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Dominant-Negative FADD Rescues the In Vivo Fitness of a Cytomegalovirus Lacking an Antiapoptotic Viral Gene▽

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Genes that inhibit apoptosis have been described for many DNA viruses. Herpesviruses often contain even more than one gene to control cell death. Apoptosis inhibition by viral genes is postulated to contribute to viral fitness, although a formal proof is pending. To address this question, we studied the mouse cytomegalovirus (MCMV) protein M36, which binds to caspase-8 and blocks death receptor-induced apoptosis. The growth of MCMV recombinants lacking M36 (ΔM36) was attenuated in vitro and in vivo. In vitro, caspase inhibition by zVAD-fmk blocked apoptosis in ΔM36-infected macrophages and rescued the growth of the mutant. In vivo, ΔM36 infection foci in liver tissue contained significantly more apoptotic hepatocytes and Kupffer cells than did revertant virus foci, and apoptosis occurred during the early phase of virus replication prior to virion assembly. To further delineate the mode of M36 function, we replaced the M36 gene with a dominant-negative FADD (FADD^{DN}) in an MCMV recombinant. FADD^{DN} was expressed in cells infected with the recombinant and blocked the death-receptor pathway, replacing the antiapoptotic function of M36. Most importantly, FADD^{DN} rescued ΔM36 virus replication, both in vitro and in vivo. These findings have identified the biological role of M36 and define apoptosis inhibition as a key determinant of viral fitness.

Cell dying is a complex process that can occur through a number of different mechanisms, which are partially overlapping and redundant (18). Apoptosis was originally defined as programmed cell death, characterized by nuclear and cytoplasmic condensation, DNA fragmentation, and membrane blebbing (22). More recently, it has been proposed that caspase activation is the defining molecular feature of apoptotic cell death (24). Two apoptotic pathways have been identified, both leading to caspase activation and cell death. The cell-intrinsic mitochondrial pathway is regulated by the Bcl2 family of proteins and is activated by cell stress (42). The death receptor pathway responds to extracellular signals (41). In this pathway, effector cells of the immune system release, or express on their surface, defined ligands that bind to receptors on target cells and induce apoptosis in them (31). These receptors, jointly termed death receptors, include FAS, the tumor necrosis factor receptor p55 (TNFRp55), and the TNF-related apoptosis inducing ligand (TRAIL) receptor (TRAIL-R). The death domain in the cytoplasmic tail of death receptors recruits the adaptor protein FAS-associated via death domain (FADD), which in turn recruits caspase-8 to the death-inducing signaling complex (DISC), allowing caspase-8 to oligomerize and to induce death signal initiation.

Cell death upon viral infection limits the ability of viruses to replicate in cells. Accordingly, prevention of apoptosis should confer a survival advantage to viruses (19). Antiapoptotic viral genes have been identified in several unrelated dsDNA viruses, including adenoviruses (47), baculoviruses (12, 14), poxviruses (4), and herpesviruses (20), indicating that the inhibition of apoptosis is an important evolutionary principle adopted by viruses. The inhibition of apoptosis by the adenoviral gene E1B-19k or by the human cytomegalovirus (HCMV) gene UL37x1 has been shown to promote viral replication in cell culture (10, 36). It has also been proposed that apoptosis inhibition may be required for the immortalization of cells by Epstein-Barr virus (1). On the other hand, apoptosis inhibition lowers the virulence of Sindbis virus (39). Furthermore, reduced mouse cytomegalovirus (MCMV) growth was shown in mice lacking TRAIL receptors (15), indicating that death receptor signaling may promote virus replication indirectly through inhibition of innate immunity. Therefore, it is not predictable a priori if apoptosis inhibition promotes or inhibits virus replication in a host in vivo.

A number of antiapoptotic genes have been described in CMVs (2, 5, 6, 16, 17, 28, 29, 40), members of the betaherpesvirus family. HCMV is an important opportunistic pathogen (34) and codes for approximately 200 genes. Many among these genes are dedicated to the subversion of antiviral host immunity (30). Three genes have been described as antiapoptotic, namely, UL37x1 (17) (viral mitochondrion-localized inhibitor of apoptosis [vMIA]), the UL36 (40) gene (viral inhibitor of caspase 8 activation [vICA]), and the newly described UL38 gene (43). Functional analogues of UL36 and UL37x1 genes have been identified in primate and rodent betaherpesviruses (28), suggesting that the inhibition of apoptosis is an evolutionary conserved function in CMVs.

Due to strict species specificity, in vivo models of CMV pathogenesis rely on the infection of animals with their respective natural CMVs. The best characterized among these mod-

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els is the infection of mice with MCMV. Four genes in MCMV have been associated with apoptosis control, namely, M36 (29), M41 (6), M45 (5), and, more recently, M38.5 (27), the proposed homologue of HCMV UL37x1 or vMIA. Cells infected with MCMV mutants that lack any of these genes undergo apoptosis. Moreover, these mutants have been shown to be attenuated in vitro (5, 6, 29) and in vivo (11, 25).

M36 of MCMV is the structural and functional homologue of HCMV UL36 or vICA. Similar to UL36 (40), it interacts with pro-caspase-8 and protects cells from death-receptor-induced apoptosis (29). We have reported that MCMV mutants lacking a functional M36 gene exhibit a selective and cell-type-specific growth attenuation (29). Infection with ΔM36-MCMV (briefly ΔM36), a mutant in which M36 was inactivated, stimulates caspase-8 and induces apoptosis upon infection of macrophages but not fibroblasts (29). In addition, ΔM36 is unable to spread from an initial site of infection to distant organs (11). On the other hand, a rhesus CMV variant with a frameshift mutation in the UL36 gene homologue was still able to replicate in vivo (28).

Viral genes associated with immune evasion can have more than one function. This holds true for immune evasive MCMV genes as well, e.g., m152 (23) and m138 (26). Therefore, it remained open to question whether the previously observed reduced fitness of ΔM36 (11) was due to the lack of the anti-apoptotic function of M36. We approached this question by replacing the M36 gene with a dominant-negative mutant of FADD. This rescued both the apoptosis inhibition and the viral growth in vitro and in vivo. Therefore, we have here clearly identified the antiapoptotic function of M36 as an important and nonredundant function of the gene in vitro and in vivo.

MATERIALS AND METHODS

Cells. The IC-21 (TIB-186; ATCC) and M2-10B4 (CRL-1972; ATCC) cell lines were grown as described previously (29). ANA-I murine macrophages (13) were maintained in Dulbecco modified minimal essential medium supplemented with 10% fetal calf serum. BALB/c murine embryonic fibroblasts (MEFs) were prepared and maintained as described previously (35).

Viruses and viral mutagenesis: M36Rev-MCMV. To generate revertant virus M36Rev-MCMV (briefly M36Rev), the M36 gene was PCR amplified with the primers M36UP (GGTGCGGCCGCTTATCCGTTTTCCCTCTA) and M36DOWN (GGTACTAGTGAGGGACGCCTGCTCCGG) from the wild-type (WT)-MCMV BACmid (45) and inserted into pOriR6K-zeo (7) by using NotI and SpeI. The resulting plasmid was used as PCR template, along with the primers HM36Rfor (GCTCATTCTTTCGGGAAAGGGGTGGAGGAG GGTCGTTTGACAGTGAAAGGA) and HM36Rrev (TTTTTTCTCCCCTC ACCCTCTCCGTCCCTTTCTTATCCGTTTTCCCTTGCGGTATTTTCTC CTTACG) to generate a linear fragment for homologous recombination. The fragment was inserted into the ΔM36 BACmid (29) by single-step ET recombination (46) to generate the M36 revertant BACmid M36Rev. This mutagenesis reintroduced the M36 coding sequence into the ΔM36 BACmid at its native position together with a zeocin resistance marker between the stop codon of M36 exon 2 and the putative poly(A) signal of the M36 transcript, which was confirmed by NsiI fragment restriction analysis (data not shown).

ΔM36-FADD^{DN}-MCMV and WT-FADD^{DN}-MCMV. A XhoI/KpnNI digest of FADD^{DN}-pCDNA3 (9) (kindly provided by K. Rueckdeschl) was cloned into pOri6K-i.e., The resulting construct was used as a template in a PCR with primers 5-FADD (TTAATCATGACAATTATAAGTGTCTTATACGCAATA CTTTTATCATAATTAAATATTAACGCTTACAATTTACGC) and 3-FADD (GAGGAATAGGAATAACTCACCACCGATTTCAGCGTCTGCCCCAAG TCTGACTGCTGATCTTCAGATCCTCTAC) to generate a linear product that was inserted into either ΔM36 or WT-MCMV BACmids in the region between open reading frames m16 and m17 (8). The correct insertion of FADD^{DN} in the BACmids was confirmed by NsiI and DraI restriction fragment analysis (data not shown). All of the newly generated BACmids were transfected into MEFs to generate infectious viral progeny as previously described. ΔM36-MCMV (29) and BAC-derived WT-MCMV (45) have been described previously.

Mice. BALB/c mice were purchased from Harlan-Winkelmann or were bred at the central laboratory animal facility of the Johannes Gutenberg University, Mainz. C57BL/6/J, tumor necrosis factor (TNF) receptor-deficient (TNFRp55$^{-/-}$), FAS$^{-/-}$, and perforin-deficient mice were bred at the central breeding facility of the School of Medicine, Rijeka.

Flow cytometry. Infected macrophages were collected at day 1 postinfection by trypsin treatment, washed, and fixed for 20 min in phosphate-buffered saline (PBS) with 10% (vol/vol) of Cellfix (BD catalog no. 340181). This was followed by a wash in PBS and a 30-min quench in 50 mM NH_4Cl-PBS. Subsequently, the cells were permeabilized in 0.1% saponin-PBS, and nonspecific binding sites were blocked with purified rabbit anti-human immunoglobulin G (IgG) polyclonal sera (Dianova), which was followed by staining with fluorescein isothiocyanate-labeled anti-caspase-3$^{active\ form}$ (BD-clone C92-605) as marker of apoptosis, or fluorescein isothiocyanate-labeled anti-human-IgG polyclonal antibodies (Dianova) as isotype control. Cells were analyzed in a Coulter EPICS XL flow cytometer.

Two-color immunohistology. For simultaneous black staining of intranuclear viral IE1 (pp76/89) protein and red staining of cytoplasmic active caspase-3 in tissue sections, a combination of the avidin-biotin complex (ABC)-peroxidase-technique and a modified alkaline phosphatase anti-alkaline phosphatase method was applied in two-color immunohistochemical (IHC) analysis. In brief, tissue was embedded in paraffin, and 2-μm sections were processed for specific staining according to standard histological procedures, including pretreatment with trypsin (1.25 g/liter at 37°C for 15 min), blocking of endogenous peroxidases with hydrogen peroxide (0.5% [vol/vol] diluted in 1:1 methanol-PBS), blocking of unspecific binding to endogenous biotin (Dako biotin blocking system; Dako-Cytomation), and blocking of unspecific antibody-binding sites with goat serum (diluted 1:10 in Tris-buffered saline [TBS]). Sections were then incubated for 1 h with the mouse monoclonal antibody (MAb) CROMA 101 (diluted 1:250 in TBS) specific for IE1, followed by biotinylated goat anti-mouse IgG (Sigma; diluted 1:200 in TBS). Black staining was achieved with ABC-peroxidase (Vectastain Elite ABC kit; Vector Laboratories) using diaminobenzidine (Sigma) as a substrate and ammonium-nickel-sulfate hexahydrate for color enhancement. For labeling of active caspase-3, heat-induced epitope retrieval was performed for 5 min in a microwave oven in 10 mM trisodium citrate-dihydrate buffer (pH 6). After blocking of unspecific binding sites with goat serum, sections were incubated with affinity-purified polyclonal rabbit IgG directed against active caspase-3 (R&D Systems; 0.5-μg/μl stock diluted 1:50 in TBS) for 18 h at 20°C, followed by alkaline phosphatase-conjugated murine IgG1 MAb (clone RG-16 [Sigma]; diluted 1:25 in TBS) specific for rabbit-IgG. After incubation for 30 min at 20°C with the alkaline phosphatase anti-alkaline phosphatase soluble complex (Sigma; diluted 1:10 in TBS), red staining was achieved with a fuchsin substrate chromogen kit (Dako-Cytomation). Light counterstaining was performed with hematoxylin.

Infectious virus quantification. Organ homogenates or tissue culture supernatants (SN) were titrated on MEFs by plaque assay (37) with modifications described previously (11).

In vitro infection protocols. All macrophage cell lines were infected at a multiplicity of infection (MOI) of 1 with the MCMV recombinants indicated. The inoculum was removed at 1 h postinfection, and cells were supplied with fresh medium or with medium that was supplemented with 0.15% (vol/vol) of zVAD-fmk (20 nM) in dimethyl sulfoxide (30 μM final concentration) or an equivalent volume of dimethyl sulfoxide. Infectious virus was quantified in cell SN by MEF plaque assay. All experiments were repeated at least twice, yielding similar results.

In vivo infection protocols. Female mice were infected with purified, tissue culture-derived virus at 6 to 10 weeks of age and housed under specific-pathogen-free conditions. Organs were sterilely dissected and stored at −80°C until use for the plaque assay. All experiments were repeated at least twice, yielding similar results. Animal experiments were approved by the Ethics Committees of the University of Rijeka, the Department of Veterinary Medicine of the State of Bavaria (approval no. 211-2531-38/99) and the State of Rhineland-Palatinate (permission no. Az. 177-07/021-29).

Statistical analysis. The significance of the difference in virus titers or numbers of apoptotic cells between two experimental groups of mice infected with different viruses (independent sets of data with sample sizes $n = 1$ and $n = 2$) was evaluated by using distribution-free Wilcoxon-Mann-Whitney (rank sum) statistics. An online calculator was used (http://elegans.swmed.edu/~leon/stats/utest.html; Ivo Dinov, Statistics Online Computational Resources, UCLA Statistics, Los Angeles, CA). Samples are not significantly different if the P value is >0.05

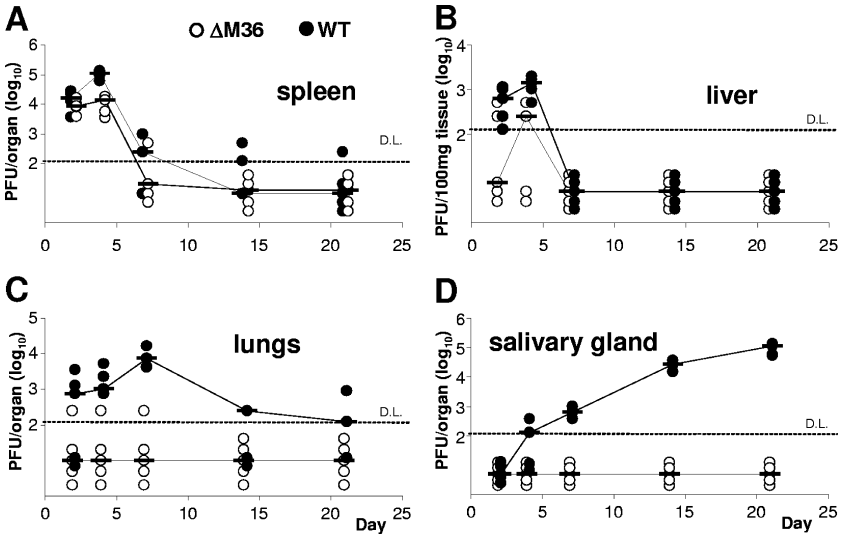


FIG. 1. ΔM36 is attenuated in vivo. BALB/c mice were infected i.p. with 5×10^4 PFU of ΔM36 (○) or WT-MCMV (●). Infectious virus in homogenates of spleen (A), liver (B), lungs (C), and salivary glands (D) was quantitated by plaque assay on days 2, 4, 7, 14, and 21 postinfection. Symbols represent individual mice, and horizontal bars marking the median values are connected. D.L., detection limit of the virus plaque assay.

(two-tailed test). For the analysis of correlation between two variables, data were arranged in 2-by-2 contingency tables, and the Fisher exact test was applied to calculate the *P* values for the null hypothesis of an independent distribution. An online calculator is available (http://home.clara.net/sisa/fisher.htm; Simple Interactive Statistical Analysis; Daan Uitenbroek, Hilversum, The Netherlands). Variables are considered to be not independent if $P < 0.01$ (two-tailed test).

RESULTS

Expression of M36 is a determinant of virus fitness in vivo. We have previously shown that ΔM36 is unable to spread to distant organs upon subcutaneous infection of immunosuppressed mice (11). To define the relevance of M36 for the virus replication in immunocompetent mice, we assessed ΔM36 growth kinetics in the spleens, livers, lungs, and salivary glands of intraperitoneally (i.p.) infected mice. ΔM36 was somewhat attenuated in the spleen (Fig. 1A), as well as in the liver (Fig. 1B), and more severely attenuated in the distantly located lungs (Fig. 1C) and salivary glands (Fig. 1D). These findings strongly suggested that M36 plays an important biological role in virus replication and/or dissemination.

M36 facilitates virus growth in macrophages by inhibiting apoptosis. MCMV mutants lacking a functional M36 gene are also attenuated in macrophages (29). To demonstrate that this attenuation was due to the lack of the viral gene M36, we generated the virus revertant M36Rev-MCMV (briefly M36Rev) by reinsertion of the M36 gene into the genome of ΔM36. Upon infection of IC-21 macrophages, M36Rev grew to WT-MCMV titers (Fig. 2A). This is strong evidence to conclude that the growth defect of ΔM36 in macrophages was indeed due to the lack of the M36 gene function. Likewise, rescue of M36Rev growth was observed also in vivo, as demonstrated for the lungs (Fig. 2B). It is worth noting that the growth deficiency phenotype of the mutant was completely reverted in virus M36Rev, as indicated by equivalent replication of WT-MCMV and M36Rev ($P = 1$; Wilcoxon-Mann-Whitney rank sum test, two-sided). In conclusion, the M36 gene product increases virus fitness not only in macrophages in vitro but also in vivo.

The growth deficit of ΔM36 in macrophages correlates with caspase-8 activation and apoptosis (29). It was not definitely clear, however, whether apoptosis inhibition is in fact the mechanism by which M36 promotes MCMV replication in macrophages. To test whether apoptosis inhibition rescues ΔM36 growth, we treated ΔM36-infected macrophages with zVAD-fmk, a pan-caspase inhibitor. The inhibition of apoptosis by zVAD-fmk was confirmed by flow cytometry specific for the active form of caspase-3 (not shown). Treatment with zVAD-fmk clearly rescued ΔM36 virus growth, resulting in virus titers comparable to WT-MCMV and M36Rev (Fig. 2C). This strongly suggested that M36 supports MCMV replication in macrophages by inhibiting caspase signaling.

Deletion of M36 enhances apoptosis in infected hepatocytes in situ at an early stage of the viral productive cycle. Hepatitis is a relevant organ manifestation of CMV disease in the immunocompromised host. Notably, ΔM36 is attenuated but can grow in vivo in hepatocytes, provided that it disseminates to the liver upon a systemic infection (Fig. 1B) or receives help from WT virus to disseminate to the liver after local coinfection (11). We therefore examined whether hepatocytes are exempt from apoptosis triggered by MCMV and do therefore not depend on M36 to complete the productive cycle. According to this hypothesis, virus replication in hepatocytes in situ should be resistant to a deletion of the M36 gene.

This question was experimentally addressed by a two-color IHC analysis for simultaneous detection of hepatocyte infection indicated by black staining of intranuclear viral IE1 protein pp76/89 and apoptosis of hepatocytes indicated by red staining of the active form of cytoplasmic effector caspase-3. Caspase-3 is an effector caspase, and in the apoptosis pathway it is located downstream of caspase-8. BALB/c mice were intravenously infected with WT-MCMV or ΔM36 (Fig. 3). To achieve a comparable degree of liver infection and viral histopathology, the mutant was administered in a 100-fold-higher dose to compensate for the dissemination deficiency. As shown

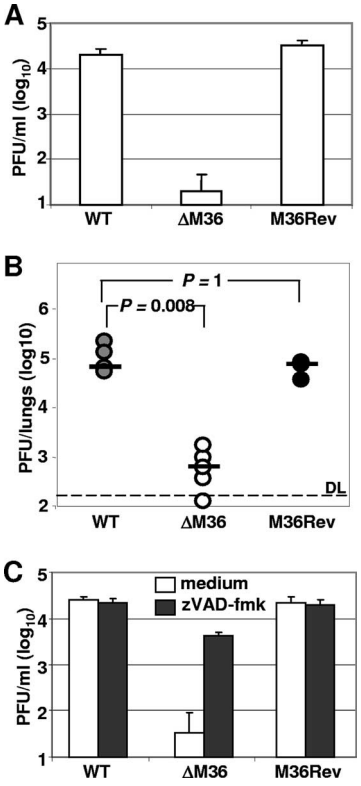


FIG. 2. M36 is required for MCMV replication in macrophages and in vivo. (A) Comparable growth of M36Rev and WT-MCMV in macrophages. Triplicate cultures of IC-21 macrophages were infected at an MOI of 1 with the viruses indicated. (B) Comparable growth of M36Rev and WT-MCMV in vivo. BALB/c mice were infected i.p. with 5×10^5 PFU of the indicated viruses. Infectious virus in lung homogenates was quantitated by plaque assay on day 5. Circles represent individual mice and horizontal bars mark the median values. DL, detection limit of the virus plaque assay. For group comparisons of interest, *P* values (Wilcoxon-Mann-Whitney rank sum test, two-tailed) are indicated. Differences are considered as significant and not significant for $P < 0.01$ and $P > 0.05$, respectively. (C) Caspase inhibition rescues ΔM36 growth. IC-21 macrophages were infected as described above. Cells were exposed to 30 μM zVAD-fmk (▨) or left untreated (□). Infectious virus in SN harvested on day 6 was titrated by plaque assay on MEFs. Histograms indicate mean values; error bars indicate standard deviations.

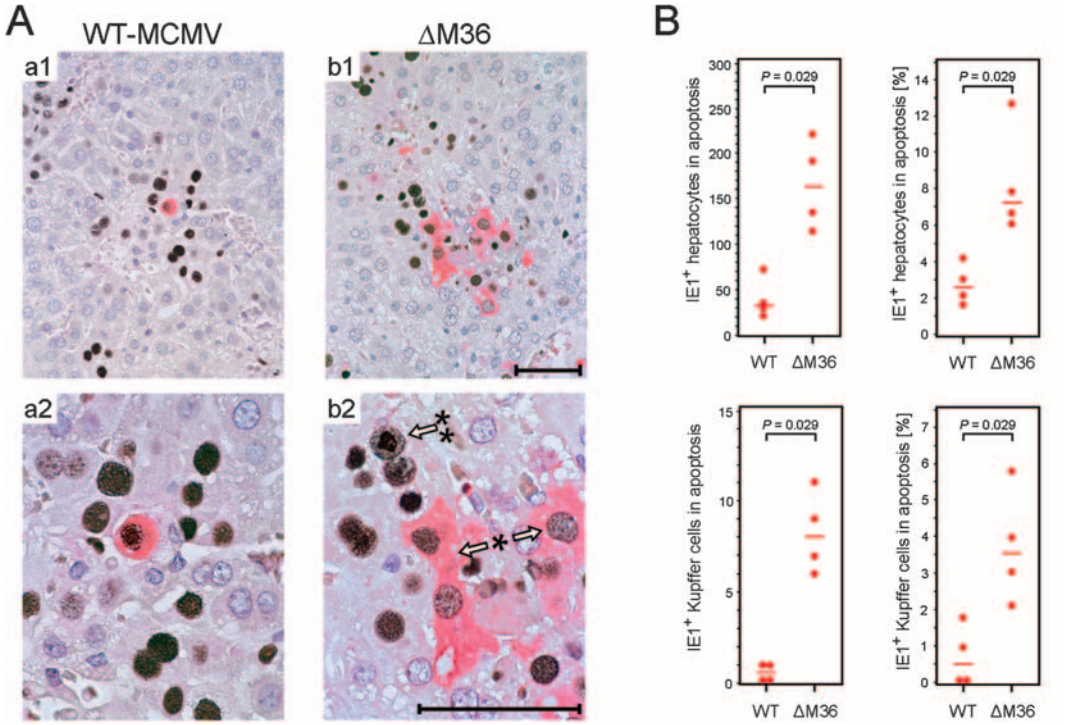
in Fig. 3Aa1 and a2, for the infection with WT-MCMV most infected hepatocytes expressing IE1, the first protein expressed by the virus after infection, were not apoptotic, but a few infected hepatocytes in the process of apoptosis were detected despite the expression of the antiapoptotic M36 gene. Note that apoptosis was not observed in uninfected hepatocytes, which shows that bystander apoptosis potentially triggered by death ligands induced during organ infection did not play a detectable role. In contrast and contrary to the original assumption, dual-stained apoptotic hepatocytes were more frequently detected within foci of infection in liver tissue infected with mutant ΔM36 (Fig. 3Ab1 and b2). The statistical significance of this finding was substantiated by quantitating $IE1^+caspase\text{-}3^+$ apoptotic cells in absolute terms per defined area of liver tissue sections and in relative terms in comparison to the overall number of infected $IE1^+$ hepatocytes (Fig. 3B). Specifically, 6 to 13% of hepatocytes infected with the mutant were found to be in apoptosis compared to only ca. 2 to 4% of hepatocytes infected with WT-MCMV. In accordance with the known phenotype of ΔM36 in macrophages in cell culture (Fig. 2C) (29), the rate of apoptosis was also found to be increased in ΔM36-infected $IE1^+$ Kupffer cells, the liver-resident macrophages (Fig. 3B). It is important to take into consideration that the true incidence of apoptosis was certainly higher, since cells already disintegrated after completion of the apoptotic process are no longer visible.

The proposed biological impact of antiapoptotic viral genes is to prolong the life span of an infected cell until the productive cycle is completed and progeny virions are released. Expression of M36 follows the early kinetics in tissue culture (data not shown), arguing that cells infected with the deletion mutant should enter apoptosis at an early stage of the productive cycle. On the other hand, one might also propose or suspect that cell death caused by a cytopathic virus is regularly associated with the activation of caspase-3. A two-color IHC analysis combined with statistical correlation analysis can solve this open question. In CMV infection of cells, the late stage of the productive cycle, is characterized by the presence of the typical intranuclear inclusion bodies representing the site of viral DNA packaging into nucleocapsids. Cells were classified into four groups according to the criteria of caspase-3 expression and expression of IE1 in the presence or absence of an inclusion body, and counted cell numbers were arranged in 2-by-2 contingency tables for correlation analysis using the Fisher exact probability test (Fig. 3C).

The result was astoundingly different for the WT and mutant. Whereas after infection with WT-MCMV, apoptosis and late stage of infection were found to be independent events ($P = 0.3515$), they were inversely correlated with very high significance ($P < 0.0001$) after infection with mutant ΔM36. Thus, in the absence of the antiapoptotic function of M36, apoptosis occurs at an early stage prior to nucleocapsid morphogenesis and the production of infectious virus. Obviously, M36 prevents apoptosis and enables the infection to proceed. However, the data also show that the requirement for M36 to prevent apoptosis is not absolute, since many hepatocytes infected with the deletion mutant reached the late stage without entering the apoptotic process despite the absence of M36, a finding that may be due to the redundance of antiapoptotic genes in MCMV (17–19, 25).

Altogether, although the requirement for M36 is less stringent in hepatocytes in vivo than for macrophages in cell culture (19), deletion of M36 clearly shows an apoptosis phenotype in the liver.

ΔM36 growth is not rescued in mice deficient for individual death receptor genes. The relevance of apoptosis inhibition by M36 for MCMV growth in vivo can only be defined by in vivo rescue experiments. Accordingly, rescue of ΔM36 replication in a genetically modified host devoid of essential apoptotic pathways would prove the relevance of apoptosis inhibition for virus in vivo replication. Unfortunately, mice lacking FADD, caspase-8, or caspase-3 cannot be used for such an analysis, since they die during gestation (44, 48–50). As an alternative approach, we therefore tested ΔM36 replication in mice devoid of individual death receptors. MCMV infection of liver tissue induces macrophages to secrete TNF-α, which contributes to cell death in foci of infection (33). Since TNF-α binding to



C

WT-MCMV

Observed	Late phase +	Late phase −	
Apoptosis +	1	26	27
Apoptosis −	133	1034	1167
	134	1060	1194

Expected	Late phase +	Late phase −	
Apoptosis +	3	24	27
Apoptosis −	131	1036	1167
	134	1060	1194

$P = 0.3515$

O = E

Random distribution

ΔM36

Observed	Late phase +	Late phase −	
Apoptosis +	4	123	127
Apoptosis −	420	1930	2350
	424	2053	2477

Expected	Late phase +	Late phase −	
Apoptosis +	22	105	127
Apoptosis −	402	1948	2350
	424	2053	2477

$P < 0.0001$

O < E

Inverse correlation

FIG. 3. Deletion of M36 increases the incidence of early apoptosis of infected hepatocytes in situ. BALB/c mice were immunocompromised by total-body gamma irradiation with a single dose of 7 Gy, and were infected intravenously (i.v.) with either 10^3 PFU of BAC-cloned WT-MCMV or with 10^5 PFU of mutant virus ΔM36. The analysis was performed on day 10. (A) Two-color IHC images of liver tissue sections with the intranuclear IE1 protein stained in black as a marker of infection and cytoplasmic active caspase-3 stained in red as a marker of apoptosis. (panels a1, overview; panel a2, detail), infection with WT-MCMV; (panels b1, overview; b2, detail), infection with ΔM36. ⋆, Arrows point to infected and apoptotic IE1$^+$Caspase-3$^+$ hepatocytes. Absence of an intranuclear inclusion body indicates an early stage of infection. ⋆⋆, The arrow points to the nucleus of an infected but nonapoptotic IE1$^+$Caspase-3$^-$ hepatocyte. The presence of an intranuclear inclusion body indicates a late phase of infection. Bars, 50 μm. (B) Quantitation of apoptotic IE1$^+$Caspase-3$^+$ hepatocytes (upper panels) and Kupffer cells (lower panels) in absolute terms for a representative tissue section area of 10 mm^2 (left panels) and relative to the total number of infected, IE1$^+$ hepatocytes and Kupffer cells, respectively (right panels). Dots represent data from four mice per group with the median value indicated. A P value of <0.05 (Wilcoxon-Mann-Whitney rank sum test, two-tailed) indicates a significant difference. (C) Correlation analysis relating apoptosis to the stage of infection. Infected, IE1$^+$ hepatocytes were counted for a total area of 10 mm^2 of liver tissue sections and were classified into four groups according to the expression of active caspase-3 (apoptosis +/−) and the presence of an intranuclear inclusion body (late phase +/−). The data were arranged in 2-by-2 contingency tables ("observed" tables) and compared to the tables expected for a random distribution ("expected" tables). The P values (two-tailed) for random distribution (null hypothesis) were calculated by using the Fisher exact test. The null hypothesis is rejected, and the variables are considered to be inversely correlated if the P value was <0.01 and if the number of double positives observed (O) is less than the number of double positives expected (E).

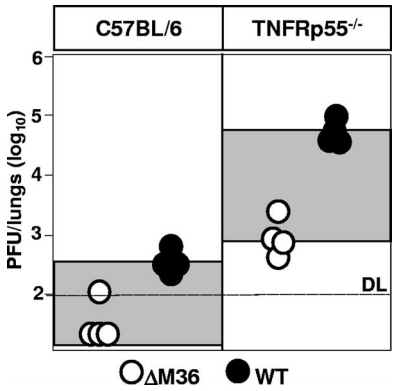


FIG. 4. ΔM36 growth is not rescued in TNFRp55$^{-/-}$ mice. TNFRp55$^{-/-}$ mice and mice of their parental strain C57BL/6 were i.v. infected with 10^6 PFU of either ΔM36 (○) or WT-MCMV (●). Virus in lung homogenates was quantified on days 3 by virus plaque assay. Circles represent individual mice. Differences between median values are highlighted by shading. DL, detection limit of the assay.

TNF receptor p55 results in caspase-8-dependent signaling and cell death, we assumed that ΔM36 growth might be restored in mice lacking the TNF receptor p55 (TNFRp55$^{-/-}$). To test this idea, we compared the growth of ΔM36 and WT-MCMV in C57BL/6 TNFRp55$^{-/-}$ mice, as well as in the parental C57BL/6 mouse strain. Because of the strong NK cell-mediated innate resistance against MCMV in mice with C57BL/6 background, infection was performed intravenously with a high virus dose. Notably, although both viruses replicated to higher titers in TNFRp55$^{-/-}$ mice, lack of TNFRp55 signaling did not rescue the growth of ΔM36 to WT levels (Fig. 4). Thus, TNF-α is not significantly implicated in the in vivo growth deficiency phenotype of ΔM36. Similar results were observed in mice deficient for other apoptosis receptors or ligands, such as FAS- and perforin-deficient mice. Although MCMV grew to higher titers in these mice compared to the respective parental mouse strain, the lack of FAS or perforin did not rescue ΔM36 growth to WT levels (data not shown). We interpreted these findings to mean that the effects of redundant proapoptotic stimuli received by a number of different death receptors could not be alleviated by the deletion of just one type of death receptor.

M36 inhibits the FADD-dependent death receptor pathway of apoptosis. Data obtained in vitro have indicated that M36 inhibits caspase-8 activation (29) and increases virus fitness by inhibiting target cell death (Fig. 2), and yet experiments in death-receptor-deficient mice apparently failed to identify a particular death receptor involved in triggering apoptosis. Apoptosis can be initiated through distinct pathways, the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. M36 and its HCMV homologue UL36 bind to caspase-8 (29, 40), an initiator caspase required for death-receptor-mediated apoptosis but not for intrinsic apoptosis. It was not excluded, however, that M36 additionally binds to other caspases and might thereby prevent intrinsic apoptosis as well. Moreover, viral proteins, such as the adenoviral E1A, are known to trigger apoptosis in a pathway that includes caspase-8 but not FADD or the upstream death receptors (32).

To test whether death receptor signaling is critically involved in cell death upon ΔM36 infection or whether any of the discussed alternative pathways applies, we constructed a mutant virus carrying a dominant-negative variant of the FADD gene (FADD^{DN}). Overexpression of FADD^{DN} in infected cells should specifically inhibit caspase-8 recruitment to the DISC and thus the extrinsic death receptor pathway, regardless of which death receptor is actually involved.

Using targeted BAC mutagenesis, we inserted FADD^{DN} under the control of the HCMV immediate-early promoter enhancer into the sequence between open reading frames m16 and m17 of the MCMV genome, a locus that was previously used for the expression of genes and shown to be dispensable for viral replication (8, 38). FADD^{DN} was introduced into the MCMV-BAC lacking the M36 gene and, for a control, also into the WT-MCMV BAC, resulting in the MCMV recombinants ΔM36-FADD^{DN} and WT-FADD^{DN}. FADD^{DN} gene insertion was controlled by BAC-DNA restriction enzyme cleavage patterns (data not shown) and expression of FADD^{DN} protein in infected fibroblasts was confirmed by Western blot analysis (data not shown). To test for FADD^{DN} functional relevance, IC-21 macrophages were infected with recombinant viruses ΔM36, M36Rev, ΔM36-FADD^{DN}, and WT-FADD^{DN} and assayed for apoptosis and viral growth. Notably, the active form of effector caspase-3, a marker of a late stage in apoptotic pathways, was found to be expressed only in cells infected with ΔM36, whereas the presence of M36 in M36Rev or of FADD^{DN} in ΔM36-FADD^{DN} equally prevented the activation of caspase-3 (Fig. 5A). Thus, FADD^{DN} compensated for the antiapoptotic function of M36 and prevented apoptosis in cells infected with ΔM36-FADD^{DN}. This finding clearly implies that alternative apoptotic pathways that would bypass FADD are not critically involved and that the apoptosis in ΔM36-infected cells occurs primarily through the extrinsic death receptor pathway.

To assess whether FADD^{DN} would also rescue the growth defect of ΔM36, IC-21 macrophages were infected with the indicated set of viruses, and virus production was measured. Expression of FADD^{DN} largely rescued the growth defect of ΔM36 (Fig. 5B), and a similar effect of FADD^{DN} on virus replication was observed also in ANA-I macrophages and in primary bone marrow macrophages derived from BALB/c and strain 129Sv mice (data not shown). Thus, the effects of FADD^{DN} on apoptosis and on virus replication are correlated, which supports the conclusion that inhibition of death receptor signaling is the mechanism by which FADD^{DN} rescues virus growth in macrophages.

FADD^{DN} rescues growth of ΔM36 in vivo. Combined, the data shown thus far have provided reasonable evidence to conclude that M36 increases virus fitness in vitro by inhibiting the FADD-dependent death receptor pathway. On the other hand, ΔM36 growth was not rescued in mice deficient for individual death receptors. Death receptor signaling converges downstream at the FADD level, but mice deficient in FADD, caspase-8, or caspase-3 cannot be tested since they die during gestation (44, 48–50). We surmised, however, that the infection of mice with viruses expressing FADD^{DN} should inhibit the death receptor pathway at sites of virus infection in vivo independent of the particular death receptor involved.

Accordingly, we compared the replication of ΔM36-FADD^{DN} and WT-FADD^{DN} in important target organs, namely, the lungs and the liver, of infected mice (Fig. 6A). While in the control groups not expressing FADD^{DN} the attenuating effect

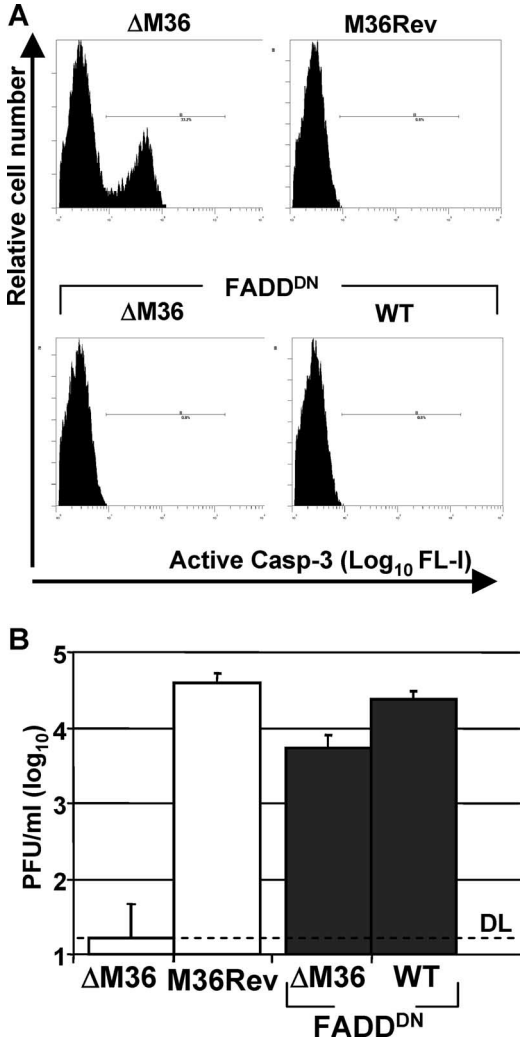


FIG. 5. Dominant-negative FADD reverses the ΔM36 phenotype in macrophage cell culture. (A) FADD^{DN} inhibits apoptosis in ΔM36-infected macrophages. IC-21 macrophages were infected with the indicated viruses at an MOI of 1. At 24 h after infection, apoptotic macrophages were quantitated by cytofluorometric analysis using the expression of active caspase-3 (abscissa) as a marker of apoptosis. FL-I, fluorescence intensity (B) FADD^{DN} rescues the growth of ΔM36 in macrophages. IC-21 macrophages were infected at an MOI of 1 with the indicated viruses devoid of FADD^{DN} (□) or encoding FADD^{DN} (■). Virus in SN was quantitated on day 5 after infection. Histograms represent mean values from three independent experiments. Error bars indicate standard deviations. DL, detection limit of the virus plaque assay.

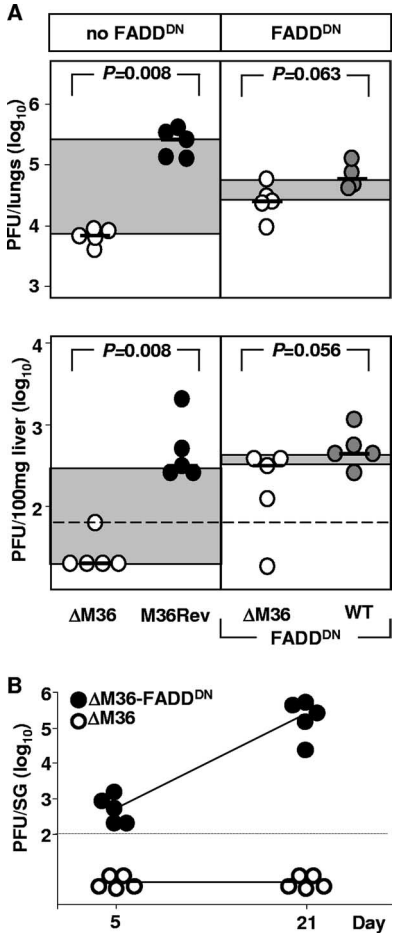


FIG. 6. Dominant-negative FADD reverses the ΔM36 phenotype in host organs. (A) BALB/c mice were infected i.v. with 3×10^5 PFU of MCMV expressing FADD^{DN} (ΔM36-FADD^{DN} or WT-FADD^{DN}) and, for a control, with the corresponding viruses not encoding FADD^{DN}. The absence of M36 is symbolized by open circles, and the presence of M36 in M36Rev and WT-MCMV (WT) is symbolized by black-filled and gray-filled circles, respectively. The amount of infectious virus in the lungs (upper panel) and liver (lower panel) was determined on day 5 after infection. Circles represent data from individual mice. Differences in median values are highlighted by gray shading. The dashed line indicates the detection limit of the virus plaque assay for liver homogenate. For group comparisons of interest, the *P* values (Wilcoxon-Mann-Whitney rank sum test, two-tailed) are indicated. Differences are considered significant and not significant for $P < 0.01$ and $P > 0.05$, respectively. (B) BALB/c mice were infected i.p. with 5×10^5 PFU of ΔM36 (○) or ΔM36-FADD^{DN} (●). Virus titers are represented on the *y* axis; times after infection are represented on the *x* axis. Infectious virus in salivary glands (SG) was determined on days 5 and 21 after infection. Circles represent data from individual mice. Median values are connected with solid lines.

of M36 deletion was again apparent from a significantly reduced replication of mutant ΔM36 in both organs ($P < 0.01$), FADD^{DN} expression largely reversed the M36 deletion phenotype, as can be concluded from similar, not significantly different ($P > 0.05$) replication of viruses ΔM36-FADD^{DN} and WT-FADD^{DN} in the lungs and liver. A comparison of M36Rev and WT-FADD^{DN} titers may suggest a mild attenuating effect of FADD^{DN} insertion on virus replication, but this effect was only marginally significant in the lungs ($P = 0.032$) and absent in the liver. Similarly, we observed that FADD^{DN} expression resulted in rescued virus replication in the salivary glands of mice at 5 and 21 days after infection (Fig. 6B).

In conclusion, M36 inhibits apoptosis in infected cells through interference with the FADD-dependent death receptor pathway. The inhibition is operative at an early stage of the viral replicative cycle, allows the infection to proceed to the release of progeny virions, and thus contributes to virus fitness in vitro and, most relevantly, also in tissues of the infected host.

DISCUSSION

Many viruses have developed genes encoding proteins with cell death suppressor functions to preclude premature cell death for completion of their productive cycle. Although previous reports have shown that apoptosis inhibition by adenovirus or herpesvirus genes promotes virus replication in cell culture (10, 36), a proposed role in vivo had remained speculative. Here we provide firm experimental evidence for the impact of a viral protein controlling extrinsic cell death on virus fitness at the sites of viral disease manifestations.

To achieve this, we developed a novel genetic approach. We compensated for the deletion of an antiapoptotic viral gene by the insertion of a dominant-negative mutant of a cellular key gene of receptor-mediated apoptosis into the viral genome. The dominant-negative mutant $FADD^{DN}$ consists only of the FADD death domain that binds to death receptors but cannot recruit caspase-8, thus inhibiting selectively the death receptor mediated pathway of apoptosis. Specifically, we document that the in vivo fitness of an MCMV mutant lacking the antiapoptotic gene M36 is rescued by insertion of $FADD^{DN}$ into the viral genome.

CMVs encode several antiapoptotic proteins and can inhibit cell death at multiple check-points (16). By using deletion mutants of antiapoptotic genes, apoptosis control has been shown to be an important in vitro determinant of host cell tropism and host species specificity of CMVs, restricting replication to defined cell types of the authentic host (5, 29) and preventing replication in cells of a heterologous host species (21).

Although apoptosis inhibition by M36 was originally defined as a determinant of macrophage tropism in vitro (29), the histological data presented here clearly document an in situ apoptosis phenotype of mutant virus ΔM36 not only in liver macrophages but also in hepatocytes, which underlines the importance of in vivo studies for conclusions on the role of viral genes in cell type tropism and viral replication fitness. The histological studies documented here provide compelling evidence that protection against death-receptor-mediated apoptosis is important in the early stages of virus replication, prior to virion assembly. In vitro studies in fibroblasts have shown that the replication cycle of MCMV takes about a day (30). That this time scale applies also to virus replication in vivo has recently been shown for endothelial cells and hepatocytes by cre-mediated conditional in vivo recombination and tracking of the spread of the recombined green fluorescent protein-expressing MCMV (U. H. Koszinowski, unpublished data). This protracted replication cycle makes CMV vulnerable to proapoptotic conditions in the host tissue environment, leading to premature target cell death.

However, apoptosis upon virus infection is not necessarily to the benefit of the host. The introduction of antiapoptotic genes into Sindbis virus, an RNA virus that does not naturally inhibit apoptosis, was found to decrease the virulence of the respective virus recombinants (39). Moreover, FAS signaling is a major contributor to immune tolerance (3), and knockout mice lacking the death receptor TRAIL can control MCMV replication better than the parental mouse strain, apparently because TRAIL signaling suppresses Toll-like receptor-mediated immune activation (15). Therefore, it is quite conceivable that optimal viral replication requires a balance between apoptosis and apoptosis inhibition.

We have seen that $FADD^{DN}$ did largely, albeit not completely, rescue virus growth in the absence of the M36 gene, although it completely abrogated the apoptosis phenotype of mutant virus ΔM36. It is open to question whether this was due to unknown additional functions that may be encoded in the viral genomic region deleted in ΔM36, positional effects that the $FADD^{DN}$ insertion may exert on neighboring genes (33), direct adverse effects of $FADD^{DN}$ expression on virus replication, or differences in M36 and $FADD^{DN}$ expression kinetics. Notably, in comparison to the replication of virus M36Rev, virus WT-$FADD^{DN}$ appeared to be moderately attenuated, at least in the lungs (recall Fig. 6), indicating that $FADD^{DN}$ insertion inhibits MCMV replication to some extent.

Here we have proven that a dominant-negative mutant of FADD, a molecular junction in the death-receptor-mediated pathway of apoptosis, can functionally replace a viral antiapoptotic gene. This novel approach has enabled us to define the biological function of gene M36 and has validated an impact of antiapoptotic viral genes on virus fitness in vivo. CMV functions influence a wide number of cellular functions and vice versa, and the analysis of the mechanistic principles of virus-host interaction continues to represent a challenge to research. Four genes of MCMV have thus far been associated with apoptosis control. As documented herein, the lack of only one of them already shows a strong phenotype in vivo. This indicates that death suppression by CMV proteins does not operate as a redundant network but rather within specific signaling pathways and with differential efficacies under conditions that can only be revealed by in vivo studies. Future work will focus on the contribution of other antiapoptotic genes and on the host-defined environments in which they exert their function. The usage of dominant-negative genes expressed by virus recombinants will likely be useful for studying further aspects of virus-host interactions.

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REFERENCES

1. **Altmann, M., and W. Hammerschmidt.** 2005. Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. PLoS Biol. **3:**e404.
2. **Andoniou, C. E., and M. A. Degli-Esposti.** 2006. Insights into the mechanisms of CMV-mediated interference with cellular apoptosis. Immunol. Cell Biol. **84:**99–106.
3. **Askenasy, N., E. S. Yolcu, I. Yaniv, and H. Shirwan.** 2005. Induction of tolerance using Fas ligand: a double-edged immunomodulator. Blood **105:** 1396–1404.
4. **Bertin, J., R. C. Armstrong, S. Ottilie, D. A. Martin, Y. Wang, S. Banks, G. H. Wang, T. G. Senkevich, E. S. Alnemri, B. Moss, M. J. Lenardo, K. J. Tomaselli, and J. I. Cohen.** 1997. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc. Natl. Acad. Sci. USA **94:**1172–1176.
5. **Brune, W., C. Menard, J. Heesemann, and U. H. Koszinowski.** 2001. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science **291:**303–305.
6. **Brune, W., M. Nevels, and T. Shenk.** 2003. Murine cytomegalovirus m41

open reading frame encodes a Golgi-localized antiapoptotic protein. J. Virol. **77:**11633–11643.
7. **Bubeck, A., M. Wagner, Z. Ruzsics, M. Lotzerich, M. Iglesias, I. R. Singh, and U. H. Koszinowski.** 2004. Comprehensive mutational analysis of a herpesvirus gene in the viral genome context reveals a region essential for virus replication. J. Virol. **78:**8026–8035.
8. **Bubic, I., M. Wagner, A. Krmpotic, T. Saulig, S. Kim, W. M. Yokoyama, S. Jonjic, and U. H. Koszinowski.** 2004. Gain of virulence caused by loss of a gene in murine cytomegalovirus. J. Virol. **78:**7536–7544.
9. **Chinnaiyan, A. M., C. G. Tepper, M. F. Seldin, K. O'Rourke, F. C. Kischkel, S. Hellbardt, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem. **271:**4961–4965.
10. **Chiou, S. K., and E. White.** 1998. Inhibition of ICE-like proteases inhibits apoptosis and increases virus production during adenovirus infection. Virology **244:**108–118.
11. **Cicin-Sain, L., J. Podlech, M. Messerle, M. J. Reddehase, and U. H. Koszinowski.** 2005. Frequent coinfection of cells explains functional in vivo complementation between cytomegalovirus variants in the multiply infected host. J. Virol. **79:**9492–9502.
12. **Clem, R. J., M. Fechheimer, and L. K. Miller.** 1991. Prevention of apoptosis by a baculovirus gene during infection of insect cells. Science **254:**1388–1390.
13. **Cox, G. W., B. J. Mathieson, L. Gandino, E. Blasi, D. Radzioch, and L. Varesio.** 1989. Heterogeneity of hematopoietic cells immortalized by v-myc/v-raf recombinant retrovirus infection of bone marrow or fetal liver. J. Natl. Cancer Inst. **81:**1492–1496.
14. **Crook, N. E., R. J. Clem, and L. K. Miller.** 1993. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J. Virol. **67:**2168–2174.
15. **Diehl, G. E., H. H. Yue, K. Hsieh, A. A. Kuang, M. Ho, L. A. Morici, L. L. Lenz, D. Cado, L. W. Riley, and A. Winoto.** 2004. TRAIL-R as a negative regulator of innate immune cell responses. Immunity **21:**877–889.
16. **Goldmacher, V. S.** 2005. Cell death suppression by cytomegaloviruses. Apoptosis **10:**251–265.
17. **Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden.** 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc. Natl. Acad. Sci. USA **96:**12536–12541.
18. **Golstein, P., and G. Kroemer.** 2005. Redundant cell death mechanisms as relics and backups. Cell Death. Differ. **12**(Suppl. 2)**:**1490–1496.
19. **Gregory, C. D., C. Dive, S. Henderson, C. A. Smith, G. T. Williams, J. Gordon, and A. B. Rickinson.** 1991. Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature **349:**612–614.
20. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65:**1107–1115.
21. **Jurak, I., and W. Brune.** 2006. Induction of apoptosis limits cytomegalovirus cross-species infection. EMBO J. **25:**2634–2642.
22. **Kerr, J. F., A. H. Wyllie, and A. R. Currie.** 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer **26:**239–257.
23. **Krmpotic, A., D. H. Busch, I. Bubic, F. Gebhardt, H. Hengel, M. Hasan, A. A. Scalzo, U. H. Koszinowski, and S. Jonjic.** 2002. MCMV glycoprotein gp40 confers virus resistance to $CD8^+$ T cells and NK cells in vivo. Nat. Immunol. **3:**529–535.
24. **Kroemer, G., W. S. El Deiry, P. Golstein, M. E. Peter, D. Vaux, P. Vandenabeele, B. Zhivotovsky, M. V. Blagosklonny, W. Malorni, R. A. Knight, M. Piacentini, S. Nagata, and G. Melino.** 2005. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death. Differ. **12**(Suppl. 2)**:**1463–1467.
25. **Lembo, D., M. Donalisio, A. Hofer, M. Cornaglia, W. Brune, U. Koszinowski, L. Thelander, and S. Landolfo.** 2004. The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J. Virol. **78:**4278–4288.
26. **Lenac, T., M. Budt, J. Arapovic, M. Hasan, A. Zimmermann, H. Simic, A. Krmpotic, M. Messerle, Z. Ruzsics, U. H. Koszinowski, H. Hengel, and S. Jonjic.** 2006. The herpesviral Fc receptor fcr-1 down-regulates the NKG2D ligands MULT-1 and H60. J. Exp. Med. **203:**1843–1850.
27. **McCormick, A. L., C. D. Meiering, G. B. Smith, and E. S. Mocarski.** 2005. Mitochondrial cell death suppressors carried by human and murine cytomegalovirus confer resistance to proteasome inhibitor-induced apoptosis. J. Virol. **79:**12205–12217.
28. **McCormick, A. L., A. Skaletskaya, P. A. Barry, E. S. Mocarski, and V. S. Goldmacher.** 2003. Differential function and expression of the viral inhibitor of caspase 8-induced apoptosis (vICA) and the viral mitochondria-localized inhibitor of apoptosis (vMIA) cell death suppressors conserved in primate and rodent cytomegaloviruses. Virology **316:**221–233.
29. **Menard, C., M. Wagner, Z. Ruzsics, K. Holak, W. Brune, A. E. Campbell, and U. H. Koszinowski.** 2003. Role of murine cytomegalovirus US22 gene family members in replication in macrophages. J. Virol. **77:**5557–5570.
30. **Mocarski, E. S., and C. T. Courcelle.** 2000. Cytomegaloviruses and their replication, p. 2629–2673. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott/The Williams & Wilkins Co., Philadelphia, PA.
31. **Moss, D. J., S. R. Burrows, G. D. Baxter, and M. F. Lavin.** 1991. T cell-T cell killing is induced by specific epitopes: evidence for an apoptotic mechanism. J. Exp. Med. **173:**681–686.
32. **Nguyen, M., P. E. Branton, S. Roy, D. W. Nicholson, E. S. Alnemri, W. C. Yeh, T. W. Mak, and G. C. Shore.** 1998. E1A-induced processing of procaspase-8 can occur independently of FADD and is inhibited by Bcl-2. J. Biol. Chem. **273:**33099–33102.
33. **Orange, J. S., T. P. Salazar-Mather, S. M. Opal, and C. A. Biron.** 1997. Mechanisms for virus-induced liver disease: tumor necrosis factor-mediated pathology independent of natural killer and T cells during murine cytomegalovirus infection. J. Virol. **71:**9248–9258.
34. **Pass, R. F.** 2000. Cytomegalovirus, p. 2675–2705. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott/The Williams & Wilkins Co., Philadelphia, PA.
35. **Podlech, J., R. Holtappels, N. K. A. Grzimek, and M. J. Reddehase.** 2002. Animal models: murine cytomegalovirus, p. 493–525. *In* S. H. E. Kaufmann and D. Kabelitz (ed.), Methods in microbiology. Academic Press, San Diego, CA.
36. **Reboredo, M., R. F. Greaves, and G. Hahn.** 2004. Human cytomegalovirus proteins encoded by UL37 exon 1 protect infected fibroblasts against virus-induced apoptosis and are required for efficient virus replication. J. Gen. Virol. **85:**3555–3567.
37. **Reddehase, M. J., F. Weiland, K. Munch, S. Jonjic, A. Luske, and U. H. Koszinowski.** 1985. Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs. J. Virol. **55:**264–273.
38. **Rupp, B., Z. Ruzsics, T. Sacher, and U. H. Koszinowski.** 2005. Conditional cytomegalovirus replication in vitro and in vivo. J. Virol. **79:**486–494.
39. **Ryan, C. A., H. R. Stennicke, V. E. Nava, J. B. Burch, J. M. Hardwick, and G. S. Salvesen.** 2002. Inhibitor specificity of recombinant and endogenous caspase-9. Biochem. J. **366:**595–601.
40. **Skaletskaya, A., L. M. Bartle, T. Chittenden, A. L. McCormick, E. S. Mocarski, and V. S. Goldmacher.** 2001. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc. Natl. Acad. Sci. USA **98:**7829–7834.
41. **Strasser, A., A. W. Harris, D. C. Huang, P. H. Krammer, and S. Cory.** 1995. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. EMBO J. **14:**6136–6147.
42. **Strasser, A., L. O'Connor, and V. M. Dixit.** 2000. Apoptosis signaling. Annu. Rev. Biochem. **69:**217–245.
43. **Terhune, S., E. Torigoi, N. Moorman, M. Silva, Z. Qian, T. Shenk, and D. Yu.** 2007. Human cytomegalovirus UL38 protein blocks apoptosis. J. Virol. **81:**3109–3123.
44. **Varfolomeev, E. E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J. S. Beckmann, I. L. Mett, D. Rebrikov, V. M. Brodianski, O. C. Kemper, O. Kollet, T. Lapidot, D. Soffer, T. Sobe, K. B. Avraham, T. Goncharov, H. Holtmann, P. Lonai, and D. Wallach.** 1998. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity **9:**267–276.
45. **Wagner, M., S. Jonjic, U. H. Koszinowski, and M. Messerle.** 1999. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J. Virol. **73:**7056–7060.
46. **Wagner, M., and U. H. Koszinowski.** 2004. Mutagenesis of viral BACs with linear PCR fragments (ET recombination). Methods Mol. Biol. **256:**257–268.
47. **White, E., P. Sabbatini, M. Debbas, W. S. Wold, D. I. Kusher, and L. R. Gooding.** 1992. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. Mol. Cell. Biol. **12:**2570–2580.
48. **Woo, M., R. Hakem, M. S. Soengas, G. S. Duncan, A. Shahinian, D. Kagi, A. Hakem, M. McCurrach, W. Khoo, S. A. Kaufman, G. Senaldi, T. Howard, S. W. Lowe, and T. W. Mak.** 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev. **12:**806–819.
49. **Yeh, W. C., J. L. Pompa, M. E. McCurrach, H. B. Shu, A. J. Elia, A. Shahinian, M. Ng, A. Wakeham, W. Khoo, K. Mitchell, W. S. El Deiry, S. W. Lowe, D. V. Goeddel, and T. W. Mak.** 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science **279:**1954–1958.
50. **Zhang, J., D. Cado, A. Chen, N. H. Kabra, and A. Winoto.** 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. Nature **392:**296–300.