

The human cytomegalovirus-encoded chemokine receptor US28 induces caspase-dependent apoptosis

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Viral subversion of apoptosis regulation plays an important role in the outcome of host/virus interactions. Although human cytomegalovirus (HCMV) encodes several immediate early (IE) antiapoptotic proteins (IE1, IE2, vMIA and vICA), no proapoptotic HCMV protein has yet been identified. Here we show that US28, a functional IE HCMV-encoded chemokine receptor, which may be involved in both viral dissemination and immune evasion, constitutively induces apoptosis in several cell types. In contrast, none of nine human cellular chemokine receptors, belonging to three different subfamilies, induced any significant level of apoptosis. US28-induced cell death involves caspase 10 and caspase 8 activation, but does not depend on the engagement of cell-surface death receptors of the tumour necrosis factor receptor/CD95 family. US28 cell-death induction is prevented by coexpression of C-FLIP, a protein that inhibits Fas-associated death domain protein (FADD)-mediated activation of caspase 10 and caspase 8, and by coexpression of the HCMV antiapoptotic protein IE1. The use of US28 mutants indicated that the DRY sequence of its third transmembrane domain, required for constitutive G-protein signalling, and the US28 intracellular terminal domain required for constitutive US28 endocytosis, are each partially required for cell-death induction. Thus, in HCMV-infected cells, US28 may function either as a chemokine receptor, a phospholipase C activator, or a proapoptotic factor, depending on expression levels of HCMV and/or cellular antiapoptotic proteins.

Programmed cell death (PCD) or apoptosis is a genetically regulated cell suicide process, central to the control of cell proliferation and differentiation and to the elimination of damaged and infected cells [1,2]. Conversely, viral subversion of PCD regulation plays an important role in the dissemination and pathogene-

sis of several viral infections [3,4]. The human cytomegalovirus (HCMV) causes severe disease in newborns and immunocompromised hosts. *In vivo*, HCMV and murine CMV induce apoptosis in various cell types through different mechanisms that may favour either viral clearance or disease development [5–8].

Abbreviations

CHO, Chinese hamster ovary cells; DED, death effector domains; FADD, Fas-associated death domain protein; GFP, green fluorescent protein; GPRC, G-protein-coupled receptor; GRK, G-protein kinase; HCMV, human cytomegalovirus; IC, intracytoplasmic domain; IE, immediate early; InsP, inositol phosphate; PCD, programmed cell death; PI, propidium iodide; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; SMC, smooth muscle cells; TM, transmembrane domain; TNFR, tumour necrosis factor receptor.

HCMV encodes several immediate early (IE) proteins, with antiapoptotic properties, namely IE1, IE2, vMIA and vICA [9–11]. IE1 and IE2 each inhibit apoptosis induced by tumour necrosis factor (TNF) α or by E1B-19 kDa protein-deficient adenovirus, and IE2, but not IE1, protects smooth muscular cells (SMC) from p53-mediated apoptosis [12]. vMIA blocks apoptosis at the mitochondria level without sharing structural homology with Bcl-2 protein family members. vICA inhibits Fas-mediated apoptosis by binding to the pro-domain of caspase 8 and preventing its activation. Despite the presence of several antiapoptotic proteins encoded by CMV, no HCMV gene product that causes apoptosis induction has been identified.

HCMV contains four open reading frames US27, US28, UL33 and UL78 that encode G-protein-coupled receptors (GPCR) [13]. US28, one of the earliest viral genes transcribed in both latently and productively HCMV-infected cells, is a functional CC chemokine receptor that can promote different functions *in vitro* [14,15]. US28 allows CMV-infected SMC migration, which could provide a molecular mechanism for CMV's implication in the progression of vascular disease [16]. US28 withdraws CC chemokines from the infected cell microenvironment [17], suggesting a potential involvement in immune evasion, and enhances cellular fusion induced by different viral envelopes, suggesting that it could participate in cell-to-cell diffusion of CMV and other viruses [18]. US28 acts also as a coreceptor for HIV [18–21].

It has been shown that US28 induces phospholipase C (PLC) and NF- κ B activation constitutively, independent of the binding of any ligand [22,23]. US28 can also undergo rapid receptor endocytosis and recycling in a ligand-independent fashion. The US28 C-terminal domain is constitutively phosphorylated by GRK family proteins, then β -arrestin recruitment attenuates constitutive signalling and allows constitutive receptor endocytosis and recycling via a clathrin-mediated mechanism [23–25].

Here we show that US28 constitutively induces apoptosis in different cell types by triggering activation of initiator caspase 8 and caspase 10, independent of cellular TNF family death receptor activation, via a pathway that appears partially dependent on the integrity of the third US28 transmembrane domain (TM) required for constitutive PLC activation and on the presence of the US28 intracellular C-terminal domain required for its internalization. Thus, depending on its expression level and on the expression level of HCMV-encoded or cellular antiapoptotic proteins, US28 may provide HCMV-infected cells with a broad functional repertoire, by acting either as

a chemokine receptor, a PLC activator, or as a proapoptotic protein.

Results

US28 expression induces apoptosis

Adherent human 293T cells were transiently transfected with an HCMV expression vector encoding either the HCMV CC chemokine receptor US28 from the laboratory strain AD169, or the human CC chemokine receptor CCR-5. As a negative control, we used an empty vector (Rc/CMV). As a positive control for apoptosis induction, cells were transfected with a vector encoding human Bax, a major proapoptotic member of the Bcl-2/Bax protein family, which acts downstream of cell-surface signalling by inducing outer membrane permeabilization of mitochondria [26]. We assessed cell death using both optical microscopy analysis of cell adherence loss (Fig. 1A) and flow cytometry analysis of nuclear DNA loss (hypodiploidy) (Fig. 1B), a typical feature of apoptosis. Both Bax and US28 expression induced cell death within 48 h, whereas transfection of CCR-5 or the empty vector Rc/CMV did not affect cell survival (Fig. 1A,B). Using flow cytometry analysis of hypodiploidy, we then performed a comparative kinetic analysis of cell death following expression of US28, Bax, the human chemokine receptors CCR-5 and CXCR-4, or the aminopeptidase CD26, which has no chemokine receptor activity (Fig. 1C). Bax expression led to rapid induction of apoptosis that was already significant 18 h after transfection and resulted in > 50% cell death by 72 h (Fig. 1C). US28 expression induced a slower kinetics of cell death that was significant at 48 h, resulting in > 35% cell death by 72 h (Fig. 1C). In contrast, neither CCR-5, CXCR-4, CD26, nor the empty vector Rc/CMV induced any significant apoptosis during 72 h after transfection. At 48 h post-transfection, we compared the effect of US28 expression on cell death induction versus that of nine other human chemokine receptors representing three different receptor subfamilies: CCR-1, -3, -4 and -5, CXCR-1, -2, -4 and -6, and CX3CR-1. Human chemokine receptors induced either no, or only moderate, apoptosis (Fig. 1D), suggesting that the capacity of US28 to trigger cell death was somehow unique among chemokine receptors. Although we did not analyse the expression levels for all the different chemokine receptors, these constructs have been extensively used in other studies from our laboratories and showed receptor expression and functionality [14,18,27] (personal communication). We also

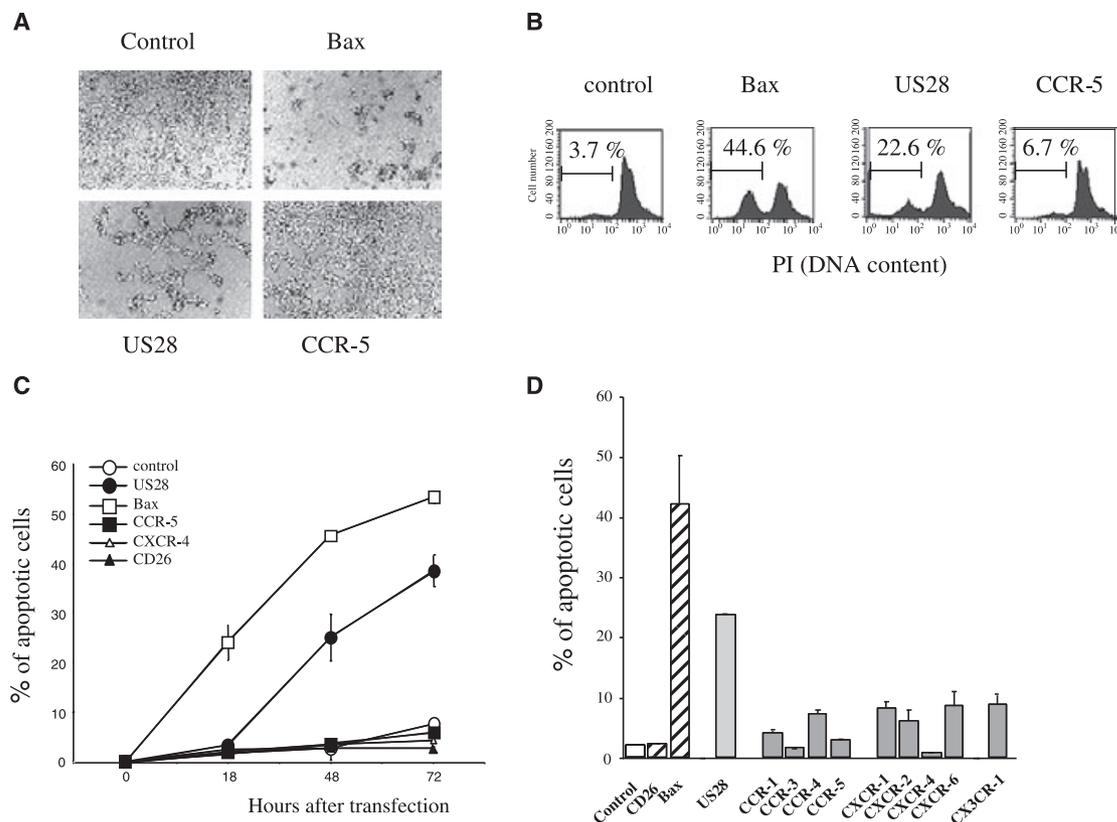
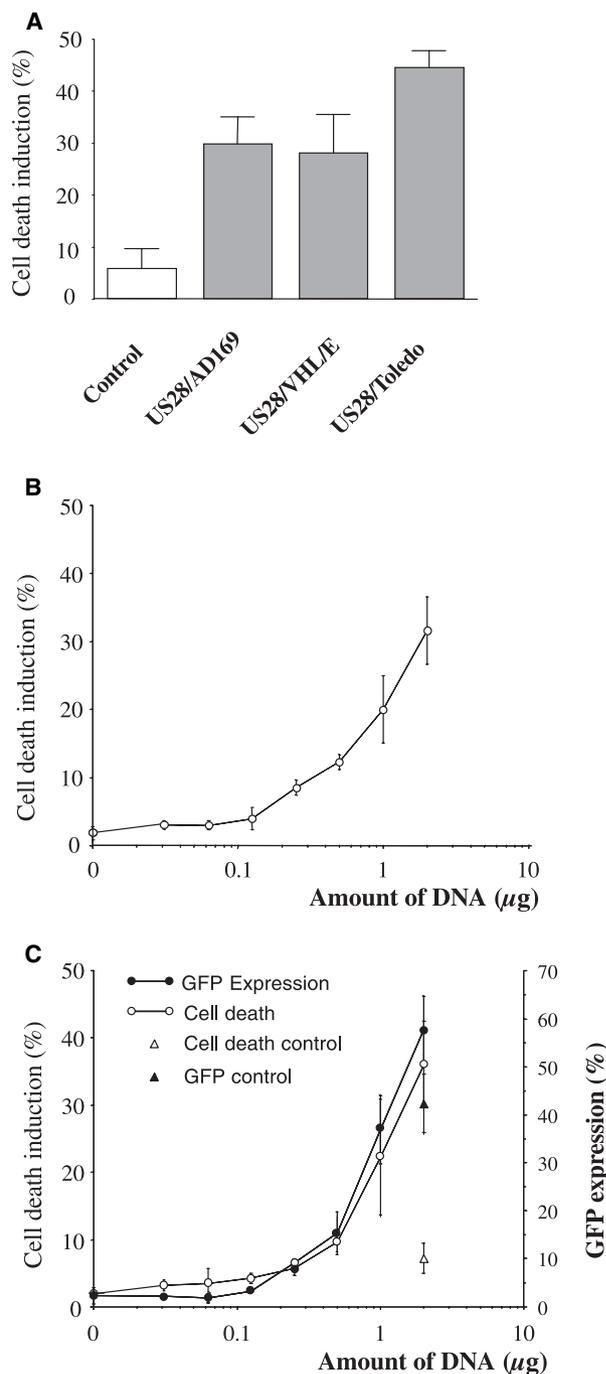


Fig. 1. Induction of apoptosis by US28 expression. Subconfluent 293T cells were transfected using the calcium phosphate precipitate method with vectors expressing either the HCMV chemokine receptors US28, various cellular chemokine receptors, the proapoptotic Bax protein as a positive control for apoptosis induction, and the aminopeptidase CD26 or the empty Rc/CMV vector as negative controls (Control). Cell death was assessed 48 h after transfection by (A) light microscopy ($\times 150$), and (B) flow cytometry analysis of DNA loss (PI staining) in adherent cells (numbers indicate the percentage of hypodiploid cells). (C) Kinetics of apoptosis (DNA loss) following transfection with Bax, US28, CD26, or the cellular chemokine receptors CCR-5 and CXCR-4. (D) Apoptosis (DNA loss) induced 48 h after transfection by Bax, US28, CD26, and nine different cellular chemokine receptors (CCR-1, -3, -4, -5, CXCR-1, -2, -4, -6 and CX3CR-1). Results are means \pm SD of one representative experiment of two (C) or three (D).

analysed cell death induced by US28 amplified from two other HCMV strains, the clinical isolate VHL/E and the laboratory isolate Toledo (Fig. 2A). Seventy-two hours after transfection, US28-VHL/E induced cell death at a level comparable with that of US28-AD169 ($\approx 30\%$), whereas US28 from the laboratory isolate Toledo induced higher levels of cell death ($\approx 45\%$) (Fig. 2A). Expression levels of US28 in fibroblasts infected with different HCMV isolates (AD169, Toledo and TB40/E) do not show significant differences [28] and, most importantly, are even higher ($\approx 1 \times 10^6$ sites per cell) than expression levels obtained with transient transfection ($\approx 2 \times 10^5$ sites per cell). Hence, cell death induction by US28, expressed at even higher levels in HCMV-infected cells, appears to be a general property of HCMV.

Cellular localization and expression level of US28

It has been shown previously that epitope-tagged versions of the US28 receptor and US28-GFP fusion protein do not modify the cellular localization of the native receptor [24]. We used N-terminally tagged US28 and CCR-5 to compare their cell-surface expression levels, and US28-GFP and CCR-5-GFP to compare their whole cellular expression levels and localization. Concerning cell-death induction, tagged chemokine receptors and GFP-fused receptors behaved like native ones (Table 1). Flow cytometry analysis using a tag-specific antibody for tagged chemokine receptors, indicated that 16 h after transfection, before the onset of US28-induced cell death, US28 and CCR-5 were expressed at similar levels: $\approx 25\%$ of the whole cell population expressed the receptors at the surface



(Table 1 and data not shown). This implies that differences in cell-death induction did not result from differences in cell-surface expression. However, flow cytometry analysis 16 h post transfection of GFP-fused receptors reveals that the overall cellular level of US28 expression is greater than that of CCR-5 (52.8 versus 35.1), suggesting that US28 is expressed mostly in the intracellular compartment, as previously described

Fig. 2. Effects of US28 from different CMV isolates on cell death, and effects of different expression levels of US28 on cell death. (A) Subconfluent 293T were transfected using the calcium phosphate method by expression vectors of US28-AD169, US28-VHL/E and US28-Toledo. Flow cytometry analysis of DNA loss (PI staining) (numbers indicate the percentage of hypodiploid cells) was performed 72 h post transfection. (B) Transfection as in (A) of different amounts (from 0 to 2 µg for 4 wells of 24-well plates) of expression vector of US28. Cell were detached 72 h later, and analysed for DNA loss (PI staining). (C) Transfection as in (A) of different amounts (from 0 to 2 µg for 4 wells of 24-well plates) of expression vector US28-GFP, and as control, of 2 µg of CCR-5-GFP vector. Cell were detached 72 h later, and analysed using flow cytometry for DNA loss (PI staining), and GFP expression. Results are means \pm SD of triplicates in two independent experiments (A-C).

[24,25]. Using US28 and US28-GFP, we then explored the effect of different amounts of transfected DNA on both US28 whole-cell expression and cell-death induction levels in 293T cells. Transfection of 2 µg of each vector induced 30–35% of cell death after 72 h, and US28-GFP expression (% of GFP + cells) appeared to be \approx 60% (Fig. 2B,C). Comparison of PCD induced by US28-GFP and CCR-5-GFP when transfected cells express similar level of GFP indicates that a high expression level of US28, compared with that of CCR-5, was not directly responsible for US28-mediated cell-death induction.

Apoptosis appeared only in US28-GFP expressing cells, which represented > 50% of the whole GFP + population (Fig. S1). This suggests that US28 does not trigger cell death by a diffusible factor, because survival of untransfected cells is not affected by neighbouring US28-expressing cells. Nuclei were stained with Hoescht 24 and 48 h post transfection of 293T cells. After 24 h no apoptotic nuclei were detected (Fig. S2). After 48 h, some US28-GFP+, but not CCR-5-GFP+, cells showed shrinkage with apoptotic nuclei. This could be partially inhibited in presence of the pan caspase inhibitor z-VAD-fmk (Fig. S2).

US28 induces cell death in different cell types

Because US28-GFP induced cell death only in the US28-GFP+ cells, we investigated US28-GFP-mediated cell death in different cell lines using flow cytometry analysis of hypodiploidy in the GFP+ cell population. US28-GFP expression 72 h post transfection, was 70.88, 22.8 and 6.60% in 293T, HeLa and Cos cells, respectively, and cell death appeared, respectively, in 40.87, 64.34 and 42.44% of US28-GFP+ cells (Table 2). In each case the pan caspase inhibitor z-VAD-fmk partially inhibited cell death.

Table 1. Comparative analysis of cell-surface expression or whole-cell expression, and cell-death induction, by epitope-tagged US28 or CCR-5 and by US28-GFP or CCR-5-GFP fusion proteins.

Vectors ^a	Surface expression ^b	Whole cell expression ^c	
	(% myc + cells)	(% of GFP + cells)	% US28-mediated apoptosis ^d
Rc/CMV-Tag	5.5		20.0 ± 1.0
Tag-US28	24.3		115.0 ± 0.8
Tag-CCR-5	28.8		21.6 ± 2.1
Rc/CMV		0.6	12.9 ± 8.1
GFP		73.4	7.2 ± 0.9
US28-GFP		52.8	114.2 ± 11.6
CCR-5-GFP		35.1	24 ± 5.8

^a 293T cells were transfected with US28, CCR-5 or the empty Rc/CMV vector, each carrying an N-terminal myc tag, or with GFP, US28-GFP, CCR-5-GFP, or the empty Rc/CMV vector. ^b Flow cytometry analysis of cell-surface expression was performed 16 h after transfection using a monoclonal antibody specific for the myc tag. Results are from one representative experiment. ^c Flow cytometry analysis of the whole cell expression was performed 16 h after transfection. Results are from one representative experiment. ^d Flow cytometry analysis of apoptosis (nuclear DNA loss) was assessed 48 h after transfection using PI staining. Results are in means ± SD of at least two independent experiments.

US28-induced apoptosis is repressed by protein kinase inhibitors

Signal transduction following ligand binding to CC chemokine receptors, including US28, involves activation of *Pertussis* toxin (PTX)-sensitive $G\alpha_i$ proteins [7, 41]. PTX had no proapoptotic activity on mock transfected cells, and no inhibitory effect on cell death induced by US28 expression in the absence of any added CC chemokine (or on Bax-induced death, used

as a control) (Fig. 3A). Addition of the US28 ligands, CC chemokine RANTES or the CX3C chemokine fractalkine (50 nM), did not modify US28-induced apoptosis (data not shown). This suggests that US28-mediated death signalling is not a default pathway triggered in the absence of ligand. A protein tyrosine kinase (PTK) pathway has been shown to be necessary for SMC migration induced by US28, which, LIKE cell death, is not sensitive to PTX [16]. The tyrosine PTK inhibitor, Genistein, and the phosphoinositol 3-kinase inhibitor, LY 294002, reduced US28 cell death (Fig. 3A), suggesting that the US28 cell-death pathway uses various kinase families, some of which may be necessary for US28-induced SMC migration.

US28-induced apoptosis is repressed by the HCMV immediate early protein IE1, and depends on caspase 10 and caspase 8 activation

Execution of PCD involves two pathways that usually operate together and amplify each other [32, 38]. One is triggered by the activation of initiator caspases, such as caspase 10 and caspase 8, downstream of the engagement of cell-surface death receptors of the CD95/tumour necrosis factor receptor (TNFR) family, leading to the recruitment of the adapter protein FADD, and subsequently to caspase-dependent death [29]. The other, triggered by various proapoptotic stimuli, including p53 activation, requires a mitochondria-dependent step, under the control of antiapoptotic and proapoptotic members of the Bcl-2/Bax protein family, which can induce either caspase-dependent or caspase-independent death [26]. Bax represents an example of a proapoptotic protein that induces mitochondria-dependent, caspase-independent PCD [30]. To further

Table 2. Cell death induction using US28-GFP in 293T, Cos-7 and HeLa cells.

Cells ^a	Vectors	Whole population ^b		Cells death in GFP + population ^c	
		% of cell death	% of GFP + cells	–	+ Z-VAD
293T	GFP	2.15 ± 0.26	99.54 ± 0.11	2.16 ± 0.26	–
	CCR5-GFP	7.22 ± 1.76	42.34 ± 6.00	17.05 ± 3.67	–
	US28-GFP	34.26 ± 3.48	70.83 ± 4.51	48.51 ± 5.78	24.76 ± 3.8
HeLa	CCR5-GFP	–	10.00 ± 1.66	9.00 ± 1.40	–
	US28-GFP	–	22.88 ± 7.48	64.34 ± 12.23	28.49 ± 6.5
Cos-7	CCR5-GFP	–	4.51 ± 0.40	11.60 ± 2.40	–
	US28-GFP	–	6.60 ± 1.21	42.44 ± 14.38	11.76 ± 4.6

^a Cells were transfected using the calcium phosphate method. Per cent GFP + cells obtained with the empty Rc/CMV vector: 1.87 ± 2.39% for 293T cells, 2.13 ± 0.40% for HeLa cells, and 2.59 ± 0.86% for Cos-7 cells. ^b Flow cytometry analysis of whole-cell GFP expression and of cell death induction was assessed 72 h after transfection. Results are from a representative experiment. ^c Analysis for red (x-axis) and green (y-axis) fluorescence allows to determine the percentage of GFP + apoptotic cells. The pan caspases-inhibitor z-VAD-fmk was used at the concentration of 50 μM.

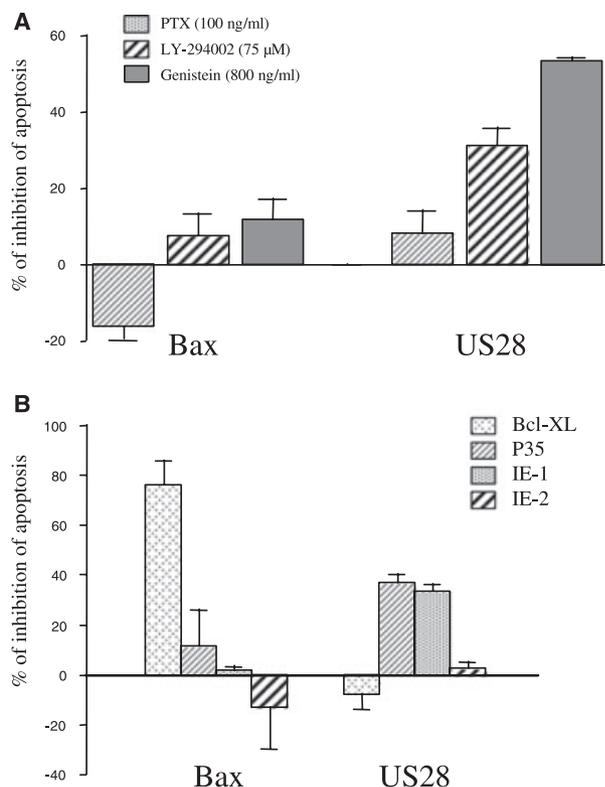


Fig. 3. Inhibition of US28-induced apoptosis. (A) 293T cells were transfected with vectors expressing Bax or US28. PTX, an inhibitor of $G_{\alpha i}$ protein subunits, LY294002, an inhibitor of phosphatidylinositol 3-kinase, and Genistein a inhibitor of tyrosine protein kinases were added 4 h after transfection. All compounds were used at maximal nontoxic concentrations as determined in preliminary experiments. (B) 293T cells were cotransfected with either Bax or US28, and either Bcl-XL, which protects against Bax-induced apoptosis, P35, a baculovirus-encoded pan-caspase inhibitor, or the HCMV encoded immediate early proteins IE1, or IE2. Percentages of inhibition of apoptosis were assessed as described in Experimental procedures. Results are means \pm SD from one of three representative experiments.

explore the pathway involved in US28-mediated cell death, we examined the effect of coexpressing various proteins with US28: Bcl-XL, an antiapoptotic member of the Bcl-2 family that prevents mitochondria-dependent death, p35, a baculovirus-encoded pan-caspase inhibitor [31] and two different HCMV-encoded antiapoptotic proteins, IE1 and IE2 that have been reported to prevent TNFR-mediated apoptosis [11]. IE2, but not IE1, also prevents p53-mediated death [12]. As controls, we examined the effect of coexpressing these proteins on Bax-induced death.

Bcl-XL expression almost completely prevented Bax-induced cell death, as previously described [26], but had no effect on US28-induced cell death (Fig. 3B).

p35, as expected, had little effect on Bax-mediated cell death, but reduced US28-mediated death. Neither IE1 nor IE2 showed any effect on Bax-mediated cell death, while IE1, but not IE2, reduced US28-mediated cell death (Fig. 3B). These results suggest that US28, in contrast to Bax, may induce a caspase-dependent, mitochondria-independent death pathway, which can be blocked either by HCMV-IE1 expression or by caspase inhibitors.

To further explore the potential role of caspase activation in US28-mediated death signalling, we used the pan-caspase inhibitory peptide, z-VAD-fmk. US28-induced cell death was inhibited in a dose-dependent manner by z-VAD-fmk (Fig. 4A). Flow cytometry analysis using tagged US28 indicated that this inhibitory effect was not due to downregulation of US28 surface expression (data not shown). This was further confirmed by investigating the effect of z-VAD-fmk on the capacity of US28, like that of CCR-5, to function as a coreceptor for R5-tropic HIV1 strains [18–21]. Using a cell-fusion assay, involving cocultures of control, US28- or CCR-5-expressing HeLa P4 reporter cells and HIV1 ADA envelope-expressing HeLa cells, we found that z-VAD-fmk induced a 100% increase in the ability of US28 to function as an HIV coreceptor, while reducing by $\approx 30\%$ that of CCR-5 (Fig. 4B). Although we found that the level of US28 surface expression was similar to that of CCR-5 (Table 1), US28–HIV coreceptor function has been reported to be less than half as efficient as that of CCR-5 in HeLa P4 cells [18,19]. In the presence of a pan-caspase inhibitor, US28 was a more efficient coreceptor for HIV than CCR-5 (Fig. 4B), suggesting that the HIV coreceptor activity of US28 may have been underestimated previously because of its proapoptotic activity [18–21].

We further explored the caspase activation pathway triggered by US28 expression using selective caspase inhibitory peptides. Inhibitors of death receptor-coupled initiator caspases 10 and 8 and inhibitors of downstream executionary caspases 3 and 6 showed inhibitory effects similar to that of z-VAD-fmk (Fig. 4C). In contrast, inhibition of caspase 9, the initiator caspase activated downstream of mitochondria permeabilization, or inhibition of caspase 1, a pro-inflammatory caspase, had no effect on US28-induced cell death (Fig. 4C). A comparative kinetic study of the time window in which caspase 10 and 8 inhibitors prevent cell death following US28 expression indicated that the inhibitory effect of the caspase 10 inhibitor was lost several hours before that of the caspase 8 inhibitor (Fig. 4D). This suggests that caspase 10 activation may either occur upstream of caspase 8 activa-

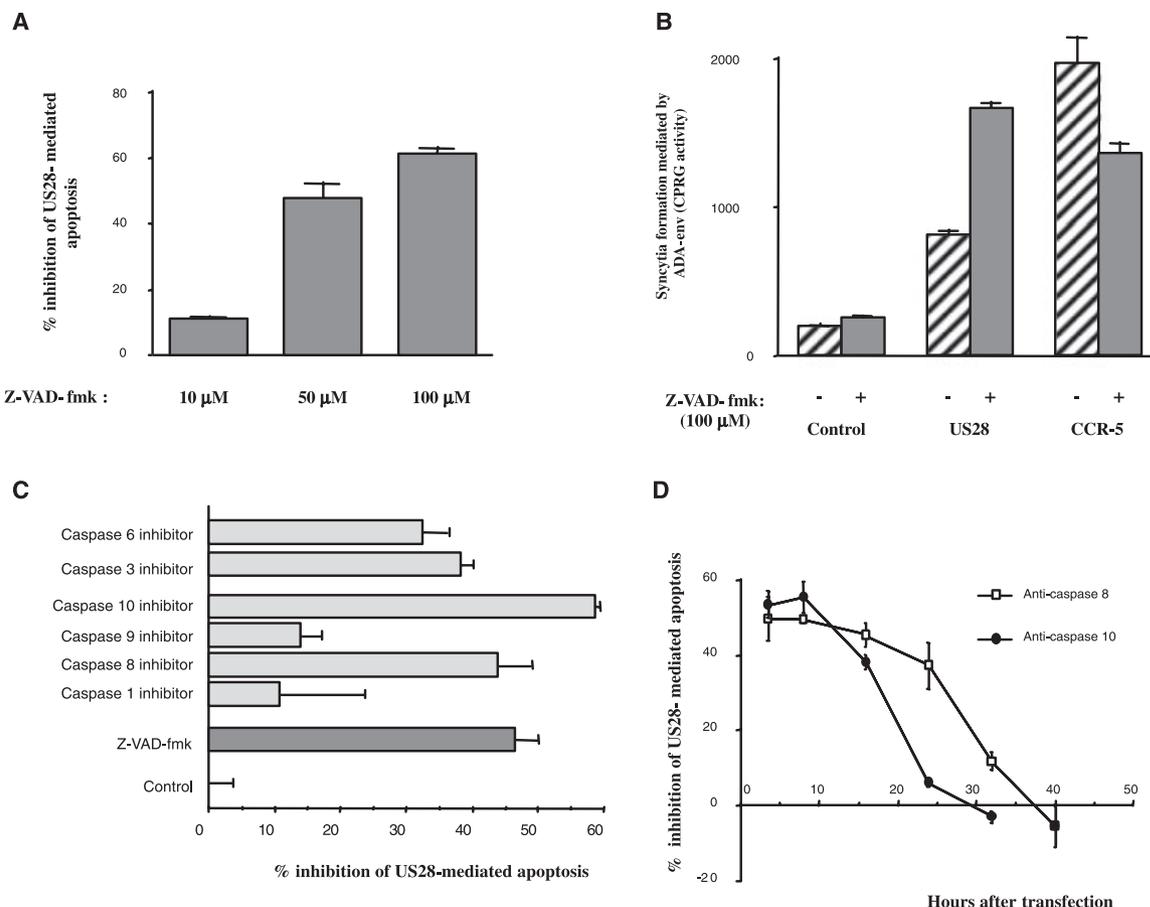


Fig. 4. Inhibition of US28-induced apoptosis by caspase inhibitors. (A) Apoptosis was assessed in 293T cells 48 h after US28 transfection, in the presence of various concentrations of the pan-caspase inhibitor, z-VAD-fmk. (B) HeLa P4 cells, stably expressing CD4, were transfected by the calcium phosphate method with US28, CCR-5 or empty (Control) vectors and cocultured with HeLaEnv/ADA cells, stably expressing HIV envelope, in the presence or absence of z-VAD-fmk. Syncytia formation is an indicator of HIV-coreceptor activity and receptor surface expression. (C) Apoptosis was assessed in 293T cells 48 h after US28 transfection in the presence or absence, of either the pan-caspase inhibitor z-VAD-fmk, or of various selective caspase inhibitors. (D) Kinetic analysis of the inhibitory effect of caspase 8 and caspase 10 inhibitors on apoptosis induced by US28 transfection in 293T cells. Percentages of inhibition of apoptosis in (A, C, D), and numbers of syncytia in (B) were determined as described in Experimental procedures. Results are means \pm SD from one representative experiment out of three (A) or two (B–D).

tion in response to US28 expression, or be more effective in inducing death. We assessed caspase 3 and 8 activities 24 h post transfection, by caspase 3-mediated cleavage of z-MCA-VDQMDGWK(DNP)-NH₂, and caspase 8-mediated cleavage of z-IETD-AFC, over a time course of 90 min (Fig. S3). Caspase 3 and 8 activities were significantly higher in US28-expressing cells than in controls (Rc/CMV) (Fig. S3), whereas 16 h after transfection both activities were weak and similar in US28-expressing cells and controls (data not shown). Taken together, these data imply that US28 triggers a death pathway that depends on activation of initiator caspases 10 and 8, leading rapidly to executionary caspase activity without requiring a mitochondria-dependent step.

US28 cell-death induction is prevented by C-FLIP expression, but not by death receptor neutralization

Activation of caspases 10 and 8 downstream of death signalling by cell-surface receptors of the TNFR family occurs through recruitment of these caspases to death effector domains (DED) containing adapter proteins [29,32,33]. This process is inhibited by cellular members of the FLIP family and their viral homologues [34]. We established HeLa cells stably expressing cellular C-FLIP to explore the effect of C-FLIP on US28-GFP cell-death induction. As shown in both Table 2 and Fig. 5A, expression of US28 in wild-type HeLa cells induced 3 days after US28 transfection, cell death

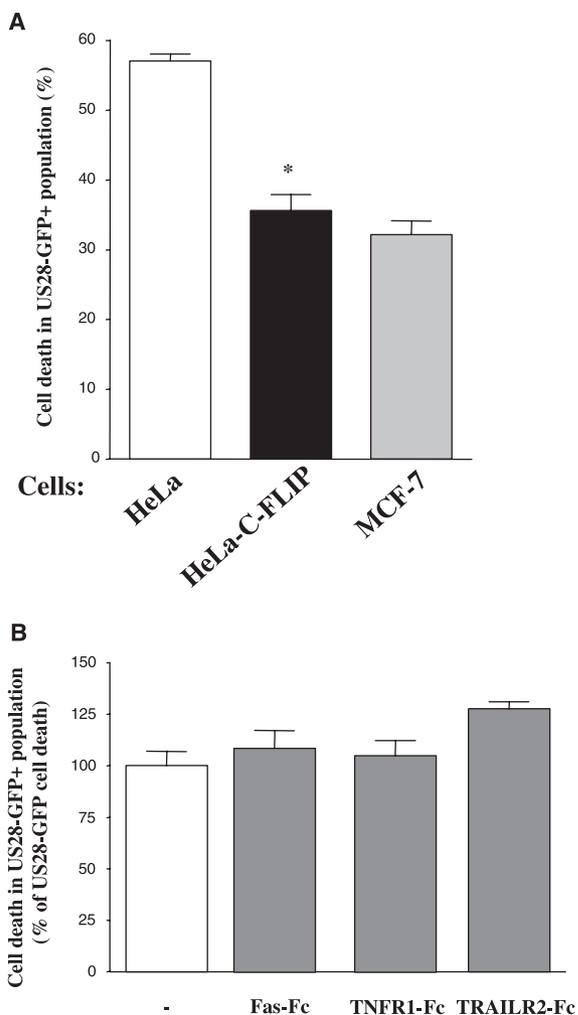


Fig. 5. US28 cell death is inhibited by C-FLIP expression and does not involve members of the TNFR family. (A) Subconfluent HeLa cells, or C-FLIP stably expressing HeLa cells, or as control MCF-7 cells, were transfected using the calcium phosphate method with vectors expressing, US28-GFP or the control vector Rc/CMV vector. Cells were detached 72 h later, and analysed for red PI staining (DNA loss), and GFP fluorescence. Results are the percentage of apoptotic US28-GFP+ cells. (B) Subconfluent 293T cells were transfected by the calcium phosphate method with vectors expressing, US28-GFP or the control vector Rc/CMV vector, in presence or absence of decoy receptors ($30 \mu\text{g}\cdot\text{mL}^{-1}$) that block receptor-ligand interactions. Shown are the percentages of US28-dying cells as assessed by PI staining. *Difference between US28 cell death induction in HeLa and HeLa-C-FLIP cells are statistically significant (*t*-test, $P < 0.001$). Triplicate samples were run at each time point and data represent means \pm SD of two independent experiments (A), and of one representative experiment of two (B).

in $\approx 60\%$ of US28-GFP+ HeLa cells (Fig. 5A). In contrast, in HeLa-C-FLIP cells, US28 expression induced death in only $\approx 35\%$ cells (Fig. 5A), indicating that C-FLIP reduces US28-induced cell death. US28-

induced cell death also appeared strongly reduced in the breast cancer cell line MCF-7 (Fig. 5A), defective in the expression of the apoptosis effector caspase 3 [35], which is necessary for US28-mediated apoptosis.

The death receptor family is composed of different death ligand/receptor pairs of the TNF/TNFR family, such as CD95 (Fas)/CD95L, TNF/TNFR1, TNF/TNFR2, TRAIL/TRAILR1 and TRAIL/TRAILR2 [29]. Decoy receptors have previously been shown to block the interaction between these ligands and their receptors, and ligand-mediated cell death. Treatment of US28-transfected 293T cells with the decoy receptors CD95-Fc, which neutralizes CD95L, TNFR1-Fc, which neutralizes TNF, or TRAILR2-Fc, which neutralizes TRAIL, did not prevent cell death observed 3 days after US28 transfection (Fig. 5B). These data suggest that US28 triggers a cell-death pathway that can be blocked by C-FLIP expression, but which is independent of the engagement of members of the TNF receptor family by their ligands.

Role of constitutive activity and the C-terminal cytoplasmic domain of US28 in induction of apoptosis

Previously, we have shown that US28 constitutively activates several intracellular pathways, resulting in an increase in inositol phosphate (InsP) accumulation as well as NF- κ B-driven transcription [22]. Our results indicated that these effects are mediated via US28 coupling to G proteins of the q/11 family [22]. Interestingly, several studies have demonstrated that enhancement of Gq activity can lead to apoptosis induction via the activation of a protein kinase C (PKC)-dependent pathway [36–38]. To investigate whether the activation of this signalling route plays a role in US28-mediated cell death, we tested the apoptotic effect induced by a signalling deficient mutant, referred to as US28-R129A. US28-R129A carries a mutation in the DRY sequence at the bottom of TM-3, a motif that is highly conserved in class A G-protein-coupled receptors (GPCRs) and plays a pivotal role in G-protein activation [39].

US28-R129A appears unable to induce InsP turnover in HEK293T cells (see Fig. 6A in comparison to US28-wild type), although its level of expression at the cell surface is similar to US28-WT, as determined by ^{125}I -labelled-CX3CL1/fractalkine binding (Fig. 6B). Interestingly, US28-R129A-induced apoptosis was significantly reduced (Fig. 6C) indicating that activation of Gq/11 proteins is involved in US28-mediated cell death. However, the residual apoptotic effect of US28-R129A clearly denotes that additional pathways play a role in the effects observed with US28-WT.

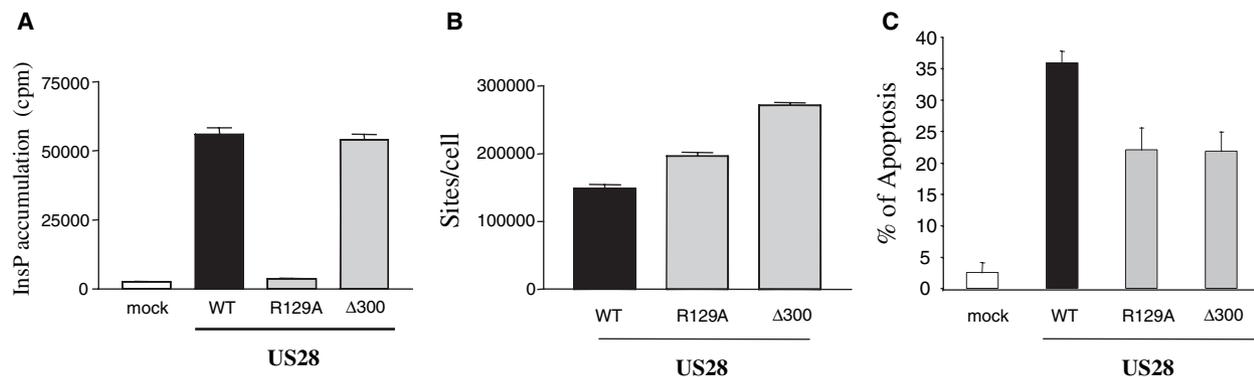


Fig. 6. Cell-death induction by the US28 mutants R129A and $\Delta 300$. (A) InsP accumulation induced by US28, US28-R129A and US28- $\Delta 300$, was evaluated in 293T cells, as described in Experimental procedures. (B) Surface expression of the different US28 mutants was monitored in HEK 293T cells by binding of ^{125}I -labelled fractalkine/CX3CL1. Data are normalized as binding sites per cell. (C) Cell-death induction by US28, US28-R129A or US28- $\Delta 300$, was evaluated 72 h post transfection in 293T cells as in Fig. 1B. Results are means \pm SD of three independent experiments.

Previous studies have shown that the US28 receptor undergoes constitutive endocytosis and recycling at the cell surface [24]. This phenomenon depends on constitutive phosphorylation of serines in the C-terminus of US28 by GRKs and consequent recruitment of β -arrestin-2 to the plasma membrane [23,40]. We have previously shown that deletion of the C-terminal of US28 generates a receptor (US28- $\Delta 300$) which is unable to constitutively internalize and is fully localized at the cell membrane [25]. As can be seen in Fig. 6A, US28- $\Delta 300$ constitutively couples to $G_{\alpha q/11}$ proteins, similarly to WT receptor, indicating that constitutive activity and internalization profiles are distinct properties of US28. Consistent with the idea that this receptor mutant does not undergo constitutive internalization, its expression levels at the cell surface are significantly higher than US28-WT (Fig. 6B). The apoptotic effect induced by US28- $\Delta 300$ is reduced in comparison with WT (Fig. 6C), especially if considering that the receptor mutant has higher expression levels. These results indicate that the C tail of US28, responsible for receptor internalization profiles, is also involved in cell-death induction.

Discussion

To our knowledge, our findings provide the first identification of a virally encoded chemokine receptor which constitutively induces cell death. Apoptosis triggered by the HCMV CC chemokine receptor US28 depends on caspase activation and appears independent of both the mitochondria-dependent death pathway and the engagement by their ligands of death receptors of the TNFR family. US28-induced cell death appears unique among chemokine receptors, because nine human

chemokine receptors belonging to three different sub-families do not induce significant constitutive death. Although engagement of the human chemokine receptor CXCR-4 by its chemokine (SDF-1) and/or viral ligand (HIV envelope) has been reported to induce apoptosis in some cell types, no constitutive death induction by a chemokine receptor in the absence of ligand has yet been identified.

Our results were obtained in cells transfected with US28, a system that allowed us to analyse the behaviour of different US28 mutants and compare it with other human chemokine receptors. A critique often made to this type of studies is that receptor expression levels are often not physiological in transfected cells. However, this is not the case for US28, because infection of permissive cells with HCMV results in even higher levels of US28 than in our study [28], due to the strength of CMV promoters for early genes.

In vitro, survival of HCMV-infected cells may result from neutralization of US28-induced cell death by the different HCMV antiapoptotic proteins (IE1, IE2, vMIA, vICA). However, cell death induced by US28 could be responsible of CMV apoptosis observed *in vivo* in various cells.

Our finding that US28 constitutively induces apoptosis might explain our inability to obtain stable US28 expression in a variety of cell lines, including the Chinese hamster ovarian (CHO) cell line, four human myeloid cell lines (THP1, U937, HL60 and K562), the HEK293T cell line, and one human glioma cell line permissive for CMV infection (U373-MG) (unpublished results). We also observed that the only HeLa clone, which we previously obtained and reported as stably expressing US28 [19], could not be maintained for long periods in culture, while we easily obtained

and maintained stable CCR-3 or CCR-5 transfectants (data not shown). However, we were able to stably express US28 in the murine NIH 3T3 and SVEC cells, suggesting that the cell-death-inducing activity of US28 is strongly dependent on the cellular environment. Two different US28 stably expressing HEK293 cell lines and one US28 stably expressing K562 cell line have been described by others [41,42]. After obtaining one US28-expressing HEK293 cell line [41], we found it behaved like our previously reported HeLa clone, namely we could not maintain it in culture (data not shown).

Our findings using US28 mutants suggest that both US28 constitutive signalling through Gq/11 proteins and US28 receptor internalization play a role in apoptosis induction. Results suggest that the two signalling pathways are independent and additive. Interestingly, the involvement of such pathways in apoptosis has been previously reported in other models of cell-death induction. Indeed, several studies have demonstrated that enhancement of Gq activity induces apoptosis of cultured cells and cardiac myocytes in transgenic mice [36,37]. Moreover, transfection of a constitutively active mutant of G α q into COS-7 and CHO cells was found to induce apoptosis through a PKC-dependent pathway [38]. Also, increasing evidence indicates that GPCRs can modulate several intracellular signals through mechanisms that are independent from G-protein activation. In particular, GPCR interaction with arrestin proteins is actively involved in signal transduction, in particular activation of the nonreceptor tyrosine kinase SRC and mitogen-activated protein kinase [43,44].

Our findings indicate that apoptosis induced by US28 expression can be repressed by concomitant expression of either the antiapoptotic HCMV-encoded immediate early protein IE1, the cellular protein C-FLIP, or by the use of synthetic caspase inhibitors. Interestingly, both IE1, and FLIP proteins prevent apoptosis induction by ligand-mediated engagement of two cell-surface death receptors, TNFR and CD95 that trigger death via recruitment of DED-containing adapter proteins which lead to recruitment and activation of caspase 10 and/or caspase 8. C-FLIP represses such death signalling through direct interference with adapter protein-mediated recruitment and activation of caspase 10 and 8. To investigate the possible involvement of TNF family ligand and death receptors in US28 cell death, we used soluble recombinant CD95, TNFR2 and TRAIL extracellular domains fused to the immunoglobulin Fc domain, which inhibit apoptosis induced by CD95L, TNF and TRAIL, respectively, by acting as soluble decoy receptors. All these decoy

receptors failed to protect cells from US28-induced cell death, suggesting that US28-induced cell death does not depend on such death receptor engagement by their ligands. Caspase-mediated cell death induction independent of TNFR family death receptors has also been reported in a process called Anoikis, which is a caspase-dependent apoptosis induced by loss of integrin-mediated adhesion to extracellular matrix in the absence of any other death-inducing stimuli [45].

Our finding that US28-induced apoptosis is prevented by IE1 expression suggests that HCMV might function, at least partially, according to the adenovirus paradigm of viral-mediated regulation of cell survival and cell death. Expression of an adenovirus gene product required for viral replication, e.g. E1A, induces cell death thereby aborting infection unless additional antiapoptotic viral gene products, e.g. E1B, are expressed that allow infected cell survival [46]. Accordingly, US28, one of the earliest HCMV gene products expressed in both latently and productively infected cells [47,48], might lead to apoptosis as a default pathway, unless IE1 is also expressed, allowing repression of cell-death induction. In contrast to the adenovirus E1A protein, however, the HCMV US28 protein does not appear to be required for the HCMV cycle, at least *in vitro* [17].

In vivo, the function of US28 as a chemokine receptor has been proposed to provide HCMV with several potential advantages, including viral dissemination through RANTES-mediated chemotaxis of infected cells [16], immune evasion via the clearance of proinflammatory chemokines from the environment of infected cells [17], and possibly activation of the infected cell for its latency or replication by constitutive PLC signalling [41]. Our finding that US28 can induce constitutive death implies that repression of apoptosis induction might be a prerequisite for its function as a chemokine receptor or cellular activator, and also raises the question of the potential role that US28-mediated death induction may play in HCMV/host interactions. US28-induced apoptosis of infected cells could contribute to viral dissemination, once active viral replication has been achieved, but also to the immune control of systemic infection by stimulating antiviral CD8 T-cell responses through ingestion of these apoptotic cells by dendritic cells [49].

All β and most of γ herpesviruses encode chemokine receptor homologues [15]. Our finding that one of them, US28, is involved in constitutive death induction, raises the question of whether this represents an exception or rather a particular example of a general strategy of viral subversion of chemokine receptor functions. It should be noted that proof of the concept

of constitutive receptor-mediated cell-death induction is difficult to obtain, because extreme ligand promiscuity may be an alternative mechanism allowing viral chemokine receptors to signal in the presence of as yet unknown chemokines. Whatever the mechanism(s) involved, the virally encoded chemokine receptor US28 might provide infected cells with unique functional properties, which differ from those conferred by their human cellular homologues.

Finally, viral chemokine receptor death signalling might represent an opportunity for therapy. Indeed, our finding that the expression of IE1 can repress US28-induced apoptosis could enhance interest in current strategies which focus on IE1 as a drug target in HCMV-related disorders [50].

Experimental procedures

Reagents

The pan-caspase inhibitor z-VAD-fmk was purchased from Bachem (Voisins-le-Bretonneux, France). The selective caspase inhibitors, z-WEHD-fmk (caspase 1), z-DEVD-fmk (caspase 3), z-VEID-fmk (caspase 6), z-IEYD-fmk (caspase 8), z-LEHD-fmk (caspase 9) and z-AEVD-fmk (caspase 10), and chemokines RANTES and Fractalkine were from R&D Laboratories (Abingdon, UK). Pertussis toxin was purchased from Sigma Chemical Co. (St. Louis, MO), Genistein from Alexis Corp (San Diego, CA), and LY-294002 from Calbiochem (La Jolla, CA). Human CD95-Fc immunoglobulin fusion protein, which binds CD95L, TRAILR2-Fc, which binds TRAIL, and TNFR1-Fc, which binds TNF α and lymphotoxin α , were provided by P. Schneider.

Cells

All cell lines were grown in Dulbecco's modified Eagle's medium containing 10% de complemented fetal bovine serum and antibiotics (60 mg·mL⁻¹ penicillin and 100 mg·mL⁻¹ streptomycin). HeLa-tat-Env/ADA cells stably express HIV R5-tropic ADA strain envelope protein, and HeLa P4 cells stably express CD4 and long-terminal repeat (LTR)-LacZ. P4 cells were cultured in the presence of G418 neomycin, and HeLa-tat-Env/ADA cells in presence of methotrexate.

Expression vectors

Expression vectors of US28-AD169, US28-VHL/E, US28-Toledo, and cellular chemokine receptors have been described elsewhere [14,18,27,51]. Bax, Bcl-XL, and p35 expression plasmids were obtained by cloning the cDNAs into pcDNA3. Bax and Bcl-XL cDNAs were provided by S. Korsmeyer (Departments of Pathology and Medicine,

Harvard Medical School, Boston, MA) and p35 cDNA by J. Abrams (Departments of Pharmacology and Biochemistry, University of Texas South-Western Medical, Dallas, TX). Expression vectors for CMV IE1 and IE2 were obtained from R. LaFemina (Department of Antiviral Research, Merck Research Laboratories, West Point, PA). C-FLIP expression vector was obtained by J. Tschopp (Department of Biochemistry, University of Lausanne, Epalinges, Switzerland) [52].

US28 mutants, US28-GFP and CCR-5-GFP fusion proteins

US28-WT and US28-R129A from CMV-AD169 strain and US28-R129A subcloned in pcDEF3 have been previously described [53]. The C-terminus truncation mutant US28- Δ 300 was generated by PCR according to previous literature [25] and subcloned in pcDEF3 vector. Enhanced green fluorescent protein (EGFP) fusion constructs were created using the pEGFP-N1 vector (Clontech Laboratories, Palo Alto, CA) by ligation of PCR fragments amplified from US28 and CCR-5 between *Xho*I and *Hind*III sites.

Detection of tagged chemokine receptors and EGFP

For flow cytometry analysis of myc-tagged receptors, 293T cells were analysed 16 h after transfection with tagged chemokine receptor vectors after cell detachment with phosphate-buffered saline (NaCl/P_i) containing 1 mM EDTA. Cells (10⁵) were pelleted and incubated for 1 h at 4°C with Cy3-conjugated anti-Myc IgG, 9E10 (Sigma) at 0.5–2 μ g·mL⁻¹ in NaCl/P_i + 1% bovine serum albumin (washing buffer). After washing, cells were analysed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Measurement of apoptosis

293T cells were seeded in 24-well plates (2 \times 10⁴ cells per well), incubated overnight at 37 °C in complete medium, then transfected by the calcium phosphate technique (0.5 μ g of plasmid DNA per well, or 0.3 μ g of each plasmid DNA per well in cotransfection experiment). Adherent cells were recovered at indicated time points post transfection by incubation in NaCl/P_i containing 1 mM EDTA, and washed in NaCl/P_i. DNA content loss (hypodiploidy) was assessed by flow cytometry analysis of propidium iodide (PI) (2 μ g·mL⁻¹) (Sigma) staining in the presence of 1% saponin, using a FACScan flow cytometer (Becton Dickinson). Caspase inhibitors or other inhibitory molecules were added to cells 4 h after transfection. Because expression vectors were under the control of the CMV enhancer/promoter, the effect of inhibitors on this enhancer/promoter

was verified in parallel using cells transfected with a control CMV vector expressing LacZ (pCMV-LacZ) and β -galactosidase activity was assessed as previously described [54]. None of the inhibitors affected β -galactosidase expression.

In others cell lines, cell-death induction was monitored after transfection of US28-GFP or CCR-5-GFP fusion proteins, or GFP alone, 3 days after transfection in 24-well plates by determining the amount of DNA loss (hypodiploidy) in GFP+ cells by flow cytometry analysis.

HIV-Env syncytia formation assay

Briefly, HeLa P4 cells (10^5 cells per well), stably transfected with CD4 and a *lacZ* gene under the control of the HIV-LTR, were seeded in six-well plates and transfected with 3 μ g of DNA per well using the calcium phosphate technique. Syncytia formation was assessed 48 h after transfection using HeLa-Tat-Env-ADA cells, as previously described [18,19]. β -Galactosidase activity, generated upon syncytia formation, was quantified as described previously [54].

Inositol phosphate accumulation

293T cells were plated in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected by the calcium phosphate method. Eight hours after transfection, the medium was replaced with inositol-free Dulbecco's modified Eagle's medium supplemented with 10% dialysed fetal bovine serum and *myo*-[2- 3 H]inositol (3 μ Ci·mL $^{-1}$). After overnight incubation, the labelling medium was aspirated, cells were washed for 10 min with Dulbecco's modified Eagle's medium containing 25 mM Hepes (pH 7.4) and 20 mM LiCl, then incubated for 1 h in the same medium. Incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min incubation on ice, InsP were isolated by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and measured by liquid scintillation.

Binding experiments

Labelling of fractalkine/CX3CL1 (residues 1–72; Peprotech, Rocky Hill, USA) with 125 I using the IODOGEN method (Pierce, Rockford, IL) was performed as described previously [55]. Two days after transfection, HEK293T cells were incubated with 0.6 nM 125 I-labelled fractalkine in binding buffer (50 mM Hepes pH 7.4, 1 mM CaCl $_2$, 5 mM MgCl $_2$ and 0.5% bovine serum albumin) for 3 h at 4 °C. After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined in the presence of 0.1 μ M cold chemokine. Data were analysed with the program GRAPHPAD PRISM (San Diego, CA).

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Supplementary material

The following material is available online:

Fig. S1. Detection of GFP-fused chemokine receptors and cell-death induction. Subconfluent 293T cells were transfected by the calcium phosphate method with vectors expressing (A) the control Rc/CMV vector, (B) the GFP, and (C) CCR-5-GFP or (D) US28-GFP fusion proteins. Cells were detached 72 h later, and analysed using flow cytometry for DNA loss (PI staining) (x-axis), and green GFP fluorescence (y-axis). The percentages of living GFP + cells are indicated on the right and the percentages of apoptotic GFP + cells on the left.

Fig. S2. Fluorescent microscope analysis of GFP and GFP-fused chemokine receptors US28 and CCR-5. Cells were examined 48 h post transfection after strain-

ing with HOESCHT 33342, in the presence or in absence of z-VAD-fmk in US28–GFP transfected cells. The arrow indicates typical nuclear chromatin condensation and fragmentation in dying cells.

Fig. S3. Caspase 3 and 8 activation by US28. Caspase 3 and 8 activation is expressed as raw fluorescence units as a result of z-MCA-VDQMDGWK(DNP)-NH₂ and z-IETD-AFC hydrolysis, respectively. 293T

cells (2×10^6) were transfected with 10 μ g of US28 expression vector or the empty vector Rc/CMV as a control. Transfected cells were incubated 24 h later with each substrate at 25 °C for 0 min (immediately following mixing) and 90 min later. No significant difference was obtained at time 0. Duplicate samples were run at each time point and data represent means \pm SD of two experiments.