Human cytomegalovirus US9 protein contains an N-terminal signal sequence and a C-terminal mitochondrial localization domain, and does not alter cellular sensitivity to apoptosis

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The human cytomegalovirus (CMV) US2–US11 genomic region contains a cluster of genes whose products interfere with antigen presentation by the major histocompatibility complex (MHC) proteins. Although included in this cluster, the US9 gene encodes a glycoprotein that does not affect MHC activity and whose function is still largely uncharacterized. An in silico analysis of the US9 amino-acid sequence uncovered the presence of an N-terminal signal sequence (SS) and a C-terminal transmembrane domain containing the specific hallmarks of known mitochondrial localization sequences (MLS). Expression of full-length US9 and of US9 deletion mutants fused to GFP revealed that the N-terminal SS mediates US9 targeting to the endoplasmic reticulum (ER) and that the C-terminal MLS is both necessary and sufficient to direct US9 to mitochondria in the absence of a functional SS. This dual localization suggested a possible role for US9 in protection from apoptosis triggered by ER-to-mitochondria signalling. Fibroblasts infected with the US2–US11 deletion mutant virus RV798 or with the parental strain AD169varATCC were equally susceptible to death triggered by exposure to tumour necrosis factor (TNF)-α, tunicamycin, thapsigargin, brefeldin A, lonidamine and carbonyl cyanide m-chlorophenyl hydrazone, but were 1.6-fold more sensitive to apoptosis induced by hygromycin B. Expression of US9 in human embryonic kidney 293T cells or in fibroblasts, however, did not protect cells from hygromycin B-mediated death. Together, these results classify US9 as the first CMV-encoded protein to contain an N-terminal SS and a C-terminal MLS, and suggest a completely novel role for this protein during infection.

INTRODUCTION

Human cytomegalovirus (CMV) is a very prevalent betaherpesvirus that poses serious medical concerns in immunocompromised individuals (Mocarski et al., 2007). The ability to evade or modulate host immune responses, coupled with the capacity to establish latency after primary infection, is at the basis of the tremendous success of CMV as a human pathogen.

The US2–US11 genomic region has been shown to be dispensable for viral replication in primary human fibroblasts (HF) (Jones & Muzithras, 1992; Kollert-Jons et al., 1991), and to encode five endoplasmic reticulum (ER)-resident glycoproteins, US2, US3, US6, US10 and US11, that disrupt antigen presentation by the major histocompatibility complex (MHC) class I and class II proteins (Lin et al., 2007). Despite sharing some very limited homology with US6, US10 and US11, US9 does not bind to or reduce MHC cell surface levels (Hegde et al., 2002, 2006; Pereira et al., 1995). US9 was originally described as a member of the US6 family of proteins, which includes the products of the US6–US11 genes. These proteins contain markedly hydrophobic sequences at both the N- and C-termini, and were predicted to insert into cellular membranes (Weston & Barrell, 1986). US9 intracellular localization was initially examined in non-polarized Madin–Darby canine kidney cells constitutively expressing a C-terminal-tagged form of US9 from strain AD169varATCC (AD). In non-polarized cells, accumulation was observed in the ER and Golgi compartments, while cell polarization resulted in additional staining along lateral membranes (Maidji et al., 1998).

ER localization was subsequently reported also in human endometrial carcinoma 1A cells infected with replication-defective adenovirus vectors expressing AD US9, and
stained with anti-US9 polyclonal antibodies (Huber et al., 2002). In this study, however, no evidence was found of US9 reaching the Golgi or accumulating at the plasma membrane. Attempts at detecting US9 in CMV-infected HF by immunofluorescence microscopy were unsuccessful, probably on account of the low levels of expression of this protein during infection (Huber et al., 2002).

A clear relationship between US9 expression and virus yields has not been established. Two different US2–US11 deletion mutant viruses, RV798 (Pereira et al., 1995) and RVΔUS2-11 (Falk et al., 2002) generated in an AD background by two different methods, displayed growth properties in HF similar to wild-type virus. The AD temperature-sensitive mutant ts9 (Yamanishi & Rapp, 1979), carrying a US1–US13 deletion, also produced titres similar to AD when grown at the permissive temperature. By contrast, growth kinetics of the US9 deletion mutant virus RV61 in HF and in ARPE-19 epithelial cells were similar to those of the parental AD virus only until day 5 post-infection, with marked differences recorded at later times (Huber et al., 2002; Jones & Muzithras). Growth defects were also reported for RV80, a US8–US9 deletion mutant (Pereira et al., 1995), and for RV134, which contains the β-glucuronidase gene inserted in the US9–US10 intergenic region, but expresses both US10 (Jones et al., 1991) and US9 (Huber et al., 2002). Thus, additional factors besides the lack of US9 expression appeared to be responsible for the observed decreases in virus yields, and in the absence of rescued versions of the US9 deletion mutants, no definitive conclusion could be drawn as to the effects of US9 on viral cycle progression (Huber et al., 2002).

Compared with AD, RV61 and RV80 produced smaller plaques on polarized ARPE-19 cells after 3 weeks of culture (Huber et al., 2002; Maidji et al., 1996). It was thus proposed that US9 could facilitate virion transmission across lateral membranes of polarized cells (Maidji et al., 1996). The small-plaque phenotype on ARPE-19 cells, however, was subsequently reported also for mutants expressing US9 (RV134 and a US2–US3 deletion virus), leading to the suggestion that mutations other than those in the US9 gene might be responsible for the reduction in plaque size and viral yields of these recombinants (Huber et al., 2002).

In this work, we sought to gain insights into US9 function by predicting protein localization motifs in silico, and by establishing their role in directing US9 to specific intracellular locations. We show for the first time that the N terminus of US9 contains a signal sequence (SS) mediating localization to the ER, while the C terminus contains a mitochondrial localization sequence (MLS) that is necessary and sufficient to direct US9 to mitochondria. A dual localization to the ER and mitochondria has been described for CMV viral mitochondria-localized inhibitor of apoptosis (vMIA), a broadly acting inhibitor of cell death that also disrupts mitochondrial reticular networks during infection (McCormick et al., 2003; McCormick, 2008). Despite the localization similarities with vMIA, US9 did not change the morphology of mitochondria and did not protect cells from apoptosis during infection.

**METHODS**

**In silico amino acid sequence analyses.** The following programs were used: SignalP3.0 (Bendtsen et al., 2004), MEMbrane protein Structure And Topology 3 (MEMSAT3) (Jones, 2007), Kyte–Doolittle Hydrophathy Plot (Kyte & Doolittle, 1982), TargetP1.1 (Emanuelsson et al., 2000) and PSORTII (Nakai & Horton, 1999).

**Cells, viruses and drugs.** Primary HF and human embryonic kidney (HEK) 293T cells (gifts from E. S. Mocarski, Emory Vaccine Center, Atlanta, GA, USA) and HeLa S3 cells (a gift from D. Litchfield, The University of Western Ontario, London, ON, Canada) were cultured in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen) supplemented with 10% Fetal Clone III (HyClone), 4 mM HEPES, 1 mM sodium pyruvate, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (all from Gibco Invitrogen). Human CMV strains AD169varATCC, RV798, TB410/E and Toledo were kind gifts from E. S. Mocarski and were originally obtained from the American Type Culture Collection (ATCC), T. Jones (Wyeth Research, Pearl River, NY, USA), S. Plotkin (Aventis Pasteur, Swiftwater, PA, USA) and C. Sinzger (University of Tübingen, Tübingen, Germany), respectively. Propagation, purification and titre determination of all strains were performed as described previously (Hertel et al., 2003). Tumour necrosis factor (TNF)-z was purchased from PeproTech and was resuspended in water. All other drugs were purchased from Sigma and were resuspended as follows: cycloheximide (Cx), tunicamycin (tunica), thapsigargin (thapsi) and lonidamine (lonida) in DMSO, brefeldin A (bref) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) in methanol, and hygromycin B (hygro) in water.

**Cell transfection, retrovirus production and cell transduction.** HEK293T cell transfection was carried out using Polyfect (Qiagen). Retroviral particles were generated, purified and stored as described previously (Hertel et al., 2007). For retrovirus transduction, 70–80% confluent HeLa cells or 100% confluent HF were split at a 1:4 ratio in 24-well plates and were exposed to retroviral particles in the presence of 5 µg Polybrene ml⁻¹ (Sigma) 24 h after seeding. Cells were tested for protein expression at 24 h post-transduction.

**HF infection with CMV and drug treatments.** Confluent HF were infected at confluency with purified virions at a m.o.i of 5. After virus adsorption for 1 h at 37 °C, the inoculum was removed, and cells were washed twice prior to the addition of fresh medium. At 24 h post-infection (h p.i.), medium containing each drug or its respective solvent was added to cells. The number of viable cells after 24 h of treatment was determined by counting trypan blue-excluding cells with a haemocytometer. The following final drug concentrations were used: TNF-α 2.5 ng ml⁻¹ with 1.25 µg Cx ml⁻¹, TNF-α 5 ng ml⁻¹ with 2.5 µg Cx ml⁻¹, TNF-α 10 ng ml⁻¹ with 5 µg Cx ml⁻¹, 250 µM lonidamine, 20 µM CCCP, 2 µM thapsi, 6.2 µg tunica ml⁻¹, 5 or 10 µg bref ml⁻¹ and 800 µg hygro ml⁻¹.

**Plasmid construction.** Details of plasmids generated by PCR are reported in Supplementary Table S1 available in JGV Online. LGFP GFPnoSTOP was created by replacing the green fluorescent protein (GFP) ORF in LGFP (McCormick et al., 2005) with a GFP ORF lacking the termination codon TAA. LNCX-mRFP was produced by inserting the monomeric red fluorescent protein (mRFP) ORF amplified by PCR from the pRSET B-mRFP vector (a kind gift from G. Dekaban, Robarts Research Institute, London, ON, Canada) into
the BamHI and SalI restriction sites (rs) of LNCX (Miller & Rosman, 1989). The US9AD and US9Tol ORFs were amplified by PCR from cDNA libraries generated by reverse-transcription of total RNA extracted from AD- or Toledo-infected HF. To create LGFP GFP–US9AD and LGFP GFP–US9Tol, US9AD and US9Tol cDNA were inserted into LGFP GFPNstop in-frame with the C terminus of GFP using the NotI and SalI rs. To create LGFP US9AD–GFP, US9AD cDNA was cloned into LGFP in-frame with the N terminus of GFP using the BamHI rs. LGFP US9AD1–31–GFP and LGFP US9AD1–31–GFP were produced by replacing GFP in LGFP with 5’-end-truncated versions of the US9AD–GFP ORF, amplified by PCR from LGFP US9AD–GFP. LGFP GFP–US9AD1–31–GFP was produced by replacing the GFP ORF in LGFP with 3’-terminus versions of the US9–US9AD ORF, amplified by PCR from LGFP GFP–US9AD. To generate LGFP GFP–aa198–247, the US9 ORF region corresponding to nucleotides 590–744 was amplified by PCR and cloned into LGFP GFPNstop in-frame with the C terminus of GFP using the NotI and SalI rs. To generate LGFP SRSKESL–GFP, the GFP ORF was amplified by PCR using a forward primer containing the ATG codon followed by US9 nucleotides 73–93. LCNX US9AD198–247–mRFP was generated by PCR amplification of the US9 ORF region corresponding to amino acids 1–198 and cloning of the product into LCNX mRFP in-frame with the N terminus of mRFP using the BamHI rs. To produce LCNX US9, the US9 ORF was excised from LGFP GFP–US9 and inserted into the NotI site of LCNX. The correct sequence of all constructs was confirmed by nucleotide sequencing.

Mito Tracker Red and immunofluorescence staining analyses. For mitochondrial labelling, cells were exposed to 200 nM Mito Tracker Red CMXRos (Molecular Probes) for 5 min at 37 °C, washed with PBS, incubated in fresh medium for 30 min at 37 °C, fixed with 3.7 % formaldehyde in PBS for 15 min at 37 °C and mounted. For immunofluorescence staining of transfected or transduced cells, samples were fixed, permeabilized and blocked as described previously (Hertel & Mocarski, 2004), before incubation with rabbit anti-calreticulin antibodies (1:300, Stressgen) or rabbit anti-Translocase of outer membrane 20 antibodies (Tom20; 1:100, Santa Cruz Biotechnology) for 1 h at room temperature. Samples were washed in PBS/0.05 % Tween-20 and incubated with Alexa-Fluor 594- or Alexa-Fluor 488-conjugated goat anti-rabbit antibodies (1:100, Molecular Probes) for 1 h at room temperature. For dual staining of infected cells for Tom20 and for the viral nuclear proteins immediate-early 1 and 2 (IE1/IE2), fixed cells were first incubated with the anti-Tom20 antibody and the Alexa-Fluor 594-conjugated goat anti-rabbit antibody, then blocked with normal mouse immunoglobulin G (1:100; Caltag), and finally stained with a FITC-conjugated anti-IE1/IE2 monoclonal antibody (1:800; MAb810F, Chemicon). Samples were viewed on a Zeiss LSM510 META/ConfoCor2 laser-scanning confocal microscope equipped with Zeiss LSM-510 META image processing software.

RESULTS

US9 amino acid sequence analysis

A CLUSTAL W global alignment of the US9 amino acid sequence from strain AD with sequences from 21 other human CMV strains and isolates showed US9 to be an exceptionally invariant, highly conserved protein (Fig. 1a). US9 from two strains (Towne and Toledo) and three isolates (v900, 452 and v851) appears to be truncated at the C terminus by 15 aa. A Kyte–Doolittle hydropathy plot of AD US9 revealed four regions of at least 20 aa with hydrophobicity values greater than 0 (Fig. 1a and b, shaded boxes). On the basis of the reported accumulation of US9 in the ER of non-polarized epithelial cells (Huber et al., 2002; Maidji et al., 1998), we hypothesized that the most N-terminal of these regions could function as an SS, targeting US9 to the ER. An SS spanning amino acids 1–24 was indeed predicted by SignalP3.0 with a probability score of 0.93 and was perfectly conserved in all US9 sequences (Fig. 1a, black line). The most C-terminal region, composed of amino acids 194–233, exhibited hydrophobicity values peaking above 1.6, a strong indication that it might contain a transmembrane domain (TMD). Accordingly, the MEMSAT3 program predicted the presence of a TMD in this region (amino acids 196–223), composed of a central helix flanked by an outside and an inside cap (Fig. 1a, indicated by H, O and I, respectively). The TMD amino acid sequence appeared to be identical in all strains and isolates, with the exception of TR-BAC which carries a conservative hydrophobic amino acid difference (C 217 to Y) that does not alter the TMD prediction. The sequence from amino acids 198–221 was also highly conserved in chimpanzee CMV US9 (80.8 % identity and 100 % similarity scores), suggesting that the TMD could be functionally essential during infection of primate hosts. Upon close examination, the TMD sequence appeared to perfectly match a consensus targeting signal of known tail-anchored, mitochondrial outer membrane (MOM) proteins (Rapaport, 2003), with a short stretch of amino acids with overall positive net charge (RLFAEER, positively charged amino acids are in bold type), followed by 29 aa with modest hydrophobicity (YVVLVQFKVALFS-FGQVACCYVLRWI) and another stretch of amino acids with overall positive net charge (RPWVRGHRRA). Finally, two subcellular localization prediction programs, TargetP1.1 and PSORTII, assigned a secretory pathway localization to the full-length protein and a mitochondrial localization to US9 lacking the putative SS. Thus, the in silico analysis of US9 amino acid sequences revealed that this protein might contain two separate localization domains, an N-terminal SS directing it to the ER and a C-terminal TMD targeting it to mitochondria.

US9 intracellular localization

To test the role of the predicted SS and TMD in directing US9 to the ER and to mitochondria, we sought to interfere with the function of either the SS or the TMD by fusing GFP to the N- or C-terminus of AD US9. The effects, if any, of the C-terminal 15 amino acids on regulating US9 trafficking were also examined by creating an N-terminal GFP fusion of Toledo US9 (GFP–US9Tol). US9AD–GFP, GFP–US9AD and GFP–US9Tol were expressed in HEK293T, HeLa and HF cells. Consistent with the reported US9 localization in epithelial cells (Huber et al., 2002; Maidji et al., 1998), US9AD–GFP was found to co-localize with calreticulin in the ER of transfected HEK293T cells (Fig. 2a–c), but not with the MOM-resident protein Tom20 (Fig. 2d–f). In contrast, signals from both GFP–US9AD and GFP–US9Tol did not overlap with calreticulin...
(Fig. 2g–i and not shown), but accumulated instead at mitochondria (Fig. 2j–r), co-localizing with signal from Mito Tracker Red (Fig. 2l and o, insert) or Tom20 (Fig. 2r). Mitochondrial localization was observed irrespective of the presence (GFP–US9AD) or absence (GFP–US9Tol) of the C-terminal 15 amino acids, showing that this sequence does not contribute to, or otherwise influence, mitochondrial targeting. Similar localization patterns were observed in HEK293T, HeLa and HF cells (not shown), indicating that these patterns are unlikely to be cell-type-specific or dependent on overexpression in HEK293T cells.

Consistent with previous literature reports (Huber et al., 2002), we failed to detect US9 in CMV-infected HF by immunofluorescence analysis, despite extensive attempts at staining infected cells harvested at various times post-infection and using both anti-US9 antibodies employed in previous work. Although overlap of the GFP fluorescence...
and the US9 signal was observed in HEK293T cells expressing US9AD–GFP or GFP–US9AD and stained with the only anti-US9 antibodies currently available (Huber et al., 2002), the presence of a strong background signal did not allow for accurate detection of US9 subcellular localization in cells expressing untagged US9.

Localization of US9AD–GFP and of GFP–US9AD remained unchanged at 24, 48 and 72 h after AD infection of HF expressing either protein (results not shown), suggesting that their targeting to the ER and mitochondria is unlikely to be affected by the presence of other viral proteins.

Identification of US9 localization domains

To identify the amino acid sequences responsible for directing US9 to the ER or to mitochondria, we determined the intracellular localization of a series of US9 deletion mutants. Removal of amino acids 1–24 from US9AD–GFP (US9ADΔ1–24–GFP) completely abrogated ER targeting (Fig. 3a–c), confirming that amino acids 1–24 constitute a functional SS. Quite interestingly, SS-deleted US9AD–GFP localized to mitochondria (Fig. 3d–f). Dual localization to the ER and mitochondria of some viral and cellular proteins has been shown to depend on cryptic MLS, located in tight juxtaposition to the SS. In contrast, mitochondrial tail-anchored (TA) proteins appear to contain C-terminal MLS. To establish the location of the domain targeting US9 to mitochondria, we generated two deletion mutants, one lacking the first seven amino acids following the SS (US9ADΔ1–31–GFP) and the other lacking the C-terminal TMD (GFP–US9ADΔ198–247).

Deletion of amino acids 1–31 did not substantially alter mitochondrial targeting (Fig. 3g–i). US9 amino acids 25–31 were also unable to independently confer mitochondrial localization to GFP when fused at its N terminus (SRSKESL–GFP, Fig. 3j–l). In contrast, deletion of the C-terminal TMD sequence from GFP–US9AD completely abrogated mitochondrial targeting (Fig. 3m–o), while its addition to the C terminus of GFP (GFP–aa198–247) directed GFP to mitochondria (Fig. 3p–r). These data suggest that US9 has a bimodal localization to the ER and mitochondria and show that amino acids 198–232 are both necessary and sufficient for mitochondrial targeting.

As US9 does not contain the canonical ER retention signal KDEL (Pelham, 1990), we wondered whether the TMD could also act as an ER membrane-anchoring motif. GFP–US9ADΔ1–31–GFP did not show ER localization (Fig. 3s–u), but this mutant was also probably unable to reach the ER due to the masking of the SS by GFP. A US9AD mutant lacking the TMD and containing an unmasked SS (US9ADΔ198–247–mRFP), however, still retained ER localization (Fig. 3v–x), suggesting that the TMD is exclusively required for mitochondrial retention. Fig. 3(y) shows a summary of US9 deletion mutants and their localization.

The US2–US11 genes do not contribute to viral disruption of mitochondrial networks

Localization results suggested that US9 distributes to both the ER and mitochondria, like vMIA. To determine whether US9 might share with vMIA the ability to alter mitochondrial morphology, HF infected with either AD or...
RV798 for 24, 48 and 72 h were stained for Tom20 and for
IE1/IE2. RV798 was chosen because it did not exhibit any
replication defects in HF, reaching titres similar to those of
AD (data not shown). In contrast, the specific US9 deletion
mutant RV61 appears to contain additional adventitious
mutations that reduce viral yields (Huber et al., 2002). The

**Fig. 3.** Confocal immunofluorescence microscopy of HEK293T cells expressing US9AD mutants or GFP fusions with specific
US9 domains, and summary of observed protein localizations. Transiently transfected HEK293T were stained with an anti-
calreticulin antibody (a–c and s–x) or with Mito Tracker Red (d–r). Green, GFP fluorescence or calreticulin (w); red, Mito Tracker
Red, calreticulin or mRFP (v); yellow, green and red overlap. Bars, 10 μm. (y) Schematic representation of US9 deletion mutants
localization. The SS, the SRSKESL sequence and the MLS are depicted by hatched, black and grey boxes, respectively. G, GFP.
appearance of mitochondria in RV798-infected cells was indistinguishable from that of mitochondria in AD-infected cells at each of the times tested (not shown), suggesting that the presence of the US2–US11 genes did not modify or participate in the disruption of mitochondrial networks mediated by vMIA. In addition, no alteration in mitochondrial morphology was observed in GFP–US9-expressing HEK293T, HeLa or HF cells as compared with neighbouring, non-expressing cells (results not shown), further confirming that US9 does not modify mitochondrial morphology.

**Presence of the US2–US11 genomic region confers protection from hygro-mediated apoptosis**

To assess whether US9 might influence the susceptibility of infected cells to cell death, HF cells infected with AD, RV798 or TB40/E were exposed to the extrinsic cell death pathway stimulator TNF-α, to the ER-stress-triggering compounds thapsi, tunica, bref and hygro, or to the mitochondrial-function-disrupting agents lonida and CCCP. While TB40/E encodes functional versions of the antiapoptotic proteins vMIA, viral inhibitor of caspase activation (vICA) (Skaletskaya et al., 2001) and pUL38 (Moorman et al., 2008; Terhune et al., 2007) and carries additional genes in the UL-b genomic region (Sinzger et al., 2008), AD encodes a nonfunctional vICA (Skaletskaya et al., 2001) and contains a longer UL-b’ region. Inclusion of all three strains thus allowed us to evaluate the contribution of the US2–US11 genes in protection from cell death in a vMIA+/pUL38+/vICA–genetic background (AD) and to compare the behaviour of AD to that of TB40/E, a virus carrying a more complete set of genes (Sinzger et al., 2008).

At 24 h p.i., mock-infected HF and HF infected with AD, RV798 or TB40/E (m.o.i of 5) were exposed to each drug or to their respective solvents for 24 h. The percentage of live drug-treated cells with respect to each solvent was calculated (Fig. 4 a and c) and the survival fitness of TB40/E or RV798 relative to AD was scored as the ratio between the percentage of surviving TB40/E- or RV798-infected cells and the percentage of surviving AD-infected cells (Fig. 4b and d).

Three different concentrations of TNF-α were used, and for each a 2:1 ratio with the concentration of Cx was maintained. The percentage of viable mock-, AD- and RV798-infected cells decreased as the concentration of TNF-α/Cx increased, whereas survival of TB40/E-infected cells remained close to 80% at each dose (Fig. 4a). While the RV798/AD ratios were close to 1 for each condition, the TB40/E/AD ratios showed a TNF-α/Cx dose-dependent increase from 1.3 ± 0.4 to 2.2 ± 1 (Fig. 4b), indicating that vICA, but none of the US2–US11 genes, contributed to protection from death following activation of the extrinsic pathway.

Cells infected with each virus also showed slight differences in their ability to survive death induced by lonida, with TB40/E/AD and RV798/AD ratios of 1.3 and 0.8, respectively (Fig. 4b). This suggested that expression of vICA and of one or more of the US2–US11 genes conferred a slight advantage in protection from apoptosis induced by the dissipation of the mitochondrial inner transmembrane potential. By contrast, RV798- and AD-infected HF
appeared similarly sensitive to death induced by CCCP. Survival of AD- and RV798-infected HF exposed to thapsi, tunica or bref was similar and slightly lower than survival of TB40/E-infected HF (Fig. 4c), indicating that none of the genomic differences in these strains contributed to survival from apoptosis following ER-stress. In contrast and quite surprisingly, RV798-infected HF were about 1.7-fold less protected than AD-infected HF when exposed to hygro (RV798/AD ratio = 0.62 ± 0.17, Fig. 4d), with statistically significant (P = 0.0028) differences scored for each virus in four independent experiments. This indicated that expression of one or more of the US2–US11 genes might protect infected cells from death induced by the accumulation of mistranslated proteins in the ER, but not from apoptosis triggered by other types of ER-stress.

While US3 expression is maximal at 4 h p.i. and substantially reduced thereafter, expression of US10 and US11, of US8 and US9, and of US6 and US7 peaks at 8, 24 and 48 h p.i., respectively (Jones et al., 1991; Tenney & Colberg-Poley, 1991; Weston, 1988). To assess whether addition of hygro at different times p.i. would alter the susceptibility of infected cells to apoptosis, AD- or RV798-infected HF were exposed to hygro from 24 to 48, 48 to 72 and 72 to 96 h p.i. and the number of surviving cells was counted. The RV798/AD average ratio values obtained for three separate wells in one experiment were remarkably consistent for each time point (0.65 at 24 h p.i., 0.72 at 48 h p.i. and 0.71 at 72 h p.i.), indicating that neither the decreased expression of US10 and US11 at early times nor the onset of US6 and US7 transcription at late times had a major effect on the ability of infected cells to survive hygro-induced death.

**Expression of GFP–US9, US9–GFP or US9 does not protect cells from hygro-induced apoptosis**

To establish whether US9 was directly responsible for protection from hygro-induced cell death, HEK293T cells expressing GFP, US9AD–GFP, GFP–US9AD and US9 were exposed to hygro for 16, 20 and 24 h, and the number of surviving cells was counted (Fig. 5). The total number of cells in each of the untreated samples increased progressively with time and in a similar way for each cell population, indicating that expression of US9 did not affect cell viability or proliferation rates any differently than expression of GFP. In contrast, the number of cells surviving exposure to hygro in each population remained fairly constant during the entire time-course. Cells expressing US9AD–GFP, GFP–US9AD and US9 did not appear to have any major survival advantage when compared with cells expressing GFP alone. To determine if these results were specific to HEK293T cells only, the number of retrovirus-transduced HF expressing GFP, US9AD–GFP, GFP–US9AD or US9 and surviving exposure to hygro for 24 and 48 h was counted. Transduced HF appeared to be equally susceptible to death irrespective of the protein expressed (results not shown), suggesting that

**DISCUSSION**

Canonical ER targeting occurs co-translationally and is mediated by N-terminal hydrophobic SS that are first recognized by the signal recognition particle (SRP), and subsequently cleaved during ER translocation of the nascent polypeptide. Two different groups have described US9 accumulation in the ER of epithelial cells (Huber et al., 2002; Maidji et al., 1998). Here we report ER localization of US9 in two additional epithelial cell lines (HEK293T and HeLa) and in cells of mesenchymal origin (HF), and we provide the first experimental evidence that amino acids 1–24 act as a SS for US9. Two SS-deletion mutants failed to reach the ER (Fig. 3a–c and g–i), indicating that recognition by the SRP is required for ER entry. SS cleavage usually occurs if a short-chain amino acid is present at position −1 and charged amino acids are absent at position −3 N-terminal of the cleavage site (Martoglio & Dobberstein, 1998). SignalP3.0 predicted the existence of two potential sites with the correct amino acid configuration for cleavage (VLS-S and SRS-K), suggesting that US9 SS is likely to be removed, yielding a luminal ER protein.

Quite interestingly, both SS-deleted mutants did not remain in the cytoplasm, but accumulated in mitochondria (Fig. 3d–f and g–i). Canonical mitochondrial matrix-targeted proteins possess cleavable N-terminal presequences that allow for post-translational targeting of the peptide to mitochondria (Kanaji et al., 2000; Young et al., 2003). GFP–US9AD, GFP–US9Tol and US9AD1–24–GFP, however, still reached the MOM, clearly demonstrating that US9 SS is not an N-terminal presequence.

**Fig. 5.** Survival of HEK293T cells expressing GFP, GFP–US9, US9–GFP or US9 after exposure to hygro for 16, 20 or 24 h. HEK293T cells transiently transfected with LGFP, LGFP GFP-US9AD, LGFP US9AD–GFP or with LNCX US9 were exposed to 800 µg hygro ml⁻¹ or to water (control) for the indicated times and the number of surviving cells was counted. Means ± sd of four independent experiments are shown.

US9 does not exert anti-apoptotic functions when expressed in either HEK293T or HF cells.
vMIA was shown to distribute to both the ER and mitochondria through an N-terminal bipartite signal consisting of four basic amino acids following the non-cleavable, strongly hydrophobic SS (Mavinakere & Colberg-Poley, 2004). US9 SS is also followed by three basic amino acids and we wondered if they might constitute a cryptic MLS. Deletion of the first 31 amino acids of US9, however, still resulted in mitochondrial accumulation of the truncated protein, implying that US9 MLS was not located in the N terminus of the protein.

TA proteins insert into the ER membrane or into the MOM via C-terminal hydrophobic domains, with the N terminus exposed to the cytosol. Proteins of this class do not contain an SS and are delivered to their final destination via post-translational mechanisms (Borgese et al., 1993, 2007; Kutay et al., 1993). The US9 C-terminal TMD possesses the typical hallmarks of MOM-resident TA proteins, and a mitochondrial localization for US9AD1–24 and US9AD1–31 was predicted by two subcellular localization prediction programs. Removal of the 49 C-terminal amino acids of US9 completely abolished mitochondrial accumulation, while their addition to the C terminus of GFP targeted it to the MOM (Fig. 3m–r). Thus, the presence of a C-terminal MLS that is both necessary and sufficient for MOM targeting indicates that US9 belongs to the family of mitochondrial TA proteins.

Discrimination of TA proteins between ER and mitochondrial membranes appears to rely in part on the amino acid composition of the C-terminal domain. As US9 does not contain a canonical ER retention motif and does not appear to be an ER-signal-anchored protein, we tested the possibility that the C-terminal anchor might act as an ER membrane insertion motif. A C terminus truncation mutant lacking the TMD but containing a functional SS, however, was still retained in the ER, demonstrating that the TMD is not required for ER targeting (Fig. 3v–x). Collectively, these data show that US9 may be the first member of a new class of TA proteins with dual localization to the ER and to the MOM.

Whether US9 is targeted to both intracellular locations during infection and if so, under which conditions, remains to be established. As the SS is the first part of the polypeptide to emerge from the ribosome, it is likely that the nascent protein will be initially cotranslationally translocated into the ER lumen. From here, US9 could then be delivered to mitochondria through the mitochondrion-associated membranes, as is the case for vMIA (Bozidis et al., 2008; Colberg-Poley et al., 2000). Unlike vMIA, US9 does not contain an N-terminal bipartite signal and may anchor to the MOM through the C-terminal MLS. This anchoring could be facilitated by the association with molecular chaperones, and we recently found that US9 can co-precipitate hsp90B (L. Hertel, unpublished).

Some Bcl-2 family members with dual targeting to the ER and mitochondria have recently been shown to participate in ER-to-mitochondria signalling following alterations in calcium homeostasis and in protein processing pathways (Hetz & Glimcher, 2008; Thomenius & Distelhorst, 2003). We therefore wondered if cells infected with RV798 could be more susceptible than AD- or TB40/E-infected cells to death triggered by different ER stress inducers. No statistically significant differences, however, were observed among the survival capabilities of cells infected with each virus and treated with thapsi, tunica and bref, indicating that neither the US2–US11 proteins nor viCA play important roles in preventing apoptosis triggered by ER-to-mitochondria signalling. The observed increased susceptibility of RV798-infected cells to hygro-mediated apoptosis was also not due to the absence of US9, as expression of US9 in HEK293T cells and in HF was insufficient to confer protection. Thus, it is possible that protection from hygro-mediated cell death is provided by a protein other than US9 encoded by the US2–US11 region.

Loss of inner mitochondrial potential and release of cytochrome c inevitably lead to cell death, unless these events are inhibited by the action of antiapoptotic proteins, such as Bcl-2 and Bcl-xL. Several viruses encode mitochondrial proteins that mimic the activity of Bcl-2. To test whether US9 might belong to this group, we treated infected cells with compounds that interfere with normal mitochondrial membrane functions. As expected, expression of viCA conferred protection against TNF-x-induced cell death. In contrast, the presence of the US2–US11 genes was irrelevant for protection, indicating that US9 is not a Bcl-2 functional homologue and that none of the US2–US11 proteins contribute to protection from mitochondria-mediated apoptosis.

In this study we identify US9 as the first CMV-encoded protein to contain an N-terminal SS and a C-terminal MLS. Both domains could function to direct US9 to specific intracellular compartments during infection, although the mechanisms mediating this process remain unknown. Despite a potential dual localization to the ER and mitochondria, US9 did not have anti-apoptotic functions in the cell types and conditions tested. It is possible that US9 has a role in survival of other cell types or in protection from specific stimuli not tested in our analyses, similar to the recently described role of CMV J2.7 RNA (Reeves et al., 2007). Alternatively, US9 might be involved in facilitating or disrupting the ER-to-mitochondria trafficking of specific cellular proteins, or in controlling some as yet unknown function involving both intracellular compartments. Efforts to determine US9 function are currently under way.

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