV proteins in TGN membranes are sufficient to enable wrapping of IMV to IEV, but actin tail formation was reduced greatly. This indicates differing requirements for these two processes, as noted with other EEV mutants; for instance, the Δ A36R mutant (Parkinson and Smith, 1994; Sanderson *et al.*, 1998; Wolffe *et al.*, 1998) and the B5R mutants lacking SCR domains (Herrera *et al.*, 1998; Mathew *et al.*, 1998) are able to wrap IMV normally but unable to initiate actin tail formation. The intermediate plaque size of vB5R-K₂X₂ was consistent with its reduced ability to initiate actin tail formation, because actin tails are considered important for cell-to-cell spread of virus. vB5R-K₂X₂ made more actin tails than v Δ B5R but less than WT; hence the plaque size was intermediate between these two viruses.

The amount, buoyant density, and infectivity of EEV produced by vB5R-K₂X₂ were indistinguishable from WT

despite the few actin tails and the absence of B5R from EEV. It is unclear how normal amounts of EEV are made when actin tail formation is reduced greatly, but several other EEV mutants make normal or enhanced levels of EEV and have decreased formation of actin tails (McIntosh and Smith, 1996; Wolffe *et al.*, 1997; Herrera *et al.*, 1998; Mathew *et al.*, 1998; Roper *et al.*, 1998; Sanderson *et al.*, 1998). There is, therefore, an actin-independent pathway for EEV formation. This might involve the movement of IEV particles to the surface in an actin-independent manner or the direct budding of IMV through the plasma membrane as observed with the IHD-W strain of VV (Tsutsui, 1983). The electron microscopy of vB5R-K₂X₂-infected cells did not provide evidence for the latter, although there were more incompletely wrapped IMV particles and an increased number of virions (IEV or CEV) contained within a single outer envelope. A possible explanation for this phenotype might be that there is less efficient interaction between vB5R-K₂X₂ IMV particles and wrapping membranes compared with WT IMV. Consequently, vB5R-K₂X₂ IMV particles accumulate before wrapping, and subsequently multiple virions are wrapped in the same membranes. The normal infectivity of EEV lacking B5R indicated that B5R is not required for EEV infectivity despite B5R being reported to be a target for antibodies that neutralise EEV infectivity (Galmiche *et al.*, 1999).

In conclusion, we report here the effects of redirecting the B5R protein from the TGN to the ER on VV morphogenesis and dissemination. Despite the location of the B5R in the ER, IMV particles were wrapped normally to form IEV. However, the great majority of IEV particles were deficient in B5R and did not induce actin tails so that the plaque size was reduced. However, the formation of infectious EEV was not reduced, demonstrating that the EEV particles can infect new cells in the absence of B5R.

MATERIALS AND METHODS

Cells and viruses

TK⁻143B, RK₁₃, HeLa, and BS-C-1 cells were grown in Dulbecco's modified Eagle's medium in 10% FBS. VV strain Western Reserve (WR) and the recombinants derived from WR, v Δ B5R (Engelstad and Smith, 1993), and vB5R/TK (Mathew *et al.*, 1998) have been described previously.

Plasmids

Plasmid pEM9 contains the 5' end of the B5R gene (including the B5R promoter) inserted within the VV thymidine kinase (TK) gene (Mathew *et al.*, 1998) and was used to construct plasmids encoding the entire B5R ORF fused at the C-terminus to either the K₂X₂ or K₂X₄ motifs. The 3' end of the B5R gene encoding the 180 C-terminal

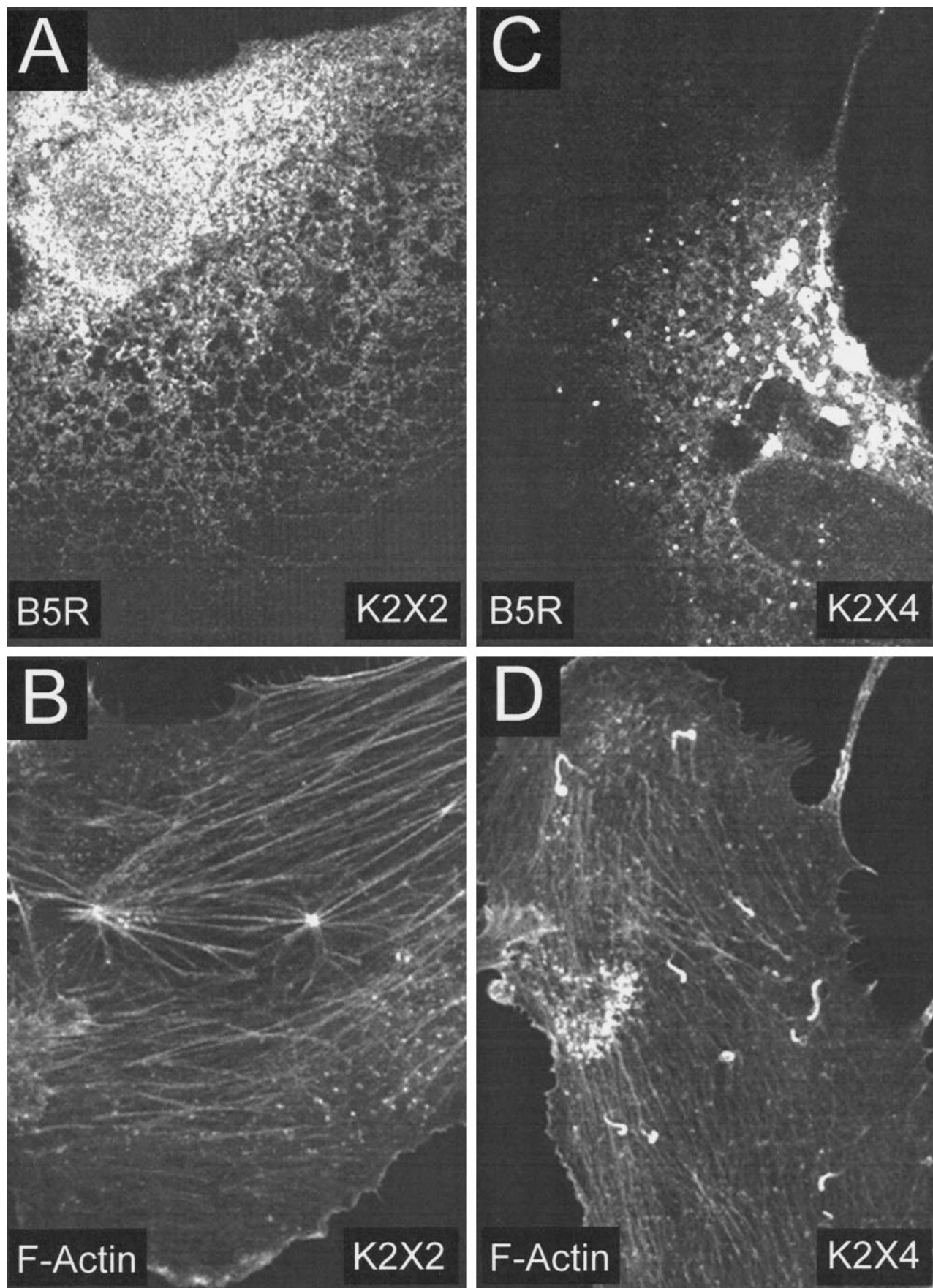


FIG. 7. Detection of actin tails in virus-infected cells. BS-C-1 cells were infected with the indicated viruses and then processed for double immunofluorescent microscopy as described in Materials and Methods.

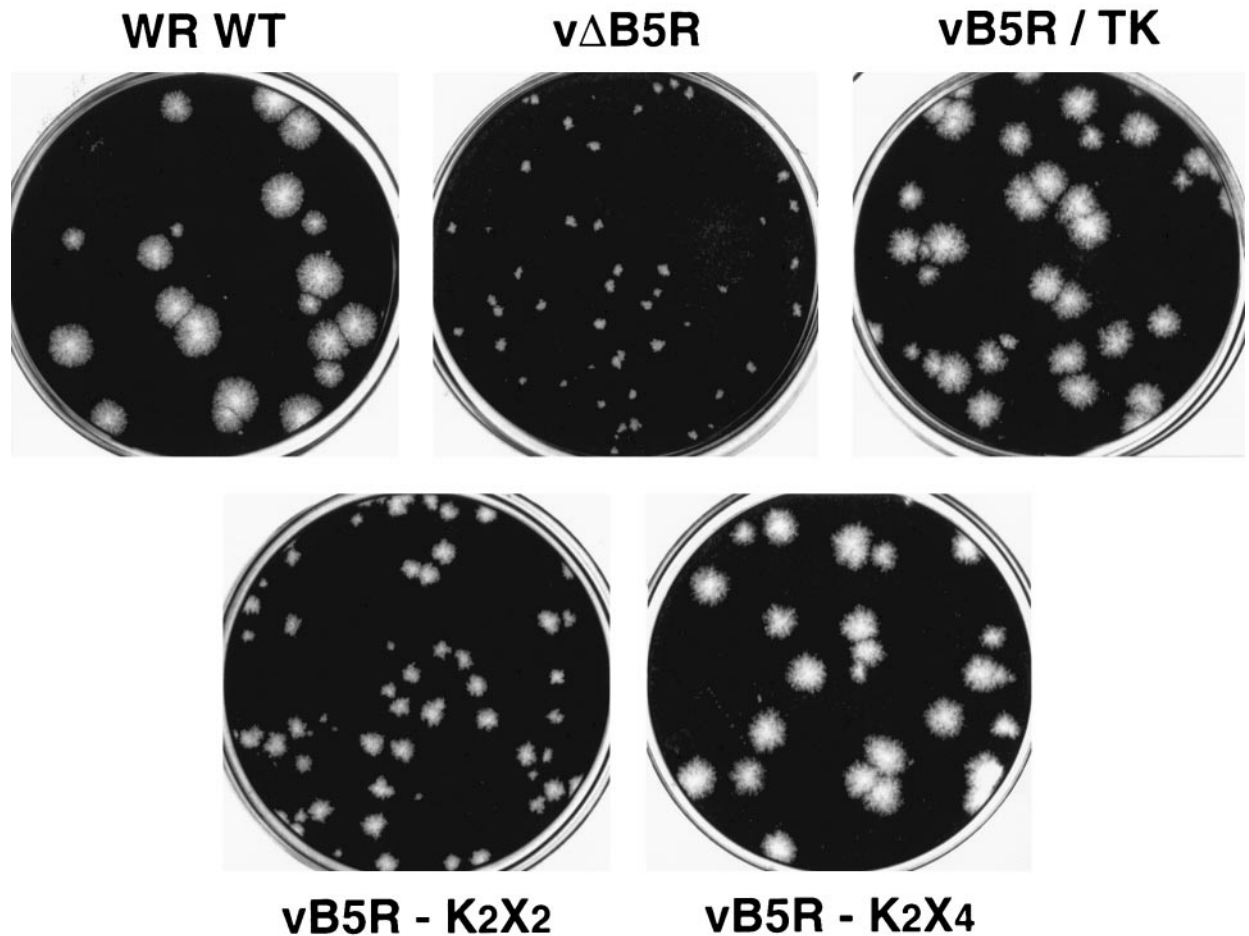


FIG. 8. Plaque sizes produced by different viruses. Monolayers of BS-C-1 cells were infected with approximately 50 PFU of the indicated viruses and incubated in MEM containing 1.5% carboxymethyl cellulose and 2.5% FBS. Five days later the overlay was removed, and the cells were stained with crystal violet.

amino acids fused to the K_2X_2 or K_2X_4 motifs was amplified by PCR using plasmid pSTH4 as the template (Howard, 1991) and oligonucleotides TACGAAGTGAAT-TCCACCAT (forward primer) and either CCCGAATTCT-TAgaggtctcttcttCGGTAGCAATTTATGGAAGTT or CCC-GAATTCTTAagagcgaggctcttcttCGGTAGCAATTTATGGAAGTT as reverse primers for the K_2X_2 or K_2X_4 motifs, respectively. Underlined, bold, or lowercase nucleotides represent the *EcoRI* site, translational termination codon, or K_2X_2 or K_2X_4 motifs, respectively. PCR fragments of 706 and 712 bp were isolated, digested with *EcoRI*, and cloned into pEM9 that had been digested with *EcoRI*. The resultant plasmids, pEM16 and pEM17, contained the entire B5R gene with either KKSL (B5R- K_2X_2) or KKSLAL (B5R- K_2X_4) fused to the C-terminus inserted within the VV TK gene.

Construction of recombinant VVs

Plasmids pEM16 or pEM17 were transfected into $v\Delta B5R$ -infected cells, and TK negative recombinant viruses were isolated by plaque assay on TK⁻ 143 cells as

described previously (Mathew *et al.*, 1998). These viruses were plaque purified three times, amplified, and named $vB5R-K_2X_2$ and $vB5R-K_2X_4$, respectively.

Immunofluorescence

BS-C-1 cells were infected at 1 PFU/cell and processed for indirect immunofluorescence at 14 h p.i. (unless stated otherwise) as described previously (Sander-son *et al.*, 1998). Mouse MAb AB1.1 directed against the VV D8L gene product (diluted 1:300) (Parkinson and Smith, 1994), rabbit polyclonal antibody against bovine Gal trans (diluted 1:100) (Lopez *et al.*, 1991), or mouse MAb ID3 directed against PDI (hybridoma culture supernatant diluted 1:100) (Vaux *et al.*, 1990; Fricker *et al.*, 1997), and rat MAbs against VV B5R (19C2, hybridoma culture supernatant diluted 1:8) (Hiller and Weber, 1985) and VV F13L (15B6, hybridoma culture supernatant diluted 1:5) (Hiller and Weber, 1985) were used as primary antibodies. Bound antibodies were detected with fluorescein B isothiocyanate-conjugated goat anti-mouse Ig or rhodamine-conjugated donkey anti-rat or anti-rabbit Ig

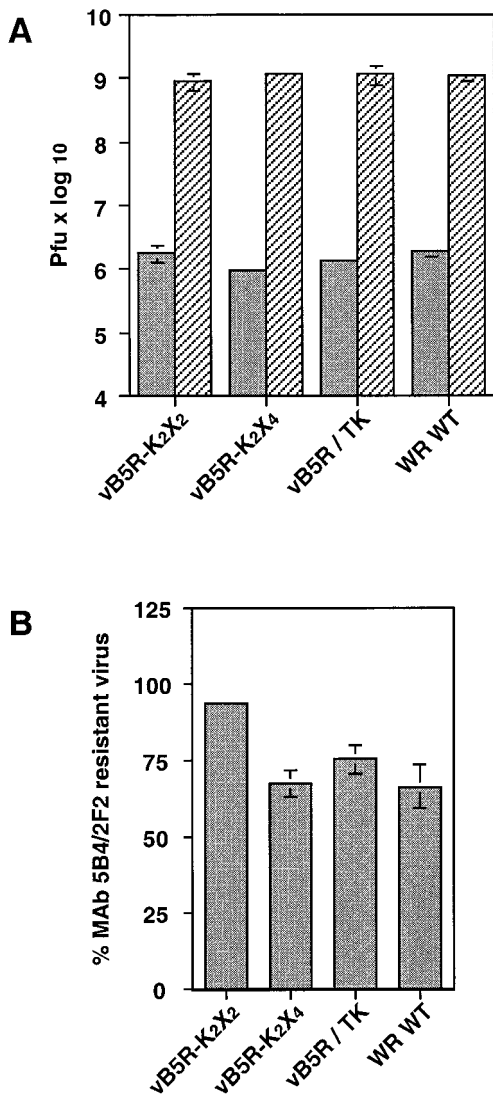


FIG. 9. Growth of VVs in cell culture. (A) RK₁₃ cells were infected with the indicated viruses at 1 PFU/cell. At 24 h p.i. the titre of virus present in the infected cells (hatched bars) and the culture supernatant (stippled bars) was determined by plaque assay on BS-C-1 cells as described in Materials and Methods. (B) Percentage of infectivity in the cell supernatant that is resistant to neutralisation by MAb 5B4/2F2.

(both diluted 1:50) (Jackson Laboratories). Filamentous actin was detected by direct staining with tetramethylrhodamine B isothiocyanate-phalloidin (Sigma, Poole, UK). Images of a single focal plane were collected using a Bio-Rad MRC 1024 laser scanning confocal microscope and were processed with Adobe TM Photoshop Software.

Flow cytometry

TK⁻143 cells were infected at 5 PFU/cell. At 12 h p.i. the cells were washed in PBS, incubated in 2 mM EDTA in PBS for 20 min at 37°C, and lifted from the Petri dish by pipetting. Cells were incubated for 1.5 h on ice with undiluted tissue culture supernatant from either rat hy-

bridoma 19C2 (anti-B5R) or OX11 (an isotype-matched rat MAb) (Hunt and Fowler, 1981). Cells were washed with PBS containing 10% heat-inactivated FBS and then incubated for 2 h at 4°C with fluorescein B isothiocyanate-conjugated sheep anti-rat IgG (diluted 1:100) (Serotech). After further washing in PBS containing 10% heat-inactivated FBS, the cells were fixed in PBS containing 4% paraformaldehyde and analysed by flow cytometry as described previously (Vanderplasschen and Smith, 1997).

Immunoblotting

BS-C-1 cells were infected at 10 PFU/cell in the presence or absence of tunicamycin (10 µg/ml). At 16 h p.i., the cells were washed in PBS, scraped from the dish, collected by centrifugation (2000 rpm, 10 min, 4°C), and dissolved in RIPA buffer minus SDS (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris-HCl, pH 8). Protein samples were then mixed with protein loading buffer, resolved by SDS-PAGE (12% gel), and transferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal antibody against the B5R protein (Engelstad *et al.*, 1992) (diluted 1:2000) or with mouse MAb AB1.1 against VV D8L protein (Parkinson and Smith, 1994) (diluted 1:2000), and bound antibody was detected by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (diluted 1:2000) (Sigma, Dorset, UK) followed by chemiluminescence reagents (Amersham) as directed by the manufacturer. When membranes were to be reprobed with a second antibody they were first stripped by incubation in 100 mM 2β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7 (50°C, 30 min with gentle agitation), and then washed in PBS containing 0.1% Tween 20.

Electron microscopy

HeLa cells were infected at 5 PFU/cell for 2 h at 37°C. Unbound virus was then washed away, and the cells were incubated at 37°C in MEM containing 2.5% FBS for 10 h before being washed in ice-cold PBS and fixed in 0.5% glutaraldehyde in 200 mM sodium cacodylate (pH 7.4) for 30 min at room temperature. After fixation, cells were washed in 200 mM sodium cacodylate and post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 60 min at room temperature. Samples were then washed in water, incubated overnight at 4°C in Mg²⁺-uranyl acetate, washed again in water, dehydrated in ethanol, and flat-embedded in Epon. Sections were cut parallel to the surface of the dish, lead citrate was added as a contrast agent, and the sections were examined on a Zeiss Omega EM 912 electron microscope (LEO Electron Microscope Ltd.) equipped with a Proscan-cooled, CCD camera (1024 × 1024 pixels) and a Dage-MTI SIT 66 low-level light camera. The captured images were processed using Adobe Photoshop TM software.

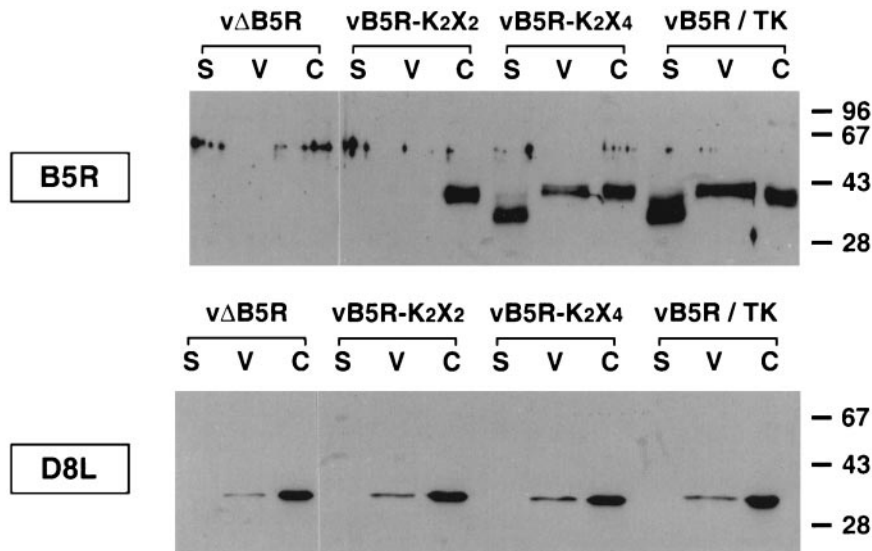


FIG. 10. Immunoblots showing the B5R and D8L proteins present in infected cells (C), released virus particles (V), and clarified culture supernatant (S). RK₁₃ cells were infected at 10 PFU/cell and cultured in MEM without serum. At 24 h p.i. the supernatants were centrifuged at low speed (2000 rpm, 10 min, 4°C) to pellet detached cells and then at high speed (14,000 rpm, 80 min, 4°C) to pellet virus particles. Soluble protein present in the culture supernatant was precipitated by the addition of trichloroacetic acid and recovered by centrifugation (14,000 rpm, 80 min, 4°C). Infected cells were scraped from the cell monolayer into PBS and recovered by centrifugation (2000 rpm, 10 min, 4°C). Pellets were combined with pellets obtained from low-speed centrifugation of the culture supernatant and dissolved in RIPA buffer lacking SDS. Protein samples were resolved by SDS-PAGE and immunoblotted as described in Materials and Methods. To obtain comparable signals, protein in the soluble supernatant from 4×10^5 cells (S), virus from 1×10^6 cells (V), or cell lysates from 2×10^4 cells were loaded onto the same gel. Upper panel shows an immunoblot with anti-B5R antibody. Lower panel shows the same membrane reprobed with anti-D8L MAb. The sizes of molecular weight markers are shown in kDa.

Immunoprecipitation and pulse-chase analysis

BS-C-1 cells were infected at 5 PFU/cell. At 6 h p.i., the cells were washed with MEM lacking methionine and cysteine (prewarmed to 37°C) and incubated in the same medium for 30 min. Cells were then labeled metabolically with the same medium supplemented with 25 μ Ci of Promix (a mixture of [³⁵S]methionine and [³⁵S]cysteine, 1000 Ci/mmol, Amersham, UK) and 25 μ Ci of [³⁵S]cysteine (600 Ci/mmol, New England Nuclear) for 5 min. Cells were harvested immediately or chased for 20, 40, 60, or 80 min in MEM supplemented with 2 mM cysteine and methionine. At the time of harvest, cells were washed on ice with ice-cold PBS and lysed in RIPA buffer minus SDS. The lysates were centrifuged for 10 min at 4000 rpm in a Beckman GPR centrifuge, and then SDS was added to the supernatant to a final concentration of 0.1%. Samples were incubated with rabbit polyclonal antibody to the VV B5R protein (Engelstad *et al.*, 1992). Immune complexes were immunoprecipitated with protein A-conjugated Sepharose beads, and the immunoprecipitates were incubated for 3 h at 37°C in the presence or absence of 1000 units of EndoH_f (New England Biolabs). Samples were resolved by SDS-PAGE (12% gel), and the dried gel was analysed by autoradiography.

Analysis of virus growth properties

RK₁₃ cells were infected at 1 PFU/cell, and after 2 h the cells were washed with MEM containing 2.5% FBS (pre-

warmed to 37°C) and then incubated in the same medium. At 24 h p.i. the culture supernatant was collected and centrifuged to remove detached cells (2000 rpm, 10 min, 4°C). The virus present in the clarified supernatant was titrated by plaque assay on BS-C-1 cells before or after incubation for 1 h at 4°C with MAb 5B4/2F2 directed against the IMV surface protein A27L (Czerny and Mahnel, 1990). MAb was added to the samples after they had been diluted to approximately 200 infectious particles (determined from prior pilot experiments) per unit volume so that the ratio of MAb to infectious virus particles in the test sample was constant. This concentration of MAb was shown to neutralise 86–92% of purified IMV particles (data not shown). The titre of cell-associated virus was determined by scraping the infected cells from the dish into PBS and collecting the cells through centrifugation (2000 rpm, 10 min, 4°C). The cell pellet was combined with the pellet obtained during centrifugation of the culture supernatant, resuspended in PBS, frozen and thawed three times, and sonicated, and then the virus infectivity was determined by plaque assay on BS-C-1 cells.

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