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# The ribonucleotide reductase R1 subunits of herpes simplex virus types 1 and 2 protect cells against TNF $\alpha$ - and FasL-induced apoptosis by interacting with caspase-8

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**Abstract** We previously reported that HSV-2 R1, the R1 subunit (ICP10; *UL39*) of herpes simplex virus type-2 ribonucleotide reductase, protects cells against apoptosis induced by the death receptor (DR) ligands tumor necrosis factor-alpha- (TNF $\alpha$ ) and Fas ligand (FasL) by interrupting DR-mediated signaling at, or upstream of, caspase-8 activation. Further investigation of the molecular mechanism underlying HSV-2 R1 protection showed that extracellular-regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3-K)/Akt, NF- $\kappa$ B and JNK survival pathways do not play a major role in this antiapoptotic function. Interaction studies revealed that HSV-2 R1 interacted constitutively with caspase-8. The HSV-2 R1 deletion mutant R1(1-834)-GFP and Epstein-Barr virus (EBV) R1, which did not protect against apoptosis induced by DR ligands, did not interact with caspase-8, indicating that

interaction is required for protection. HSV-2 R1 impaired caspase-8 activation induced by caspase-8 over-expression, suggesting that interaction between the two proteins prevents caspase-8 dimerization/activation. HSV-2 R1 bound to caspase-8 directly through its prodomain but did not interact with either its caspase domain or Fas-associated death domain protein (FADD). Interaction between HSV-2 R1 and caspase-8 disrupted FADD-caspase-8 binding. We further demonstrated that individually expressed HSV-1 R1 (ICP6) shares, with HSV-2 R1, the ability to bind caspase-8 and to protect cells against DR-induced apoptosis. Finally, as the long-lived Fas protein remained stable during the early period of infection, experiments with the HSV-1 *UL39* deletion mutant ICP6 $\Delta$  showed that HSV-1 R1 could be essential for the protection of HSV-1-infected cells against FasL.

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## Introduction

The tumor necrosis factor (TNF) superfamily of ligands, acting through cognate death receptors (DRs), plays a critical role in innate and adaptive immune responses to viruses. The antiviral activity of several of these ligands often correlates with their ability to induce apoptosis initiated by the triggering of DRs [1–3]. TNF $\alpha$ , which acts mainly through binding to TNF receptor 1 (TNFR1), has been reported to have antiviral activity against herpes simplex viruses (HSVs) [4, 5]. HSV-1-infected mice knocked-out for TNF $\alpha$  develop HSV encephalitis due to an impaired immune response which fails to clear the virus from the brain [6]. Fas ligand (FasL), expressed on cytotoxic T lymphocytes and natural killer cells, is also important in the elimination of infected cells. Recently, Fas-mediated apoptosis of HSV-2-infected cells involving CD4<sup>+</sup> cytotoxic T lymphocytes was shown to be crucial for the defense against lethal infection in mice [3]. The importance for HSVs to counteract DR activation is furthermore evidenced by the diverse strategies used by these viruses to block DR signaling pathways. At least two viral proteins, the ribonucleotide reductase (RR) R1 subunit of HSV-2 (HSV-2 R1) and the virion host shutoff protein (vhs), have been hypothesized to interfere with these pathways [7, 8].

Ligand binding to DRs triggers the recruitment of death domain (DD)-containing adaptor proteins to the DD of receptors, resulting in the formation of membrane-bound signaling complexes. Important differences exist between Fas and TNFR1 activation, not only in terms of signaling complexes but also function, Fas exhibiting mainly proapoptotic behavior, and TNFR1, mostly proinflammatory activity. Upon FasL-Fas binding, Fas-induced apoptosis involves recruitment of the Fas-associated death domain protein FADD to Fas. Receptor-bound FADD forms a death-inducing signaling complex (DISC) with caspase-8. Aggregation of proteins in DISC stimulates autocatalytic processing of caspase 8 and triggers the caspase cascade [reviewed in 9]. Cellular FLICE-inhibitory protein (c-FLIP), by inhibiting the recruitment of caspase-8 to DISCs, negatively modulates the caspase cascade [10, 11]. FLIP was originally identified as a viral gene, viral FLIP (v-FLIP) being present in  $\gamma$ -herpes viruses such as equine herpes virus-2, herpes virus saimiri and human herpes virus-8 (HHV-8) [reviewed in 12].

TNF $\alpha$ -induced signaling involves the formation of two sequential signaling complexes [13, 14]. Upon TNF $\alpha$  binding to TNFR1, a first complex consisting of TNF $\alpha$  receptor-associated death domain protein (TRADD), Ser/Thr kinase receptor-interacting protein 1 (RIP1) and TNF $\alpha$  receptor-associated factor 2 (TRAF2) is formed [reviewed in 15]. This membrane-associated complex (complex I)

rapidly leads to the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), mitogen-activated protein kinase (MAPK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), p38 MAPK and ERK1/2. Activation of these pathways stimulates the transcription of genes that regulate proliferation, inflammation or apoptosis, including the key antiapoptotic cellular inhibitor of apoptosis (cIAPs) and c-FLIP [reviewed in 16, 17]. In a second step, TRADD and RIP1 form an intracellular complex (complex II) with FADD. FADD recruits caspase-8 by death-effector domain (DED) interaction, allowing the dimerization and activation of caspase-8 that initiates the apoptotic process [13, 14]. The outcome of TNFR1 stimulation depends on the balance between signals triggered by both complexes, the activation of NF- $\kappa$ B by complex I playing an essential role in protecting cells against apoptosis [18]. With several cell lines, such as HeLa cells, induction of apoptosis by TNF $\alpha$  critically depends on the presence of cycloheximide (CHX), which, by drastically reducing the levels of the short-lived proteins c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub>, increases caspase-8–FADD interaction [10].

HSV RRs convert ribonucleotide diphosphates to corresponding deoxyribonucleotides, allowing virus replication in non-dividing cells [19]. The association of two homodimeric subunits, denoted as R1 and R2, forms the holoenzyme. HSV R1s carry the RR domain and a unique NH<sub>2</sub> domain of about 400 amino acids dispensable for RR activity [20, 21]. Several non-RR related functions have been ascribed to HSV R1s. (i) Numerous reports indicate that a protein kinase activity could be intrinsic to the NH<sub>2</sub> domain of HSV-2 R1, but not to that of HSV-1 R1 [22]. However, extensive biochemical studies on HSV-1 R1 and HSV-2 R1 provided strong evidence that both proteins are devoid of intrinsic kinase activity [23, 24]. (ii) A stretch exhibiting weak similarity with the  $\alpha$ -crystallin domain of the small heat shock proteins (sHsps) has been detected in the HSV R1s N terminus [25, 26], leading to the observation that purified HSV-2 R1 exhibits a chaperone activity similar to Hsp27 *in vitro* [26]. (iii) HSV-1 R1 has been associated with the promotion of protein translation in growth-arrested cells by stimulating assembly of the translation initiation complex eIF4F through its binding to eIF4G [27]. (iv) Finally, antiapoptotic properties have been associated to HSV R1s, most often to HSV-2 R1 [7, reviewed in 25].

The Aurelian group has provided evidence that HSV-2 R1 could impair apoptosis induced by the mitochondrial pathway through its ability to activate the MEK/ERK1/2 and PI3-K/Akt pathways [reviewed in 25]. Our group has shown that the individually expressed HSV-2 R1 blocks DR-induced apoptosis but does not impair apoptosis triggered via the mitochondrial pathway [7]. The RR domain of HSV-2 R1, but not the NH<sub>2</sub> domain, is essential for protection against TNF $\alpha$ -induced apoptosis [28]. HSV-2

R1, which does not act as an enzymatic inhibitor of active caspase-8, interrupts DR-mediated signaling at, or upstream of, caspase-8 activation [7]. As two HSV-1 *UL39* deletion mutants, ICP6 $\Delta$  and hrR3, exhibited a 50% reduction in protection from TNF $\alpha$ , we had proposed that HSV-1 R1 could be important for protecting HSV-infected cells against this DR ligand. However, as 50% of cells infected with R1 deletion mutants were resistant to TNF $\alpha$ , we also postulated that other viral protein(s) contribute(s) to protection. Since deletion of the *vhs* gene reduced antiapoptotic potential by 30%, we hypothesized that *vhs* protein, by decreasing the amount of DRs at the cell surface, could play a significant role in resistance [7]. Our hypothesis was later substantiated by a report showing that, owing to the very short half-life of TNFR1 protein, *vhs* causes a rapid reduction of TNFR1 early after HSV-1 infection [8].

Here, our efforts to better understand how HSV-2 R1 behave at the molecular level to prevent TNF $\alpha$ -induced apoptosis led us to show that HSV-2 R1 interacts constitutively with caspase-8 through its prodomain. Evidence is provided that the interaction between the two proteins impairs caspase-8 dimerization/activation and disrupts FADD-caspase-8 binding. Extending our study to HSV-1 R1, we demonstrated that individually expressed HSV-1 R1 shares with HSV-2 R1 the ability to protect cells against TNF $\alpha$ - and FasL-induced apoptosis and to bind to caspase-8. Moreover, we provided evidence that HSV-1 R1 could play an essential role in the protection of HSV-1-infected cells against FasL. These results suggest that HSV R1s are viral caspase-8 inhibitors that are functionally similar to viral inhibitor of caspase-8 activation (vICA), the UL36 gene product of cytomegalovirus (CMV).

## Materials and methods

### Cell lines

The conditions for culture of HeLa, A549-tTA and A549-tTA-HSV-R1-GFP cells have been described [7, 28]. When cultured in the absence of anhydrotetracyclin (Tet), A549-tTA-HSV-R1-GFP cells express HSV-2 R1 fused to green fluorescent protein (GFP) [28].

### Transfection and immunofluorescence

HeLa cells were seeded one day before transfection in 6-well plates at a concentration of  $2 \times 10^5$  per well or in 100-mm dishes at  $1.5 \times 10^6$  per dish. The calcium phosphate technique [7] was used to transfect 10  $\mu$ g per well and 50  $\mu$ g per dish of the plasmids pAdCMV5 (empty), pAdCMV5-R1 (HSV-2 R1); pLBPF1-GST (GST),

pLBPF1-GST-R1 (GST-HSV-1 R1) (kindly provided by A. Pearson); pcDNA3 (empty), pcDNA3-HA-EBV R1 (HA-EBV R1) [29]; pEGFP C1 (GFP) and plasmids encoding for caspase-8 GFP (casp-8 GFP), caspase-8 C360S GFP (casp-8 C360S GFP), caspase-8 DED-AB (1-209) GFP (casp-8 DED-AB GFP), caspase-8 CD (210-479) GFP (casp-8 CD GFP) and FADD YFP [30]. Immunofluorescence was performed on cells fixed with paraformaldehyde as previously described [31].

### Infection

The previously-described adenovirus (Ad) recombinants AdCMV5-GFP, AdCMV5-R1, AdTR5-GFP, AdTR5-R1-GFP and AdTR5-R1(1-834)-GFP express GFP, HSV-2 R1, GFP, HSV-2 R1-GFP and HSV-2 R1(1-834)-GFP, respectively [7]. HeLa cells ( $2 \times 10^5$  cells/well,  $2.5 \times 10^6$  cells/100-mm dish) were infected at 15 plaque forming units (PFU)/cell with AdCMV5-R1 or AdCMV5-GFP. A549-tTA cells ( $2.5 \times 10^6$  cells/100-mm dish) were infected with AdTR5-R1-GFP (10 PFU/cell), AdTR5-R1(1-834)-GFP (50 PFU/cell) or AdTR5-GFP (25 PFU/cell). For infection followed by transfection, HeLa cells ( $2 \times 10^5$  cells/well) were infected with AdCMV5-R1 or AdTR5-CuO (10 PFU/cell) for 8 h before transfection. For HSV infections, HeLa and A549-tTA cells ( $2.5 \times 10^6$  cells/100-mm dish) were infected at 10 PFU/cell with the HSV-1 R1 null mutant ICP6 $\Delta$  [19] or its parental wild type (WT) HSV-1 strain KOS.

### Apoptosis and caspase assays

Apoptosis was induced by adding to the cellular medium either CHX (15  $\mu$ g/ml) plus human recombinant TNF $\alpha$  (2.5 ng/ml; Sigma) or CHX (15  $\mu$ g/ml; Sigma) alone as control, or hexameric FasL (Fc:FasL, a human recombinant FasL fused at the C terminus of the Fc domain of IgG1) [32]. After 6–8 h of treatment, the percentage of apoptotic cells was scored by observation under an inverted microscope in at least five randomly-selected fields, as described previously [7]. Apoptotic cells showed several of the following morphological features: cytoplasmic shrinkage, membrane blebbing, rounding up, ballooning, acquisition of refringence, detachment from the substratum and loss of membrane integrity. Attached and detached cells were collected, washed twice with PBS and lysed in buffer appropriate for subsequent assays. Caspase-3/7 activity (DEVD-AFC cleavage) was measured as described [7].

### Immunoprecipitation

After infection, transfection and/or treatment, attached and detached cells were collected, washed twice with PBS, and

lysed at 4°C for 15 min in a buffer containing 0.5% Nonidet-P40, 0.5% Triton X-100, 50 mM Hepes (pH 7.5), 1 mM EDTA, 150 mM NaCl, 10% glycerol, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche), followed by centrifugation (10 min at 10,000×g at 4°C). Lysates were pre-cleared by incubation with protein G Sepharose 4B beads (Amersham Biosciences) for 2 h at 4°C, followed by centrifugation. Anti-GFP antiserum or caspase-8 monoclonal antibody (mAb) 1C15 (kindly provided by Peter and co-workers [33]) was incubated with protein G Sepharose 4B beads for 2 h at 4°C. Pre-cleared lysates were incubated with antibody-coated beads for 2 h at 4°C. The beads were recovered by centrifugation, washed five times with lysis buffer, and immunoprecipitated. Proteins were eluted by boiling in sodium dodecyl sulphate (SDS) sample-loading buffer. As controls, pre-cleared lysates were incubated for 2 h at 4°C with protein G Sepharose beads alone.

#### In vitro interaction assays

Glutathione-S-transferase (GST), GST caspase-8 (GST casp-8) [34], GST caspase-8 DED-AB (amino acids 1–180) (GST casp-8 DED-AB) and GST caspase-8 CD (amino acids 181–478) (GST casp-8 CD) [35] were produced in *Escherichia coli* BL21DE3 after induction by isopropyl  $\beta$ -D-1-thiogalactopyranoside (0.1 mM) at 37°C. Recombinant GST fusion proteins were purified by affinity absorption with glutathione Sepharose 4B beads (Amersham). GST fusion proteins pre-coupled to glutathione Sepharose 4B beads (Amersham Biosciences) were incubated at 4°C for 1 h with 1  $\mu$ g of HSV-2 R1 purified by peptidaffinity from production in bacteria (pET-R1) or in human 293S cells (BM5-R1) [24]. The beads were recovered by centrifugation and washed five times. Bound proteins were eluted by boiling in SDS sample-loading buffer and immunoblotted with anti-R1 serum 168R1.

#### Protein extraction and immunoblot analysis

Whole cell extracts were prepared by lysis in SDS buffer (2% SDS, protease inhibitor cocktail and phosphatase inhibitor cocktail), followed by brief sonication and centrifugation. Conditions for cytosolic and nuclear protein fraction extraction have been described previously [36]. Protein content was analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting [37]. mAbs against caspase-8 (1C12) and phospho-p42/44 ERK1/2 (Thr202/Tyr204; E10), and polyclonal antibodies against p42/44 ERK1/2, Akt, phospho-Akt (Thr308), JNK and phospho-JNK (Thr183/Tyr185), were from Cell Signaling Technology. mAbs directed against FADD (A66-2), TRADD (clone 37) and RIP1 (G322-2) were from BD Biosciences.

Anti-TNFR1 (H-5),  $\alpha$ -tubulin, I $\kappa$ B- $\alpha$  (C-21), NF- $\kappa$ B p65 (C-21) and GFP (B-2) mAbs were from Santa Cruz Biotechnologies. mAb AC15 against  $\beta$ -actin was from Abcam, and c-FLIP (NF6), from Alexis Biochemicals. Polyclonal anti-R1 serum 168R1 and polyclonal anti-R2 serum P9 served for HSV R1s and HSV R2s detection, respectively [7, 38]. Polyclonal antiserum against GFP was used for HSV-2 R1-GFP and GFP detection [28]. Immunoblotting was quantified with Quantity One software (Bio-Rad).

## Results

The ERK, Akt, NF- $\kappa$ B and JNK survival pathways do not play a major role in protection from TNF $\alpha$ -induced apoptosis.

#### ERK1/2 and Akt

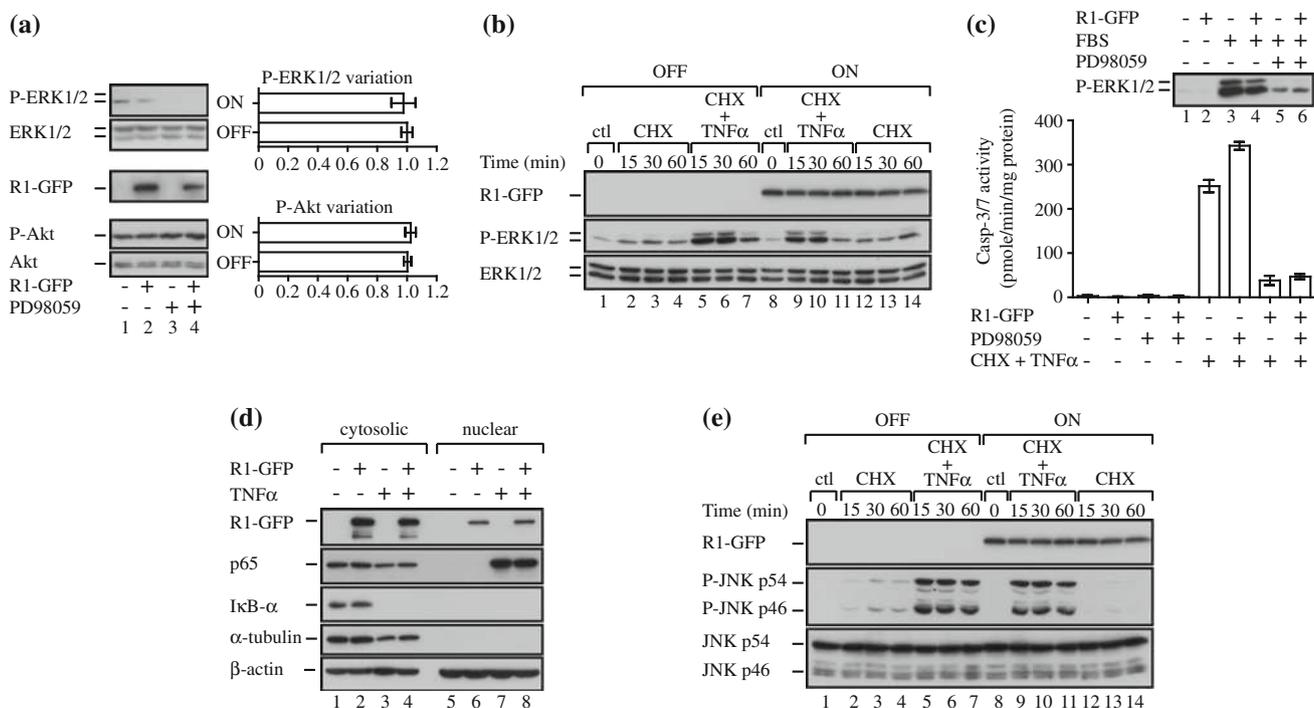
The role of the ERK1/2 and PI3-K/Akt signaling pathways in controlling apoptosis is well known [reviewed in 39, 40], and it has been shown that ERK1/2 or PI3-K/Akt activation can confer resistance to ligands of the TNF superfamily [41, 42]. Moreover, it has been reported that cells constitutively expressing HSV-2 R1 exhibit activation of the ERK1/2 signaling pathway [43] and that such activation could be involved in HSV-2 R1 antiapoptotic activity [22].

To study the effect of HSV-2 R1 on ERK1/2 and Akt phosphorylation, we first took advantage of our A549-tTA-HSV-R1-GFP cell line, in which expression of the chimeric protein HSV-2 R1-GFP is controlled by TR5, a Tet-responsive promoter. When these cells were kept in the OFF state in the presence of Tet, HSV-2 R1-GFP expression was shut down and apoptotic morphology, as scored by microscopic observation, was induced in more than 90% of the cells by CHX + TNF $\alpha$  treatment. Switching ON HSV-2 R1-GFP expression upon Tet removal for 24 h induced HSV-2 R1-GFP expression and more than 90% of the cells exhibited healthy morphology as previously described [28]. ERK1/2 and Akt activation was measured with antibodies recognizing the activated/phosphorylated forms of Thr202/Tyr204-ERK1/2 and Ser473-Akt in A549-tTA-HSV-R1-GFP cell extracts at 24 h after switching on HSV-2 R1-GFP expression. As depicted in Fig. 1a (lanes 1 and 2), HSV-2 R1-GFP expression did not significantly affect the level of either phospho-ERK1/2 or phospho-Akt. This was confirmed by densitometric analyses of immunoblots from five experiments (graph bars, right panel). As this result was contradictory to the above-mentioned report of ERK1/2 activation in 293 cells, we examined the phospho-ERK1/2 level in 293 cells inducibly expressing HSV-2 R1 (293-rtTA-HSV-2-R1 cells). Once again, we did not detect significant ERK1/2 activation after switching on HSV-2 R1 expression (data not shown).

TNF $\alpha$  is known to induce transient cell type-dependent activation of ERK1/2 [44]. To determine whether HSV-2 R1 could influence this transient activation, we investigated the effect of switching on HSV-2 R1-GFP expression for 48 h in A549-tTA-HSV-R1-GFP cells on the time course of TNF $\alpha$ -induced ERK1/2 phosphorylation. As expected, in cells kept in the OFF state, CHX + TNF $\alpha$  elicited an increase in ERK1/2 phosphorylation that peaked between 15 and 30 min after treatment (Fig. 1b, lanes 5–7). HSV-2 R1-GFP-expressing cells treated with CHX + TNF $\alpha$  exhibited a pattern similar to that of control cells in terms of level and time course of ERK1/2 phosphorylation (Fig. 1b, lanes 9–11).

To further assess the involvement of the ERK1/2 pathway in HSV-2 R1 protection from TNF $\alpha$ -induced apoptosis, we examined whether PD98059, a specific pharmacological inhibitor of mitogenic-extracellular signal-regulated kinase

1/2 (MEK1/2) [45] would decrease the HSV-2 R1 protective effect. PD98059 did not increase the low level of apoptosis (<10%) seen in CHX + TNF $\alpha$  treated cells when HSV-2 R1-GFP was expressed. Measurements of caspase-3/7 activity showed that the presence of PD98059 during CHX + TNF $\alpha$  treatment did not increase the low level of activity seen in the presence of HSV-2 R1-GFP (Fig. 1c, graph bars). These results indicated that the MEK1/2 inhibitor did not alter the protection afforded by HSV-2 R1-GFP expression. The efficiency of the inhibitor was ascertained by showing that it caused the disappearance of basal phospho-ERK1/2 level in serum-starved cells (Fig. 1a, lanes 3 and 4) and a strong reduction of ERK1/2 phosphorylation induced by serum addition (Fig. 1c, upper panel). Similar experiments with LY294002, a specific inhibitor of PI3-K [46], did not disclose any effect on



**Fig. 1** ERK1/2, Akt, NF- $\kappa$ B and JNK survival pathways do not play a major role in HSV-2 R1 protection against TNF $\alpha$ -induced apoptosis. **a** A549-tTA-HSV-R1-GFP cells, grown for 5 h in medium containing (OFF) or not (ON) Tet and 10% FBS, were cultured in 0.5% FBS for 18 h before being treated or not with PD98059 for 90 min. Total protein extracts were analyzed by immunoblotting for HSV-2 R1-GFP, phospho-ERK1/2 (Thr202/Tyr204, P-ERK1/2), phospho-Akt (Ser473, P-Akt), Akt and ERK1/2. P-ERK1/2 and P-Akt variations (mean  $\pm$  SE,  $n = 10$ ) in ON samples versus OFF samples were quantified by densitometric analyses of immunoblots from five experiments performed in duplicate. **b** Cells were cultured as in (a) and treated with CHX or CHX + TNF $\alpha$  for the indicated periods. Total protein extracts were analyzed by immunoblotting for HSV-2 R1-GFP, phospho-ERK1/2 (Thr202/Tyr204, P-ERK1/2) and ERK1/2. Immunoblotting is representative of three experiments performed in duplicate. **c** Cells were incubated or not with PD98059 for 90 min as

in (a) and treated with CHX + TNF $\alpha$  for 5 h before harvesting for caspase-3/7 activity determination. The inserted immunoblot shows the efficacy of PD98059 in inhibiting ERK1/2 phosphorylation induced by 7 min treatment with medium containing 10% FBS. Immunoblots and caspase assays (mean  $\pm$  SE,  $n = 6$ ) are representative of three experiments performed in duplicate. **d** Cells were grown as in (a) before being treated or not with TNF $\alpha$  for 30 min. Cytosolic and nuclear fractions were prepared and analyzed by immunoblotting for HSV-2 R1-GFP, NF- $\kappa$ B p65 (p65) and I $\kappa$ B- $\alpha$ . Protein loading was assessed by probing for  $\beta$ -actin, and cytosolic contamination of nuclear fraction, by probing for  $\alpha$ -tubulin. **e** Cell lysates prepared as in (b) were analyzed by immunoblotting for HSV-2 R1-GFP, phospho-JNK (Thr183/Tyr185, P-JNK) and JNK. The immunoblots are representative of three experiments performed in duplicate

HSV-2 R1 protection against TNF $\alpha$ -induced apoptosis (data not shown). Altogether, these results suggested that the MEK/ERK1/2 and PI3-K/Akt pathways do not play a major role in HSV-2 R1 protection against TNF $\alpha$ .

#### NF- $\kappa$ B and JNK

As HHV-8 v-FLIP/K13 protein, which can interfere with TNF $\alpha$ -induced apoptosis, exhibits the ability to constitutively activate NF- $\kappa$ B by interacting with I $\kappa$ B kinase complex (IKK) [12], we sought to determine whether HSV-2 R1 would similarly activate NF- $\kappa$ B signaling. Moreover, as complex I formation after TNF $\alpha$  stimulation is known to lead to the rapid degradation of I $\kappa$ B proteins and to the phosphorylation of NF- $\kappa$ B dimeric transcription factor, which translocates to the nucleus to activate many antiapoptotic genes [reviewed in 9], it was also interesting to study the effect of HSV-2 R1 on NF- $\kappa$ B activation by TNF $\alpha$ . To this end, we evaluated the impact of turning on HSV-2 R1-GFP expression in our A549-tTA-HSV-R1-GFP cells on I $\kappa$ B- $\alpha$  degradation (Fig. 1d) and phosphorylation of the major NF- $\kappa$ B subunit p65 on Ser536 (not shown). Nuclear translocation of p65 was also examined both by cellular fractionation (Fig. 1d, lanes 1–8) and immunofluorescence staining (Fig. S1). HSV-2 R1-GFP did not affect basal levels of I $\kappa$ B- $\alpha$  and p65 (Fig. 1d, lanes 1 and 2), and p65 cytosolic localization (Fig. 1d, lanes 5 and 6). I $\kappa$ B- $\alpha$  degradation induced by TNF $\alpha$  (Fig. 1d, lanes 3 and 4) and consequent phosphorylation (data not shown), and nuclear translocation of NF- $\kappa$ B p65 occurred also normally in the presence of HSV-2 R1-GFP (Figs. 1d, lanes 7 and 8; S1). These results showed that HSV-2 R1-GFP expression did not either constitutively activate NF- $\kappa$ B or affect NF- $\kappa$ B activation by TNF $\alpha$ .

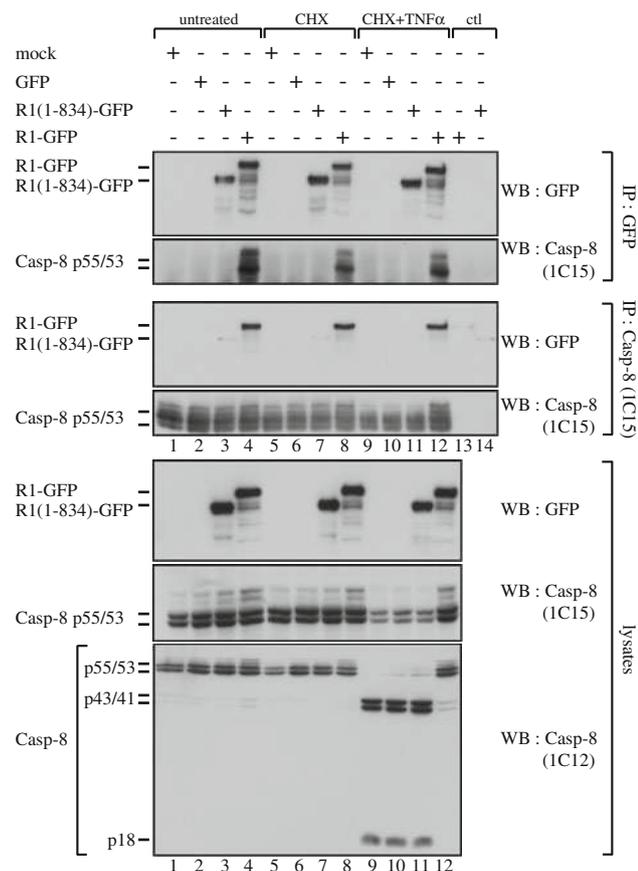
After TNF $\alpha$  treatment, activation of the JNK pathway by complex I has been described as being either anti- or proapoptotic, depending on the cellular context [47]. Figure 1e illustrates that the TNF $\alpha$ -induced increase in JNK(Thr183/Tyr185) phosphorylation, which peaked at 30 min, was not influenced by HSV-2 R1-GFP expression (compare lanes 5–7 to lanes 9–11). Altogether, these results suggested that the protection against TNF $\alpha$ -induced apoptosis afforded by HSV-2 R1 does not involve alteration in the NF- $\kappa$ B and JNK signaling pathways.

Caspase-8 interacts with antiapoptotic HSV-2 R1 but not with an HSV-2 R1 deletion mutant devoid of antiapoptotic activity against TNF $\alpha$

Since we had demonstrated that HSV-2 R1 protects cells against TNF $\alpha$ -induced apoptosis at, or upstream of, caspase-8 activation [7], we first examined whether HSV-2 R1 expression could modify the accumulation of key proteins

involved in the assembly of death complexes and consequently decrease cellular sensitivity to apoptosis. The results detailed in supplementary data showed that HSV-2 R1 does not modify the accumulation of Fas, FADD, TRADD and RIP1, and does not alter the expression or stability of c-FLIP isoforms c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> (Fig. S2). Many antiapoptotic proteins, containing a DED or not, act at the level of death complex assembly by binding with one or more components of these death complexes [reviewed in 48]. Thus, we speculated that HSV R1s could interact with caspase-8 and/or FADD to prevent its activation by DR stimulation, as do other viral inhibitors of apoptosis [49, 50]. The first indication for such an interaction came from size exclusion chromatography, showing that caspase-8 constitutively co-elutes in high-molecular-weight fractions with HSV-2 R1 (data not shown).

To obtain direct evidence that HSV-2 R1 has the ability to interact physically with caspase-8 and/or FADD, co-immunoprecipitation experiments were performed with GFP-tagged HSV-2 R1 expressed in A549-tTA cells after infection with AdTR5-R1-GFP. AdTR5-GFP-infected cells served as controls for the presence of GFP-tag. To examine whether the interaction with caspase-8 correlates with the antiapoptotic function of HSV-2 R1, we also used HSV-2 R1(1-834)-GFP, a deletion mutant unable to protect against TNF $\alpha$  [28]. A549-tTA cells, either mock-, AdTR5-GFP-, AdTR5-R1(1-834)-GFP- or AdTR5-R1-GFP-infected, were treated or not with CHX or CHX + TNF $\alpha$  for 8 h. When treated with CHX + TNF $\alpha$ , >90% of mock-infected, GFP- or HSV-2 R1(1-834)-GFP-expressing cells exhibited apoptotic morphology whereas >95% of HSV-2 R1-GFP-expressing cells were protected as previously described [28]. These morphological observations were confirmed by an immunoblot for caspase-8 (Fig. 2, panels lysates) showing a large decrease in caspase-8 (p55/53) level with a concomitant increase of p43/41 and p18 cleavage products of caspase-8 in all CHX + TNF $\alpha$ -treated cells (lanes 9–11) except for HSV-2 R1-GFP-expressing cells where caspase-8 cleavage was prevented (lane 12). HSV-2 R1-GFP, HSV-2 R1(1-834)-GFP and GFP were immunoprecipitated from lysates of A549-tTA-infected cells with anti-GFP antiserum (Fig. 2, panels IP: GFP). Caspase-8 was immunoprecipitated from the same lysates with the mAb 1C15 (Fig. 2, panels IP: casp-8). Immunoblot analyses were performed with anti-GFP or anti-caspase-8 mAb 1C15. Full-length caspase-8 (casp-8 p55/53) was detected in GFP immunoprecipitates (panels IP: GFP) from HSV-2 R1-GFP-expressing cells (lanes 4, 8 and 12) but not in control cells either mock- (lanes 1, 5 and 9) or GFP-infected (lanes 2, 6 and 10). Even though both HSV-2 R1-GFP and HSV-2 R1(1-834)-GFP were expressed at similar levels (panels lysates), the latter failed to co-precipitate with caspase-8, regardless of the treatment (lanes 3, 7 and 11). In the reverse co-immunoprecipitation



**Fig. 2** HSV-2 R1, but not the inactive mutant R1(1-834)-GFP, co-immunoprecipitates with caspase-8. A549-tTA cells were infected with AdTR5-R1-GFP (R1-GFP), AdTR5-R1(1-834)-GFP (R1(1-834)-GFP) or AdTR5-GFP (GFP) and, 24 h later, were treated or not (untreated) with CHX or CHX + TNF $\alpha$ . After 8 h, the cells were harvested, and GFP-tagged proteins or caspase-8 were immunoprecipitated with anti-GFP antibody or anti-caspase-8 mAb 1C15. As precipitation control (ctl), pre-cleared lysates were incubated with G-Sepharose beads without antibody. Immunoprecipitates (*panels IP*) and cell lysates (*panels lysates*) were analyzed by immunoblotting for GFP-tagged proteins and caspase-8. The immunoblots are representative of three experiments

experiment (*panels IP: casp-8*), HSV-2 R1-GFP (lanes 4, 8 and 12) but not HSV-2 R1(1-834)-GFP (lanes 3, 7 and 11) was specifically detected in caspase-8 immunoprecipitates, the low amount of HSV-2 R1(1-834)-GFP seen in all R1(1-834)-GFP samples being due to the unspecific precipitation of mutant protein with beads (see *ctl* lanes 13 and 14). Similar co-immunoprecipitation experiments with A549-tTA-HSV-R1-GFP cells confirmed the interaction between HSV-2 R1 and caspase-8 (data not shown).

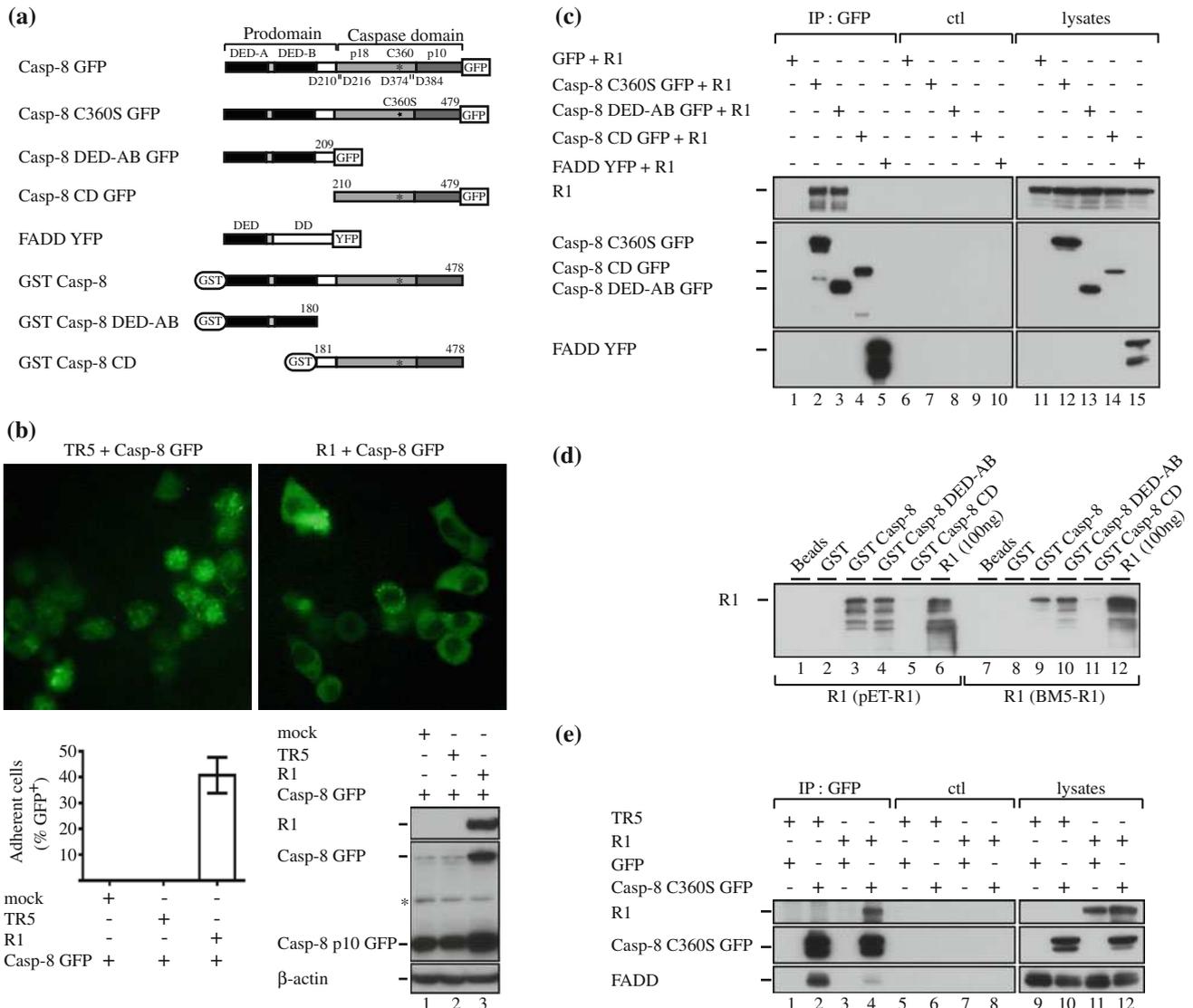
#### HSV-2 R1 impairs apoptosis induced by caspase-8 over-expression

It is well known that caspase-8 over-expression induces apoptosis [51]. In this context, apoptosis is thought to be the

outcome of caspase-8 dimerization through catalytic domain interaction, as single point mutants in the dimer interface, which are unable to undergo dimerization, exhibit greatly reduced apoptosis induction [52]. To determine whether HSV-2 R1 could prevent apoptosis induced by caspase-8 over-expression, we transfected into HeLa cells a GFP-tagged version of caspase-8 (casp-8 GFP depicted in Fig. 3a) known to potently induce apoptosis in these cells [30]. Observations by fluorescence microscopy at 24 h following casp-8 GFP transfection showed that all the GFP-positive cells in control dishes (mock-infected or pre-infected with an empty control Ad recombinant) were rounded up and floating in the medium (Fig. 3b). In these detached cells, the fluorescence was seen as diffuse throughout apoptotic bodies and blebs, as well as concentrated in large aggregates (Fig. 3b, photographs). In sharp contrast, when cells had been infected with AdCMV5-R1 prior to transfection, more than 40% of GFP-positive cells remained adherent with healthy morphology (Fig. 3b). In these adherent cells, fluorescence appeared diffuse throughout the cytoplasm, as reported in conditions where apoptosis induced by casp-8 GFP was inhibited by z-VAD-fmk [30]. Immunoblotting with anti-GFP antibody showed that casp-8 GFP (~85 kDa) was nearly completely absent in control cells, which accumulated the processed casp-8 p10 GFP product (~42 kDa) (Fig. 3b, lanes 1 and 2). Strikingly, cells expressing HSV-2 R1 accumulated high levels of unprocessed casp-8 GFP (Fig. 3b, lane 3), indicating that HSV-2 R1 can impair casp-8 GFP activation. To determine whether casp-8 GFP interacted with HSV-2 R1 as did endogenous caspase-8, cells were infected with AdCMV5-R1 and 12 h later transfected with a plasmid encoding catalytically-inactive caspase-8 C360S GFP (casp-8 C360S GFP) (depicted in Fig. 3a) to avoid caspase-8 autoprocessing. Immunoprecipitation with anti-GFP antiserum showed that HSV-2 R1 co-immunoprecipitated with casp-8 C360S GFP, as expected (Fig. 3c, lane 2). The results demonstrating that HSV-2 R1 impairs caspase-8 activation induced by its over-expression suggested that the interaction between the two proteins impedes caspase-8 dimerization.

#### HSV-2 R1 interacts directly with caspase-8 and the DEDs of caspase-8 are essential for this interaction

As purified HSV-2 R1 does not inhibit active recombinant human caspase-8, which consists of the processed caspase domain [7], we hypothesized that it interacts with the DEDs of caspase-8. To ascertain that, two caspase-8 deletion mutants C-terminally fused to GFP (casp-8 DED-AB GFP and casp-8 CD GFP depicted in Fig. 3a) were tested. In addition, to determine whether the interaction was specific to caspase-8 DEDs, FADD fused to YFP



**Fig. 3** HSV-2 R1 interacts directly with caspase-8 through its two tandem DEDs. **a** Schematic representation of the fusion proteins used in this study. Death-effector domains by DED, death domains by DD, and caspase domain by CD. The aspartic residue processed during caspase-8 maturation and the active site cysteine (C360) are shown. **b** HSV-2 R1 protects cells against caspase-8 GFP-induced apoptosis. HeLa cells were mock-infected or infected with AdCMV5-R1 (R1) or AdTR5CuO (TR5). After 8 h, they were transfected with plasmid encoding for casp-8 GFP. Twenty-four hours later, GFP-positive cells were observed under fluorescence microscopy, and the percentages (mean  $\pm$  SE,  $n = 6$ ) of GFP-positive apoptotic cells were determined. Cell lysates were analyzed by immunoblotting for HSV-2 R1, GFP-tagged proteins and  $\beta$ -actin. The data are representative of three independent experiments performed in duplicate. The asterisk indicates a non-specific band. **c** HSV-2 R1 interacts with caspase-8 through its DEDs but not with FADD. HeLa cells were infected with AdCMV5-R1 (R1) and transfected 12 h later with the plasmid encoding GFP, casp-8 C360S GFP, casp-8 DED-AB GFP, casp-8 CD GFP or FADD YFP and harvested 30 h later. GFP-tagged proteins were immunoprecipitated with polyclonal anti-GFP

antibody. As immunoprecipitation control (ctl), pre-cleared lysates were incubated with G-Sepharose beads without antibody. Immunoprecipitates (IP) and cell lysates (lysates) were analyzed by immunoblotting for HSV-2 R1 and GFP-tagged proteins. Since the anti-GFP mAb B-2 has low affinity for YFP, FADD YFP was revealed with the anti-FADD mAb A66-2. **d** HSV-2 R1 directly interacts with caspase-8 through its DEDs but not with its caspase domain. Beads coupled to GST, GST casp-8, GST casp-8 DED-AB or GST casp-8 CD were incubated with 1  $\mu$ g of purified HSV-2 R1 (pET-R1 or BM5-R1), and proteins bound to beads were analyzed by immunoblotting with the anti-R1 serum 168R1. As control for unspecific binding, 1  $\mu$ g of each purified R1 was incubated with uncoupled beads (Beads) or GST-coupled beads (GST). R1 was quantified by loading 100 ng of each R1. **e** The interaction between HSV-2 R1 and caspase-8 disrupts FADD-caspase-8 binding. HeLa cells were infected as in (b) and transfected to express GFP or casp-8 C360S GFP. GFP-tagged proteins were immunoprecipitated as in (c). Immunoprecipitates (IP) and cell lysates (lysates) were analyzed by immunoblotting for HSV-2 R1, FADD and GFP-tagged proteins. The immunoblots are representative of two experiments

(FADD YFP depicted in Fig. 3a) was included in the analysis. Immunoprecipitation with anti-GFP antiserum (Fig. 3c) showed that HSV-2 R1 co-immunoprecipitated with casp-8 DED-AB GFP (lane 3), but not with casp-8 CD GFP (lane 4) or with FADD YFP (lane 5). Consistent with these results, we also observed that HSV-2 R1 could block the formation of previously-described [30] cytoplasmic filaments in casp-8 DED-AB GFP-expressing cells but not those seen in FADD YFP-expressing cells (Fig. S3). Our findings suggested that HSV-2 R1 could interact with DED-AB of caspase-8 in a way that inhibits the formation of filaments but not with the DED of FADD or with the caspase domain of caspase-8.

To better establish that HSV-2 R1 interacted with casp-8 DED-AB, GST pull-down experiments (Fig. 3d) were performed with GST-tagged deletion mutants of caspase-8 produced in *E. coli* and with purified HSV-2 R1 produced in either bacteria (pET-R1, lanes 1–6) or human cells (BM5-R1, lanes 7–12). Purified HSV-2 R1, whether produced in bacteria or in mammalian cells, could be efficiently pulled-down with either GST casp-8 (lanes 3 and 9) or GST casp-8 DED-AB (lanes 4 and 10) but not with GST casp-8 CD (lanes 5 and 11). These results demonstrated not only that HSV-2 R1 directly interacts with DED-AB of caspase-8, without requiring additional cofactors, but also that eukaryotic post-translational modifications of HSV-2 R1 are not required for the interaction.

#### Binding of HSV-2 R1 to caspase-8 disrupts FADD–caspase-8 interaction

To gain additional insight into the mechanism by which HSV R1 interaction with caspase-8 prevented its activation, we investigated whether HSV-2 R1 could affect the interaction between caspase-8 and FADD. To this end, HeLa cells were infected with AdCMV5-R1 or an empty Ad recombinant for 16 h before transfecting GFP or casp-8 C360S GFP to avoid caspase-8 autoprocessing (Fig. 3e). As expected, when immunoprecipitation was performed with anti-GFP antiserum, FADD co-precipitated with casp-8 C360S GFP (lane 2) but not with GFP (lane 1). When cells were infected with AdCMV5-R1 prior to transfection (lanes 3 and 4), the interaction between FADD and casp-8 C360S GFP was greatly reduced (compare lanes 2 and 4) and HSV-2 R1 was present in the immunoprecipitate (lane 4). This result indicated that HSV-2 R1 binding to caspase-8 disrupts FADD–caspase-8 interaction.

#### Individually expressed HSV-1 R1 protects cells against TNF $\alpha$ - and FasL-induced apoptosis

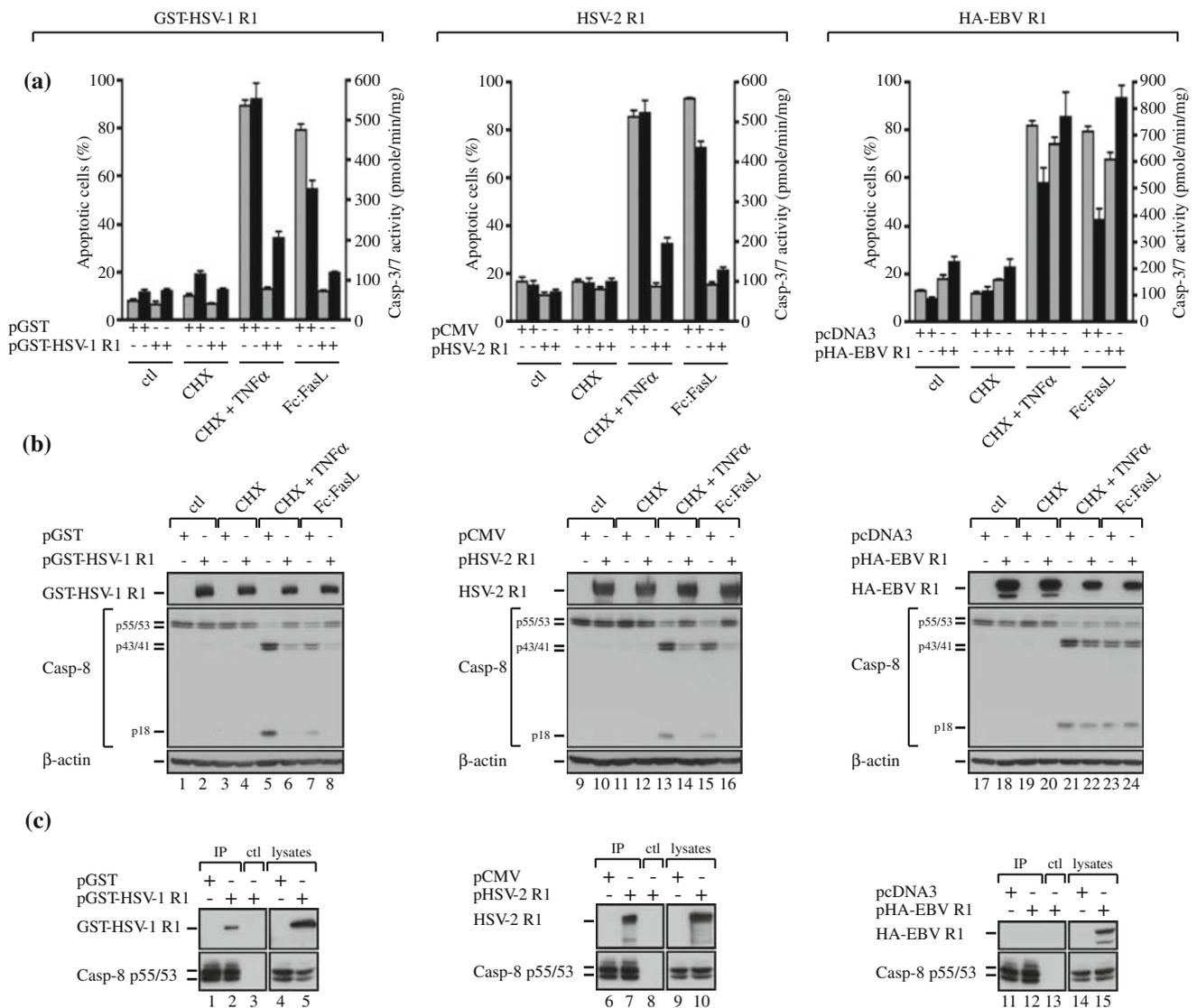
Our previous observation that HSV-1 *UL39* deletion mutants exhibited half of the WT virus antiapoptotic

potential against TNF $\alpha$  had provided indirect evidence that HSV-1 R1 could be antiapoptotic against DR ligands [7]. To firmly establish that it is the case and also to determine whether HSV-1 R1 could, like HSV-2 R1, interact with caspase-8, HeLa cells were transfected with an expression plasmid encoding HSV-1 R1 fused to GST or, for comparison, with an HSV-2 R1 expression plasmid. In these experiments a plasmid encoding EBV R1 (an RR active subunit) was also tested. Treatments with CHX + TNF $\alpha$  or highly cytotoxic hexameric FasL for 6 h (Fc:FasL, [32]) induced apoptotic morphology in more than 80% of control HeLa cells (Fig. 4a) as previously reported [7, 53]. Both HSV R1s reduced by more than 5-fold the % of morphologically apoptotic cells, but HA-EBV R1 was without significant effect. To confirm the morphological observations, caspase-8 activation was assessed by immunoblotting (Fig. 4b), whereas caspase-3/7 activation was evaluated by *in vitro* enzymatic assay with DEVD-AFC as substrate (Fig. 4a). Both HSV-1 R1 and HSV-2 R1, but not EBV-R1, greatly reduced caspase-8 (lanes 6, 8; 14, 16; 22, 24) and caspase-3/7 activation produced by DR triggering (lanes 5, 7; 13, 15; 21, 23). Note that the 168R1 serum, which was raised against purified HSV-2 R1, recognized less efficiently HSV-1 R1 than HSV-2 R1 (C. Guillbault and Y. Langelier, unpublished observations). As immunofluorescence staining revealed that >75% of cells were positive for the HA tag, it is unlikely that a poor transfection efficiency would be the cause of the lack of protection by the HA tagged EBV R1. Altogether these results indicated that the R1s of both types of HSVs protect HeLa cells against TNF $\alpha$  and FasL whereas that of EBV does not exhibit this property.

To assess interaction with caspase-8, immunoprecipitation was performed with the mAb 1C15. Figure 4c showed that, like HSV-2 R1 (lane 7), HSV-1 R1 (lane 2) coprecipitated with caspase-8 but not EBV R1 (lane 12). These results demonstrated that HSV-1 R1 and HSV-2 R1 but not EBV R1 interact with caspase-8.

#### The R1 null mutant ICP6 $\Delta$ does not protect cells against FasL

Determining the importance of HSV-1 R1 in protecting HSV-infected cells from TNF $\alpha$  was complicated by the *vhs* effect on the short-lived TNFR1. As Fas has been described to be much more stable than TNFR1 [54] and given the antiapoptotic potential of individually expressed HSV-1 R1 against DR-induced apoptosis, we thought that more conclusive data could be obtained for FasL-induced apoptosis. Because the effect of HSV infection on Fas level had never been studied, we first compared the fate of Fas and TNFR1 in total lysates of HeLa cells infected with WT HSV-1 KOS or its R1 null mutant ICP6 $\Delta$  for up to 8 h. As seen in Fig. 5a for KOS (lanes 2–6) and ICP6 $\Delta$  (lanes 7–11)



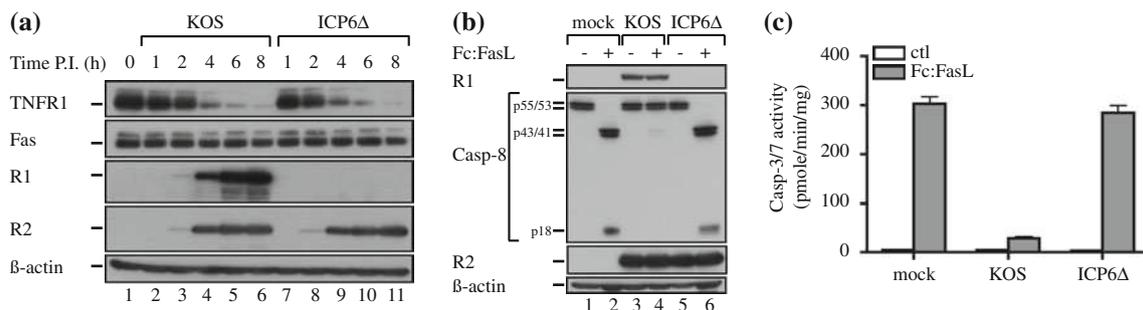
**Fig. 4** HSV-1 R1 inhibits DR-induced apoptosis and interacts with caspase-8. **a, b** GST-HSV-1 R1 and HSV-2 R1 inhibit TNF $\alpha$ - and FasL-induced apoptosis but not HA-EBV R1. HeLa cells were transfected with the plasmid pAdCMV5 (pCMV) or pAdCMV5-R1 (pHSV-2 R1) (*left panel*); pLBPF1-GST (pGST) or pLBPF1-GST-R1 (pGST-HSV-1 R1) (*middle panel*); pcDNA3 or pcDNA3-HA-EBV R1 (pHA-EBV R1) (*right panel*). After 48 h, the cells were untreated (ctl) or treated with CHX (15  $\mu$ g/ml), CHX + TNF $\alpha$  (2.5 ng/ml) or Fc:FasL (10 ng/ml) for 8 h. Percentages of apoptotic cells were determined by microscopic observation (**a**). Cytoplasmic cell lysates were analyzed by immunoblotting for HSV R1s, EBV R1, caspase-8

(1C12) and  $\beta$ -actin protein content (**b**) and tested for caspase-3/7 activity (**a**). The immunoblots and caspase activities (mean  $\pm$  SE,  $n = 4$ ) are representative of at least two experiments performed in duplicate. **c** HSV-1 R1 and HSV-2 R1 co-immunoprecipitate with caspase-8 but not EBV R1. Caspase-8 was immunoprecipitated with anti-caspase-8 mAb 1C15 from ctl extracts prepared in (**a, b**). Immunoprecipitates (IP) and cell lysates (lysates) were analyzed by immunoblotting for HSV R1s, HA-EBV R1 and caspase-8 (1C15). As precipitation control (ctl), pre-cleared lysates were incubated with G-Sepharose beads without antibody. The immunoblots are representative of two experiments

infection, Fas levels remained almost unaltered during this period as expected from the report on Fas stability [54]. TNFR1 decreased steadily from 2 h post-infection as previously reported [8] but, even at 8 h, it was still detectable, albeit at a very low level (lanes 6 and 11). The rate of TNFR1 disappearance was similar for both viruses, indicating that HSV-1 R1 does not affect the rate of TNFR1 reduction. The experiments were repeated for HSV-2

infection with similar results (data not shown). Two conclusions can be drawn from these observations. First, protein stability appears to be a key determinant of the fate of DRs during HSV infection. Second, even if TNFR1 protein level decreases drastically after HSV infection, this DR is still present at 8 h after infection.

As Fas levels remained almost unaltered during the first 8 h of infection, the effect of deleting the HSV-1 R1 gene



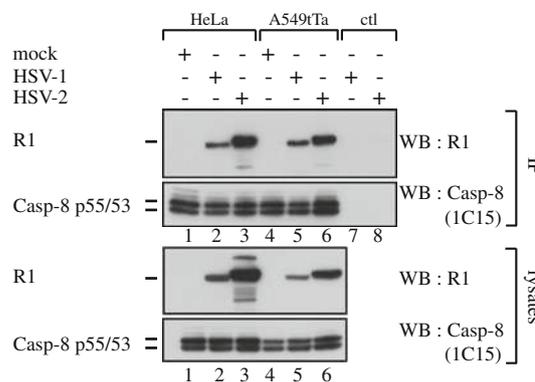
**Fig. 5** HSV-1 R1 plays a key role for protection against FasL-induced apoptosis. **a** HeLa cells were infected with the parental HSV-1 strain (KOS) or the R1 null mutant strain (ICP6Δ) and harvested at the indicated times post-infection (PI). Whole cell extracts were analyzed by immunoblotting for TNFR1, Fas, HSV-1 R1, HSV-1 R2 and β-actin. **b, c** HeLa cells were mock-infected (mock) or infected

as in **(a)** for 8 h and then treated with Fc:FasL (50 ng/ml) or not (ctl). After 6 h, the cells were harvested and cytoplasmic cell lysates were analyzed by immunoblotting for HSV-1 R1, HSV-1 R2, caspase-8 (1C12) and β-actin protein content **(b)** and tested for caspase-3/7 activity **(c)**. The immunoblots and caspase activities (mean ± SE, n = 4) are representative of two experiments performed in duplicate

on protection from FasL was studied by infecting HeLa cells for 8 h with either WT KOS or ICP6Δ and treating them for 6 h with 50 μg/ml Fc:FasL. KOS infection reduced the % of morphologically apoptotic cells from >90% in mock-infected samples to <5%. In sharp contrast, ICP6Δ infection did not alter the % of apoptosis induced by Fc:FasL (<90%). Our morphological observations were corroborated by analyses of caspase-8 and caspase-3/7 activation. Indeed, KOS infection completely prevented caspase-8 cleavage (Fig. 5b, lane 4 vs. lane 2) and caspase-3/7 activation (Fig. 5c), whereas ICP6Δ infection did not affect either caspase-8 (Fig. 5b, lane 6 vs. lane 2) or caspase-3/7 activation (Fig. 5c). These results showed that the protection afforded by HSV against Fc:FasL is completely lost by deleting R1. For all the experiments involving ICP6Δ, exponentially growing cells were used to prevent the inhibition of translation of viral mRNAs, which would occur if the cells were growth-arrested [27]. Evidence that the synthesis of viral proteins was not impaired in our ICP6Δ-infected cells was obtained from the observation that the R2 subunit of HSV RR (Fig. 5a) and the infected cell polypeptide 0 (data not shown) accumulated at similar rate in cells infected with either KOS or ICP6Δ. This made unlikely the possibility that the antiapoptotic defect of ICP6Δ against FasL is related to a lower ability of the cells to support the synthesis of viral polypeptides. Together, these results suggested that HSV-1 R1 could play an essential role in the protection against FasL.

**HSV-1 R1 and HSV-2 R1 interact with caspase-8 in the context of HSV infection**

We next determined whether HSV R1s could interact with caspase-8 in the context of HSV infection. Our previous work had revealed that protection from TNFα-induced apoptosis plateaued around 8 h of infection with both types



**Fig. 6** HSV-1 R1 and HSV-2 R1 co-immunoprecipitate with caspase-8 in the context of HSV infection. HeLa and A549-tTA cells were infected with HSV-1 (strain KOS) or HSV-2 (strain HG-52). After 8 h, the cells were harvested, and caspase-8 was immunoprecipitated with anti-caspase-8 mAb 1C15. Immunoprecipitates (*panels IP*) and cell lysates (*panels lysates*) were analyzed by immunoblotting for HSV R1s and caspase-8 (1C15). As precipitation control (ctl), pre-cleared lysates were incubated with G-Sepharose beads without antibody. The immunoblots are representative of two experiments

of HSV [7]. Hence, HeLa and A549-tTA cells were infected for 8 h with either HSV-1 or HSV-2, and caspase-8 was immunoprecipitated with the anti-caspase-8 mAb 1C15. Immunoblot analyses with anti-R1 serum 168R1 (Fig. 6) revealed that both HSV-1 R1 (lanes 2 and 5) and HSV-2 R1 (lanes 3 and 6) interacted with caspase-8 in the two cell lines tested. Altogether, these results indicate that HSV R1s can physiologically interact with caspase-8.

**Discussion**

Apoptosis triggered by stimulation of DRs is important in the host response to viral infection. To counteract host

defense mechanisms, viruses have evolved several molecular strategies to prevent caspase-8 activation after DR stimulation through the acquisition of proteins that act on different targets in this pathway [50, 55–57]. Several studies have indicated that HSVs can efficiently block apoptosis induced by DR activation [reviewed in 58]. In this regard, two HSV proteins were proposed to play important roles: the R1 subunit of HSV-2 RR which, expressed on its own, protects epithelial cells against TNF $\alpha$ - and FasL-induced apoptosis by interrupting DR-mediated signaling at, or upstream of, caspase-8 activation [7, 28], and vhs which, by destroying TNFR1 mRNA, impairs the replenishment of short-half-life TNFR1 [8]. Here, we report the following main findings on the antiapoptotic activity of HSV R1s against DR ligands. HSV-2 R1, which does not stimulate the major antiapoptotic survival pathways ERK, Akt, NF- $\kappa$ B and JNK, interacts with the prodomain of caspase-8 in a way that inhibits its dimerization/activation. The interaction between HSV-2 R1 and caspase-8 also disrupts FADD-caspase-8 binding. Individually expressed HSV-1 R1, like HSV-2 R1, has the ability to protect cells against TNF $\alpha$  and FasL and to bind to caspase-8. Strikingly, HSV-1 R1 could play an essential role for protecting infected cells against FasL.

Since some viral antiapoptotic proteins, including HSV-2 R1 [22], had been found to target the ERK1/2 and Akt signaling pathways, it was important to evaluate whether they could be involved in the antiapoptotic activity of HSV-2 R1. Three lines of evidence strongly argue against any effect of HSV-2 R1 on ERK1/2 and PI3-K/Akt pathways in the protection from TNF $\alpha$ . Firstly, HSV-2 R1 expression at a level sufficient to confer full protection against TNF $\alpha$ -induced apoptosis did not significantly affect ERK1/2 or Akt phosphorylation. Secondly, HSV-2 R1 did not modify the transient activation of ERK1/2 induced by TNF $\alpha$ . Thirdly, suppression of the ERK1/2 or Akt pathways with specific biochemical inhibitors did not influence HSV-2 R1 protection against TNF $\alpha$ -induced apoptosis. Our observation that the inducible expression of HSV-2 R1 did not increase ERK1/2 phosphorylation contrasts with previous reports that 293 or PC12 cells stably expressing HSV-2 R1 exhibit a higher level of ERK1/2 phosphorylation than their parental counterparts [43, 59]. As we have been unable to detect any increase in ERK1/2 phosphorylation upon inducible HSV-2 R1 expression in 293 cells, we have ruled out, as an explanation for this discrepancy, that the effect could be cell type-specific. It is possible that the higher constitutive ERK1/2 phosphorylation, previously observed in 293 cells stably expressing HSV-2 R1, could be due to clonal variation. Indeed, it is well-known that several established cell lines exhibit high constitutive ERK1/2 phosphorylation whereas others do not [60]. Furthermore, HSV-2 R1 does not constitutively activate

NF- $\kappa$ B and JNK or alter the activation of NF- $\kappa$ B and JNK by TNF $\alpha$ . Such observations not only indicate that these pathways are not involved in protection from TNF $\alpha$  but also strongly suggest that HSV-2 R1 does not interfere with the TNF $\alpha$ -induced formation of complex I, since assembly of the complex is required for early NF- $\kappa$ B, JNK or ERK1/2 phosphorylation. In this respect, HSV-2 R1 differs from HHV-8 v-FLIP/K13 that constitutively activates the canonical NF- $\kappa$ B pathway by interacting with IKK $\gamma$  [12].

HSV-2 R1 protection against TNF $\alpha$ -induced apoptosis differs from viral strategies affecting the expression or stability of critical proteins implicated in DR signaling [57, 61–63]. Indeed, HSV-2 R1 does not affect either the expression of c-FLIP isoforms or their stability. HSV-2 R1 does not modify the expression of TNFR1, TRADD, FADD, RIP1 and caspase-8. Even if sequences of HSV R1s do not present similarities with c- or v-FLIP, we hypothesized that HSV R1s could interact with caspase-8 to prevent its activation by DR stimulation, as do other viral inhibitors of apoptosis [49, 50]. Immunoprecipitation experiments confirmed that both individually expressed HSV-1 and HSV-2 R1 interact with caspase-8 with or without treatment with TNF $\alpha$ , demonstrating that the interaction is constitutive. Moreover, interaction between HSV R1s and caspase-8 could be detected with extracts of HSV-1- and HSV-2-infected cells, supporting a role for this interaction in the resistance of HSV-infected cells to DR-induced apoptosis [7, 64]. HSV-2 R1 binding to caspase-8 appears indispensable for the antiapoptotic activity of HSV-2 R1 since the deletion mutant HSV-2 R1(1-834)-GFP, which is devoid of antiapoptotic activity [28], does not interact with caspase-8. Moreover, EBV R1, which does not show protection from DR-ligands in our experimental system, does not interact with caspase-8.

The data, obtained with two series of caspase-8 deletion mutants, showed that HSV-2 R1 interacts with the two tandem DED-containing prodomain of caspase-8 but not with the caspase domain of caspase-8. This is in agreement with the observation that HSV-2 R1 does not protect cells against caspase-8 CD GFP-induced apoptosis (F. Dufour and Y. Langelier, unpublished observations) and with our previous finding that purified HSV-2 R1 does not inhibit the protease activity of processed recombinant caspase-8, which contains only the caspase domain [7]. GST pull-down experiments with purified proteins demonstrated that HSV-2 R1 interaction with either full-length caspase-8 or caspase-8 DED-AB is direct and does not require additional molecules. Also, as the interaction still occurred when both proteins were produced and purified from bacteria, it can be concluded that post-translational modifications specific to mammalian cells are not necessary for binding. Altogether, these data firmly established that

HSV-2 R1, by interacting constitutively and directly with caspase-8, prevents its activation. A similar mechanism of action has been described for vICA, the UL36 gene product of human CMV that is conserved in all CMV genomes sequenced to date [65]. Like HSV R1s, vICA suppresses TNF $\alpha$ - and FasL-induced apoptosis but appears to marginally inhibit cell death induced by cytotoxic drugs activating the mitochondrial apoptosis pathway [7, 50]. Even though HSV-2 R1 and vICA do not exhibit sequence similarity, both proteins associate with caspase-8 in the absence of DR activation and interact with the caspase-8 prodomain.

Since dimerization is a precondition for self-processing and caspase-8 activity [66], we speculated that HSV-2 R1 could interact with caspase-8 in a way that inhibits this event. Supporting our hypothesis, we found that HSV-2 R1 protects cells against apoptosis induced by over-expression of GFP-tagged caspase-8 that is known to be triggered by dimerization/activation of the over-expressed zymogen [51]. In the case of TNF $\alpha$ - or FasL-induced apoptosis, complex II or DISC drives dimerization and activation of monomeric caspase-8 via FADD [13, 14]. Since on one hand HSV-2 R1 interacts with the DEDs of caspase-8 and on the other hand FADD contains a DED involved in both FADD self-association and caspase-8 interaction [67], we investigated putative interaction between FADD and HSV-2 R1. We have been unable to detect, by co-immunoprecipitation, any interaction between HSV-2 R1 and endogenous or over-expressed FADD, indicating that the interaction is specific to the tandem DEDs contained in the caspase-8 prodomain. A recent report identified the caspase-8-specific binding surface on FADD and suggested preferential interaction of caspase-8 DED-B with FADD DED [67]. Since HSV-2 R1 interacts with the caspase-8 prodomain, it is conceivable that oligomerized HSV-2 R1 could sterically hinder the site of caspase-8 interaction with FADD DED. Supporting this hypothesis, we found that the interaction between over-expressed casp-8 C360S GFP and endogenous FADD is inhibited when HSV-2 R1 is expressed. It remains to be determined whether HSV R1s inhibit the recruitment of caspase-8 to the assembled complex II or DISC.

Even if most of our data were obtained with HSV-2 R1, we believe that the majority of our conclusions on the mechanism of action of this protein can be extrapolated to HSV-1 R1. Indeed, both proteins interact with caspase-8 and our previous mapping of the antiapoptotic domain of HSV-2 R1 indicates that this domain is highly conserved between both types of HSV (95% similarity [28]). We also speculate that the deficiency of EBV R1 in protection from TNF $\alpha$  and FasL would be related to the absence of the putative  $\alpha$ -crystallin domain in the shorter EBV R1, a domain shown to be important for HSV-2 R1 antiapoptotic

activity [26]. It is noteworthy that until now protection from TNF $\alpha$  has been reported only for another herpes virus R1, that of murine  $\beta$ -herpes virus CMV, also named M45 [reviewed in 68].

Extensive studies have uncovered several antiapoptotic proteins in the herpes viruses family, several of them being FLIP or Bcl-2 homologues [reviewed in 69]. HSV R1s differ structurally and functionally from these inhibitors. Indeed, HSV R1s belong with vICA to a new class of viral antiapoptotic proteins that interact directly and constitutively with caspase-8 in a way that inhibits its activation. It is highly attractive to hypothesize that, in addition to counteracting DR-induced apoptosis, HSV R1s could prevent apoptosis induced by other signals that trigger caspase-8 activation during HSV infection. This function could be particularly important in certain types of cells as it has been shown recently that both c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> are degraded during HSV infection [70].

Our previous experiments with two HSV-1 R1 deletion mutants (hrR3 and ICP6 $\Delta$ ) had suggested that the R1 of HSV-1 could play an important role in the prevention of TNF $\alpha$ -induced apoptosis during the early period of infection [7, 28]. However, during the same period, there is a rapid decrease of TNFR1 from HSV-1- and HSV-2-infected cells (Fig. 5a) [8], which should also contribute to TNF $\alpha$  resistance. It could even seem paradoxical that at 8 h post infection  $\sim$ 50% of infected cells are still sensitive to TNF $\alpha$  while they exhibit barely detectable levels of unstable TNFR1. One can hypothesize that low levels of TNFR1 are sufficient to trigger apoptosis or that TNF $\alpha$  signals through TNFR2 or through a third, unknown TNFR. The existence of such an unknown TNF receptor was invoked to explain how TNF $\alpha$  could protect against fatal HSV-1 encephalitis in TNFR1<sup>-/-</sup> TNFR2<sup>-/-</sup> mice [71]. A much clearer conclusion on the importance of HSV-1 R1 in counteracting DR-induced apoptosis was obtained here with respect to the Fas pathway. Indeed, from the observation that HSV infection does not affect Fas levels, we were able to show with the R1 deletion mutant ICP6 $\Delta$  that HSV-1 R1 could play an essential role in the protection of HSV-infected cells from FasL, a more definitive conclusion awaiting an experiment with a virus in which the deleted sequences would be restored. This finding is important not only because of the magnitude of the effect seen with the R1 deletion mutant but also because Fc:FasL was used without CHX, a more physiological treatment than CHX + TNF $\alpha$ . The importance of protection against DR ligands in HSV pathogenesis remains to be determined, but a recent report showing that Fas-FasL signaling is important in protecting mice against HSV-2 lethality suggests that HSV R1 could contribute to increase virus replication by counteracting this immune system mechanism in vivo [3].

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**Conflict of interest** The authors declare that they have no conflict of interest.

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