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Induction of apoptosis limits cytomegalovirus cross-species infection

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Cross-species infections are responsible for the majority of emerging and re-emerging viral diseases. However, little is known about the mechanisms that restrict viruses to a certain host species, and the factors viruses need to cross the species barrier and replicate in a different host. Cytomegaloviruses (CMVs) are representatives of the beta-herpesviruses that are highly species specific. They replicate only in cells of their own or a closely related species. In this study, the molecular mechanism underlying the cytomegalovirus species specificity was investigated. We show that infection of human cells with the murine cytomegalovirus (MCMV) triggers the intrinsic apoptosis pathway involving caspase-9 activation. MCMV can break the species barrier and replicate in human cells if apoptosis is blocked by Bcl-2 or a functionally analogous protein. A single gene of the human cytomegalovirus encoding a mitochondrial inhibitor of apoptosis is sufficient to allow MCMV replication in human cells. Moreover, the same principle facilitates replication of the rat cytomegalovirus in human cells. Thus, induction of apoptosis serves as an innate immune defense to inhibit cross-species infections of rodent CMVs.

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Introduction

Viruses have gone through a coevolution with their hosts, during which they have adapted to them. As a result of this adaptation, many viruses have a limited host range. Occasionally, a virus acquires a mutation or a new gene that allows it to infect and replicate in a different host species. This may lead to severe disease in the newly infected species, to a local outbreak, or even a pandemic (Weiss, 2003) such as the Spanish flu of 1918/19 (Taubenberger *et al*, 2005). Another example of a cross-species infection is the AIDS pandemic. In this case, simian immunodeficiency viruses have acquired the ability to replicate and spread in humans (Hahn *et al*, 2000).

The molecular mechanisms underlying the species barrier have only begun to be understood. In some instances, the availability of an appropriate entry receptor on the cell surface limits the virus' host range (reviewed in Baranowski *et al*, 2003). In other cases, the virus is able to enter cells of other species, but fails to block intracellular defense mechanisms that inhibit virus replication. It has recently been shown for the human immunodeficiency virus that the ability of the viral protein Vif to bind cellular cytidine deaminases limits its host range (Mariani *et al*, 2003). If not blocked by Vif, these enzymes cause a lethal hypermutation of the viral genome (Lecossier *et al*, 2003). More recent work has demonstrated that the species specificity of myxoma virus, a poxvirus of rabbits, is a consequence of the virus' inability to inhibit the interferon response in cells of foreign species (Wang *et al*, 2004). Myxoma virus can break the species barrier and replicate in murine cells if activation of the interferon response is blocked by inhibitory drugs or with interferon neutralizing antibodies. Similarly, the host range of certain paramyxoviruses and an attenuated vaccinia virus also depends on this innate immune defense (Parisien *et al*, 2002; Hornemann *et al*, 2003).

For the cytomegaloviruses (CMVs), the molecular basis of their species specificity has not been determined. These viruses belong to the β subfamily of the herpesviruses. Representatives of this subfamily have been identified in numerous animal species, and these viruses elicit similar illnesses in their respective hosts (Mocarski and Courcelle, 2001). Human cytomegalovirus (HCMV) is an opportunistic pathogen that causes generally mild infections in healthy people, but can cause severe disease in immunocompromised individuals such as transplant recipients or AIDS patients. Since their first isolation in cell culture, the CMVs have been recognized as highly species specific (Weller, 1970). They replicate only in cells of their own or a closely related species. For instance, simian CMV can replicate in human fibroblasts (Lafemina and Hayward, 1988), and HCMV can replicate in simian fibroblasts (Perot *et al*, 1992). Similarly, murine cytomegalovirus (MCMV) was shown to be infectious for rat cells (Bruggeman *et al*, 1982; Smith *et al*, 1986), but the rat cytomegalovirus did not replicate in murine fibroblasts (Bruggeman *et al*, 1982). However, cells of other more distant species are usually nonpermissive. A number of studies have shown that CMVs can enter cells of other species and express a subset of viral genes (Kim and Carp, 1972; Fioretti *et al*, 1973; Lafemina and Hayward, 1988; Garcia-Ramirez *et al*, 2001). This has led to the conclusion that the restriction to CMV replication in nonpermissive cells is associated with a postpenetration block to viral gene expression and DNA replication, but not due to a failure to enter the cell (Mocarski and Courcelle, 2001). However, the molecular mechanism for this intracellular block to viral replication has remained elusive.

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In this study, we show that MCMV infection of human cells activates programmed cell death (apoptosis). This prevents efficient viral DNA replication and expression of late genes. Inhibition of apoptosis by a Bcl-2-like protein of human CMV or a different virus is sufficient to allow replication of MCMV or the rat CMV in human cells.

Results

MCMV replicates in human 293 and 911 cells

Previous studies have shown that CMVs can enter cells of other species but do not replicate in them (Kim and Carp, 1972; Fioretti *et al*, 1973; Lafemina and Hayward, 1988). To analyze this phenomenon, we infected various human cell types with an MCMV expressing the green fluorescent protein (MCMV-GFP) at a low multiplicity of infection (MOI). MCMV-GFP infected human cells as indicated by GFP expression, but the infection did not spread from infected to uninfected neighboring cells, and the GFP expressing cells disappeared over time (not shown). Only in human embryonic kidney 293 and human embryonic retinoblast 911 cells, a cell-to-cell spread of infection was noticed: groups of green cells and occasionally even the formation of small plaques could be observed (Figure 1A and B). A growth kinetic experiment showed that human 293 and 911 cells can support MCMV replication, although virus release was delayed and reached lower titers as compared to permissive murine cells (Figure 1C). In primary human embryonic lung fibroblasts (MRC-5) and retinal pigment epithelial cells (RPE1) as well as in all other human cells analyzed, MCMV replication was not detected. RPE1 and MRC-5 cells were used for further analyses, because they are among the few cells that are permissive for HCMV. When infected at a low MOI, 293 and 911 cells did not release substantial amounts of virus into the supernatant (data not shown). Apparently, the cells infected at a low MOI produced enough virus to allow a limited spread to neighboring cells, but not enough to be detectable in the supernatant by plaque assay.

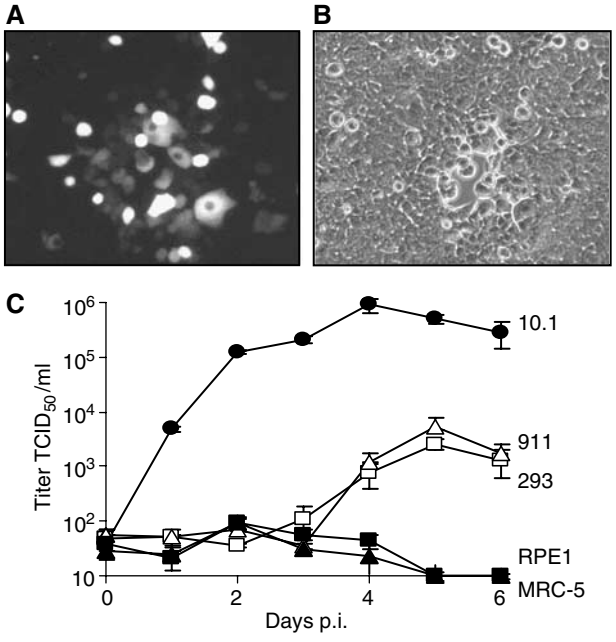

Figure 1 MCMV replication in human 293 and 911 cells. (**A**, **B**) Fluorescent and phase contrast images of 293 cells 6 days after infection with MCMV-GFP at a low MOI. (**C**) Growth kinetic of MCMV-GFP on murine 10.1 cells and human 293, 911, RPE1 and MRC-5 cells. Cells were infected at an MOI of 5 $TCID_{50}$/cell. Virus titers were determined in the supernatant.

Impaired viral gene expression and apoptosis of nonpermissive human cells

To identify a block in the cascade of viral gene expression as a possible underlying mechanism of the species barrier, the expression of an immediate-early (IE1), an early (E1), an early-late (M44) and a late (gB) gene was analyzed in permissive and nonpermissive cells (Figure 2A). In 293 cells, all kinetic classes of genes were expressed, and viral DNA was replicated, again with delay as compared to the murine cells. In the nonpermissive RPE1 cells, by contrast, expression of gB was not detectable, and DNA replication occurred only transiently. Starting at day 2 postinfection, massive cell death was observed: cells disintegrated and detached from the culture dish. The extent of cell death can be seen in a time course experiment that measured cell viability up to 120 h postinfection (Figure 2B). An apoptosis-specific assay showed that nonpermissive human cells entered apoptosis upon MCMV infection (Figure 2C). The DNA degradation associated with apoptosis probably accounts for the loss of viral DNA seen in RPE1 cells (Figure 2A). Interestingly, Kim and Carp (1972) had already observed that none of the human WI-38 fibroblasts used in their study survived an MCMV infection, but apparently this observation was not further investigated.

A viral bcl-2 homolog is sufficient to facilitate MCMV replication in human cells

Based on these observations, we asked which properties of 293 and 911 cells made them resistant to MCMV-induced apoptosis and permissive for MCMV replication. These two cell lines differ from all other human cells we have analyzed in that they were transformed with adenovirus type 5 DNA and express adenoviral E1A and E1B genes (Graham *et al*, 1977; Fallaux *et al*, 1996). The 293 cell line also contains sequences from the adenovirus E4 region, but the 911 cell line does not (Fallaux *et al*, 1996). E1A is a strong transactivator of gene expression and is proapoptotic. The E1B genes are antiapoptotic either by binding and sequestering p53 (E1B-55k) or by functioning as a viral Bcl-2-like protein (E1B-19k) (Shenk, 2001). Therefore, we transduced human RPE1 cells with retroviral vectors encoding E1B-55k or E1B-19k, in order to determine if one of these genes could make these cells permissive for MCMV replication. Transgene expression was tested by Western blot (Figure 3A) and immunofluorescence (not shown). E1B-55k had no effect on the permissivity of RPE1 cells, whereas E1B-19k inhibited MCMV-induced apoptosis and rendered RPE1 cells fully permissive for MCMV replication (Figure 3B and C). Even after low MOI infection, the virus spread across the whole monolayer and reached remarkably high titers (Figure 3D). Similarly, overexpression of the cellular bcl-2 or bcl-XL gene conferred permissivity, as did the HCMV UL37x1 gene, which encodes a mitochondria-localized inhibitor of apoptosis (vMIA), a protein with a function similar to Bcl-2 (Goldmacher *et al*, 1999; Arnoult *et al*, 2004) (Figure 3B and C). Thus, a single gene of HCMV can facilitate MCMV replication in human cells.

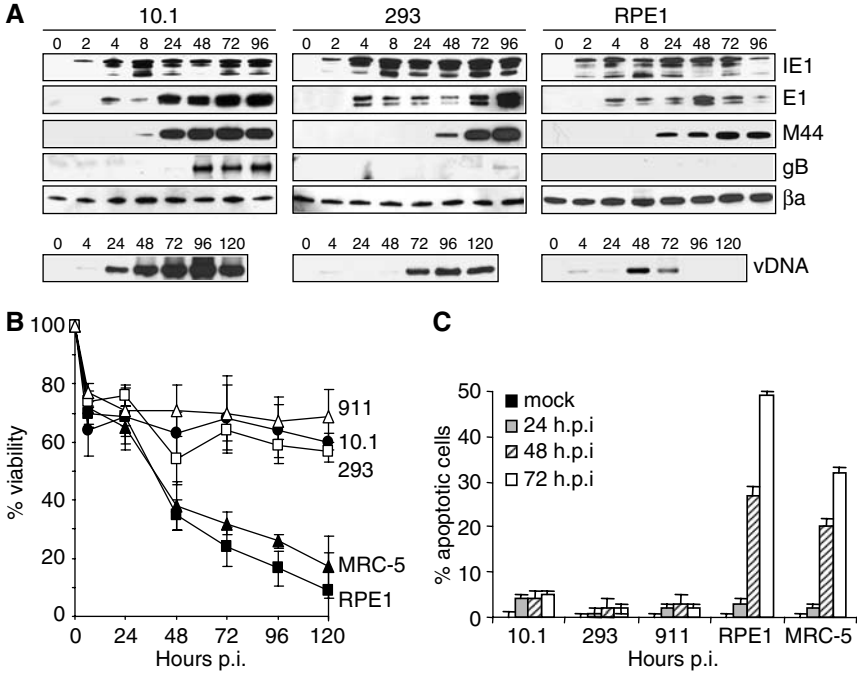

Figure 2 Impaired MCMV gene expression and apoptosis of nonpermissive human cells. (**A**) Permissive murine 10.1 cells, permissive human 293 cells, and nonpermissive human RPE1 cells were infected at an MOI of 5 $TCID_{50}$/cell. Viral immediate-early (IE1), early (E1), early-late (M44) and late (gB) gene expression was analyzed by Western blot at the indicated hours postinfection. Viral DNA (vDNA) was detected by Southern blot hybridization. Both attached and floating cells were collected for Western and Southern blot analyses. (**B**) Viability of cells infected at the same MOI was analyzed by MTT assay. (**C**) Nuclear DNA fragmentation as a sign of apoptosis was analyzed by TUNEL assay on adherent cells on coverslips. Since apoptotic cells tend to detach from the coverslips, the true percentage of apoptotic cells is higher than the percentage determined by TUNEL assay.

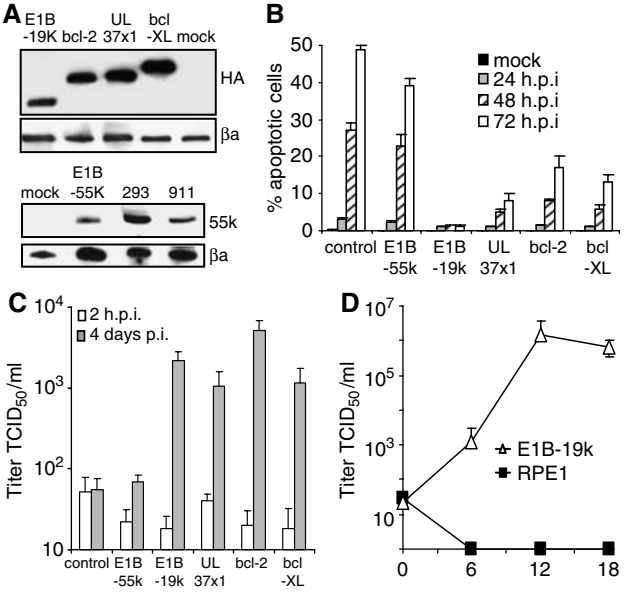

Figure 3 MCMV replication in RPE1 cells expressing E1B-19k. (**A**) Western blot of transduced RPE1 cells expressing HA-tagged E1B-19k, bcl-2, bcl-XL, UL37x1/vMIA, or untagged E1B-55k. Proteins were detected with an anti-HA or an anti-E1B-55k antibody, respectively. 293 and 911 cells served as positive controls for E1B-55k expression. βa, beta actin. (**B**) Apoptosis of transduced and control RPE1 cells after high-MOI infection with MCMV-GFP was determined by TUNEL assay as in Figure 2C. (**C**) Virus release after infection of RPE1 cells at an MOI of 5 $TCID_{50}$/cell. The 2 h value represents the residual input virus after infection and washing. (**D**) Virus release after infection of RPE1 cells expressing E1B-19k at an MOI of 0.2 $TCID_{50}$/cell.

If a bcl-2-like protein is sufficient to allow MCMV replication in cultured human cells, it should be possible to construct a recombinant MCMV that can replicate in human cells. To create space for the insertion of foreign genes, we deleted the region m02–m06 of the MCMV genome. This region contains well-characterized immune evasion genes that are necessary for MCMV virulence and pathogenicity in mice (Kleijnen *et al*, 1997; Reusch *et al*, 1999; Oliveira *et al*, 2002; Wagner *et al*, 2002). Therefore, the recombinant viruses should be nonvirulent in an immunocompetent host. A total of 12 different apoptosis-related genes and control genes, driven by a phosphoglycerate kinase (pgk) promoter, were inserted into the MCMV genome (Figure 4A and B). In addition to the adenoviral and bcl-2 family genes used in the previous experiments, a Bcl-2 homolog from Kaposi Sarcoma associated Herpesvirus (KSBcl-2) (Cheng *et al*, 1997) as well as the antiapoptotic genes crmA of cowpox virus (Miura *et al*, 1993; Tewari and Dixit, 1995), p35 of baculovirus (Xue and Horvitz, 1995), E3L of vaccinia virus (Garcia *et al*, 2002), and IE1 of HCMV (Zhu *et al*, 1995) were included. The recombinant viruses were tested for their ability to facilitate MCMV growth on human RPE1 and MRC-5 cells. Figure 4 shows that only MCMVs expressing a bcl-2-like gene were able to replicate in human cells, whereas MCMVs expressing viral genes that inhibit different checkpoints of apoptosis were not. Even after infection at a very low MOI, the recombinant viruses containing a bcl-2-like gene could form plaques on RPE1 cells (Figure 4E). The infection spread across the entire monolayer and could be passaged serially on RPE1 cells.

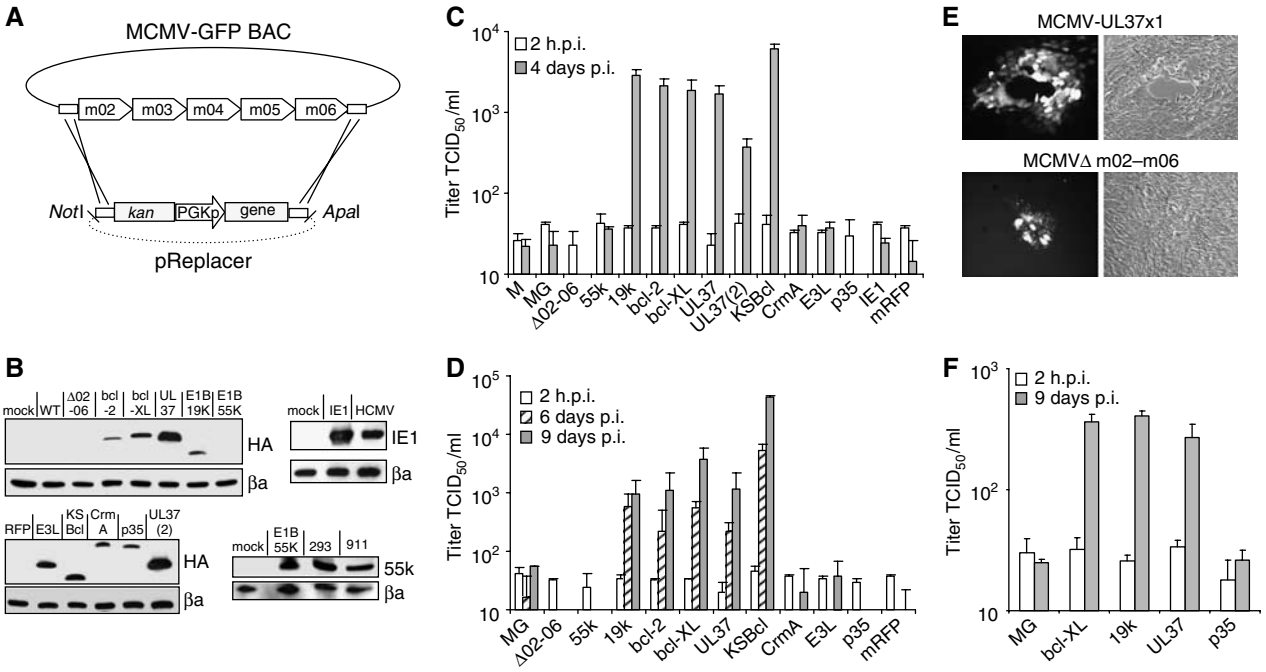

Figure 4 Growth of recombinant MCMVs in human cells. (**A**) Construction of recombinant MCMVs using the BAC technology. Inserted genes were driven by a murine pgk promoter (PGKp). (**B**) Expression of the inserted genes in cells infected with the recombinant viruses. HA-tagged proteins were detected with an anti-HA antibody. The HCMV IE1 protein and the E1B-55k protein were detected with specific antibodies. The wildtype MCMV-GFP (MG) and recombinant MCMVs carrying the monomeric red fluorescent protein (mRFP) gene or a deletion of m02–m06 only were used as controls. UL37(2) represents a recombinant virus, in which UL37x1/vMIA was inserted in place of the m152 gene. (**C**) Virus release after infection of RPE1 cells at an MOI of 5 $TCID_{50}$/cell. The 2 h values represent the residual input virus after infection and washing. M, MCMV wild-type virus. (**D**) Virus release after infection of RPE1 cells at an MOI of 0.2 $TCID_{50}$/cell. (**E**) Fluorescent and phase contrast image of RPE1 cells 8 days after a low-MOI infection with an MCMV expressing a bcl-2-like protein (MCMV-UL37x1) or a control virus (MCMVΔm02-m06). Efficient spread of the infection and plaque formation was only seen with MCMVs expressing a bcl-2-like protein. (**F**) Virus release after infection of MRC-5 cells at an MOI of 5 $TCID_{50}$/cell.

To test if the site of insertion is important, the UL37x1/vMIA gene was also inserted at a different location. It replaced the immune evasion gene m152 (Ziegler *et al*, 1997) and was driven by the endogenous m152 promoter. This recombinant virus also replicated in RPE1 cells, albeit to slightly lower titers (Figure 4C). This may reflect the fact that the m152 promoter is an early promoter and as such does not provide the gene product immediately after infection.

An MCMV mutant expressing the adenovirus E1A gene could not be generated. Murine fibroblasts transfected with the recombinant genome showed morphological signs of apoptosis, probably because E1A is proapoptotic (Shenk, 2001). The infection did not spread to neighboring cells, and thus infectious virus could not be obtained.

Activation of caspase-9 by MCMV infection

Bcl-2 and its homologs block apoptosis by inhibiting mitochondrial cytochrome *c* release and subsequent activation of caspase-9 (Kuwana and Newmeyer, 2003). Our observation that bcl-2-like proteins inhibit MCMV-induced apoptosis of human cells suggested that MCMV infection causes activation of caspase-9 and cleavage of downstream effectors such as caspase-3 and poly(ADP-ribose) polymerase (PARP). Indeed, caspases-9 and -3 and PARP were cleaved in MCMV-infected RPE1 cells (Figure 5A). Similarly, these molecules were cleaved in cells infected with an HCMV (strain AD169) in which UL37x1/vMIA was deleted. This is consistent with a previous study, which has found PARP cleavage in human fibroblasts infected with a UL37x1 mutant of AD169 (Reboredo *et al*, 2004). Caspase-9, caspase-3, and PARP were not activated when viral DNA replication was inhibited by phosphonoacetic acid (PAA), indicating that viral DNA replication or events after DNA replication were responsible for the induction of apoptosis (Figure 5A). MCMV-induced death of RPE1 was significantly reduced when broad-spectrum caspase inhibitors were added after infection (Figure 5B and C), supporting the notion that caspase-mediated cell death inhibited viral replication. zVAD-fmk at 100 μM concentration was the most effective inhibitor (Figure 5B and C). Unfortunately, Boc-D-fmk could not be used at this high concentration, because it turned out to be toxic for cells (not shown).

It has previously been shown that the cowpox virus CrmA (an inhibitor of caspase-1 and -8) and the baculovirus p35 protein (a substrate inhibitor of many caspases) can also inhibit caspase-9 in recombinant protein assays (reviewed in Ekert *et al*, 1999). However, later studies have shown that both, CrmA and p35, do not inhibit caspase-9-mediated cell death in living cells (Ryan *et al*, 2002). Nevertheless, we wanted to exclude the possibility that CrmA and p35 failed to facilitate MCMV replication (Figure 4), because the genes were inactivated or expressed at insufficient levels by the recombinant MCMVs. We also wondered, whether a recently discovered positional homolog of UL37x1 in MCMV, m38.5, might be expressed by MCMV at levels insufficient to inhibit MCMV-induced apoptosis of human cells. The m38.5 protein has a low-level sequence similarity to UL37x1/vMIA, localizes to mitochondria, and inhibits proteasome inhibitor-

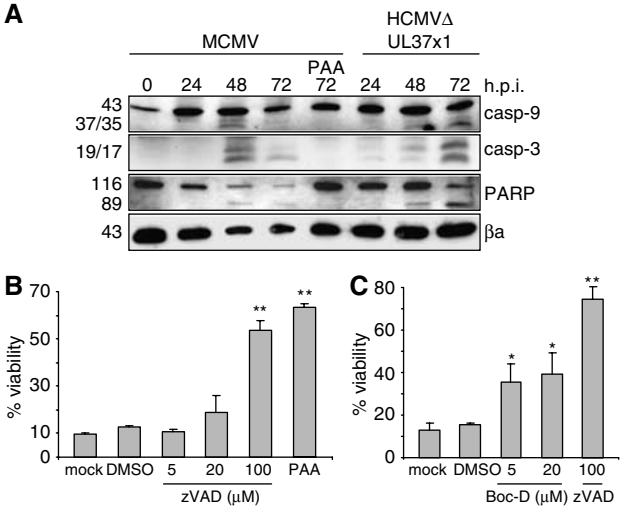

Figure 5 Caspase activation upon MCMV infection of human cells. (**A**) Human RPE1 cells were infected at an MOI of 5 $TCID_{50}$/cell. Cleavage (activation) of caspases 9 and 3 and PARP was detected at 48 and 72 h postinfection, but not in the presence of PAA. Similarly, an HCMV UL37x1 deletion mutant induced activation of these molecules. (**B, C**) Cell death triggered by MCMV infection of RPE1 cells was inhibited by broad-spectrum caspase inhibitors zVAD-fmk and Boc-D-fmk or by an inhibitor of viral DNA replication (PAA, 250 μg/ml). Significance levels were calculated using ANOVA. $*P<0.05$; $**P<0.001$.

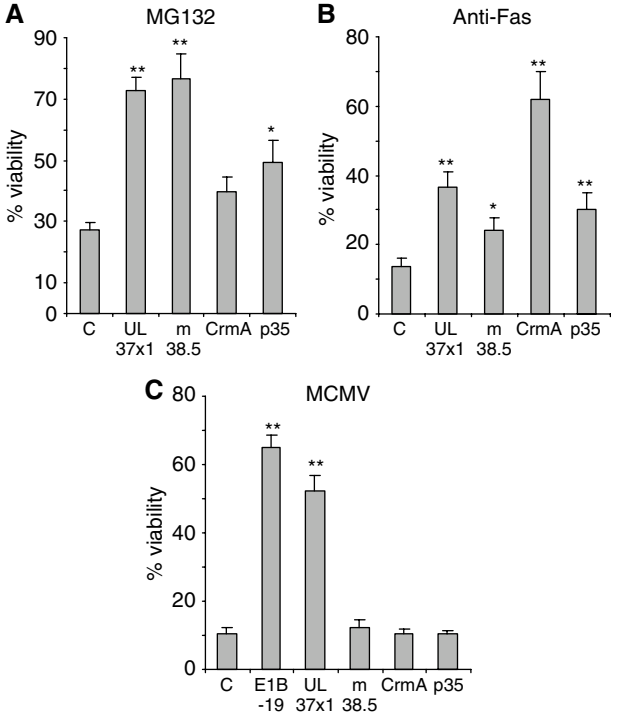

Figure 6 Transduced RPE1 cells stably expressing UL37x1/vMIA, m38.5, CrmA, or p35 were treated with (**A**) the proteasome-inhibitor MG-132 or (**B**) anti-Fas antibody plus cycloheximide, or (**C**) infected with MCMV to induce apoptosis. Cell viability was measured using an MTT assay. C, RPE1 control cells Significance levels were calculated using ANOVA. $*P<0.05$; $**P<0.001$.

mediated, but not Fas-mediated apoptosis (McCormick *et al*, 2005). However, its activity against virus-induced apoptosis and its role for MCMV replication have not been studied yet. To address these questions, we transduced RPE1 cells with retroviral vectors encoding CrmA, p35, or m38.5, respectively. Expression of the proteins was verified by Western blot (not shown). The cells were then tested for resistance against proteasome inhibitor- and Fas-mediated cell death (as carried out by McCormick *et al*, 2005) and against MCMV-induced apoptosis. Figure 6 shows that CrmA was a potent inhibitor of Fas-meditated apoptosis, whereas m38.5 protected cells from apoptosis induced by the proteasome inhibitor MG-132. The baculovirus p35 had only a moderate activity against Fas- and proteasome inhibitor-induced cell death. However, none of these three proteins protected from MCMV-induced cell death. This confirms the results obtained with the recombinant MCMVs (Figure 4). The inability of CrmA and p35 to inhibit MCMV-induced apoptosis is consistent with previous studies, which have shown that CrmA and p35 expressed by recombinant Sindbis viruses conferred only very little protection against Sindbis virus-induced neuronal cell death in a mouse model of apoptosis (Nava *et al*, 1998; Ryan *et al*, 2002). By contrast, Bcl-2 and a dominant-negative caspase-9 protected mice efficiently in the same system (Levine *et al*, 1996; Ryan *et al*, 2002). Apparently, the inhibitory activity of p35 against effector caspases (Ekert *et al*, 1999) is insufficient for blocking Sindbis virus- or cytomegalovirus-induced apoptosis.

Replication of RCMV in human cells

To analyze whether the requirement of a bcl-2-like gene is specific for MCMV or whether this principle is of more general importance, we infected RPE1 cells and RPE1 cells expressing E1B-19k with rat cytomegalovirus (RCMV, Maastricht strain). Like MCMV, RCMV also induced cell death in RPE1 cell, which could be suppressed by E1B-19k (Figure 7A). RCMV grew only in the presence of a bcl-2-like protein such as E1B-19k (Figure 7B). The amounts of virus released from RPE1-E1B-19k cells were relatively low, but the plaque formation seen in these cells after low-MOI infection clearly indicated virus replication and spread (Figure 7C). Taken together, the results suggest that the inability to inhibit apoptosis in human cells limits cross-species infections and represents an important determinant of MCMV's and RCMV's species specificity.

Discussion

Previous analyses of the CMV species specificity have indicated that the host cell restriction to CMV replication observed in nonpermissive cells is the result of a postpenetration block to viral gene expression and not of a failure to enter cells (Kim and Carp, 1972; Fioretti *et al*, 1973; Lafemina and Hayward, 1988). However, the nature of this block has remained elusive.

Inside the cell, viruses need to overcome several innate immune defenses of the cell in order to replicate and spread efficiently. These include toll-like receptor signaling, triggering of the interferon response, activation of cellular stress responses, and induction of apoptosis. Here we show that MCMV infection of human cells leads to activation of caspase-9 and induction of apoptosis. Antiapoptotic Bcl-2 family proteins can inhibit mitochondrial release of cytochrome *c* and subsequent activation of caspase-9. This study demonstrates that expression of such a protein inhibits apoptosis

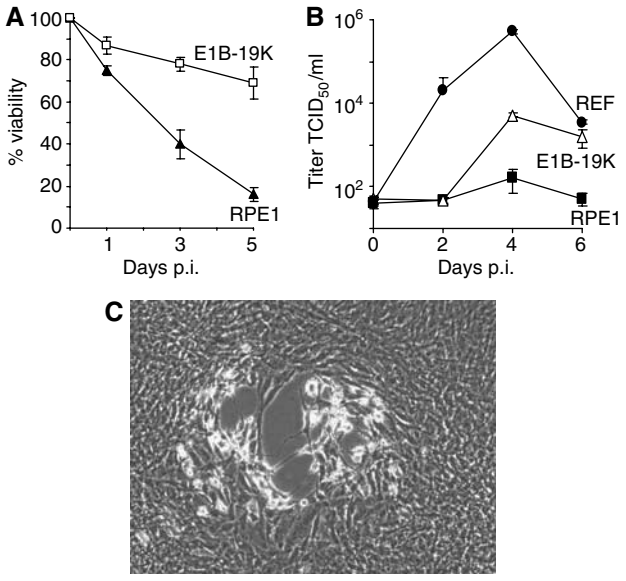

Figure 7 Growth of rat cytomegalovirus in human cells. (**A**) RPE1 cells died after infection with RCMV at an MOI of 5 $TCID_{50}$/cell, but RPE1 cells expressing E1B-19k were mostly protected from RCMV-induced cell death. (**B**) RPE1 cells, RPE1-E1B-19k cells, and rat embryo fibroblasts (REF) were infected with RCMV at an MOI of 5 $TCID_{50}$/cell, and titers in the supernatant were determined. (**C**) Phase contrast image of RPE1 cells expressing E1B-19k 7 days after low-MOI infection with RCMV. Plaque formation as an indication of virus replication and spread was only observed in RPE1-E1B-19k cells, but not in normal RPE1 cells.

induced by cytomegalovirus infection and allows MCMV replication in human cells. In this context, it should be noted that Bcl-2 and the HCMV vMIA protein can also inhibit caspase-independent cell death (Roumier *et al*, 2002). Therefore, we cannot exclude that this activity contributed to the inhibition of MCMV-induced apoptosis. However, the fact that two broad-spectrum caspase inhibitors, zVAD-fmk and Boc-D-fmk, were capable of inhibiting virus-induced cell death argues for an important role of caspases in this process. At high concentrations, zVAD-fmk can also inhibit calpains. These molecules are activated upon genotoxic stress and act upstream of caspases (Waterhouse *et al*, 1998). This suggests a possible role of calpains in MCMV-induced apoptosis and could explain why zVAD-fmk is a somewhat more potent inhibitor than Boc-D-fmk.

Our finding that the HCMV vMIA protein can facilitate MCMV replication in human cells is in agreement with previous results showing that this protein is required for efficient replication of the HCMV laboratory strain AD169*var*ATCC (Brune *et al*, 2003; Yu *et al*, 2003; Reboredo *et al*, 2004) and the clinical strain VR1814/FIX (S Hölzer and W Brune, unpublished). However, it was recently reported that vMIA is not required for efficient replication of the HCMV strain Towne, although a UL37x1 deletion mutant caused increased apoptosis of infected fibroblasts (McCormick *et al*, 2005). Since Towne and FIX both encode a functional copy of the inhibitor of caspase-8 activation (vICA), a gene product of UL36, whereas AD169 does not, the requirement of vMIA appears to be strain-dependent, but not dependent on the function of vICA.

Insertion of the UL37x1 gene into the MCMV genome is sufficient to facilitate MCMV growth in human cells. This leaves two possible explanations: either MCMV does not encode an analogous protein and does not need it for replication in murine cells, or the virus does encode such a protein, but it functions in a species-specific manner (i.e. in murine but not in human cells). A recent reevaluation of the MCMV genome sequence has identified a previously unrecognized ORF, m38.5, which is a positional homolog of UL37x1/vMIA and shows a low-level sequence similarity to this HCMV protein (McCormick *et al*, 2003). The m38.5 protein localizes to mitochondria and inhibits cell death induced by a proteasome inhibitor, but does not inhibit Fas-induced apoptosis of human HeLa cells like vMIA does (McCormick *et al*, 2005). Our results confirm this activity and show that m38.5 cannot block MCMV-induced cell death in human cells. It remains to be determined whether the m38.5 protein is responsible for inhibiting virus-induced apoptosis in murine cells, which would suggest a species-specific function. Another recent study has detected increased levels of the cellular antiapoptotic bcl-2 family protein Bfl-1/A1, but also of the proapoptotic protein Bim in MCMV-infected dendritic cells (Andoniou *et al*, 2004). Although the study did not resolve whether the increased Bfl-1/A1 levels were responsible for the observed resistance of infected cells against apoptotic stimuli, it points out the possibility that MCMV could compensate for a lack of a Bcl-2-like protein by upregulating a cellular antiapoptotic gene.

The present study shows that inhibition of apoptosis enables not only the murine but also the rat cytomegalovirus to cross the species barrier and replicate in human cells. This indicates that the mechanism identified is not unique to MCMV. It also raises the question, whether—conversely—HCMV triggers apoptosis upon infection of rodent cells. Preliminary data from our laboratory indicate that infection of murine NIH-3T3 and 10.1 fibroblasts with HCMV strains AD169 or FIX does not induce significant levels of apoptosis, even when the cells were infected at a high MOI. A possible explanation for this can be found in a previous study, which has shown that HCMV does not reach the stage of viral DNA replication in mouse cells (Lafemina and Hayward, 1988). This is consistent with the observation that an inhibitor of viral DNA replication prevents the induction of apoptosis (Reboredo *et al*, 2004; and this study). It further suggests that the species specificity of HCMV depends on an additional intracellular mechanism, which prevents the onset of DNA replication. To date, it is not known whether viral DNA replication itself induces apoptosis, or whether later processes are responsible: viral DNA replication can activate the DNA damage response, which is known to be proapoptotic (Smith and Mocarski, 2005; Sinclair *et al*, 2006). Subsequently, DNA-filled capsids have to leave the nucleus, which is associated with dissolution of the nuclear lamina (Muranyi *et al*, 2002). This process is also likely to trigger apoptosis, if it does not occur at an appropriate time during the cell cycle. Viral glycoproteins traveling through the ER and Golgi could cause 'ER stress'. This process, also known as 'unfolded protein response', has recently been shown to be activated during HCMV replication (Isler *et al*, 2005).

Considering the large size and the protracted replication cycle of the CMVs, it seems likely that these viruses have found ways to subvert many if not all aspects of innate immunity. CMV proteins inhibiting apoptosis (Zhu *et al*, 1995; Goldmacher *et al*, 1999; Brune *et al*, 2001, 2003; Skaletskaya *et al*, 2001; Ménard *et al*, 2003) and the interferon

response (reviewed in Hengel *et al*, 2005) have already been identified, and viral proteins blocking TLR signaling or the stress response are likely to follow. Analyses of viral inhibitors of the adaptive immune response encoded by HCMV and MCMV, for example, proteins downregulating MHC class I surface expression, have shown that some of the proteins do not function properly with target molecules of other species (Machold *et al*, 1997). Thus, it can be assumed that some of the viral inhibitors of the innate immune response will also operate in a species-specific manner. Inhibiting apoptosis of the infected cell is clearly a crucial task for the virus (Andoniou and Degli-Esposti, 2006) and can restrict the virus' cell tropism (Brune *et al*, 2001) and host range, as we show here. However, the fact that MCMV replicates slower and spreads less efficiently in human cells even in the presence of an antiapoptotic protein suggests that inhibition of apoptosis represents an important, but not the only limiting factor for efficient replication and spread.

We are only beginning to understand the molecular mechanisms underlying the species barrier of different viruses, and only few mechanisms have been identified to date. Failure to inhibit the interferon response was identified as a limiting factor for cross-species infections of certain paramyxo- and poxviruses (Parisien *et al*, 2002; Hornemann *et al*, 2003; Wang *et al*, 2004), which replicate in the cytoplasm. The present study shows that certain β-herpesviruses—large DNA viruses that replicate their genomes in the nucleus—induce apoptosis in cells of a foreign species, even though they can inhibit premature apoptosis in cells of their own species. A number of other viruses also depend on inhibition of apoptosis for normal replication, and consequently they encode potent cell death suppressors: Adenoviruses and γ-herpesviruses are two prominent examples (Pilder *et al*, 1984; White *et al*, 1984; Altmann and Hammerschmidt, 2005). It is possible that the species restriction of these viruses also depends (in part) on their ability to inhibit cell death in cells of a foreign species, even if in the end more than one mechanism should turn out to be involved, as it appears to be the case for human CMV.

Studies on viral species specificity teach us, how viruses counteract innate immune defenses, how these innate immune defenses operate, and how they differ from one species to another. These insights should lead to a better understanding of zoonotic infections in general.

Materials and methods

Cells

MRC-5 cells (ATCC CCL-171) are primary human embryonic lung fibroblasts (Jacobs *et al*, 1970). hTERT RPE1 cell (ATCC CRL-4000) are telomerase-immortalized retinal RPE1 (Bodnar *et al*, 1998). 293 cells (ATCC CRL-1573) are human embryonic kidney cells transformed with adenovirus 5 DNA (Graham *et al*, 1977). For the experiments in this study, the 293A subclone was used, which was selected for a flattened morphology (Invitrogen). 911 cells are human embryonic retinoblasts transformed with adenovirus 5 E1 genes (Fallaux *et al*, 1996). 10.1 and REF cells are spontaneously immortalized mouse and rat embryo fibroblasts, respectively (Burns *et al*, 1988; Harvey and Levine, 1991).

Plasmids and genes

The human bcl-2 and bcl-XL genes, adenovirus E1B-19k, cowpox virus crmA, baculovirus p35, and vaccinia virus E3L were cloned by PCR in pcDNA3 (Invitrogen), adding an HA tag to the 5′ end. Plasmids pcDNA-UL37x1HA and pcDNA-m38.5 contain the HCMV UL37x1 and the MCMV m38.5, respectively, tagged with an HA epitope at the 3′ end. pcDNA3-IE1 containing the HCMV IE1 gene was provided by Michael Nevels (University of Regensburg, Germany). Plasmid pRC-RSV-E1A containing the adenovirus E1A gene was provided by Thorsten Stiewe (University of Würzburg, Germany). The pSTK146 plasmid (Schiedner *et al*, 2000) containing the murine pgk promoter and the adenovirus 5 E1 region was provided by Stefan Kochanek (University of Ulm, Germany). The mRFP gene was excised from pRSET-mRFP1 (Campbell *et al*, 2002). The kanamycin resistance gene flanked by FRT sites was taken from pSLFRTkn (Atalay *et al*, 2002).

Retroviral transduction

The E1B-19k, E1B-55k, bcl-2, bcl-XL, and UL37x1 genes were inserted into the murine leukemia virus-based retroviral plasmid pLXSN (Clontech). Production of retroviral vectors using the Phoenix packaging cell line, and transduction of RPE1 cells was done as in previous studies (Brune *et al*, 2003). Transduced cells were selected with 700 μg/ml G418 and grown as bulk cultures without clonal selection.

Western blotting and immunofluorescence

For Western blot analysis, cells were lysed with lysis buffer containing 1% Triton X-100. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. For immunological detection, the following monoclonal antibodies were used: CROMA101 against MCMV IE1 and CROMA103 against E1 (both provided by Stipan Jonjic, University of Rijeka, Croatia), 2E8.21A against MCMV gB and 3B9.22A against M44 (both provided by Lambert Loh, University of Saskatchewan, Canada), 2A6 against E1B-55k and 1B12 against HCMV IE1 (both provided by Tom Shenk, Princeton University, USA). Antibodies against the HA epitope tag (16B12, Covance Research Products), β-actin (A5316, Sigma), caspases-3 and -9 (8G10 and 9502, Cell Signaling), and PARP (7D3–6, BD Biosciences) were purchased from suppliers as indicated. For immunofluorescence, cells were grown on coverslips, fixed with 3% paraformaldehyde, and permeabilized with 0.3% Triton X-100. Proteins were detected using the primary antibodies listed above and an AlexaFluor 594-coupled secondary antibody (Molecular Probes).

Viruses and growth kinetics

MCMV-GFP, a recombinant MCMV expressing the enhanced GFP, was constructed by Martin Messerle (Medical School Hannover, Germany) and has been used in previous studies (Brune *et al*, 2001, 2003). The Maastricht strain of RCMV (Bruggeman *et al*, 1982) was provided to us by Sebastian Voigt (Charité, Berlin, Germany). All recombinant viruses were constructed using bacterial artificial chromosome (BAC) technology (Brune *et al*, 2000) and are based on the MCMV-GFP BAC. Construction of these recombinant MCMVs has been approved by the Central Commision for Biological Safety (ZKBS) of the Federal Republic of Germany. To delete the immune evasion genes m02–m06 and insert foreign genes, a plasmid named pReplacer was constructed on the basis of pBluescriptII KS^+ (Stratagene). It contains 50 nucleotide homologies to sequences upstream of m02 and downstream of m06, a kanamycin resistance gene, a pgk promoter, and a multiple cloning site as shown in Figure 4A. Foreign genes were inserted using the multiple cloning site. The mutagenesis cassette can be excised from the backbone of pReplacer with restriction enzymes *Not*I or *Sac*II at the 5′ and *Apa*I or *Kpn*I at the 3′ end. The linear recombination substrates were used for homologous recombination in *Escherichia coli* strain DY380 containing the MCMV-GFP BAC as previously described (Brune *et al*, 2003). Recombinant MCMV genomes were analyzed by restriction digest and Southern blot. Wild-type and recombinant MCMV were grown on murine 10.1 fibroblasts essentially as described (Brune *et al*, 1999), and RCMV was propagated of REFs. Titrations were performed on the same cells using the median tissue culture infectious dose ($TCID_{50}$) method (Mahy and Kangro, 1996). For growth kinetics, cells were seeded in six-well plates and infected with MCMV at the indicated MOI. At 2 h after infection, cells were washed with PBS, and fresh medium was added. Medium was replaced at the indicated time points, and the content of virus in the supernatant was determined by titration. All growth kinetic experiments were performed in triplicate.

The HCMV mutant AD169ΔUL37x1 and the clinical HCMV strain VR1814 (FIX) have been described previously (Hahn *et al*, 2002; Brune *et al*, 2003).

Southern blot analysis

Adherent and floating (dead) cells were collected at the indicated time points, and DNA was extracted by standard procedures. Three micrograms of each DNA sample was digested with *Hin*dIII, separated on a 0.8% agarose gel, and transferred onto a nylon membrane. Hybridization with a digoxigenin-labeled probe directed against the GFP gene and chemiluminescent detection was performed using a DIG-High Prime DNA labeling and detection kit (Roche), according to the manufacturer's recommendations.

Apoptosis assays

Cell viability was determined by MTT assay, which measures mitochondrial activity, according to standard protocols. Briefly, cells were seeded in 96-well plates at 5×10^3 cells per well and treated with proapoptotic reagents or infected with MCMV. At appropriate time points after treatment, cells were incubated for 4 h with 100 µl medium containing 500 µg/ml 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The formazan crystals were solubilized with 100 µl of a 1:1 DMSO:ethanol mixture. The formazan concentration was measured at 570 nm using an ELISA plate reader. Every test was carried out with at least four replicates of each sample. Statistical analyses were carried out using the analysis of variance (ANOVA, F-test). To analyze nuclear DNA fragmentation as a late sign of apoptosis, cells were grown and infected on coverslips, fixed with 3% paraformaldehyde, and stained with a terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay kit (Roche). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). The percentage of apoptotic cells was determined by counting 500 cells in about 20 random visual fields.

For induction of apoptosis, cells were treated with 10 µM of the proteasome inhibitor MG-132 (Calbiochem) or 0.2 µg/ml anti-Fas antibody (clone 7C11, Coulter) and 10 µg/ml cycloheximide (AppliChem). To analyze caspase and PARP cleavage, cells were infected at an MOI of 5 $TCID_{50}$/ml, washed with PBS 2 h p.i., and harvested at the indicated time points. Lysates were prepared as described above, and proteins were detected by Western blot. The broad-spectrum caspase inhibitors zVAD-fmk and Boc-D-fmk were purchased from MBL International as 100 and 20 mM stock solutions in DMSO, respectively. RPE1 cells were infected at an MOI of 5. After 6 h, cells were washed and incubated with medium containing zVAD-fmk, Boc-D-fmk, DMSO, or PAA.

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