A mutational analysis of the vaccinia virus B5R protein

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A mutational analysis of the vaccinia virus (VV) B5R protein is presented. This protein is related to the regulators of complement activation (RCA) superfamily, has four short consensus repeats (SCRs) that are typical of this superfamily and is present on extracellular enveloped virus (EEV) particles. Here we have constructed VV mutants in which the cytoplasmic tail (CT) of the B5R protein is progressively truncated, and domains of the B5R protein [the SCR (short consensus repeat) domains, the transmembrane anchor region or the CT] are substituted by corresponding domains from the VV haemagglutinin (HA), another EEV protein. Analysis of these mutant viruses showed that loss of the B5R CT did not affect the formation of intracellular enveloped virus (IEV), actin tails, EEV or virus plaque size. However, if the SCR domains of the B5R protein were replaced by the corresponding region of the HA, the virus plaque size was diminished, the formation of actin tails was decreased severely and the titre of infectious EEV released from cells was reduced approximately 25-fold compared to wild-type virus and 5-fold compared to a virus lacking the entire B5R gene. Thus the linkage of HA to the B5R transmembrane and CT is deleterious for the formation and release of EEV and for cell-to-cell virus spread. In contrast, deletion or substitution of the B5R CT did not affect virus replication, although the amount of cell surface B5R was reduced compared to control.

Introduction

Vaccinia virus (VV) replicates in the cell cytoplasm (Moss, 1996) and encodes approximately 200 genes (Goebel et al., 1990). VV morphogenesis produces two forms of infectious virus called intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (Appleyard et al., 1971). IMV is more abundant and is released when the cell lyses (Ichihashi et al., 1971). EEV may represent less than 1% of progeny, is released from cells before lysis and mediates long-range dissemination of virus (Appleyard et al., 1971; Payne, 1980).

IMV particles are formed within cytoplasmic factories and subsequently are wrapped by a double layer of membrane (Ichihashi et al., 1971) derived from the early endosomes (Tooze et al., 1993) or trans-Golgi network (TGN) (Hiller & Weber, 1985; Schmelz et al., 1994) forming intracellular enveloped virus (IEV). These membranes contain several proteins that become part of the EEV outer envelope and which are absent from IMV (Hiller & Weber, 1985; Schmelz et al., 1994). IEV move to the cell surface where the outer membrane fuses with the plasma membrane forming enveloped virus that has one more membrane than IMV. These particles remain at the cell surface as cell-associated enveloped virus (CEV) or are released as EEV (Payne & Kristensson, 1982; Blasco & Moss, 1992). The movement of IEV to the cell surface was reported to be driven by the formation of actin tails attached to IEV particles (Cudmore et al., 1995, 1996). However, actin seems not to be essential for this process because CEV are formed in the presence of cytochalasin D (Payne & Kristensson, 1982), and several virus mutants form IEV and enhanced levels of EEV but not actin tails (McIntosh & Smith, 1996; Wollfe et al., 1997; Mathew et al., 1998; Roper et al., 1998; Sanderson et al., 1998). Actin tails are important for cell-to-cell virus spread because, with one exception (Herrera et al., 1998), virus mutants unable to form actin tails have a small plaque size (Blasco & Moss, 1992; Cudmore et al., 1995;
VV genes A33R, A34R, A36R, A56R, B5R and F13L encode proteins that are associated with EEV but absent from IMV (for review see Smith & Vanderplaschen, 1998). However, A36R is not present on EEV particles but is associated with fragments of cell membranes that remain attached to EEV (van Eijl et al., 2000). Deletion of each gene does not affect IMV formation or infectivity significantly, but has various effects on the formation, release and infectivity of enveloped particles. The F13L (Blasco & Moss, 1992) and B5R (Engelstad & Smith, 1993; Wolfe et al., 1993) proteins are required for the envelopment of IMV to form IEV, and the A33R (Roper et al., 1998) and A34R (Duncan & Smith, 1992) proteins were also reported to affect this process. In contrast, neither A36R nor A56R affect the formation of IEV, but A36R is needed to polymerize actin tails (Parkinson & Smith, 1994; Sanderson et al., 1998; Wolfe et al., 1998; Frischknecht et al., 1999; Röttger et al., 1999). Deletion of A36R (Parkinson & Smith, 1994), F12L (Zhang et al., 2000), B5R (Engelstad & Smith, 1993; Wolfe et al., 1993) and F13L (Blasco & Moss, 1992) genes results in a 3- to 5-, 7-, 10- or 100-fold reduction in EEV formation, respectively.

The B5R protein is a 42 kDa glycoprotein with type I membrane topology (Engelstad et al., 1992; Isaacs et al., 1992; Schmelz et al., 1994). In addition, a 35 kDa form of the B5R protein is released from infected cells due to proteolysis near the transmembrane (TM) anchor (Martinez-Pomares et al., 1993). The ectodomain (EC) of B5R contains four short consensus repeats (SCRs) that are characteristic of members of the complement control protein superfamily (Takahashi-Nishimaki et al., 1991). The B5R protein is required for the wrapping of IMV to form IEV, actin tail formation, a normal plaque size and for virus virulence (Engelstad & Smith, 1993; Wolfe et al., 1993; Sanderson et al., 1998). Viruses lacking SCR domains 2 to 4, 3 and 4, or 4 alone, produced a small plaque and approximately 60-fold more EEV than no actin tails (Mathew et al., 1998). In contrast, a virus lacking all SCR domains produced a larger plaque and actin tails (Herrera et al., 1998). The B5R TM and cytoplasmic tail (CT) were sufficient to direct human immunodeficiency virus gp120 to the outer envelope of EEV (Katz et al., 1997) and deletion of the CT was reported not to affect plaque size or EEV production (Lorenzo et al., 1999). However, retargeting the B5R protein to the endoplasmic reticulum (ER) caused a small plaque phenotype and reduction in actin tail formation (Mathew et al., 1999). EEV lacking the B5R protein is infectious (Mathew et al., 1998), although B5R is a target for neutralizing antibodies (Galmiche et al., 1999).

Here a further mutational analysis of the B5R protein is presented. The effects of progressive deletion of the B5R CT and of interchanging the EC, TM and CT domains of the B5R and VV haemagglutinin (HA) (A56R) proteins are reported. Data presented show that the CT does not affect plaque size or EEV production, but is needed for efficient transport of B5R to the plasma membrane. Substitution of the B5R TM and CT by the corresponding regions of the HA was not deleterious, but substitution of the B5R EC by the corresponding region of HA caused the formation of a small plaque, a dramatic reduction in virus-induced actin tails and 25-fold fewer EEV than wild-type virus.

**Methods**

- **Cells and viruses.** BS-C-1, RK13 and TK143 cells were grown in minimum essential medium (MEM) containing 10% foetal bovine serum (FBS). HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)–10% FBS. VV strain Western Reserve (WR) and WR mutants lacking A56R (vA56R) (Sanderson et al., 1998) or B5R (vA5BR) (Engelstad & Smith, 1993) or expressing BSR from the TK locus (vBSR/TK) (Mathew et al., 1998) were used.

- **Construction of viruses containing mutant B5R genes.** Mutant B5R genes were assembled by PCR and gene cloning. Plasmids pSTH2 and pSTH4 (Howard, 1991; Engelstad et al., 1992), which encode the HA and B5R genes respectively, were used as templates for PCRs. Each mutant BSR allele was driven by the B5R promoter and flanked by VV thymidine kinase (TK) gene sequences (Fig. 1). Gene BSR-HA CT (plasmid pEM9) contained the B5R gene with the CT replaced with those of B5R. Similarly, BSR-HA TM/CT (plasmid pEM10) contained the BSR gene with the TM and CT domains replaced with the corresponding domains of HA. Gene HA-B5R CT (plasmid pEM6) contained the HA open reading frame (ORF), with the CT replaced with the BSR CT. Similarly, HA-B5R TM/CT (plasmid pEM11) contained the HA ORF with the TM and CT domains replaced with those of BSR. Plasmids were constructed as follows.

**pEM9.** The BSR promoter, EC and TM domain were amplified by PCR using oligonucleotides 5′ CCAGCGCTATGATAAACTCATTATAACGGT 3′ and 5′ GCCCGTACCATGATAAACTAATAATACGTT 3′ (KpnI and NsiI sites underlined). The DNA fragment was digested with HindIII and KpnI and cloned into pUC19 forming pEM7. The HA CT was amplified by PCR using oligonucleotides 5′ CCGCTTACGATTAAACACGTTTCACTG 3′ (NsiI site underlined) and 5′ CCGCGGATCCCTAGATTGTTTCTTTCTCTGT 3′ (EcoRI site underlined). The fragment was digested with NsiI and EcoRI and cloned into pEM7, forming pEM8. The BSR-HA CT gene was excised from pEM8 with ClaI and EcoRI and cloned into pGS50 (Chakrabarti et al., 1985), forming pEM9.

**pEM10.** Oligonucleotides 5′ TACGAAATGATATTCACCAT 3′ (EcoRI site underlined) and 5′ TCTTACATGCGCTGATATTTGCATTATTTTCTCATTGCACTA 3′ were used to amplify 567 bp of the BSR EC upstream of the TM domain. The HA TM and CT domains were amplified using oligonucleotides 5′ CCCAGATTTCACTTGTCTGCCTTCTCTG 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCT 3′ (NsiI site underlined) and 5′ CCGCGGATCCCTAGATTGTTTCTTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined) and 5′ GAAAGGATATTTGAGCGAAACTGGAAGCTTTGAATTCCACTTCTCCTCCTCCTTCTCTGT 3′ (EcoRI site underlined). The PCR products were then spliced together by amplification with the terminal primers (containing EcoRI sites), utilizing the complementarity of the internal primers. The product was digested with EcoRI and cloned into pEM9 that had been digested with EcoRI to remove the 3′ end of the BSR- HA CT gene, forming pEM10.

**pEM6.** The BSR promoter was amplified using oligonucleotides 5′ CCGGTACCATGATAAACTCATTATAACGGT 3′ (HindIII and ClaI sites underlined) and 5′ CCGCGGATCCCTAGATTGTTTCTTTCTCTGT 3′ (BamHI site underlined), digested with HindIII and BamHI and cloned ligated into pEM10 using the EcoRI site.
into pUC19, forming pEM3. The HA EC and TM domains were amplified using oligonucleotides 5’ CCGGATCCATAATGACACGGATTACCA 3’ (BamHI site underlined) and 5’ CCGGATCCATGCATATATGTAATACGAA 3’ (KpnI site underlined) and the fragment was digested with BamHI and KpnI and cloned into pEM3 downstream of the B5R promoter, forming pEM4. Finally, the B5R CT was amplified by PCR using oligonucleotides 5’ CCCATGCAATGCAGAACAAAATAATGAC 3’ (NsiI site underlined) and 5’ CCGGATCCATGCATATATGTAATACGAA 3’ (KpnI site underlined). The product was digested with NsiI and KpnI and cloned into pEM4 downstream of the promoter and EC, forming pEM5. The HA-B5R CT gene was excised from pEM5 with Clal and EcoRI and cloned into pG50, forming pEM6.

pEM11. A DNA fragment encoding the C-terminal region of HA-B5R CT gene was excised from pEM6 with BstBI and KpnI and replaced by a DNA fragment produced as follows. Firstly, a PCR fragment encoding the C-terminal region of the HA EC was amplified using oligonucleotides 5’ CGTCGGTATTACGAATTCGGCG 3’ (BstBI site underlined) and 5’ ATGATAAGTTGCTTCTAATTTATAATTGCTAATA-TACTATAAC 3’ (EcoRI site underlined). These four PCR fragments were digested with HpaI and EcoRI and cloned into pEM9, forming pEM12, pEM13, and pEM15. These plasmids encoded either no CT, or a CT of five, eight or eleven amino acids, respectively, and were named accordingly. The sequences of all mutant B5R genes were confirmed by DNA sequencing (data not shown).

| Virus growth analysis. RK13 cells were infected at 1 p.f.u. per cell and harvested at 24 h post-infection (p.i.), as described (Mathew et al., 1999). Virus infectivity present in the culture supernatant was determined by plaque assay on BS-C-1 cells with or without prior incubation for 1 h at 4°C with MAb 5B4/2F2 (Czerny & Mahn, 1990) to neutralize IMV as described (Vanderplasschen & Smith, 1997). MAb 5B4/2F2 was added to virus samples which were diluted to approximately 200 p.f.u./μl volume, so that similar infectious virus/antibody ratios were maintained. The concentration of MAb 5B4/2F2 used was shown to neutralize 86–92% of purified IMV (data not shown). Virus infectivity associated with the cells was determined by plaque assay on BS-C-1 cells.

| Immunoblotting. BS-C-1 cells were infected at 10 p.f.u. per cell with or without tunicamycin (10 μg/ml). Extracts from cells, virions and the culture supernatant were prepared at 16 or 24 h p.i. as described (Mathew et al., 1999) and proteins were resolved by SDS–PAGE on a 12% gel before being transferred to nitrocellulose membranes. Membranes were incubated with rabbit polyclonal antibody against the B5R protein (Engelstad et al., 1992), mouse MAb AB1.1 against the VV D8L protein (Parkinson & Smith, 1994) (both diluted 1:2000) or with mouse MAb 1-H831 against the VV HA (hybridoma culture supernatant diluted 1:20) (Shida, 1986). Bound antibodies were detected with species-specific secondary antibodies conjugated to horseradish peroxidase followed by chemiluminescence reagents (Amersham) as directed by the manufacturer. Membranes were stripped and reprobed with different antibodies as described (Mathew et al., 1999).

| Immunoprecipitation and pulse–chase analysis. BS-C-1 cells were infected and labelled metabolically with 25 μCi Promix (1000 Ci/m mole; Amersham) and 25 μCi [35S] cysteine (600 Ci/mmol; New England Nuclear) as described (Mathew et al., 1999). Cells were harvested immediately or chased for 20, 40 or 60 min in MEM supplemented with 2 mM cysteine and methionine, and then immunoprecipitated with rabbit polyclonal antibody against the VV B5R protein as described (Mathew et al., 1999). Where indicated, immunoprecipitated proteins were incubated for 3 h at 37°C in the presence of 1000 units of EndoH (New England Biolabs). Samples were resolved by SDS–PAGE (12% gel), and the dried gel was analysed by autoradiography.

| Immunofluorescence. BS-C-1 cells at 20–30% confluency were infected at 10 p.f.u. per cell and processed for indirect immunofluorescence at 14 h p.i. as described previously (Sanderson et al., 1998). Mouse MAB AB1.1 (diluted 1:300) and rat MAB 19C2 against VV B5R (hybridoma culture supernatant diluted 1:8) (Schmelz et al., 1994) were used as primary antibodies. Bound antibodies were detected with fluorescein B isothiocyanate (FITC)-conjugated goat anti-mouse Ig or rhodamine-conjugated donkey anti-rat (diluted 1:50). Filamentous actin was detected using rhodamine isothiocyanate (RITC)–phalloidin.

| Electron microscopy. HeLa cells were infected at 5 p.f.u. per cell, harvested at 16 h.p.i., and processed for electron microscopy as described previously (Mathew et al., 1999).
Results

The roles of the different B5R domains were investigated by the construction of virus mutants in which the B5R EC, TM or CT was swapped for comparable domains of VV HA (Fig. 1) or in which the CT was progressively truncated. The HA was selected because, like B5R, it is a type I membrane glycoprotein that is present on EEV. The two proteins co-localize within infected cells, although greater amounts of HA are present on the cell surface (Schmelz et al., 1994). The mutant B5R genes

Fig. 2. Immunoblots showing protein expression by recombinant viruses. Cells were infected with the indicated viruses and cell extracts prepared and analysed as described in Methods. Where indicated (+) cells were infected and incubated in the presence of tunicamycin. In (a) and (b) membrane filters were probed with anti-B5R and then stripped and probed with anti-D8L. In (c) the filter was probed with anti-HA MAb and then stripped and probed with anti-D8L. Molecular mass markers are shown in kDa.
were assembled and inserted into the TK locus of vΔB5R, a virus lacking the B5R gene (Engelstad & Smith, 1993), as described in Methods. Southern blotting and PCR analysis of the virus genomes confirmed that each recombinant virus retained the mutated B5R allele of the parental virus (vAB5R) at the B5R locus, encoded their respective mutant genes from the TK locus and no other changes were observed (data not shown).

**Protein expression by recombinant viruses**

To examine the proteins made by each virus, infected cell extracts were examined by immunoblotting using a polyclonal anti-B5R antiserum (Fig. 2a, b). A 42 kDa protein was made by vB5R/TK and similar levels of slightly larger proteins were made by vB5R-HA CT and vB5R-HA TM/CT. No B5R protein was detected in mock-infected cells or cells infected by vΔB5R; however, the latter cells were infected because immunoblotting with MAb AB1.1 detected equivalent levels of D8L protein. WT B5R protein synthesized in the presence of tunicamycin was reduced in size and cells infected with viruses with a truncated CT contained similar levels of B5R protein that decreased in size as the CT tail was truncated (Fig. 2b). In the presence of tunicamycin these B5R proteins were more heterogeneous in size and lower levels were detected. This indicated that in the absence of N-linked glycans the CT influenced protein stability. The polyclonal anti-B5R antiserum was raised against the B5R EC and so could not be used to analyse the chimaeric protein made by vHA-B5R CT and vHA-B5R TM/CT and attempts to generate an antiserum against the B5R CT domain as a glutathione S-transferase–B5R CT fusion protein were unsuccessful (data not shown). Instead, HA-B5R CT and HA-B5R TM/CT proteins were detected by the HA MAb 1-H831 (Fig. 2c). The HA proteins produced by WR WT, vB5R/TK and vΔB5R were indistinguishable and migrated as a broad band of approximately 85 kDa and a less abundant 76 kDa protein as noted previously (Brown et al., 1991; Payne, 1992). No protein was detected in mock-infected cells or cells infected with vHA (Sanderson et al., 1998). In the presence of tunicamycin, the WT HA is smaller, 62 kDa (lanes...
Analysis of cell surface B5R by flow cytometry. TK-143 cells were infected with the indicated viruses at 5 p.f.u. per cell for 12 h. Cells were then detached from the plastic support by pipetting, collected by centrifugation and stained with MAb 19C2. Bound antibody was detected with FITC-conjugated sheep anti-rat antibody, and cells were fixed in paraformaldehyde and analysed by flow cytometry as described (Vanderplasschen & Smith, 1997).

6, 12 and 14) (Shida & Dales, 1981, 1982; Payne, 1992). The chimaeric HA-B5R proteins made by vHA-B5R CT and vHA-B5R TM/CT in the absence and presence of tunicamycin co-migrated with WT HA, but more HA protein was made by these viruses (lanes 7–10) and a larger protein (~120 kDa) was made by vHA-B5R TM/CT in the presence of tunicamycin that might represent HA homodimers. Immunoblotting with MAb AB1.1 detected similar amounts of D8L in cells infected by each virus indicating that the greater amounts of HA detected in the vHA-B5R CT and vHA-B5R TM/CT samples were due to the extra ectopic copy of the HA gene.

Pulse–chase analysis

The transport of the mutant B5R proteins was investigated by pulse–chase analysis followed by immunoprecipitation and digestion with EndoH (Fig. 3). EndoH cleaves high-mannose N-linked sugars that are present on proteins in the ER but not Golgi, and consequently the acquisition of resistance to endoH measures the transport of N-glycosylated proteins from the ER to the Golgi (Hsieh et al., 1983). Viruses vHA-B5R CT and vHA-B5R TM/CT were not included because these chimaeric proteins are not recognized by the anti-B5R antibody, and only the viruses with the greatest or smallest CT deletion are shown.

Immediately after pulse-labelling the B5R protein was sensitive to endoH digestion and was reduced in size by this treatment to about 40 kDa. The WT B5R protein produced by vB5R/TK became resistant to endoH within 60 min (Mathew et al., 1999), and both B5R-HA-CT and B5R-HA TM/CT proteins exhibited similar kinetics (Fig. 3). In contrast, B5R mutants expressing the least and most truncated CTs (vB5R ΔCT and vB5R CT11, respectively) gained resistance more slowly, and by 60 min the majority of these proteins remained sensitive to endoH. In addition, the vB5R ΔCT protein was more diffuse than the WT B5R protein (Fig. 2). Therefore, the CT of B5R affects both the rate of transport of B5R from the ER to Golgi and the protein stability.

Flow cytometry

The amount of cell surface B5R protein was investigated by flow cytometry (Fig. 4). Live infected cells were stained with MAb 19C2, which recognizes an epitope in SCR domain 2 (Mathew, 1998). Substitution of the B5R CT or the TM and CT with the corresponding regions of the HA did not affect transport of B5R to the cell surface (Fig. 4a). Similarly, B5R proteins with a CT of eleven or eight amino acids were transported effectively to the cell surface (Fig. 4c). In contrast, more extensive deletion of the CT caused a reduction in cell surface B5R (Fig. 4b). This is consistent with the pulse–chase analysis and may be attributable to reduced transport and/or stability of the protein.

Production of infectious virus

Mutations in B5R affect IEV and EEV formation (Introduction) and so infectious virus production by these new mutants was examined (Fig. 5). At 24 h p.i. most infectious progeny was cell-associated and was unaffected by the different B5R alleles. However, the amount of infectivity released into the culture medium varied considerably. Each B5R truncated CT mutant, vB5R-HA CT and vB5R-HA TM/CT released similar amounts of infectious particles as
vB5R/TK. In contrast, vHA-B5R CT and vHA-B5R TM/CT produced much less extracellular virus than WT WR and vB5R/TK and about 5-fold less than vAB5R. Evidently, these chimaeric proteins were more detrimental to extracellular virus production than having no B5R protein at all. To determine if the infectivity detected in the supernatant represented EEV or IMV that had been released by cell lysis, the sensitivity of virus in the culture supernatant to an IMV-neutralizing MAb (Czerny & Mahnel, 1990) was tested (Fig. 5b). Data obtained confirmed that the majority of the virus was resistant to

Fig. 5. Production of infectious virus. (a) The titre of infectious virus present in the culture supernatant (shaded bars) or associated with RK13 cells (striped bars) 24 h p.i. with the indicated viruses was determined by plaque assay on BS-C-1 cells. (b) The percentage of virus infectivity in the culture supernatant that was resistant to neutralization by MAb 5B4/2F2 is shown for each virus ± SEM (n = 3).
neutralization and thus enveloped. Notably, EEV released by viruses producing the lowest levels of EEV, vΔB5R, vHA-B5R CT and vHA-B5R TM/CT, was more resistant to MAb 5B4/2F2 than EEV made by the other viruses. Similar observations had been made previously (Mathew et al., 1998).

**Plaque phenotypes of mutant viruses**

Plaques produced under a semi-solid overlay are shown in Fig. 6. Compared to the plaques formed by vB5R/TK, the plaques formed by vB5R-HA CT and all four of the B5R truncated CT mutants were of similar size, and the plaques formed by vB5R-HA TM/CT were slightly smaller. In contrast, vHA-B5R CT and vHA-B5R TM/CT produced very small plaques that were similar to those generated by vΔB5R. None of the recombinant viruses was able to form comet-shaped plaques when incubated under liquid overlay (data not shown), consistent with the levels of EEV being similar to or less than the levels made by WT WR (Fig. 5).

**Analysis of the distribution of virions and formation of actin tails by immunofluorescence**

The ability to form a normal-sized plaque has correlated with the formation of virus-tipped actin tails (Introduction), and therefore the formation of actin tails and distribution of virions were investigated by immunofluorescence (Fig. 7). Permeabilized cells were stained with phalloidin to detect polymerized actin and with MAb AB1.1 to detect all virions. Cells infected with vB5R-HA CT and vB5R-HA TM/CT contained many virions in perinuclear regions (virus factories) and at the cell periphery (Fig. 7a, c). Cells infected with each virus contained actin tails, although it was observed consistently that fewer actin tails were made by vB5R-HA TM/CT
(Fig. 7d) than by vB5R-HA CT (Fig. 7b). Cells infected with vHA-B5R CT or vHA-B5R TM/CT contained many virions, some of which had dispersed throughout the cell but the majority remained perinuclear (Fig. 7 e, g). Notably, no actin tails were observed (Fig. 7 f, h). In these cases the actin stress fibres remained more prominent, an observation made previously with virus mutants unable to make actin tails (Sanderson et al., 1998).

To monitor the distribution of virions in cells infected by the B5R CT mutants, MAb 19C2 was used so that IEV and CEV particles were detected, as well as larger punctate structures that represent membranes used to wrap IMV particles. Results are shown only for the least and most truncated CTs, B5R ∆CT and vB5R CT11 (Fig. 7 f–h), because the other CT mutants gave similar results. These data show that the dispersal of IEV particles to the cell periphery and the formation of actin tails were not affected by truncation or deletion of the CT tail.

Electron microscopy

Cells infected with the different viruses were examined by transmission electron microscopy of Epon-embedded samples to determine if there were any differences in virus morphology. For each B5R CT mutant, vB5R-HA CT and vB5R-HA CT were noted (data not shown). However, for vHA-B5R CT and vHA-B5R TM/CT, IMV particles were mostly not wrapped to form IEV (data not shown). This latter feature is characteristic of vAB5R, which also makes a small plaque and lower levels of EEV (Engelstad & Smith, 1993). The failure to observe actin tails in cells infected by vHA-B5R CT and vHA-B5R TM/CT is therefore due largely to a failure to form IEV so that CEV particles are not present at the cell surface in a position to nucleate actin tail formation.

Incorporation of mutant B5R proteins into EEV

To determine if the B5R proteins produced by vB5R-HA CT, vB5R-HA TM/CT, vB5R ∆CT and vB5R CT11 were incorporated into EEV and whether the BR D8L protein was found in the cell lysates and released virions (data not shown). These observations suggest that the B5R CT affects, but is not essential for, the incorporation of B5R into EEV. Also, changes to the TM and CT domains did not affect the processing of the 42 kDa protein into the 35 kDa secreted version.

Discussion

Viruses expressing mutant B5R genes encoding HA domains in place of the B5R EC, TM or CT, or lacking increasing portions of the B5R CT, were analysed to investigate the relative contribution of these domains to virus morphogenesis and dissemination. The progressive truncation of the CT tail had no noticeable effect on the formation and yield of all forms of virus, production of actin tails and plaque size. This was consistent with a report examining the effect of deletion of the entire CT domain (Lorenzo et al., 1999). However, analysis of the protein processing and distribution showed that when only five amino acids of the CT remained, or when it was deleted entirely, there were lower levels of B5R present on the cell surface (Fig. 4) and the vB5R CT protein was transported from the ER more slowly (Fig. 3). In contrast, a recent report showed that more B5R reached the plasma membrane if the CT domain was deleted (Ward & Moss, 2000). A possible explanation for this discrepancy is that the report from Ward and Moss studied the expression of B5R in transfected cells without virus infection, whereas this study analysed the B5R protein location in virus-infected cells where other virus proteins are present. These truncated B5R CT proteins described here were more diffuse than control B5R protein (Figs 2 and 3), suggesting that some proteolytic digestion may have occurred. EEV produced by viruses with CT truncations still contained B5R (Fig. 8), albeit at reduced levels, but these virions remained infectious (Fig. 5). The latter observation was consistent with the report that EEV lacking B5R retained infectivity (Mathew et al., 1999); however, the B5R CT tail is conserved in all sequenced orthopoxviruses suggesting an important function.

The HA CT affects the transport of the HA protein (Shida, 1986), and we report here that transfer of the HA CT to the B5R protein produced a virus with wild-type properties. Likewise, transfer of the HA TM and CT to B5R produced a virus with properties similar to wild-type, although in this case the number of actin tails appeared to be reduced. It was reported that the signals needed for the incorporation of B5R protein into EEV were located in the TM and CT (Katz et al., 1997) and that loss of the entire CT did not prevent incorporation of B5R into EEV (Lorenzo et al., 1999). Therefore, because the B5R protein containing the HA TM and CT domains was transported and incorporated into EEV, the signals present in the C-terminal portion of the HA protein can substitute functionally for those deleted from B5R.

When the B5R CT domain or TM and CT domains were
linked to the HA EC, actin tails were not made and the virus plaque size was small. This provides another example of the linkage between actin tail formation and efficient cell-to-cell spread of virus (see Introduction). Although actin tails were not made, the distribution of virions within the cell was similar to cases in which actin tails were made. This observation is
Fig. 7. For legend see p. 1211.
Fig. 7. For legend see facing page.
derived from 4 samples were added for each fraction. Protein in the supernatant (S) was analysed by SDS–PAGE and immunoblotting with rabbit polyclonal anti-B5R antibody. To obtain similar strength signals different amounts of plastic dish into PBS and collected by centrifugation. Samples were acid and recovered by centrifugation. Cells (C) were scraped from the was removed and soluble proteins were precipitated with trichloroacetic 14000 r.p.m., 80 min, in a Beckman SW28.1 rotor. The supernatant (S) at 24 h p.i. supernatants were clarified by low-speed centrifugation EC. The reasons for this are not obvious, but a virus constructs where the HA TM and CT domains were linked to lower levels of infectious extracellular virus, lower than that required for actin tail formation was located within the lumen of the wrapping membrane. These viruses made exceptionally actin tails also indicated that the region of the B5R protein surface (M. Hollinshead, H. van Eijl, M. Law, G. Rodger & G. L. Smith, unpublished). The failure of these viruses to form consistent with the requirement for microtubules rather than actin tails for movement of enveloped virions to the cell surface (M. Hollinshead, H. van Eijl, M. Law, G. Rodger & G. L. Smith, unpublished). The failure of these viruses to form actin tails also indicated that the region of the B5R protein required for actin tail formation was located within the lumen of the wrapping membrane. These viruses made exceptionally low levels of infectious extracellular virus, lower than that made by a virus lacking the entire B5R protein. This indicated that the linkage of the HA EC to the B5R CT domain or TM and CT domains was deleterious in contrast to the converse constructs where the HA TM and CT domains were linked to the B5R EC. The reasons for this are not obvious, but a virus expressing a chimaeric protein composed of a single chain antibody fused to the N terminus of VV HA also produced lower levels of EEV than wild-type (Galmiche et al., 1997).

Possibly, the transport and/or distribution of the HA-B5R chimaeric protein made by these viruses were abnormal. This was not analysed here because the MAb 19C2 did not recognize these chimaeric proteins. Alternatively, the B5R spacer, a region of 39 amino acids between the B5R SCRs and TM, may also be required for IEV formation and EEV release. In conclusion, we show that the B5R CT is not needed for either the envelopment of IMV or the nucleation of actin tails but does affect protein transport and stability. The normal transport and stability of the B5R protein was restored by addition of the HA CT. In addition to the B5R EC domain that is known to affect actin tail formation, evidence presented here indicates that the B5R TM domain affects actin polymerization to some degree. Lastly, the linkage of the HA EC to B5R TM and CT was deleterious for virus morphogenesis and prevented envelopment of IMV to IEV and yielded lower levels of EEV than a virus lacking the B5R protein.

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