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Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors

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Viruses have evolved many distinct strategies to avoid the host's apoptotic response[1,2]. Here we describe a new family of viral inhibitors (v-FLIPs) which interfere with apoptosis signalled through death receptors[3] and which are present in several γ-herpesviruses (including Kaposi's-sarcoma-associated human herpesvirus-8), as well as in the tumorigenic human molluscipoxvirus[4]. v-FLIPs contain two death-effector domains which interact with the adaptor protein FADD[5,6], and this inhibits the recruitment and activation of the protease FLICE[7,8] by the CD95 death receptor[3]. Cells expressing v-FLIPs are protected against apoptosis induced by CD95 or by the related death receptors TRAMP[9–12] and TRAIL-R. The herpesvirus saimiri FLIP is detected late during the lytic viral replication cycle, at a time when host cells are partially protected from CD95-ligand-mediated apoptosis. Protection of virus-infected cells against death-receptor-induced apoptosis may lead to higher virus production and contribute to the persistence and oncogenicity[13] of several FLIP-encoding viruses.

In the immune system, apoptosis is used as a defence strategy to eliminate potentially harmful agents. For example, virus-specific T lymphocytes kill infected cells by delivering pro-apoptotic proteins (perforin and granzymes) or by expressing CD95 (Apo-1/Fas) ligand and triggering cell death of target cells via CD95 (ref. 14). Alternatively, apoptosis of infected cells may be cell-autonomous; cells detect the presence of certain viruses and respond by committing suicide. To allow increased virus replication, some viruses carry genes whose products are able to interfere with the host's apoptotic machinery[1]. There is increasing evidence that apoptotic cell death requires the activation of members of the ICE-like family of cysteine proteases (caspases)[15] and, not surprisingly, apoptosis inhibitory proteins that block these effector proteases are found in some viruses—for example, CrmA in the cowpox virus or p35 in baculovirus[1]. Other viral anti-apoptotic genes resemble the mammalian *bcl-2* gene[1].

The most direct pathway leading to the activation of ICE-like proteases and thus to cell death appears to be triggered by some members of the tumour-necrosis-factor receptor (TNF-R) superfamily, CD95, TNFR-1 and TRAMP (ws1/DR-3/Apo-3)[3,9–12] (collectively called death receptors). These receptors relay death signals through a cytoplasmic sequence motif called the death domain (DD), which interacts with the DD of the adaptor molecules FADD and/or TRADD[3], recruiting them to the membrane. FADD then associates with the ICE-like protease FLICE (caspase-8, Mch5, MACH)[7,8] through death-effector domains (DEDs) present at the carboxy terminus of FADD and the amino terminus of FLICE, leading to the assembly of a receptor-associated death-inducing signalling complex (DISC)[16]. DISC-associated FLICE subsequently initiates proteolytic activation of other ICE family members, which in turn leads to apoptosis[7,8,17].

Deletion mutants of FADD (containing only the DD) or FLICE (containing two DEDs) can act as dominant-negative inhibitors[8,18] early in the signalling pathway of death receptors, raising the possibility that structurally related natural inhibitors of apoptosis may exist. We therefore screened public databases with a generalized profile[19] constructed from DEDs of human and murine FADD, FLICE and Mch4 (ref. 20; caspase-10). Using this profile, the ORF E8 within the genome of equine herpesvirus-2 (EHV-2) was found to contain two sequence motives with highly significant homology to DEDs ($P < 10^{-2}$). E8 has ~23% sequence identity to the N-terminal part of FLICE and Mch4, and the presence of two copies of bona fide DEDs is evident from the selective conservation of a specific set of amino acids. EHV-2 is a γ-herpesvirus, and subsequent database rescreens revealed that other γ-herpesviruses, that is, bovine herpesvirus-4, herpesvirus saimiri (HVS), human herpesvirus-8 (HHV-8) and the human molluscipoxvirus (molluscum contagiosum virus, MCV), also contain ORFs predicted to code for proteins consisting of two DED motives (Fig. 1). We call these proteins v-FLIPs (for viral FLICE-inhibitory proteins; see below).

Considering the homology between the two DEDs of the v-FLIPs and the two DEDs found at the N terminus of FLICE, we reasoned that FLIP members may bind to FADD or a FADD-like molecule, thereby interfering with death receptor signalling. To test this hypothesis, 293T cells were transfected with expression vectors encoding FADD and N-terminally Flag-tagged FLIP from EHV-2 (Flag-E8). It was found that the 23K E8-FLIP did indeed interact with FADD in coimmunoprecipitation experiments (Fig. 2a). Moreover, when E8 and FADD were cotransfected with a Myc-tagged CD95 construct (encompassing the cytoplasmic domain including the DD), FADD bound to E8-FLIP also interacted with CD95 (Fig. 2a), indicating that FADD/E8-FLIP complex formation (mediated through DEDs) still permitted the CD95/FADD interaction (through DDs) to occur. Similar results were obtained with MCV(ORF159L)-FLIP (Fig. 2b) and HVS (ORF71)-FLIP (data not

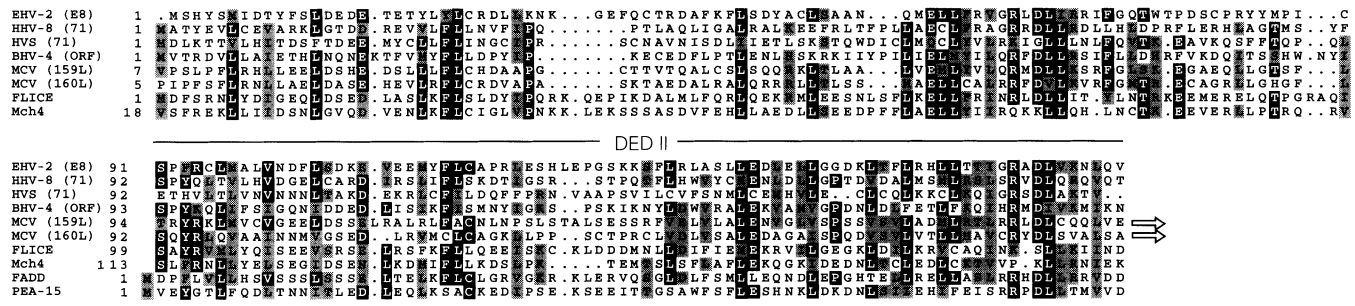


Figure 1 Amino-acid sequence alignment of the viral FLIPs and the DED containing proteins FADD, FLICE, Mch4 (GenBank (GB) accession number: U60519) and PEA-15 (GB X86809). FLIPs were found in equine herpesvirus-2 (EHV-2; GB: U20824, bovine herpesvirus-4 (BHV-4; GB: Z46385), herpesvirus saimiri (HVS; GB: X64346), human herpesvirus-8 (HHV-8)[29] and molluscum contagiosum virus (MCV, two distinct genes, GB: U60315). The previously published sequence of HHV-8 ORF 71 (K13)[29] contains a frameshift within the second DED at codon 130, thus predicting a protein of 140 amino acids. The complete sequence of 189 amino acids is shown here (GB: U90534). For each block of aligned sequences, black boxes indicate ≥50% amino-acid sequence identity; grey shading indicates ≥50% sequence similarity through conservative amino-acid substitutions. The arrows at the C terminus of the second DED of the MCV-FLIP sequences refer to their C-terminal extensions of 66 (159L) and 202 (160L) amino acids, respectively.

shown). Thus, v-FLIPs interact with the adaptor molecule FADD, thereby forming a complex with CD95.

As some FLIP-encoding viruses are lymphotropic, we next asked whether in the human Raji B cell line stably transfected with E8-FLIP, the viral protein would incorporate into the CD95-associated death-inducing signalling complex (DISC). To address this question, we generated Flag-E8-expressing Raji clones (RE8/11 and RE8/19) and control clones transfected with vector only (RCo/1 and RCo/3) (Fig. 2c). Two-dimensional gel electrophoresis analysis of anti-CD95 immunoprecipitates obtained from these clones revealed a 23K protein with the theoretically expected p*I* value of 5.0 of Flag-E8 (Fig. 2d). This protein also comigrated with Flag-E8 immunopreciptiated from E8-expressing cells, and associated with the DISC in a ligand-dependent manner (data not shown). To test whether the association of E8-FLIP with the anti-CD95 immunoprecipitates of activated cells would interfere with the recruitment of any of the DISC components CAP 1–6 (refs 16, 17), DISC formation was analysed in detail (Fig. 2e). In control RCo/3 cells, the CAP proteins CAP1 (FADD), CAP2 (hyperphosphorylated FADD), CAP3 (unknown protein containing the N terminus of FLICE), CAP4 (pro-FLICE), and CAP5 and CAP6 (the cleaved FLICE prodomain as an indicator for FLICE activation by the DISC[17]) were associated with CD95. In the E8-FLIP transfected RE8/19 cells however, the DISC was incomplete. Recruitment of CAP1 (FADD) was unchanged, whereas that of CAP4 (FLICE) and CAP3 was strongly reduced. Furthermore, the CAP5 and CAP6 spots were undetectable, indicating that the inhibition of apoptosis by E8-FLIP occurred at the level of FLICE recruitment and activation. FLICE is only activated when incorporated into the DISC[17]. Proteolytic processing by the DISC of exogenously added FLICE *in vitro* was highly decreased in the E8-FLIP transfected clone (Fig. 2f), which correlates with the reduced FLICE incorporation into the DISC (Fig. 2e).

As FLICE has been proposed to be implicated in CD95-death signalling, we anticipated that the inhibition of FLICE recruitment to the DISC should result in a decreased sensitivity to CD95 agonists. Indeed, when apoptosis was induced by anti-CD95 treatment in Raji B-cell clones, E8-FLIP-expressing clones showed a considerably decreased susceptibility to aptoptosis relative to controls (Fig. 3a). E8-FLIP expression also interfered with CD95L-induced cell death in transfected Jurkat human leukaemic T cells, where the amount of E8-FLIP present correlated with the relative resistance to death (Fig. 3b). In contrast, E8-FLIP expression did not alter the susceptibility of the clones to apoptosis induced by staurosporine or growth factor withdrawal (data not shown). In 293T human embryonic kidney cells, overexpression of CD95 led to massive cellular death, which was efficiently reduced by co-expression of E8-FLIP to an extent that is comparable to that obtained by the general ICE-like protease inhibitor z-VAD-fmk (Fig. 3c). Other v-FLIPs (MHV-159L and HVS-71) revealed similar inhibitory activities (Fig. 3d, and data not shown).

TRAMP (DR3/wsl/Apo-3) has been identified as a third member of the death receptor subfamily (containing DD) of TNF receptors[9–12]. Like the TNFR-1, TRAMP binds FLICE through association of the adaptor proteins TRADD and FADD. Although the ligand of TRAMP remains to be identified, overexpression of the receptor itself in 293T cells induces massive cell death, which is blocked by coexpression of E8-FLIP, 71-FLIP or 159L-FLIP (Fig. 3a, f, g).

TRAIL is an orphan member of the TNF family of ligands that potently induces apoptosis in various cell lines of haematopoietic origin, including Jurkat T cells[21]. In view of the functional and structural similarity of TRAIL to CD95L, it is likely that the sequence of the uncharacterized receptor for TRAIL (TRAIL-R) and CD95 are homologous and that their death signalling pathways are similar. Indeed, the Jurkat clone with the highest level of E8-FLIP (JE8/13; Fig. 3b), incubated with increasing concentrations of recombinant soluble TRAIL (sTRAIL), was completely resistant to sTRAIL-mediated apoptosis, whereas clones JE8/1 and JE8/10 (harbouring intermediate levels of E8-FLIP) were partially protected (Fig. 3h).

Finally, we selected the HVS-71-FLIP to determine at which stages of the viral replication cycle FLIP is expressed. In a marmoset T-cell line transformed with HVS (P-1079), which produces low quantities of viral particles[22], a 71-FLIP-containing mRNA of 5 kb was detected (Fig. 4a). Moreover, when fully permissive OMK cells were infected with the cytopathic HVS strains C488 or A11, 71-FLIP transcripts were detected one day before massive cellular lysis occurred (Fig. 4a). Expression of 71-FLIP coincided with increased resistance to CD95L-mediated apoptosis (Fig. 4b). Thus, 71-FLIP is expressed late in the lytic replication cycle of HVS and may protect infected cells from premature apoptosis induced by the viral overload, similar to the role proposed for other viral inhibitors such as the baculoviral p35 and IAPs, and the adenoviral Bcl-2 homologue E1B19K (ref. 1). Loss of function of these viral genes leads to a decrease in the productivity of infection of these viruses, which is accountable by an increase in apoptosis of the infected cells[2].

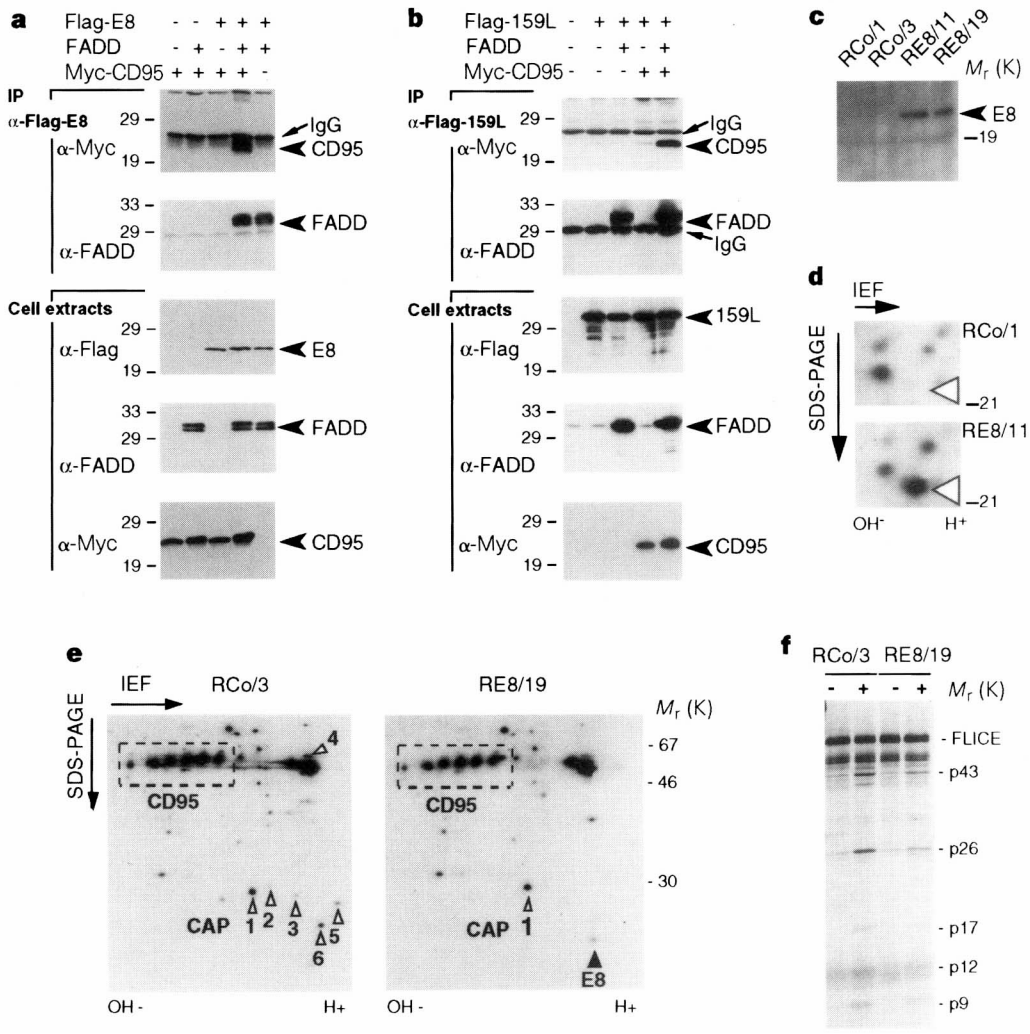
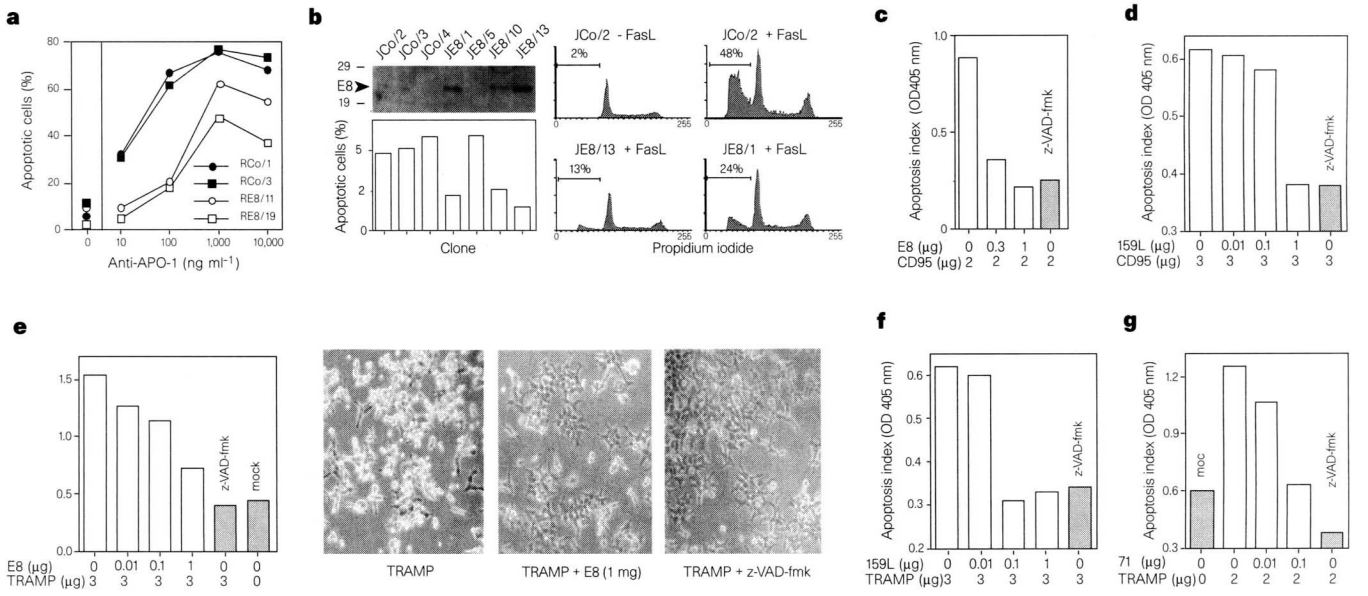


Figure 2 v-FLIPs interfere with CD95 signaling by binding to FADD. **a**, 293T cells were transiently transfected with 4 μg of each expression vector encoding Flag-E8-FLIP, FADD and Myc-CD95 as indicated, or empty vector to keep the total amount of plasmid constant. Cells were lysed 30 h after transfection, and anti-Flag immunoprecipitates or total cell extracts were analysed for the presence of FADD, Flag-E8 or Myc-CD95 by western blotting as indicated. **b**, The association of MCV-159L-FLIP with FADD and CD95 was analysed as described in **a**. **c**, Flag-E8-FLIP expressing Raji clones RE8/11 and RE8/19, as well as the control clones RCo/1 and RCo/3 were tested for E8 expression by western blotting using an anti-Flag antibody. **d**, Analysis of anti-Apo-1 immunoprecipitates from ^{35}S-labelled control (upper panel) or Flag-E8-FLIP-expressing (lower panel) Raji clones by 2D gel electrophoresis and autoradiography. The arrow indicates the migration position of a 23K protein of p*I* 5.0 found in the E8-FLIP-expressing clone RE8/11 and absent in the control clone RCo/1. **e**, Apo-1 immunoprecipitates from ^{35}S-labelled, anti-Apo-1-treated clones RCo/3 and RE8/19 were analysed by 2D gel electrophoresis and autoradiography. Numbers refer to CAP proteins: CAP1 and CAP2 (FADD), CAP3, CAP4 (FLICE), CAP5 and CAP6 (the prodomains of FLICE as a result of proteolytic cleavage). A filled arrowhead indicates the position of E8. **f**, FLICE-processing activity associated with the DISC in E8 expressing and control Raji cells. Cells were treated with anti-Apo-1 for 5 min, and Apo-1 immunoprecipitates from treated (+) or untreated (−) cells were analysed for FLICE processing activity by incubation with *in vitro* translated ^{35}S-labelled FLICE. FLICE-specific cleavage products (p43, p26, p17, p12 and p9)[17] were detected by SDS-PAGE and autoradiography.

Interestingly, all FLIP encoding γ-herpesviruses also have a Bcl-2 homologue. The anti-apoptotic Bcl-2 family members block cell death induced by growth factor deprivation, γ-irradiation and cytotoxic drugs[23,24]. However, in contrast to the v-FLIPs, these proteins have a less potent effect on CD95-mediated apoptosis of lymphoid cell lines. Some viruses may thus take advantage of two complementary anti-apoptotic functions provided on the one hand by a Bcl-2 homologue and on the other by v-FLIP.

Tissue homeostasis is maintained through the delicate balance of cell growth and apoptosis. Recent evidence indicates that tumour cells such as melanomas and hepatomas do not respond to CD95L-mediated apoptosis owing either to the downregulation of CD95 expression or to a blockade in the CD95 signalling pathway[25,26]. Thus, by interference with CD95 and other death-receptor-mediated signals, we propose that v-FLIPs may not only facilitate viral spread and persistence, but that they also contribute to the transforming capacity of some herepesviruses. This possibility is supported by the fact that MCV produces slow-growing epidermal neoplasms that persist for long periods with little immune response. HVS causes tumours in New World primates, and tight epidemiological links indicate that HHV-8 is an infectious cofactor for Kaposi's sarcoma and primary effusion lymphoma[13]. Future investigations should address the possible presence of v-FLIPs in tumours caused by viruses.

Note added in proof: Related findings have been reported by J. Bertin *et al.*[30]. □



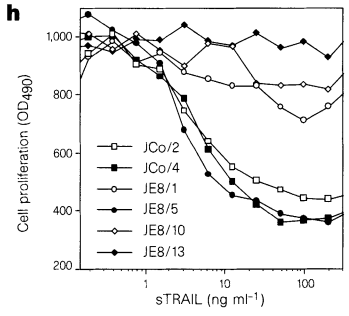


Figure 3 v-FLIPs protect cells from death receptor-induced apoptosis. **a**, The susceptibility of E8-FLIP expressing (RE8/11 and RE8/19) and control (RCo/1 and RCo/3) Raji clones to anti-Apo-1-mediated apoptosis was determined by the incubation of cells with the indicated concentration of anti-Apo-1 in medium for 16 h at 37 °C. Propidium iodide staining and flow cytometry were used to assess the percentage of apoptotic nuclei. **b**, Cellular extracts of E8-transfected (JE8/1, JE8/5, JE8/10 and JE8/13) and control (JCo/2, JCo/3 and JCo/4) Jurkat clones were analysed for Flag-E8-FLIP expression by anti-Flag western blotting. The susceptibility of the E8-FLIP-transfected and control clones to CD95L-induced apoptosis was determined by incubating cells for 3 h art 37 °C with supernatants of neuronal cells expressing or not CD95L[27]. The fraction of apoptotic cells was determined as in **a**. **c** and **d**, 293T cells were transiently transfected with the indicated amounts of an expression vector for the human CD95 with or without EHV-2 (E8)-FLIP (**c**) or MCV (ORF159L)-FLIP (**d**) expression vectors. Where indicated, 25 μM z-VAD-fmk was added to the cell culture after transfection. Quantitative analysis of cell death (induced by the overexpressed CD95 in the absence of CD95L) was done using a histone–DNA complex-release assay and represented as apoptotic index (relative amount of DNA–histone complexes released into the cytoplasm). In a typical transfection experiment, 50–90% of cells underwent apoptosis upon transfection with death receptor expression vectors. Results are from one experiment of three with similar results. **e**, 293T cells were transiently transfected with the indicated amounts of expression vector for human TRAMP and Flag-E8-FLIP or with a control vector lacking an insert and analysed as in **c**. Photographs were taken 30 h after the onset of transfection. **f** and **g**, 293T cells were transiently transfected with the indicated amounts of an expression vector for TRAMP together with (ORF159L)-FLIP (**f**) and HVS-(ORF71)-FLIP (**g**) and analysed as in **c**. **h**, E8 expressing Jurkat clones (JE8/1, JE8/10 and JE8/13) and non-expressing (JE8/5) or puromycin resistant control clones (JCo/2 and JCo/4) were incubated with the indicated concentrations of recombinant Flag-tagged sTRAIL for 20 h. Cell viability was determined using a cell proliferation assay.

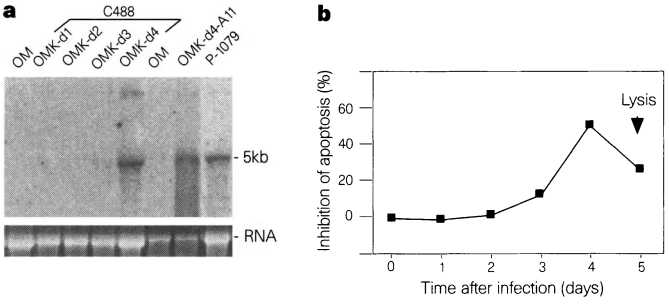


Figure 4 HVS (ORF71)-FLIP is expressed during the lytic cycle and in semipermissive virus-producing cells. **a**, Northern blot analysis of permissive OMK cells infected with the HVS strain C488 (days indicated refer to the time after infection) or with strain A11 (late conditions of infection). The marmoset monkey-derived semipermissive T cell line (P-1079) produces low titres of HVS particles. **b**, Resistance to CD95L-induced apoptosis in OMK cells expressing HVS-(ORF71)-FLIP. CD95L was added to cells infected with HVS and cell death was compared to non-infected cells. The difference in the susceptibility to CD95L-induced apoptosis (determined by the histone–DNA release assay) is shown. A virus-induced cytopathic effect was evident on day 4 affecting <10% of cells; on day 5 a large proportion (~50%) of the cells not exposed to CD95L was lysed due to viral replication. Results are from one experiment of two with similar results.

Methods

Cell lines and reagents. Human embryonic 293T cells, the human leukaemia Jurkat T-cell line and the human Burkitt lymphoma B-cell line Raji were grown as described[9]. Monoclonal antibodies used in immunoprecipitations and for western blotting included: an anti-Flag antibody and anti-Flag agarose (Kodak International Biotechnologies), an anti-FADD antibody (Transduction Laboratories), and an antibody directed against the Myc epitope (9E10, Sigma). Soluble human TRAIL (amino acids 95–281) was generated by PCR from the EST clone 117926 (Genbank accession number T90422) using oligonucleotides JT403 5′-TCAGCTGCCAGACCTCTGAGGAAAC-3′ and JT469 5′-ACTAGTTAGCCAACTAAAAAG-3′; and was cloned into a modified pQE-16 vector (Qiagen) containing the Flag sequence and a linker

(GPGQVQLQ). Protein expression in M15pRep4 bacteria was induced with 0.5 mM IPTG.

Expression vectors. The complete ORF E8 of EHV-2 (viral DNA was a gift from A. J. Davison), ORF 159L of MCV (the plasmid was a gift from G. Darai) and ORF 71 of HVS were amplified by PCR and inserted in frame with an N-terminal Flag epitope into the *Eco*RI site of a vector derived from pCR-3 (Invitrogen). The Flag-E8-FLIP construct was subcloned into the pSRαpuro vector (a gift from R. Sekaly) and then used to create stable puromycin-resistant transfectants of Jurkat and Raji cells. An expression vector for the Myc-tagged cytoplasmic domain of the murine CD95 receptor was generated by insertion of a PCR fragment corresponding to amino acids 166–306 in frame with an N-terminal Myc epitope into a pCR-3 derived vector. The expression vector for human TRAMP and FADD in pCR-3 has been previously described[9]. Human full-length CD95 was subcloned as a *Hin*dIII/*Xba*I fragment into pCR-3 and used for transfection of 293T cells.

Cell transfection. Stable puromycin-resistant transfectants of Jurkat and Raji cells were generated by electroporation of 8×10^6 cells in 800 μl HeBS, mixed with 20 μg SRαpuro plasmid with or without Flag-E8 insert, at 250 V and 960 μF. 48 h after transfection, cells were seeded at 10,000 cells per well in flat bottom 96-well plates under selection with 5 μg ml^{-1} puromycin (Sigma). 293T cells were seeded at $1–2 \times 10^6$ cells per 10-cm plate or $3–6 \times 10^5$ cells per 5-cm plate and transfected the next day by the calcium phosphate precipitation method. The precipitate was left on cells for 8 h, and cells were collected 26–30 h after transfection.

Cell lysis and co-immunoprecipitation. Cell lysis and co-immunoprecipitation of the various tagged proteins were carried out as described[9]. Jurkat and Raji clones or 293T cells were checked for protein expression by anti-tag western blot analysis of postnuclear cell lysates of equivalent protein content. Metabolic labelling of Raji cells with ^{35}S, anti-CD95 immunoprecipitations and 2D-gel electrophoresis were performed as previously described[16]. Analysis of DISC-associated FLICE activity was assessed as before[17].

Apoptosis assays. The analysis of apoptosis induced by CD95L was carried out as follows: puromycin-resistant Jurkat clones ($\sim 3 \times 10^5$ cells per 500 μl) were incubated for 3 h at 37 °C with 50 μl supernatant from neuro-2a cells transfected with a murine CD95L expression vector or with control supernatant from cells transfected with mock vector[27]. The susceptibility of Raji clones to anti-CD95-induced apoptosis was analysed by incubation of cells (5×10^5 cells ml^{-1}) with varying concentrations of anti-Apo-1 mAb[16] in medium for 16 h at 37 °C. Apoptosis was measured by quantifying DNA fragmentation as previously described[28]. Apoptosis of transiently transfected 293T cells was monitored by the cell-death detection ELISA (Boehringer Mannheim), which detects the presence of soluble histone–DNA complexes. The survival of E8-transfected and control Jurkat clones to TRAIL-induced cell death was tested by incubating cells at 50,000 cells per well in 100 μl with the indicated concentrations of recombinant TRAIL and 1 μg ml^{-1} anti-Flag mAb for 20 h. Proliferating cells were subsequently quantified with the Celltiter 96 AQ proliferation assay (Promega) following the manufacturer's instructions.

Herpesvirus saimiri cultures and transcript analysis. The *in vitro* culture of virus and northern blot analysis of transcripts were carried out as described[22]. The effect of HVS infection on CD95-mediated cell death of owl monkey kidney (OMK) cells was assayed by seeding the cells at 10^4 cells per well in 96-well flat-bottomed plates. Two days later, half of the wells were infected at a multiplicity of infection of ~1. Recombinant sCD95L (0.3 μg ml^{-1})[9] was added together with enhancer (Alexis, San Diego) at different times after infection. Material was collected 20 h later and analysed for the presence of histone–DNA complexes as described.

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erratum

Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein

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Due to an error in the reproduction process, the labelling in the right-hand panel of Fig. 5b of this letter was incorrect. The cell type involved is not $Rb^{+/+}$ 3T3, but $Rb^{-/-}$ 3T3, as indicated in the text and the figure legend.