

Vaccinia Virus Gene B7R Encodes an 18-kDa Protein That is Resident in the Endoplasmic Reticulum and Affects Virus Virulence

Nicola Price, David C. Tscharke, Michael Hollinshead, and Geoffrey L. Smith¹

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, United Kingdom

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This paper presents a characterisation of vaccinia virus (VV) gene B7R that was predicted to encode a polypeptide of 182 amino acids with an N-terminal signal peptide. *In vitro* transcription and translation analysis showed the B7R gene product was a 21-kDa protein that, in the presence of microsomes, was processed into an 18-kDa mature form. The 18-kDa form associated with the microsomal membranes and was within the lumen of the vesicle where it was inaccessible to exogenous protease or an antibody raised against the B7R C terminus. Within VV-infected cells, the 18-kDa form of B7R was detected late during infection in the endoplasmic reticulum where it colocalised with protein disulphide isomerase. The B7R protein was detected neither in the culture supernatant nor associated with virus particles. A virus deletion mutant lacking the B7R gene and a revertant virus were constructed. Compared to wild-type and revertant viruses, the deletion mutant replicated normally in cell culture and had unaltered virulence in a murine intranasal model of infection. However, the deletion mutant was attenuated in a murine intradermal model where it induced a smaller lesion than the control viruses. © 2000 Academic Press

Key Words: vaccinia virus; endoplasmic reticulum; virus virulence; B7R gene.

INTRODUCTION

Poxviruses form a family of large, eukaryotic, dsDNA viruses that replicate in the cytoplasm and encode their own enzymes for transcription and DNA replication (Moss, 1996; Traktman, 1996). The 192-kb genome of vaccinia virus (VV) strain Copenhagen has been sequenced and encodes ~200 genes (Goebel *et al.*, 1990). The central portion of the genome contains genes that mostly are essential for virus replication (Johnson *et al.*, 1993), while both termini contain nonessential genes (Perkus *et al.*, 1991) that may affect virus host range, virulence, or the host response to infection (Buller and Palumbo, 1991; Smith *et al.*, 1997).

The B7R ORF is located near the right terminus of the VV strain Copenhagen genome and is predicted to encode a protein containing 182 amino acids with a molecular weight of 21.3 kDa (Goebel *et al.*, 1990). In VV strain Western Reserve (WR), the ORF is conserved (100% amino acid identity) and contains a hydrophobic region near the N terminus that might represent a signal sequence (Howard *et al.*, 1991). This observation, together with the absence of any other potential hydrophobic transmembrane domain, suggested the B7R protein might be secreted. The B7R ORF is conserved in VV strain modified virus Ankara (ORF 175 shares 97% amino acid identity with the B7R in VV Copenhagen and WR) (Antoine *et al.*, 1998) and also has counterparts in some

other orthopoxviruses. It shares 97.8% amino acid identity with the B6R ORF of cowpox virus (Shchelkunov *et al.*, 1998) but is broken in variola virus strains Bangladesh-1975 and India-1967 (Shchelkunov *et al.*, 1995). There are also related ORFs in the leporipoxviruses: the VV WR B7R protein shares 21.4% amino acid identity over 126 amino acids with the C-terminal domain of the T2 protein of Shope fibroma virus (Howard *et al.*, 1991). The function of the C-terminal domain of the closely related myxoma virus (M)-T2 protein is unknown, but it is postulated to have a role in intracellular trafficking of the protein (Schreiber and McFadden, 1996). The N-terminal, cysteine-rich domains of the M-T2 protein bind TNF- α and inhibit apoptosis of infected lymphocytes (Schreiber *et al.*, 1997).

Membrane and soluble proteins leave the ER not only by bulk flow, the default pathway (Wieland *et al.*, 1987) but, in certain cases, by selective interactions of the proteins (via receptors, in the case of soluble proteins) with the ER-derived coat protein (COPII)-covered vesicles (Schimmoller *et al.*, 1995). Further sorting occurs by retrieval of ER proteins from post-ER compartments (Pelham, 1988) or by active retention of proteins in the ER (Duvet *et al.*, 1998). Proteins are retained in the ER because either they contain an ER retention/retrieval sequence or they bind to other proteins that have an ER retention/retrieval sequence (Gething *et al.*, 1986; Sato *et al.*, 1996).

The B7R gene was chosen for study because it is located in a region of the VV genome that is rich in immunomodulators, it encodes a protein that may be

¹ To whom reprint requests should be addressed. Fax: +44-1865-275501. E-mail: glsmith@molbiol.ox.ac.uk.

secreted, and has amino acid similarity to the leporipox-virus T2 proteins that have immunomodulatory functions. Here we have characterised the VV B7R protein and show that it is resident in the endoplasmic reticulum (ER) and does not affect virus growth properties *in vitro*. However, loss of the gene attenuates the virus in a murine intradermal model.

RESULTS

The B7R gene encodes an 18-kDa protein expressed late during infection

To help identify and characterise the function of the B7R gene product, a virus deletion mutant lacking 60% of the ORF (v Δ B7R), a plaque purified wild-type virus (vWTB7R), and a revertant virus in which the B7R gene was reinserted into v Δ B7R (vB7R-rev) were constructed as described under Materials and Methods. Southern blotting and PCR analysis of these recombinant virus genomes confirmed the genome structures were as predicted (data not shown).

The B7R nucleotide sequence contains an early transcription termination signal (TTTTTNT) (Yuen and Moss, 1987) partway through the ORF and a potential late transcriptional start site (TAAATG) (Rosel *et al.*, 1986; Weir and Moss, 1987; Davison and Moss, 1989) at the beginning of the ORF, suggesting that the gene might be transcribed late during infection. To test this experimentally, RNA was extracted from infected cells at different times after infection and was examined by Northern blotting with an anti-sense, B7R-specific, RNA probe that had been generated *in vitro* (Materials and Methods) (Fig. 1A). This probe detected RNAs of heterogeneous length from 4 h p.i. (lanes 2–4), but not from mock-infected cells (lane 5). Because VV late mRNAs are heterogeneous in length due to a failure to terminate at specific sites (Moss, 1996), the late RNAs could represent transcripts generated from promoters of either upstream genes or from the B7R gene itself. No B7R mRNA was detected from cells infected in the presence of either cycloheximide (an inhibitor of protein synthesis) (lane 6) or araC (an inhibitor of DNA replication) (lane 7), indicating that the B7R gene is transcribed only late during infection. The probe also detected RNA from v Δ B7R-infected cells, but at lower levels (lane 8), presumably because of the deletion of the majority of the B7R ORF to which the probe would hybridise.

The expression of the B7R protein in virus-infected cells was examined using a rabbit polyclonal antiserum raised to the hydrophilic domain of B7R (amino acids 20–182) that was expressed as a fusion protein in *Escherichia coli*. The B7R-specific Ig was affinity purified as described under Materials and Methods and called α -B7R-1 Ig. Cellular extracts were prepared at different times p.i. from TK⁻143B cells that had been either mock-infected (Fig. 1B, lane 1) or infected with vWTB7R in the

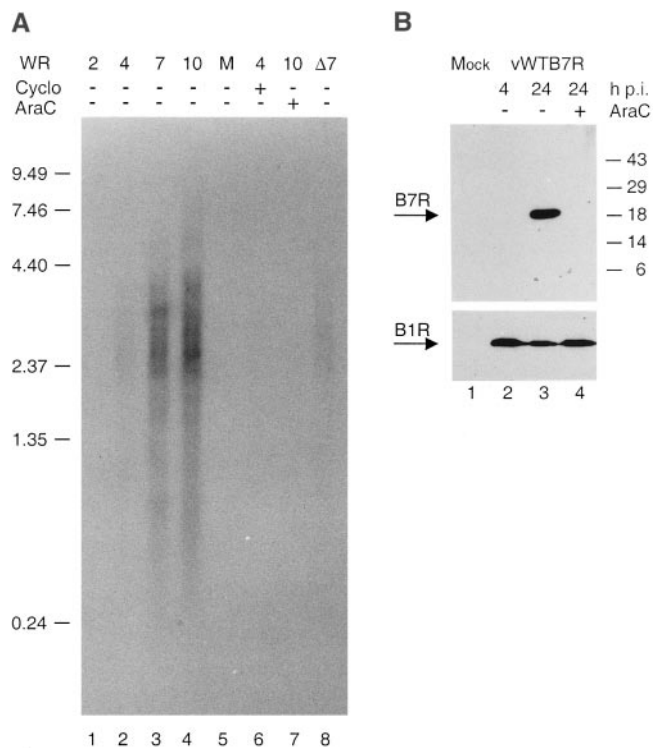


FIG. 1. (A) Northern blot analysis of B7R mRNA. RNA from mock-infected cells (M) or cells infected either with v Δ B7R (Δ 7) or VV strain WR for the indicated times (top row, h) in the presence or absence of 100 μ g/ml cycloheximide (cyclo) or 40 μ g/ml araC, was resolved on a 1.2% agarose gel and transferred to a nylon filter. The filter was hybridized with an antisense, ssRNA probe specific for the B7R ORF (Materials and Methods). Positions of size markers are shown in kb. (B) Immunoblot analysis of the B7R protein synthesised in the presence or absence of araC. Monolayers of TK⁻143B cells were mock-infected (lane 1) or infected with vWTB7R at 10 PFU/cell and harvested at 4 h p.i. (lane 2) or 24 h p.i. in the absence (lane 3) or presence (lane 4) of 40 μ g/ml araC. Samples were analysed by immunoblotting with α -B7R-1 Ig or with α -B1R antibody as described under Materials and Methods. Positions of molecular weight markers are indicated in kDa.

presence (lane 4) or absence (lanes 2 and 3) of araC and were immunoblotted with α -B7R-1 (Fig. 1B, top). A protein of 18 kDa was detected only at 24 h p.i. in the absence of araC (lane 3). A rabbit polyclonal antiserum against the B1R protein (α -B1R antibody) (Banham and Smith, 1992) was used on parallel blots to show that virus-specific protein was present in lanes 2–4 (Fig. 1B, bottom). These data show that B7R encodes an 18-kDa protein that is expressed late during VV infection, in agreement with the transcriptional analysis above.

The B7R protein is unstable in the presence of glycosylation inhibitors

To determine whether the B7R protein is modified by glycosylation, cells were infected in the presence or absence of either tunicamycin, an inhibitor of N-linked glycosylation, or monensin, an inhibitor of O-linked glycosylation and vesicular transport (Fig. 2). Extracts from

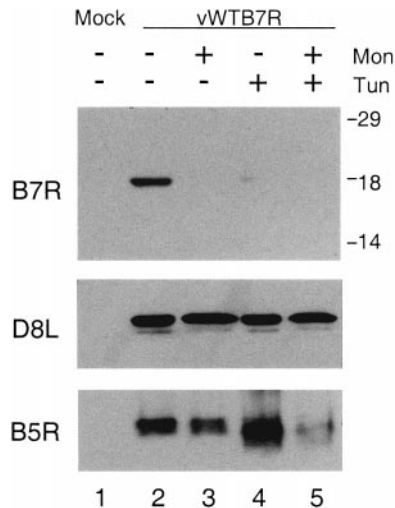


FIG. 2. Immunoblot analysis of the B7R protein in the presence or absence of glycosylation inhibitors. Monolayers of TK⁻143B cells were mock-infected (lane 1) or infected with vWTB7R at 10 PFU/cell and incubated for 24 h in the presence of no drug (lane 2), 1 μ M monensin (Mon, lane 3), 10 μ g/ml tunicamycin (Tun, lane 4), or tunicamycin and monensin (lane 5). Cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose and reacted with the α -B7R-1 Ig (top) and bound Ig was detected as described under Materials and Methods. Parallel blots were probed with MAb AB1.1 (anti-D8L) (middle) and rabbit polyclonal α -B5R antibody (bottom). Positions of molecular weight markers are indicated in kDa.

mock or vWTB7R-infected cells were prepared at 24 h p.i. and analysed by immunoblotting with α -B7R-1 Ig (Fig. 2, top). Parallel blots were also probed with a MAb AB1.1 against the VV D8L protein (Parkinson and Smith, 1994) (middle) and a rabbit polyclonal antiserum against the B5R protein (Engelstad *et al.*, 1992) (bottom). The B7R protein was not detected in the presence of these glycosylation inhibitors, whereas the level of D8L protein was unaltered under these conditions. The B5R protein is modified by N-linked glycosylation so that in the presence of tunicamycin its size decreases from 42 to 40 kDa. It is unlikely the B7R protein is glycosylated because pulse-chase analysis and immunoprecipitation of B7R showed no increase in size of the protein during the chase period (data not shown), and there is no N-linked glycosylation motif (NXS/T) in the ORF (Howard *et al.*, 1991). This result might suggest that the stability of B7R in the cell is dependent on the glycosylation of another protein.

The B7R protein is a nonstructural, intracellular protein

The presence of the B7R protein in infected cells, culture supernatants, and virions was examined by immunoblotting. Cells were mock-infected or infected with vWTB7R, v Δ B7R, or vB7R-rev, and both cells and supernatants were harvested at 24 h p.i. (Fig. 3A). The supernatant was centrifuged to remove any cellular debris and

virus particles, and proteins in the clarified supernatant were concentrated by TCA precipitation. Cellular and supernatant samples were then analysed by immunoblotting using α -B7R-1 Ig. Blots were washed and then reprobed with a rabbit polyclonal α -B5R antibody (Engelstad *et al.*, 1992) that recognises the cellular 42-kDa and secreted 35-kDa B5R proteins. The α -B7R-1 Ig detected an 18-kDa protein in fractions of cells infected with either vWTB7R or vB7R-rev but not v Δ B7R. The B7R protein was not evident in the supernatant of infected cells in contrast to the 35-kDa B5R protein that was detected easily in the supernatant.

To determine whether the B7R protein was present in

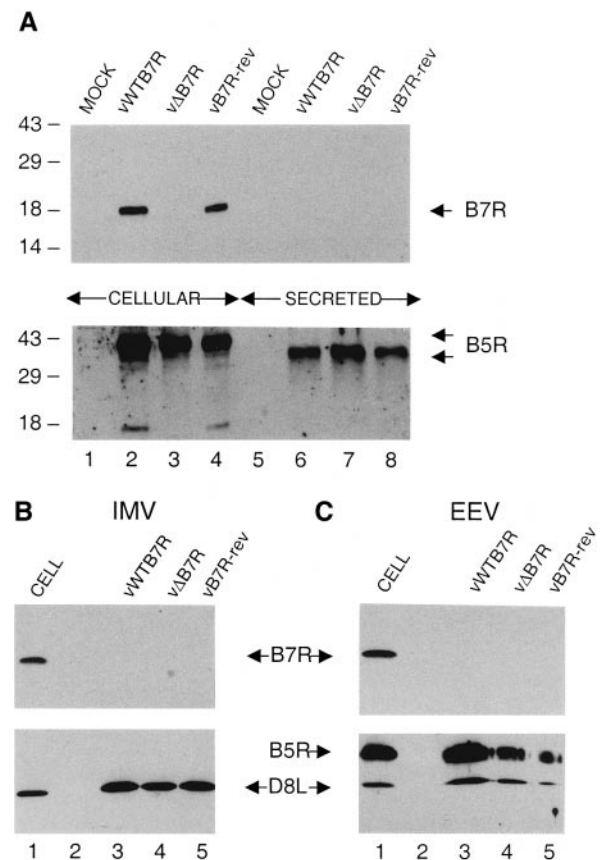


FIG. 3. Immunoblot analysis of B7R protein. (A) B7R is an intracellular protein. BS-C-1 cells were mock-infected (lanes 1 and 5) or infected with vWTB7R (lanes 2 and 6), v Δ B7R (lanes 3 and 7), or vB7R-rev (lanes 4 and 8) at 2 PFU/ml. Cellular (lanes 1–4) and supernatant (lanes 5–8) fractions were harvested and processed as described under Materials and Methods. The blot was reacted with α -B7R-1 Ig (top), and bound Ig was detected as described under Materials and Methods. The blot was washed and reprobed with the rabbit α -B5R antibody (bottom). Positions of molecular weight markers are indicated in kDa. The arrows point to the B7R protein (top) and the cellular and secreted forms of B5R (bottom). (B and C) The B7R protein is not present in VV particles. Purified IMV (B) or EEV (C) proteins of vWTB7R (lane 3), v Δ B7R (lane 4), or vB7R-rev (lane 5) and extracts from vWTB7R-infected cells (lane 1) harvested at 24 h p.i., were immunoblotted with α -B7R-1 Ig (top). The blots were reprobed with the mouse α -D8L MAb AB1.1 (B, bottom) or with both mouse α -D8L MAb AB1.1 and rabbit α -B5R antibody (C, bottom). Positions of molecular weight markers are indicated in kDa.

virus particles, vWTB7R, v Δ B7R, and vB7R-rev were grown in RK₁₃ cells, and EEV was purified from the culture supernatant by sucrose density gradient centrifugation. IMV was purified from cytoplasmic extracts in parallel. Solubilised virus proteins were then analysed by immunoblotting with α -B7R-1 Ig (Figs. 3B and 3C). This antibody did not detect the B7R protein in either IMV (Fig. 3B) or EEV (Fig. 3C) samples, although it detected the B7R protein in infected cell extracts. As a control, the blots were washed and reprobed with MAb AB1.1 (anti-D8L) to confirm equivalent protein loading. For the EEV samples, the α -B5R antibody was also included to confirm the presence of EEV protein in the samples. Evidently, B7R is not incorporated into virus particles.

The B7R protein is resident in the endoplasmic reticulum

The intracellular location of B7R was investigated by immunofluorescence. The α -B7R-1 Ig did not give a signal in immunofluorescence, and therefore a new polyclonal rabbit antiserum (α -B7R-2) was raised against a synthetic peptide representing the C-terminal 10 amino acids of the B7R protein (Materials and Methods). In cells infected with vWTB7R, α -B7R-2 showed a reticular staining pattern (Figs. 4D and 4G) that was absent in v Δ B7R-infected cells (Fig. 4A). Staining the same cells with a mouse MAb 1D3 against protein disulphide isomerase (PDI) (Vaux *et al.*, 1990), showed a similar staining pattern (Figs. 4B, 4E, and 4H). B7R and PDI were shown to be coincident by merging the two images (Figs. 4F and 4I), confirming that the B7R protein is resident in the ER.

Additional evidence that B7R is an ER protein was obtained by immunohistochemistry. BS-C-1 cells were infected with either vWTB7R or v Δ B7R and at 18 h p.i. were fixed and permeabilised and then incubated with α -B7R-2 antibody. Bound antibody was detected using an HRP-conjugated secondary antibody and DAB staining (Materials and Methods). Sections of cells (80- to 150-nm thick) were then analysed by electron microscopy (Fig. 5). Cells infected with v Δ B7R showed little staining (Figs. 5B and 5D), whereas the vWTB7R-infected cells showed a reticular and nuclear envelope staining pattern (Figs. 5A, 5C, and 5E). The virus factories (V), Golgi (G), and plasma membrane (PM) were unstained (Figs. 5A, 5C, 5E, and 5F), whereas the nuclear envelope (Fig. 5C) and fenestrated ER (Fig. 5E) showed staining in vWTB7R-infected cells. The inset in Fig. 5F illustrates the unstained Golgi at a higher magnification.

These data, taken together with the lack of secretion of B7R from infected cells, its absence from virions, and its colocalisation with PDI, support the conclusion that B7R is resident in the ER.

Analyses of the B7R protein synthesised *in vitro*

To determine whether the B7R protein was associated with membranes and, if so, to examine the membrane topology, the B7R protein was synthesised *in vitro* by transcription and translation in the presence or absence of microsomal membranes (Fig. 6). In the absence of microsomes, a 21-kDa protein was produced (Fig. 6A, lane 4) and this protein remained in the soluble fraction (S) after centrifugation (Fig. 6A, lane 6). The protein was not observed when translation was performed in the absence of a B7R-specific transcript (data not shown). When canine pancreatic microsomes were included in the translation reaction, an 18-kDa protein was also produced (Fig. 6A, lane 1) that sedimented with the microsomes into the pellet (P) during centrifugation (Fig. 6A, lane 2). The size of this protein is consistent with its formation by proteolytic cleavage of the 21-kDa form to remove the signal peptide. The 21-kDa protein is also associated partly with the membrane fraction (Fig. 6A, lanes 2 and 3), suggesting that the microsomes do not process all the 21-kDa protein quickly. In control translation reactions, yeast α -factor (Promega) increased in size from 19-kDa in the absence of microsomes to 30 kDa in the presence of microsomes. In the latter case, the protein sedimented with microsomes during centrifugation consistent with its presence in the lumen of the vesicle (data not shown).

To assess whether the 18- or 21-kDa form of B7R produced *in vitro* comigrated with the form identified in virus-infected cells, both reticulocyte and cellular lysates were subjected to immunoprecipitation with α -B7R-2 antibody followed by SDS-PAGE (Fig. 6B). The 18-kDa B7R protein that was found in virus-infected cells (Fig. 6B, lane 1) comigrated with the 18-kDa form of B7R produced *in vitro* in the presence of microsomes (Fig. 6B, lane 2) rather than the 21-kDa protein formed in the absence of microsomes (Fig. 6B, lane 3).

The association of the B7R protein with microsomes and its processing could have been either cotranslational or posttranslational. To investigate this, the B7R transcript was translated in the presence or absence of microsomes, and then microsomes were added to the latter sample in the presence of cycloheximide to inhibit new protein synthesis (Fig. 6C). Processing of 21-kDa protein to the 18-kDa form occurred only when the microsomes were present during translation of the protein (Fig. 6C, lane 1) and not when the microsomes were added to preformed B7R protein (Fig. 6C, lane 2).

The B7R protein sedimented with microsomes during centrifugation, and this could have been because it was an integral membrane protein, membrane-associated protein, or luminal protein. To examine how B7R was associated with microsomes, microsomes containing the B7R protein were extracted with either Triton X-100 or sodium carbonate pH 11 and then separated into soluble

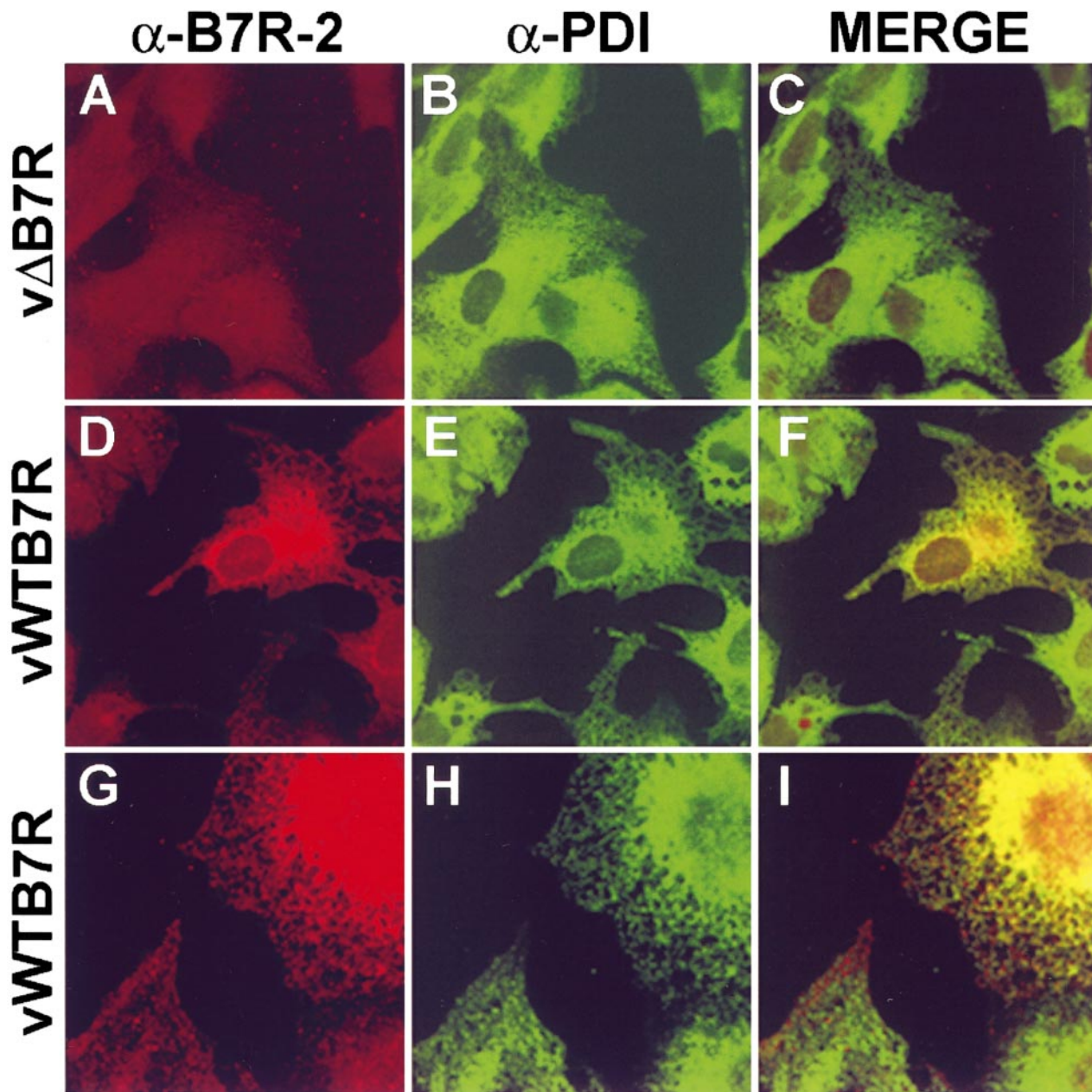
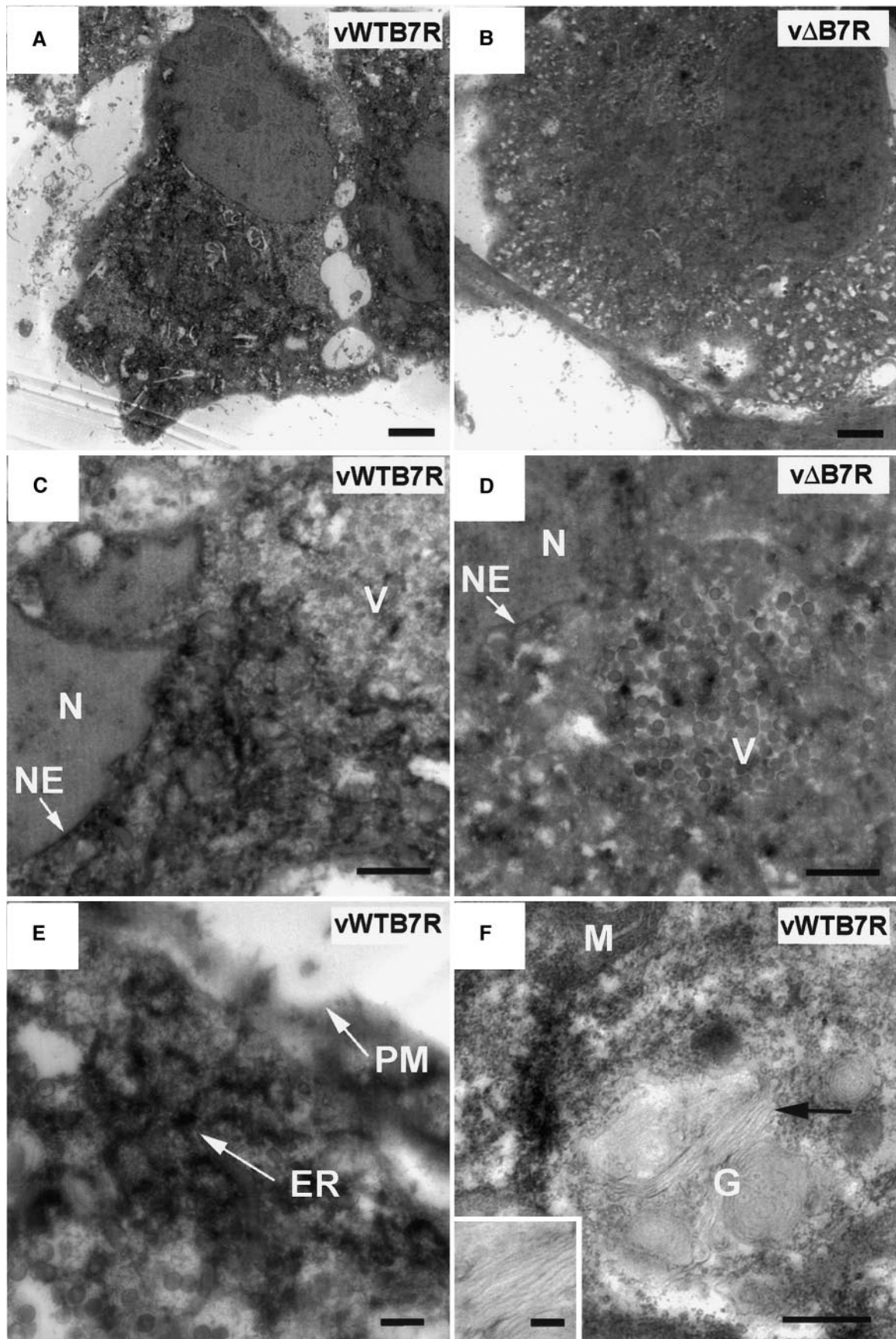


FIG. 4. Immunofluorescence of infected cells showing the ER localisation of the B7R protein. BS-C-1 cells were infected at 2 PFU/cell with either vWTB7R (D-I) or vΔB7R (A-C) for 12 h. Cells were then fixed, permeabilised, and incubated with both polyclonal rabbit α -B7R-2 antibody (A, D, and G) and mouse α -PDI MAb 1D3 (B, E, and H), and bound Ig was detected with FITC- or rhodamine-conjugated secondary antibodies as described under Materials and Methods. Merged images of (A and B), (D and E), and (G and H) are shown in (C), (F), and (I), respectively. Magnification, $\times 385$ (A-F), $\times 963$ (G-I).

(S) and pellet (P) fractions by centrifugation (Fig. 6D). Sodium carbonate punctures microsomal membranes and solubilises membrane-associated proteins, whereas integral membrane proteins remain with the membrane. Triton X-100 disrupts and solubilises both integral membrane and membrane-associated proteins. As controls, an integral membrane protein, VV A38L (Parkinson *et al.*, 1995), and a luminal protein, yeast α -factor, were translated and analysed in parallel. Both α -factor and A38L were solubilised by Triton X-100 (Fig. 6D, lane 5),

whereas α -factor was solubilised by sodium carbonate and A38L was not (Fig. 6D, lanes 2 and 3). In comparison, the B7R protein was nearly all solubilised by Triton X-100 (Fig. 6D, lane 5) but was distributed equally between soluble and pellet fractions after sodium carbonate extraction (Fig. 6D, lanes 2 and 3). The behaviour of the B7R protein is characteristic of neither an integral membrane nor a soluble protein but of a membrane-associated protein.

The membrane topology of B7R was examined by



adding trypsin to microsomes in the presence or absence of Triton X-100 (Fig. 6E). The B7R protein and yeast α -factor were protected from digestion by trypsin (Fig. 6E, lane 4), but when Triton X-100 was added to disrupt the membrane, both proteins were digested (Fig. 6E, lane 5), showing that each is located on the luminal side of the membrane. This topology was confirmed (Fig. 6F) by using the α -B7R-2 antibody, raised against the C terminus of B7R, to immunoprecipitate the translation products in the presence or absence of Triton X-100. In the absence of Triton X-100, the α -B7R-2 antibody was unable to immunoprecipitate the B7R protein (Fig. 6F, lane 2), but in the presence of Triton X-100 to disrupt the membrane, both the 18- and 21-kDa B7R gene products were immunoprecipitated (Fig. 6F, lane 1). These data indicate that B7R is located on the luminal side of the microsomal membrane and that proteolysis does not occur at the C terminus.

Loss of the B7R gene has no effect on virus replication *in vitro*

The isolation of v Δ B7R showed that the B7R gene was nonessential for replication of VV strain WR *in vitro*, as predicted by its location near the right terminus of the genome (Johnson *et al.*, 1993). To determine whether loss of B7R affects virus replication in cell culture, the formation of infectious virus was measured after infection of BS-C-1 cells at 0.01 PFU/cell (Fig. 7). Under these conditions, there was no difference between the replication of vWTB7R, v Δ B7R, or vB7R-rev for either cell-associated (Fig. 7B) or extracellular virus (Fig. 7A). The three viruses also shared similar growth characteristics after infection of BS-C-1, Jurkat E6.1, and THP-1 cells at 10 PFU/cell (data not shown). The size and morphology of plaques formed by v Δ B7R on BS-C-1 cells was unaltered compared to control viruses (Fig. 7C).

Loss of the B7R gene attenuates virus virulence in a murine intradermal model of infection

Although the B7R ORF is nonessential for the replication of VV *in vitro*, the gene product might contribute to virus virulence *in vivo*. This was assessed first in a murine intranasal model of infection, but no difference in the weight loss or mortalities of animals infected with v Δ B7R compared to vWTB7R or vB7R-rev was observed (data not shown). The virulence of v Δ B7R was then assessed in a murine intradermal model. In this model,

inoculation of 10^4 – 10^6 PFU of VV strain WR leads to a localised and limited infection in the ear pinnae with little infectious virus disseminating to local lymph nodes or other organs (Tscharke and Smith, 1999). The size of the lesion on the ear pinnae can be measured easily. Mouse ears were injected intradermally with 5×10^4 PFU/ear of vWTB7R, v Δ B7R, and vB7R-rev, and the diameter of resulting lesions was measured daily for groups of at least six mice. No animal exhibited signs of systemic infection, but mice infected with v Δ B7R had smaller lesions from 7 days p.i. compared with mice inoculated with vWTB7R or vB7R-rev (Fig. 8A). The Mann–Whitney *U*-test showed a significant difference ($P < 0.05$) between the mean values for the v Δ B7R-infected group compared to each other group on days 7–11. In addition, the unpaired Student's *t* test showed a significant difference ($P < 0.009$) when pooled sample means across days 7–15 were compared. The maximal lesion size was reached at 10 days p.i. in the v Δ B7R-infected mice and a day later in mice inoculated with vWTB7R or vB7R-rev. The titer of infectious virus in ears at various times postinfection was determined by plaque assay of ground tissue samples from groups of three mice at each time point (Fig. 8B). For each virus, the titer reached a peak of $\sim 5 \times 10^6$ PFU/ear at 5 days p.i. and then declined. There was no significant difference in virus titer after infection with the different viruses, at least at the times measured and with these group sizes.

DISCUSSION

This paper provides a characterisation of the B7R gene of VV strain WR. Data presented show that the gene is transcribed late during infection and encodes an 18-kDa protein that is not present in virus particles nor the supernatant of infected cells but is located within the ER of infected cells. *In vitro* translation in the presence of microsomal membranes showed that B7R is present within the vesicle as a membrane-associated protein. A virus mutant lacking the majority of the B7R ORF showed normal replication in cell culture but was attenuated in a mouse dermal model of infection.

Expression of the B7R protein by *in vitro* transcription and translation showed that the protein was synthesised as a 21-kDa primary translation product, which, in the presence of microsomal membranes, was processed into an 18-kDa form that comigrated with the B7R polypeptide found in VV-infected cells. This reduction in

FIG. 5. Pre-embedding immunocytochemistry of infected cells showing the ER localisation of the B7R protein. BS-C-1 cells were infected at 5 PFU/cell with either vWTB7R (A, C, E, and F) or v Δ B7R (B and D) for 18 h. Cells were then fixed, permeabilised, and incubated with the rabbit α -B7R-2 antibody, followed by HRP-conjugated goat α -rabbit Ig and then DAB. Finally, the cells were processed for electron microscopy as described under Materials and Methods. The length of the scale bars represents 4000 nm in (A and B), 2000 nm in (C and D), 800 nm in (E), and 400 nm in (F; the inset scale bar is 100 nm). N, nucleus; NE, nuclear envelope; V, virus factory; ER, endoplasmic reticulum; PM, plasma membrane; M, mitochondria; and G, Golgi. The black arrow in (F) points to the area of the Golgi magnified in the inset.

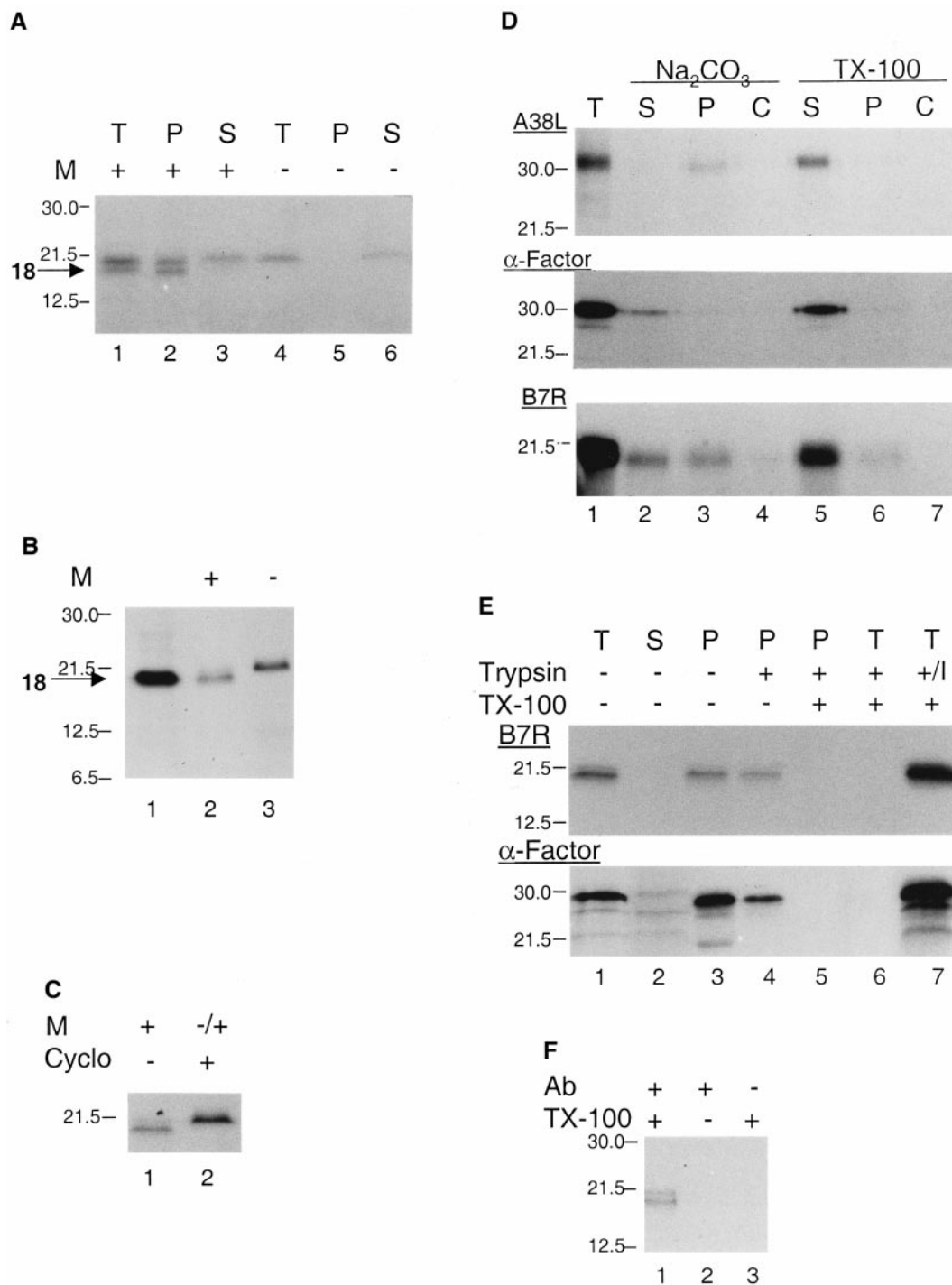


FIG. 6. *In vitro* translation of the B7R protein. (A) B7R protein was translated *in vitro* in the presence (lanes 1–3) or absence (lanes 4–6) of canine pancreatic microsomes. Soluble and membrane components of the total (T) *in vitro* translation reaction were separated into supernatant (S) and pellet (P) fractions by centrifugation. (B) Immunoprecipitation of the B7R protein from the *in vitro* translation reaction and from virus-infected cells. ³⁵S-met- and ³⁵S-cys-labeled lysates from cells infected with vWTB7R (lane 1) or the B7R protein translated *in vitro* in the presence (+) or absence (–) of microsomal membranes, were immunoprecipitated with the α -B7R-2 antibody. (C) Signal sequence cleavage occurs as a cotranslational event. B7R protein was translated *in vitro* in the presence (+) or absence (–) of microsomal membranes for 1 h. In the presence of 100 μ g/ml cycloheximide, microsomal membranes were then added to the sample that hitherto lacked membranes (–/+) and incubation was continued for a further 1 h. The proteins were then immunoprecipitated with the α -B7R-2 antibody. (D) Detergent and sodium carbonate (Na₂CO₃) extraction of the B7R protein. Yeast α -factor (middle) and VV proteins A38L (top) and B7R (bottom) were translated *in vitro* in the presence of microsomal membranes. Membranes from each translation reaction were then harvested by centrifugation (T) (lane 1). In each case, the membrane pellet was divided into two, and one half was treated with 0.1 M Na₂CO₃ pH 11 (lanes 2–4) and the other with 0.1% Triton X-100 (lanes 5–7). The samples were then floated over a sucrose cushion and centrifuged to separate the soluble (S) fraction (lanes 2 and 5) from the insoluble (P) fraction (lanes 3 and 6). The sucrose cushion (C)

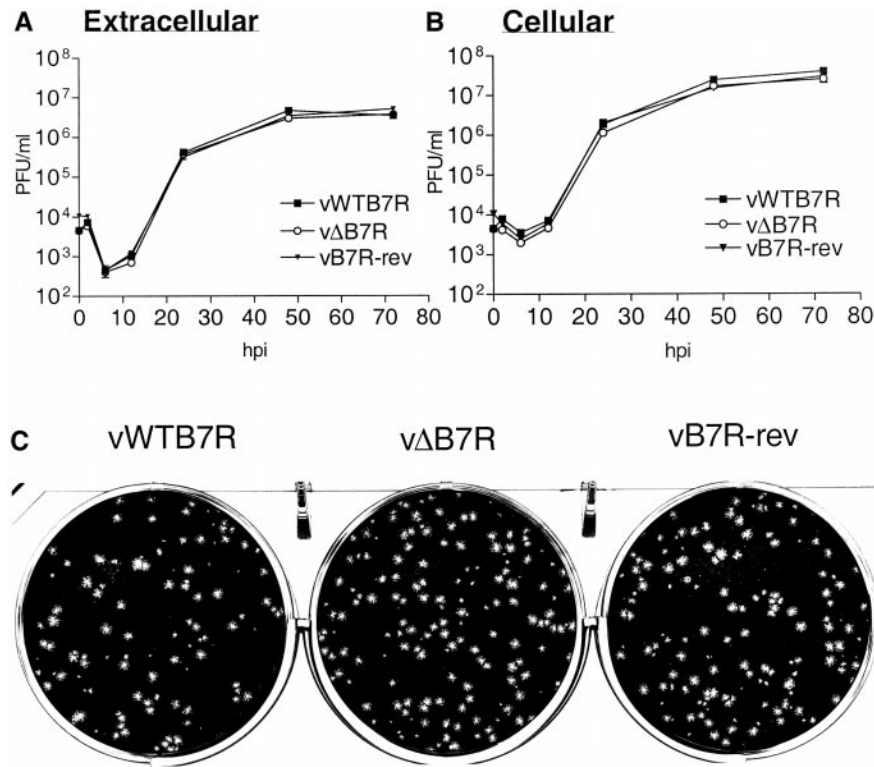


FIG. 7. Growth of vΔB7R in cell culture. (A and B) BS-C-1 cells were infected at 0.01 PFU/cell with vWTB7R (closed boxes), vΔB7R (open circles), or vB7R-rev (closed triangles). Samples of the culture supernatant and cells was removed at different times p.i. and their virus infectivity was titrated by plaque assay on BS-C-1 cells. The data shown are the means \pm SE from the duplicate samples. (C) Plaque phenotype of vΔB7R. BS-C-1 cells were infected with vWTB7R, vΔB7R, and vB7R-rev and overlaid with DMEM culture medium supplemented with 2.5% FBS and 1.5% carboxymethyl cellulose for 2 days prior to staining with 0.1% crystal violet in 15% ethanol.

size is likely to result from cleavage of the hydrophobic N-terminal signal peptide sequence because both the 18- and 21-kDa forms were recognised by an antibody directed against the C-terminal 10 amino acids. The 18-kDa form was shown to be present within the lumen of the vesicles because it was inaccessible to digestion by exogenous proteinase or recognition by specific antibody unless the membrane was disrupted by addition of detergent. The protein showed properties that were characteristic of a membrane-associated protein rather than an integral membrane protein. This suggests that the association of the B7R protein with membranes might be either direct, or via interaction with another (nonvirus) protein. The latter possibility is supported by

the observation that, although the B7R protein does not have any sites for addition of N-linked carbohydrate, in the presence of tunicamycin it was unstable and was not detected in infected cells by immunoblotting. Thus in infected cells, the B7R protein might interact with a cellular or virus glycoprotein that is unstable in the absence of N-linked carbohydrate.

The B7R protein synthesised in virus-infected cells was characterised with the aid of two anti-B7R antibodies. The first was raised against a GST-B7R fusion protein, and after affinity purification of the B7R-specific Ig, this reagent was suitable for immunoblotting analyses. These analyses showed that the protein was an intracellular, nonstructural protein of 18-kDa that was ex-

was also collected and analysed. (E) Trypsin treatment of membrane-associated B7R protein. Yeast α -factor (bottom) and B7R (top) were translated *in vitro* in the presence of microsomal membranes. The total fraction (T) was divided into three parts, one of which was incubated in the absence of either trypsin or Triton X-100 (lane 1), the second was incubated with both trypsin and Triton X-100 (lane 6), and the third was incubated with Triton X-100 and trypsin that had been inactivated by pre-treatment with trypsin inhibitor (lane 7). Control translation reactions, in the presence of microsomal membranes, were separated into supernatant (S) (lane 2) and pellet (P) (lanes 3–5) by centrifugation. The pellet was divided into three equal parts, one of which was incubated in the absence of either trypsin or Triton X-100 (lane 3), the second was incubated with trypsin but not Triton X-100 (lane 4), and a third was incubated with both trypsin and Triton X-100 (lane 5). (F) B7R associated with microsomes is not accessible to α -B7R-2 antibody. The B7R protein was translated *in vitro* in the presence of microsomes and the sample was incubated in the presence (lanes 1 and 3) or absence (lane 2) of Triton X-100. Immunoprecipitation was performed using the α -B7R-2 antibody (lanes 1 and 2) or no antibody (lane 3). All samples were resolved by SDS-PAGE and autoradiographs were prepared. The positions of molecular weight markers are indicated.

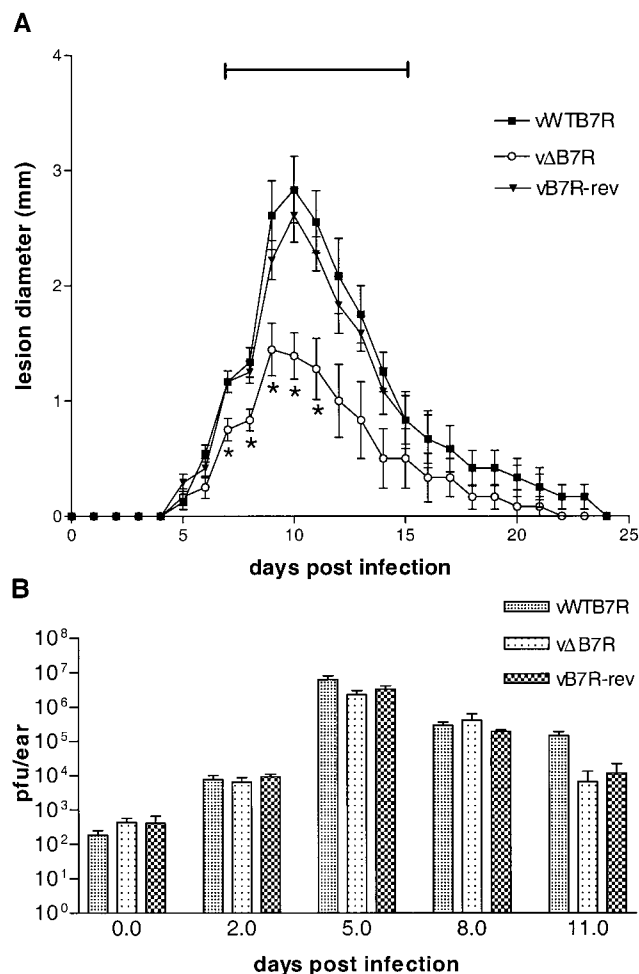


FIG. 8. Virulence of vΔB7R in mice. Lesion diameter (A) and virus titer (B) in groups of female BALB/c mice injected intradermally in the left ear pinna with 5×10^4 PFU of vWTB7R (closed boxes), vΔB7R (open circles), or vB7R-rev (closed triangles). (A) The data points represent the means \pm SE from groups of 12 mice up to day 8, 9 mice up to day 11, and 6 mice thereafter. The horizontal bar represents the comparison of pooled sample means between the vΔB7R group of mice and the vWTB7R or vB7R-rev group across days 7–15 (unpaired Student's *t* test, $P < 0.009$). The stars represent days on which a comparison of sample means of vΔB7R vs vWTB7R or vB7R-rev showed significant differences (Mann–Whitney *U* test, $P < 0.05$). (B) On the indicated days after infection, three animals infected with each virus were killed, and the titer of infectious virus in the ear was determined by plaque assay on duplicate BS-C-1 cells. Virus titers are expressed as PFU/ear. The bars represent the means \pm SE of the virus titers.

pressed by all 16 VV strains tested and by cowpox virus strains Brighton Red and elephantpox but not camelpox virus (data not shown). Published nucleotide sequence data show that the ORF is disrupted in variola major viruses (Shchelkunov *et al.*, 1995). Nonetheless, the conservation in all VV strains and in cowpox virus and the presence of related ORFs in leporipoxviruses suggests an important function for the protein.

The second antibody was raised against a 10-amino-acid synthetic peptide from the C terminus of the protein

and was used for immunoprecipitation, immunofluorescence, and immunocytochemistry. Immunofluorescence showed that the B7R protein had a reticular distribution and colocalised with PDI in the ER. Immunocytochemistry confirmed the presence of B7R in the ER and revealed that it was absent from virus particles, virus factories, the Golgi complex, and plasma membrane.

Proteins are retained in the ER because they contain an ER retention or retrieval sequence or they bind to other proteins that contain these signals (Gething *et al.*, 1986; Sato *et al.*, 1996). Several signals cause proteins to accumulate in the ER: (i) KDEL, or variants of this, at the C terminus of soluble proteins (Munro and Pelham, 1987; Andres *et al.*, 1990); (ii) RR near the cytoplasmic N terminus of type 2 membrane proteins (Schutze *et al.*, 1994); (iii) K at position -3 and -4 or -5 of the C terminus of type 1 membrane proteins (Jackson *et al.*, 1990); (iv) transmembrane domains that rely on the protein sequence for retrieval (Sato *et al.*, 1996; Rayner and Pelham, 1997); and lastly (v) the transmembrane domain of hepatitis C virus E1 and E2 proteins is responsible for retention in the ER without recycling through the Golgi (Duvet *et al.*, 1998).

The mechanism by which B7R is retained in the ER is unknown. Although the N terminus contains a KK motif and a hydrophobic sequence, these are likely to be removed by proteolytic cleavage, and the protein does not behave as an integral membrane protein. There is a $-NNEL$ (a modification of KDEL) motif at amino acid positions 49–52, but this sequence is not known to retain proteins in the ER. So the retention of B7R in the ER might be due to either an unknown retention signal or via the interaction with another protein(s). The former possibility can be assessed by a mutational analysis of B7R.

To address the role of B7R in virus infection, a recombinant virus lacking 60% of the ORF was constructed and compared with wild-type and revertant viruses. The isolation of the deletion mutant indicated that the B7R gene was nonessential for virus replication in cell culture and analyses of the growth properties and plaque phenotype showed no differences from wild type. However, in a murine intradermal model, the B7R protein affected the outcome of infection and the deletion mutant induced smaller lesions than control viruses. The mechanism by which an intracellular protein resident in the ER affects virus virulence *in vivo*, without affecting virus replication *per se*, is unknown and interesting. One possibility is that the interaction of B7R with another protein prevents the secretion or cell surface expression of protein(s) important for the immune response to infection. Alternatively, the B7R protein might affect apoptosis of infected cells. In the leporipoxviruses, there is a precedent for an ER protein playing a role in pathogenesis where the T4 protein of myxoma virus inhibits apoptosis of infected cells (Barry *et al.*, 1997). However, preliminary experiments comparing the VV B7R deletion mutant with wild-

type and revertant viruses found no alteration in apoptosis in Jurkat cells (data not shown).

In summary, the B7R gene encodes an 18-kDa protein that is retained in the ER by either a novel retention signal or by interaction with another ER-resident protein. The loss of the B7R gene results in virus attenuation *in vivo* by an unknown mechanism.

MATERIALS AND METHODS

Cells and viruses

Monkey kidney BS-C-1 and CV-1 cells, rabbit kidney (RK)₁₃ cells, human osteosarcoma TK⁻143B cells and HeLa D980R cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco). VV strain WR and recombinants derived from it were grown in RK₁₃ cells. Working stocks of virus were prepared by sedimentation of cytoplasmic extracts of cells through a sucrose cushion as described (Mackett *et al.*, 1985). Virus infectivity was titrated by plaque assay on BS-C-1 cells (Mackett *et al.*, 1985). Purified IMV and EEV were prepared from RK₁₃ cells 2 days p.i. at 0.1 PFU/cell as described (Parkinson and Smith, 1994).

Plasmid constructions

Plasmid pNP1 was used to express a glutathione-S-transferase (GST)-B7R fusion protein in *E. coli*. The B7R ORF, lacking the N-terminal hydrophobic sequence (20 amino acids), was amplified by polymerase chain reaction (PCR) from pSTH1 DNA (Howard *et al.*, 1991) to produce a fragment of 506 bp. The forward oligonucleotide 5'-TATCGGATCCAATAATGAGTACACTCCG-3' contains a *Bam*HI site (underlined) and starts 60 bp downstream of the B7R initiation codon, whereas the reverse oligonucleotide 5'-GAGCGAATTC**TTAAAAATCATATTTGA**-3' has an *Eco*RI site (underlined) and the termination codon (complement of) is shown in bold. The PCR fragment was digested with *Bam*HI and *Eco*RI and then was ligated into the *Bam*HI- and *Eco*RI-cut pGEX-2T expression vector (Smith and Johnson, 1988) to form pNP1. The nucleotide sequence of the cloned PCR fragment was verified by DNA sequencing.

Plasmid pNP3 was used as the template for *in vitro* transcription and also to create the single-stranded (ss) RNA probe for use in Northern blotting. Plasmid pSTH1, containing the full B7R ORF, was digested with *Spe*I and then made blunt-ended by treatment with Klenow enzyme in the presence of dNTPs. A 685-bp DNA fragment containing the B7R ORF was ligated into the plasmid pGEM-4Z (Promega) that had been digested with *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase (CIP), to form pNP3. The orientation of the B7R gene in the vector was established by digestion with *Pst*I and agarose gel electrophoresis. This showed the B7R ORF was under the control of the T7 promoter.

Plasmid pNP5 containing the full B7R ORF and flanking sequences was constructed to make the B7R revertant virus by transient dominant selection (TDS) (Falkner and Moss, 1990). An *Eco*RV 1564-bp fragment, containing the entire B7R gene and 535 bp upstream and 480 bp downstream, was excised from pSTH1. This was cloned into pSJH7 (Hughes *et al.*, 1991), which had been digested with *Pst*I, made blunt-ended by treatment with T4 DNA polymerase, and dephosphorylated with CIP, to form plasmid pNP5.

Plasmid pNP6 was used to make the B7R deletion virus by TDS. Plasmid pNP5 was digested with *Bst*BI and *Pst*I to excise a 344-bp fragment from the B7R gene (nucleotides 56–400, 60% of the ORF). The remaining plasmid was made blunt-ended by treatment with T4 DNA polymerase in the presence of dNTPs and religated to form plasmid pNP6.

Construction of a B7R deletion mutant and revertant virus

A virus deletion mutant lacking 60% of the B7R ORF was constructed by TDS (Falkner and Moss, 1990) using the *Ecogpt* gene as the transient selectable marker (Boyle and Coupar, 1988; Falkner and Moss, 1988) as described (Kettle *et al.*, 1995). CV-1 cells were infected with VV strain WR at 0.05 PFU/cell and transfected with pNP6. MPA-resistant plaques were isolated, plaque purified once more, and used to infect the *hprt*⁻ HeLa cell line D980R (Kerr and Smith, 1991) in the presence of 1 µg/ml 6-thioguanine (6-TG) (Isaacs *et al.*, 1990). This selected against *Ecogpt* expression and produced both a wild-type virus (WVB7R) and a deletion B7R virus (vΔB7R).

The revertant virus was constructed using TDS as described above. CV-1 cells were infected with vΔB7R at 0.05 PFU/cell and transfected with pNP5 DNA. The MPA-resistant intermediate plaque was grown on D980R cells with 6-TG to form a revertant virus (vB7R-rev) in which the B7R locus had been restored to that of wild-type virus.

Production and purification of polyclonal antisera

Two polyclonal rabbit antisera to the B7R protein were produced. The first, called α-B7R-1 Ig, was raised against B7R amino acids 20–182 (lacking the hydrophobic N terminus) expressed from plasmid pNP1 in *E. coli* strain TG1 as a GST-B7R fusion protein (Smith and Johnson, 1988), and the antiserum produced was affinity purified. New Zealand white rabbits were immunised subcutaneously with ~375 µg of the GST-B7R fusion protein in complete Freund's adjuvant, followed by similar injections in incomplete Freund's adjuvant at 3- and then 6-week intervals. Serum samples were collected 10–14 days after each boost. The immunoglobulin (Ig) fraction of anti-B7R serum was purified over a protein-A-Sepharose column (Sigma) and eluted at pH 3 (Harlow and

Lane, 1988). This fraction was then affinity purified further. First, the Ig was passed through a column of GST coupled to glutathione-agarose (Smith and Johnson, 1988). After addition of the Ig fraction to this column, the resulting flow-through was collected and applied to a GST-B7R-glutathione-agarose column. Bound Ig was eluted at pH 3 to produce α -B7R-1 Ig.

The second antibody was called α -B7R-2 and was raised against synthetic peptide SNTYYSKYDF (B7R amino acids 173–182) coupled to keyhole limpet hemocyanin by subcutaneous injection of 500 μ g of antigen in complete Freund's adjuvant. This same amount of antigen, in incomplete Freund's adjuvant, was used in booster doses at 2- and then 4-week intervals. Serum samples were taken 10–14 days after each boost. Prior to the use of this antibody in immunofluorescence, the antiserum was adsorbed against BS-C-1 cells that were infected at 5PFU/cell with v Δ B7R for 12 h.

Northern blot analysis

RNA was extracted from RK₁₃ cells that had been mock-infected or infected with VV WR at 10 PFU/cell in the presence or absence of 100 μ g/ml cycloheximide (Cyclo) or 40 μ g/ml cytosine β -D-arabinofuranoside (araC) and prepared according to established protocols (Ausubel *et al.*, 1990). Samples of the RNA (5 μ g) were denatured in 0.5 \times MOPS buffer containing 50% deionised formamide and 6.5% formaldehyde at 65°C for 10 min. After electrophoresis through a 1.2% agarose gel and transfer to a nylon Hybond N+ membrane (Parkinson and Smith, 1994), the membrane was hybridised with a ³²P-labeled, ssRNA probe. The anti-sense (complementary) ssRNA probe was produced from the *Hind*III-linearised vector pNP3 by the use of an *in vitro* transcription kit (Promega Riboprobe) in the presence of SP6 RNA polymerase.

Immunoblotting

Cells were infected with vWTB7R at 10 PFU/cell or mock-infected for 1 h and then incubated in the presence or absence of 40 μ g/ml araC, 10 μ g/ml tunicamycin, or 1 μ M monensin for 24 h in DMEM with 2.5% FBS. Extracts of infected cells were prepared and analysed by immunoblotting as described (Parkinson and Smith, 1994). Blots were incubated with rabbit α -B7R-1 Ig (diluted 1:100) in PBST-5% overnight at 4°C. The rabbit polyclonal antisera against VV proteins B5R (Engelstad *et al.*, 1992) or B1R (Banham and Smith, 1992) or the murine MAb AB1.1 against VV protein D8L (Parkinson and Smith, 1994) were diluted at 1:2500 in PBST-5%, and membranes were incubated with these antibodies for 1.5 h at RT where indicated. Following washes in PBST, bound Ig was detected by incubation with a 1:2000 dilution of a horse-

radish peroxidase (HRP)-conjugated goat α -rabbit or α -mouse Ig (Sigma) in PBST-5% for 1 h at RT. After washing in PBST, immune complexes were detected using the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's protocol.

For analysis of virion proteins, 25 μ g of IMV or EEV (purified as described above), or extracts from vWTB7R-infected cells harvested at 24 h p.i., were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted as above.

To analyse proteins in the supernatant of infected cells, BS-C-1 cell monolayers were mock-infected or infected with vWTB7R, v Δ B7R, or vB7R-rev at 2 PFU/cell. After 1 h, the unbound virus was removed and the cells were washed with PBS and incubated in DMEM. Cellular and supernatant fractions were harvested at 24 h p.i. The supernatant was centrifuged at low speed (800 *g*, 4°C, 5 min in a Beckman GPR centrifuge, GH 3.7 rotor) to remove detached cells and then at high speed (17,500 *g*, 4°C, 2 h in a Beckman L8-M ultracentrifuge, SW 41 rotor) to remove EEV particles. Proteins in the clarified supernatant were precipitated by 10% trichloroacetic acid (TCA), redissolved in protein loading buffer, analysed by SDS-PAGE alongside extracts from infected cells, transferred to nitrocellulose membranes, and immunoblotted as above.

Immunofluorescence

BS-C-1 cells were grown on glass coverslips and were infected with vWTB7R or v Δ B7R at 2 PFU/cell. At 12 h p.i. the cells were washed three times in ice-cold PBS and then were fixed at 4°C with freshly prepared 4% (w/v) and then 8% PFA in 250 mM HEPES, pH 7.4 for 10 and 50 min, respectively. The cells were washed in PBS, blocked with 50 mM ammonium chloride for 10 min at RT, and permeabilised with 0.2% Triton X-100 in PBS for 5 min at RT. The cells were washed three times with 10% FBS/PBS and blocked with this solution for 1 h at RT. For double labeling, the rabbit polyclonal α -B7R-2 antibody and mouse MAb 1D3 against PDI (Vaux *et al.*, 1990) were diluted (1:100 or 1:50, respectively) in 10% FBS/PBS and added to cells for 45 min at RT. Cells were rinsed again with 10% FBS/PBS prior to incubation for 30 min at RT with rhodamine-conjugated goat α -rabbit IgG or FITC-conjugated goat α -mouse IgG (both Sigma) at a 1:200 dilution in 10% FBS/PBS. The cells were rinsed three times in PBS and once with H₂O, and the coverslips were mounted in Mowiol (Hoechst). Samples were analysed using a Bio-Rad MCR 1024 laser scanning confocal microscope and images were collected either with COMOS-7 or with Lasersharp-4 and processed with Adobe Photoshop TM software.

Pre-embedding immunocytochemistry

BS-C-1 cells were grown on glass coverslips and infected with vWTB7R or v Δ B7R at 5 PFU/cell. At 18 h p.i. the cells were washed three times in ice-cold PBS and fixed at 4°C with freshly prepared 4% (w/v) and then 8% PFA in 250 mM HEPES, pH 7.4, for 10 and 50 min, respectively. The cells were washed in PBS, blocked with 50 mM ammonium chloride for 10 min at RT and then permeabilised with 0.1% saponin in 10% FBS/PBS for 30 min at RT. The α -B7R-2 antibody was diluted 1:100 in 0.1% saponin/10% FBS/PBS and added to the cells for 2 h at RT. Cells were rinsed three times with 0.1% saponin/10% FBS/PBS prior to incubation with a HRP-conjugated goat α -rabbit Ig (Sigma) at a dilution of 1:50 in 0.1% saponin/10% FBS/PBS for 2 h at RT. The cells were left overnight at 4°C prior to incubation in diaminobenzidine (DAB) for 2 min, and in DAB/H₂O₂/200 mM sodium cacodylate pH 7.4 for 30 min. The reaction was stopped with 200 mM sodium cacodylate pH 7.4, and the sample was postfixed in 1% osmium tetroxide/1.5% potassium ferrocyanide for 60 min at RT. After being washed in water and then incubated in 0.5% magnesium uranyl acetate overnight at 4°C, the cells were washed again in water and then dehydrated in ethanol and flat embedded in Epon. Sections of 80–150 nm were cut parallel to the surface of the coverslip and stained for 30 s in lead citrate as a contrast agent. Sections were examined in a Zeiss Omega EM 912 electron microscope (LEO Electron Microscope Ltd.), equipped with a Proscan cooled, CCD camera (1024 × 1024 pixels). Digital images were captured with the integrated Soft Imaging Software (SIS) image package (SIS Imaging Software, GmbH, Munster, Germany) and were processed using Adobe Photoshop TM software.

In vitro transcription and translation

For *in vitro* transcription, the designated gene was cloned into the pGEM-3Z or pGEM-4Z vector (Promega) under control of the T7 promoter. Plasmid pNP3 contains the B7R gene in pGEM-4Z, and the VV A38L gene was cloned into pGEM3Z (Parkinson *et al.*, 1995). These plasmids were linearised downstream of the coding region by digestion with restriction endonucleases *Eco*RI and *Hind*III, respectively, and transcription and translation reactions were performed using Promega riboprobe and reticulocyte lysate kits (as directed by the manufacturer). Canine pancreatic microsomes were also obtained from Promega.

The B7R mRNA was translated *in vitro* in the presence or absence of microsomes for 2 h at 30°C, and then soluble or membrane components were separated by centrifugation at 13,000 *g* at 4°C for 45 min. The membrane sample was dissolved in reticulocyte lysate buffer, and all samples were then lysed in protein loading buffer (Laemmli, 1970), boiled at 100°C for

5 min, and resolved by SDS-PAGE (all gels were 15% polyacrylamide).

To determine whether proteins were co- or posttranslationally associated with microsomes, *in vitro* translation was performed in the presence or absence of microsomes for 1 h, and then microsomes were added to the sample hitherto lacking microsomes, in the presence of 100 μ g/ml cycloheximide. The incubation was continued for 1 h at 30°C, after which labeled B7R protein was immunoprecipitated using 10 μ l of α -B7R-2 antibody as described below. Samples were then analysed by SDS-PAGE.

Immunoprecipitation

B7R protein synthesised in vitro. Following *in vitro* translation in the presence or absence of membranes, samples were mixed with 1 ml of RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 1% SDS, and 50 mM Tris-HCl, pH 7.5), vortexed, and incubated on ice for 10 min prior to homogenisation in a 1-ml syringe with a 26-gauge needle. Insoluble material was removed by centrifugation at 13,000 *g* for 10 min, and the soluble fraction was then added to a further 4 ml of RIPA buffer together with 10 μ l of α -B7R-2 antibody for 12 h at 4°C. Alternatively, B7R protein was translated *in vitro* in the presence of microsomes and the reaction mix was incubated in the presence or absence of 0.1% Triton X-100 in 25 mM CaCl₂ and 50 mM Tris-HCl, pH 8. Immunoprecipitation was performed using the α -B7R-2 antibody or no antibody in the 25 mM CaCl₂, 50 mM Tris-HCl, pH 8, for 12 h at 4°C. For all immunoprecipitations 7 mg of protein-A-Sepharose beads were added for 3 h at 4°C. The samples were washed 3 times in RIPA buffer or in 25 mM CaCl₂ and 50 mM Tris-HCl, pH 8 (as appropriate), resuspended in protein loading buffer, boiled at 100°C for 5 min and resolved by SDS-PAGE.

B7R protein synthesised in infected cells. TK⁻143B cells were infected with vWTB7R at 10 PFU/cell for 1 h. Unbound virus was removed and the cells were incubated in DMEM with 2.5% FBS. At 8 h p.i. the cells were starved for 30 min in medium lacking methionine and cysteine and then radio-labeled with 50 μ Ci of Promix ([³⁵S]met and [³⁵S]cys) (Amersham) and 50 μ Ci of L-[³⁵S]cysteine (NEN Life Science Products) for 1 h. Cells were washed three times in PBS and harvested in 1 ml of RIPA buffer. Samples were then mixed, homogenised, and centrifuged as for proteins synthesised *in vitro* (see above). Each soluble fraction was incubated with 10 μ l of preimmune rabbit serum for 3 h at 4°C, followed by protein-A-sepharose for 3 h at 4°C, and immune complexes were removed by centrifugation. The B7R protein was then immunoprecipitated with α -B7R-2 antibody followed by protein-A-sepharose as described above.

Extraction of proteins with Na₂CO₃ or Triton X-100

After *in vitro* translation of yeast α -factor, or VV proteins B7R or A38L in the presence of microsomes, membranes and soluble fractions were separated by centrifugation at 13,000 *g* at 4°C for 45 min. The membrane pellet was resuspended in PBS, divided into two, and one half was treated with 0.1 M Na₂CO₃, pH 11, and the other half treated with 0.1% Triton X-100 each for 30 min at 4°C. The reaction mixture was then floated over a 10% (w/v) sucrose cushion and centrifuged at 13,000 *g*, 4°C, 45 min to separate the soluble and insoluble fractions.

Treatment of microsomes with trypsin

Yeast α -factor and VV B7R protein were translated in the presence of microsomes *in vitro*, and each sample was divided into three parts. One was incubated without either trypsin or Triton X-100, the second was incubated with both 0.1 mg/ml trypsin and 0.1% Triton X-100, and the third was incubated with 0.1% Triton X-100 and 0.1 mg/ml trypsin that had been inactivated by pretreatment with 1 mg/ml trypsin inhibitor (Sigma). Incubations were continued for 45 min at 37°C. Parallel translation reactions, in the presence of microsomal membranes, were separated into soluble and pellet fractions by centrifugation at 13,000 *g*, 4°C for 45 min. The membrane pellet was divided into three equal parts, one of which was incubated without either trypsin or Triton X-100, the second was incubated with 0.1 mg/ml trypsin but not Triton X-100, and a third was incubated with both 0.1 mg/ml trypsin and 0.1% Triton X-100.

Virus growth curves

BS-C-1 cells were infected at 0.01 PFU/cell in DMEM with 2.5% FBS for 1 h, washed with PBS to remove unbound virus and then incubated in DMEM with 2.5% FBS. At various times postinfection, the culture supernatant was removed and centrifuged at 800 *g* at 4°C for 5 min to pellet-detached cells, and the supernatant retained as the EEV fraction. Cells were washed with PBS, scraped into DMEM with 2.5% FBS, added to the pelleted cells from above, freeze-thawed three times, and sonicated for 15 s to produce the cell-associated virus sample. Virus titers were determined by plaque assay on BS-C-1 cells.

Virulence assays in mice

Groups of 5 BALB/c mice were inoculated intranasally under general anaesthesia with either 10⁴, 10⁵, or 10⁶ PFU of vWTB7R, v Δ B7R, or vB7R-rev in 25 μ l PBS. Mice were weighed daily before and after infection, and the mean weights for each group of animals were calculated and compared with the mean weight of the same group of animals on day 0. Animals that had lost 30% of their body weight were killed by cervical dislocation.

Female BALB/c mice, 8 weeks old, were anaesthetised and injected intradermally in the left ear pinnae with 5 \times 10⁴ PFU of virus diluted in 10 μ l of PBS. The diameter of lesions produced on infected ears was estimated daily to the nearest 0.5 mm using a micrometer. The titer of infectious virus in ears at various times after inoculation was determined as described (Tscharke and Smith, 1999). Aliquots of the diluted viruses that were used for infection of mice were titrated on BS-C-1 cells to confirm the dose administered.

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