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The vaccinia virus B9R protein is a 6 kDa intracellular protein that is non-essential for virus replication and virulence

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Vaccinia virus (VV) strain Western Reserve gene B9R is shown to encode an intracellular 6 kDa protein that is expressed late during the infectious cycle. *In vitro* transcription and translation produced two polypeptides in the presence of microsomal membranes, but only the larger protein in the absence of membranes. The smaller protein sedimented with microsomes during centrifugation, suggesting it was inserted into the lipid membrane or into the microsomal lumen via the N-terminal hydrophobic signal sequence that was subsequently cleaved proteolytically. A VV mutant lacking B9R was constructed and found to replicate normally in cell culture and two *in vivo* models.

Vaccinia virus (VV) is the prototype orthopoxvirus and replicates in the cytoplasm (Moss, 1996). It has a dsDNA genome of approximately 200 kb that has been sequenced for strains Copenhagen (Goebel *et al.*, 1990), Tian Tan (accession no. AF095689), modified vaccinia virus Ankara (MVA) (Antoine *et al.*, 1998) and most of Western Reserve (WR) (Smith *et al.*, 1991, and references therein). Genes within the central region are highly conserved between VV strains and other orthopoxviruses, whereas genes near the termini are more variable and often are non-essential for virus replication in cell culture (Perkus *et al.*, 1990). Proteins encoded in these regions affect host range, virulence and immunomodulation (Smith *et al.*, 1997).

The right end of the VV genome encodes many proteins that affect virus virulence or act as immunomodulators. For instance, VV protein B7R is an intracellular protein that is retained in the endoplasmic reticulum and affects virus virulence (Price *et al.*, 2000), VV B8R is a secreted protein that binds interferon-γ (Alcamí & Smith, 1995), B13R is an intracellular inhibitor of caspase 1 and apoptosis (Kettle *et al.*, 1997), and VV B15R is a secreted protein that binds interleukin-1β (Alcamí & Smith, 1992). To continue our characterization of genes from this region that encode proteins with hydrophobic amino acid sequences suggesting membrane-association or secretion, we have studied the B9R gene.

VV WR gene B9R is predicted to encode a 77 amino acid primary translation product of 8·8 kDa (Smith *et al.*, 1991) that is conserved in VV strains Copenhagen and Tian Tan (there is a single amino acid substitution, Q21R, between WR and Copenhagen), and is 5 amino acids shorter in strain MVA due to deletion of amino acids 61–65. The WR protein has a hydrophobic N terminus, which is predicted to function as a signal peptide (Howard *et al.*, 1991). B9R has amino acid similarity to the N-terminal region of larger proteins in leporipoxviruses (Shope fibroma virus, myxoma virus and rabbit fibroma virus) (Upton *et al.*, 1987), two strains of capripoxvirus (Gershon & Black, 1989) and cowpox virus GRI-90 (Shchelkunov *et al.*, 1998), but in variola virus strains Harvey-1947 (Aguado *et al.*, 1992), Bangladesh-1975 (Massung *et al.*, 1994) and India-1970 (Shchelkunov *et al.*, 1994) the gene is disrupted into smaller fragments. The related 240 amino acid protein from myxoma virus (called M-T4) is located in the endoplasmic reticulum and affects virus virulence (Barry *et al.*, 1997).

To identify the B9R protein in VV-infected cells, amino acids 20–77 (excluding the hydrophobic N terminus) were expressed in *E. coli* as a glutathione *S*-transferase fusion protein, purified and used to immunize New Zealand White rabbits as described for protein B7R (Price *et al.*, 2000). An Ig fraction was obtained from the serum and was affinity purified on a B9R–GST affinity column. This antibody was used in immunoblotting. Fig. 1*a* shows that a 6 kDa protein was synthesized in cells infected with a virus containing the B9R ORF but not in cells infected with a B9R deletion mutant (vΔB9R) (see below) or mock-infected cells. The protein was not detected in the supernatant of infected cells, suggesting that only low levels, if any, of the B9R protein are released from cells (data not

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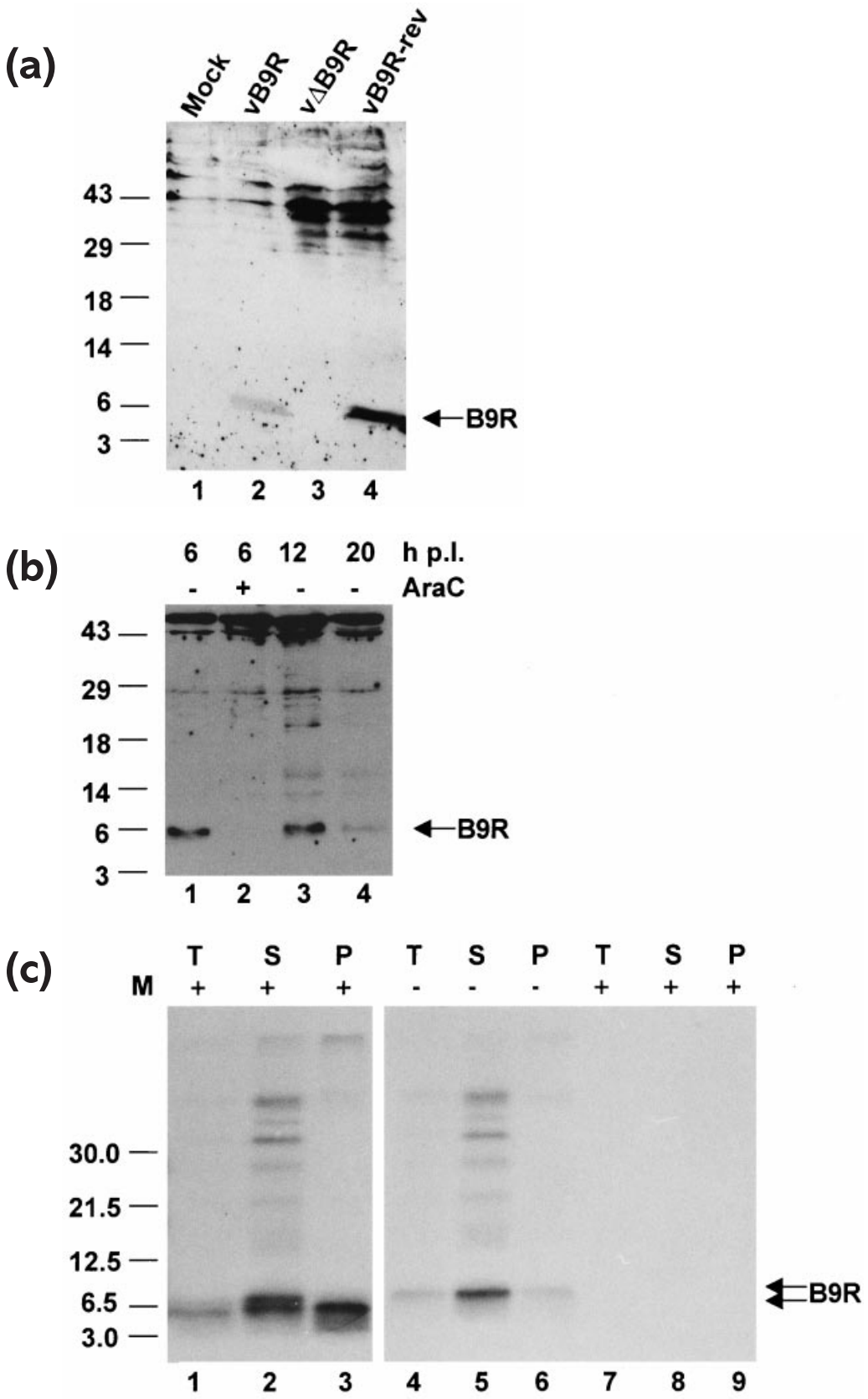

Fig. 1. For legend see facing page.

shown). This demonstrated that the protein was a 6 kDa intracellular protein.

The time of expression of B9R during the virus life-cycle was investigated. Cells were infected in the presence or absence of cytosine arabinoside (AraC) and cell extracts were immunoblotted with the anti-B9R IgG (Fig. 1b). The protein was made only in the absence of AraC and therefore is expressed late during the infectious cycle, after DNA replication has commenced. Northern blotting of RNA from infected cells detected B9R-specific transcripts of heterogeneous length that were found late during infection and which were not present after infection in the presence of cycloheximide or AraC (data not shown).

The presence of an N-terminal hydrophobic signal peptide suggested that the protein might be associated with membranes. Attempts to analyse this within infected cells were unsuccessful and therefore we used *in vitro* transcription and translation in the presence or absence of microsomal membranes. Plasmid pSTH1 (Howard, 1991) was digested with *Hpa*I and a 356 bp fragment containing the B9R ORF and downstream sequences was cloned into plasmid pGEM-4Z at the *Sma*I site downstream of the SP6 RNA polymerase promoter, forming plasmid pNP4. After transcription and translation (Price *et al*., 2000), B9R proteins were immunoprecipitated with anti-B9R Ig and analysed by SDS–PAGE and autoradiography (Fig. 1c). A protein of approximately 8 kDa was detected when the mRNA was translated in the absence of membranes (lanes 4–6), but in the presence of membranes (lanes 1–3) an additional slightly smaller protein (6 kDa) was detected. To determine if either of these proteins was associated with membranes, the translation products were fractionated by centrifugation into soluble (S) and pelleted (P) fractions, the latter containing the microsomes. Only the smaller protein was associated with membranes. This analysis did not determine whether the smaller protein was inserted into the vesicle lumen or whether it was an integral membrane protein. However, we suggest the former is more probable because the reduction in size of the translation product associated with membranes is consistent with the protein being cleaved proteolytically to remove the N-terminal signal peptide.

The role of the B9R protein in the virus life-cycle was investigated by the construction of a deletion mutant that lacked amino acids 1–29 (vΔB9R). The remaining part of the gene was retained as it overlapped the putative promoter region of gene B10R; however, amino acids 30–77 would not be expressed because of the lack of an in-frame methionine codon (Smith *et al*., 1991). The deletion virus was constructed by transient dominant selection using established methods (Falkner & Moss, 1990). The transfer plasmid (pNP7) was constructed by PCR amplification of the flanking regions of the B9R gene using pSTH1 DNA as template and oligonucleotides 5′ CCCGAATTCGTGTGCGTGACAGCACAGGGAGCC 3′ (*Eco*RI site underlined) and 5′ CTATTTTCAATCCCCCTTGAAAATGGTTAGAG 3′ for the upstream flanking region and 5′ CCATTTTCAAGGGGGATTGAAAATAGGG 3′ and 5′ CCCGGATCCCGGTAACGGATAGACCTCCGGC 3′ (*Bam*HI site underlined) for the downstream flanking region. The fragments were amplified separately, and joined together by splicing by overlap extension (Horton *et al*., 1989) into a single fragment that was digested with *Eco*RI and *Bam*HI and ligated into pSJH7 (Hughes *et al*., 1991). This plasmid contains the *E. coli* xanthine guanine phosphoribosyltransferase (Ecogpt) gene under the control of the VV 7.5K promoter. pNP7 was transfected into cells infected with VV strain WR and a mycophenolic acid-resistant intermediate virus isolate was plaque-purified on BS-C-1 cells in the presence of mycophenolic acid, xanthine and hypoxanthine (Falkner & Moss, 1988). This was then plaque-purified on HeLa D980R cells in the presence of 6-thioguanine (Isaacs *et al*., 1990; Kerr & Smith, 1991) to resolve the intermediate virus into deletion mutant (vΔB9R) and plaque-purified wild-type virus (vB9R). A revertant virus was constructed by reinsertion of the B9R gene at its natural locus. A 908 bp *Eco*RV fragment containing the entire B9R gene and flanking sequences was excised from plasmid pSTH1 and cloned into pSJH7 at the *Sma*I site forming pNP8. This plasmid was transfected into cells infected with vΔB9R and a revertant virus was isolated by transient dominant selection (as above) and called vB9R-rev.

The genomic structure of the recombinant viruses was analysed by Southern blotting and PCR. Virus DNA was extracted from purified intracellular mature virus and

Fig. 1. Characterization of the B9R protein. (a and b) Immunoblots. Human TK$^-$143 cells were infected at 10 p.f.u. per cell with the indicated viruses and at 6 h post-infection (p.i.), cell extracts were prepared and proteins were analysed by SDS–PAGE (15% gel) and immunoblotting. After transfer to nitrocellulose filters (Towbin *et al*., 1979), the B9R protein was detected by incubation with anti-B9R Ig (diluted 1:100) followed by horseradish peroxidase-conjugated goat anti-rabbit Ig (diluted 1:2000) and chemiluminescence reagents as described by the manufacturer (Amersham). Molecular size markers are indicated in kDa. (b) The B9R protein is expressed late during infection. Cells were infected with VV WR as in (a) and harvested at 6 h p.i. in the presence (lane 2) or absence (lane 1) of 40 μg/ml AraC, or at 12 h (lane 3) or 20 h p.i. (lane 4) in the absence of AraC. Samples were processed as in (a). (c) *In vitro* translation of B9R. The B9R gene was transcribed *in vitro* using plasmid pNP4 as template and the transcripts were then translated *in vitro* in the presence (lanes 1–3) or absence (lanes 4–6) of canine pancreatic microsomes. Alternatively, parallel reactions were performed with the reticulocyte lysate in the absence of exogenous RNA (lanes 7–9). An aliquot of the reaction sample was retained (T) prior to separation of the reaction products into soluble (S) and membrane pellet (P) fractions by centrifugation (13000 r.p.m., 4 °C, 45 min). The samples were then immunoprecipitated with anti-B9R Ig and proteins were resolved by SDS–PAGE (15% gel) after which an autoradiograph was prepared. The positions of size markers are indicated in kDa and the B9R proteins by arrows.

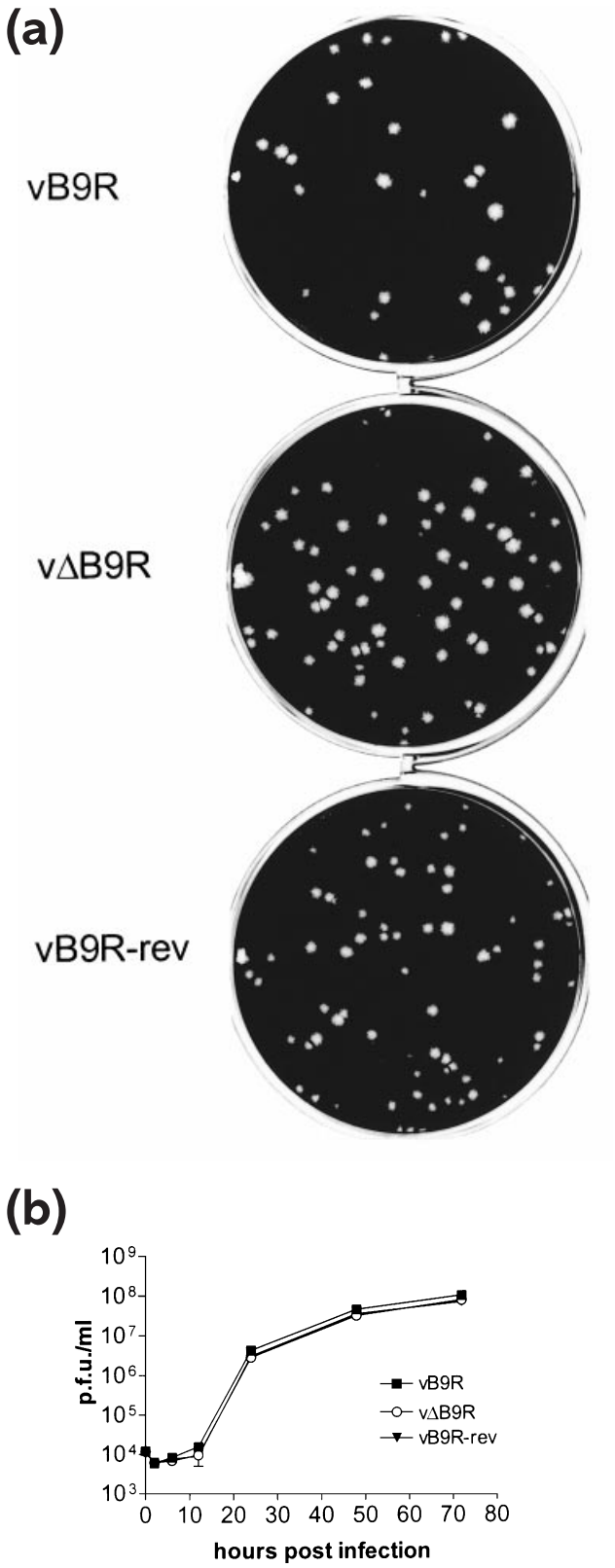

Fig. 2. Growth properties of vΔB9R in cell culture. (a) Plaque formation. RK_{13} cells were infected with the indicated viruses, overlaid with DMEM containing 2·5% foetal bovine serum and 1·5% carboxymethyl cellulose and stained 2 days later with 0·1% crystal violet in 15% ethanol. (b) Multi-step growth curve. BS-C-1 cells were infected with the indicated viruses at 0·01 p.f.u. per cell and at the indicated times p.i. the cells were scraped into the culture medium, freeze–thawed three times, sonicated and the infectivity of the released virus was titrated by plaque assay on monolayers of BS-C-1 cells.

was digested with *Hpa*I, separated by electrophoresis on an agarose gel and blotted onto nitrocellulose membranes. The membranes were probed with a fluorescein-labelled 908 bp DNA fragment representing the 231 bp B9R ORF and 416 and 261 bp upstream and downstream, respectively. This probe detected a 360 bp fragment (representing the wild-type B9R allele) with WT and vB9R-rev, but a 270 bp fragment with vΔB9R (data not shown). This probe also detected other DNA fragments, which flank the B9R locus, in all viruses. PCR analysis with oligonucleotide primers that flanked the B9R gene also showed the presence of the 90 bp deletion in vΔB9R compared to WT and revertant viruses (data not shown). Furthermore, additional PCR analysis showed that the genes flanking B9R were unaltered. Collectively, these data indicated that the viruses had the predicted genome structures.

The isolation of the deletion mutant indicated that the B9R gene was non-essential for virus replication in cell culture. However, to investigate if the growth of the mutant virus was altered, the plaque size was compared with wild-type and revertant viruses in cell culture. The plaque size of vΔB9R on RK_{13} cells was unaltered compared to control viruses (Fig. 2a) and similar data were obtained for $TK^{-}143$ and BS-C-1 cells (data not shown). Next, the yields of infectious virus were determined in growth curve analysis after low m.o.i. (0·01 p.f.u. per cell) (Fig. 2b). These data showed that the yield of deletion mutant was unaltered compared to controls and similar data were obtained after high m.o.i. (10 p.f.u. per cell) (data not shown).

There are several examples of VV genes that are non-essential for virus replication in cell culture but which affect virus virulence *in vivo*. Therefore, the virulence of the deletion mutant was investigated in two *in vivo* models. First, the virulence was tested in a murine intranasal model (Williamson *et al.*, 1990). Following infection of groups of mice with 10^4 (data not shown) or 10^5 p.f.u. of wild-type, deletion and revertant viruses, the weight loss and signs of illness in each group were indistinguishable (Fig. 3a,b). Second, the virulence was assessed in a murine intradermal model (Tscharke & Smith, 1999). In this model, the lesion size (Fig. 3c) was unaltered compared to controls. Therefore in each model loss of the B9R gene did not affect virus virulence.

In conclusion, the protein encoded by the VV strain WR B9R gene has been characterized and its function investigated *in vitro* and *in vivo*. Data presented show that the protein is an intracellular 6 kDa polypeptide that is expressed late during infection but which does not contribute to virus replication in cell culture or virus virulence in two mouse models. In the presence of microsomal membranes, *in vitro* translation of B9R mRNA produced 6 and 8 kDa forms of the B9R protein

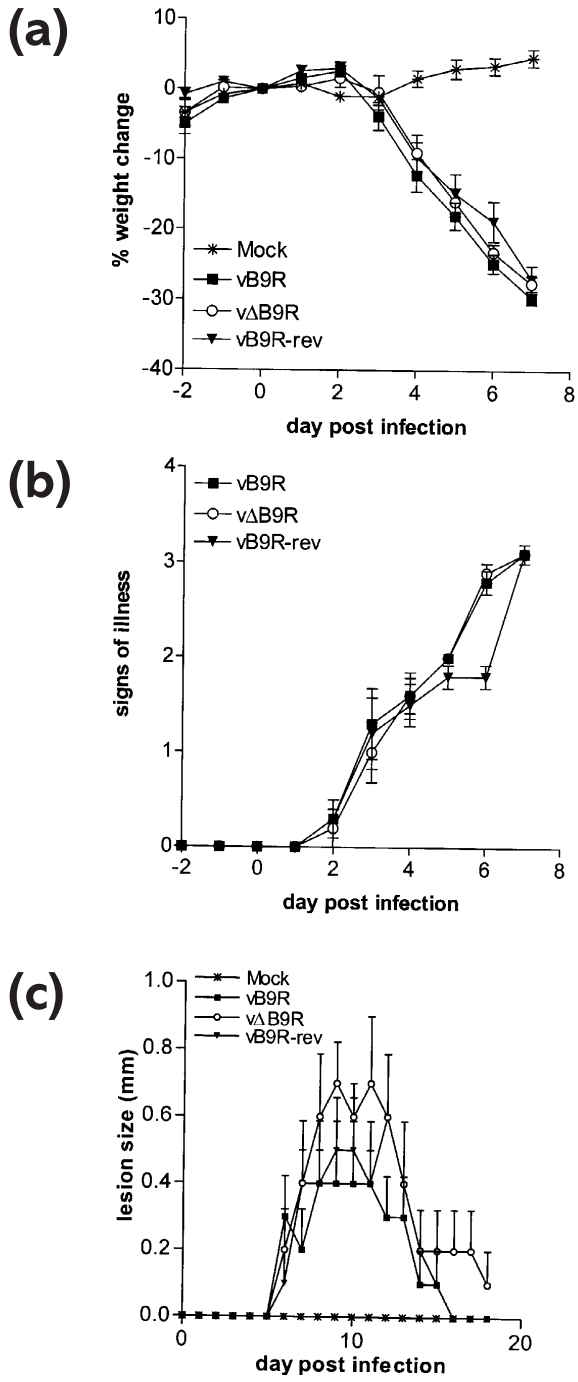

Fig. 3. Virulence of vΔB9R. (a and b) Intranasal infection. Groups of five female BALB/c mice (6–8 weeks old) were anaesthetized and infected intranasally with 10^5 p.f.u. of the indicated viruses. (a) On each day the animals were weighed and the mean weight calculated and expressed as a percentage of the mean weight of the group of animals on day zero. Animals were sacrificed by cervical dislocation when their body weight was reduced to 70% of original. (b) The signs of illness (ruffled fur, hunched backs and reduced mobility) were scored each day as described previously (Alcamí & Smith, 1992) and the mean scores ± SEMs are shown. (c) Intradermal infection. Groups of mice (as in a) were injected with 5×10^4 p.f.u. of the indicated viruses into the left ear pinnae. The diameter of the lesion was estimated daily to the nearest micrometer. Data points are the mean ± SEM for each group.

whereas only an 8 kDa protein was seen in the absence of microsomes. The smaller protein was associated with membranes and may have been proteolytically cleaved to remove the N-terminal signal peptide. The function of the B9R protein therefore remains unknown. Cowpox virus, Shope fibroma virus, myxoma virus and capripoxvirus encode larger proteins related to B9R. Of these, the myxoma virus protein (M-T4) has been characterized and found to be an intracellular protein located in the endoplasmic reticulum and which contributes to virulence (Barry *et al.*, 1997). The related proteins of similar length also contain a KDEL-like motif at their C terminus (absent from B9R) that might cause their retention in the endoplasmic reticulum where they might have a similar function to M-T4. It is possible that the VV B9R gene represents a fragment of the larger gene found in other viruses and is non-functional. While this is possible, it is notable that the B9R gene is conserved in all strains of VV for which sequence data exist and so B9R might have a function that has not been revealed by the experiments described here.

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