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The Ribonucleotide Reductase R1 Subunits of Herpes Simplex Virus 1 and 2 Protect Cells against Poly(I · C)-Induced Apoptosis▽

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We recently provided evidence that the ribonucleotide reductase R1 subunits of herpes simplex virus types 1 and 2 (HSV-1 and -2) protect cells against tumor necrosis factor alpha- and Fas ligand-induced apoptosis by interacting with caspase 8. Double-stranded RNA (dsRNA) is a viral intermediate known to initiate innate antiviral responses. Poly(I · C), a synthetic analogue of viral dsRNA, rapidly triggers caspase 8 activation and apoptosis in HeLa cells. Here, we report that HeLa cells after HSV-1 and HSV-2 infection were quickly protected from apoptosis caused by either extracellular poly(I · C) combined with cycloheximide or transfected poly(I · C). Cells infected with the HSV-1 R1 deletion mutant ICP6Δ were killed by poly(I · C), indicating that HSV-1 R1 plays a key role in antiapoptotic responses to poly(I · C). Individually expressed HSV R1s counteracted caspase 8 activation by poly(I · C). In addition to their binding to caspase 8, HSV R1s also interacted constitutively with receptor-interacting protein 1 (RIP1) when expressed either individually or with other viral proteins during HSV infection. R1(1-834)-green fluorescent protein (GFP), an HSV-2 R1 deletion mutant protein devoid of antiapoptotic activity, did not interact with caspase 8 and RIP1, suggesting that these interactions are required for protection against poly(I · C). HSV-2 R1 inhibited the interaction between the Toll/interleukin-1 receptor domain-containing adaptor-inducing beta interferon (IFN-β) (TRIF) and RIP1, an interaction that is essential for apoptosis triggered by extracellular poly(I · C) plus cycloheximide or TRIF overexpression. TRIF silencing reduced poly(I · C)-triggered caspase 8 activation in mock- and ICP6Δ-infected cells, confirming that TRIF is involved in poly(I · C)-induced apoptosis. Thus, by interacting with caspase 8 and RIP1, HSV R1s impair the apoptotic host defense mechanism prompted by dsRNA.

Cells have an innate capacity to sense virus infections and to trigger potent antiviral countermeasures to limit viral replication and spreading. Two major components of this antiviral defense are (i) a protective response that leads to the synthesis of cytokines, including interferons (IFNs), to alert and protect neighboring cells (17) and (ii) a suicidal response of infected cells to restrict both the period and cellular components available for virus multiplication (42). Viruses, including herpes simplex viruses (HSVs), have evolved a large variety of strategies to evade both IFN and cell death responses (19, 59, 62). Despite virus-encoded inhibitors of cell death, the suicide program occurs in most human viral infections (12), such as encephalitis caused by HSV replication in the brain (14, 60). HSVs encode different cell death suppressors, several of them conferring resistance to apoptosis elicited by the process of viral replication itself and/or by extrinsic stimuli linked to immune effector cell cytotoxicity or activation of death receptors (25). Among the viral genes involved in the control of apoptosis, *UL39*, which encodes the R1 subunit of HSV ribonucleotide reductase (HSV R1), protects epithelial cells from apoptosis induced by the death receptor ligands tumor necrosis factor alpha (TNF-α) and Fas ligand (FasL) (15, 44).

Double-stranded RNA (dsRNA), one of the major viral products involved in the cellular sensing of viral invasion, triggers both IFN and apoptosis responses (17, 29). During HSV infection, dsRNAs are produced by overlapping convergent transcription and accumulate in large amounts in infected cells from the onset of HSV genome expression (31, 73). Intracellular dsRNAs generated during infection can be released into the extracellular space, from where they can stimulate an antiviral response in neighboring cells. dsRNAs are detected by cellular sensors that, through activation of specific signaling cascades, elicit inflammatory gene expression and/or apoptosis. Depending on their cellular localization, viral dsRNAs and poly(I · C), a synthetic dsRNA commonly employed to mimic viral dsRNAs, are recognized by either membrane (cytosolic or endosomal)-bound Toll-like receptor 3 (TLR3) or cytosolic sensors, including dsRNA-dependent protein kinase (PKR) and retinoic acid-inducible gene (RIG)-like receptors (RLRs), RIG-I, and melanoma differentiation-associated gene 5 (MDA5) (21, 65).

A simplified schematic representation of signaling pathways involved in dsRNA/poly(I · C)-induced apoptosis is shown in Fig. 1. TLR3 detects extracellular or endosomal viral dsRNA, experimentally reproduced by adding poly(I · C) to the culture

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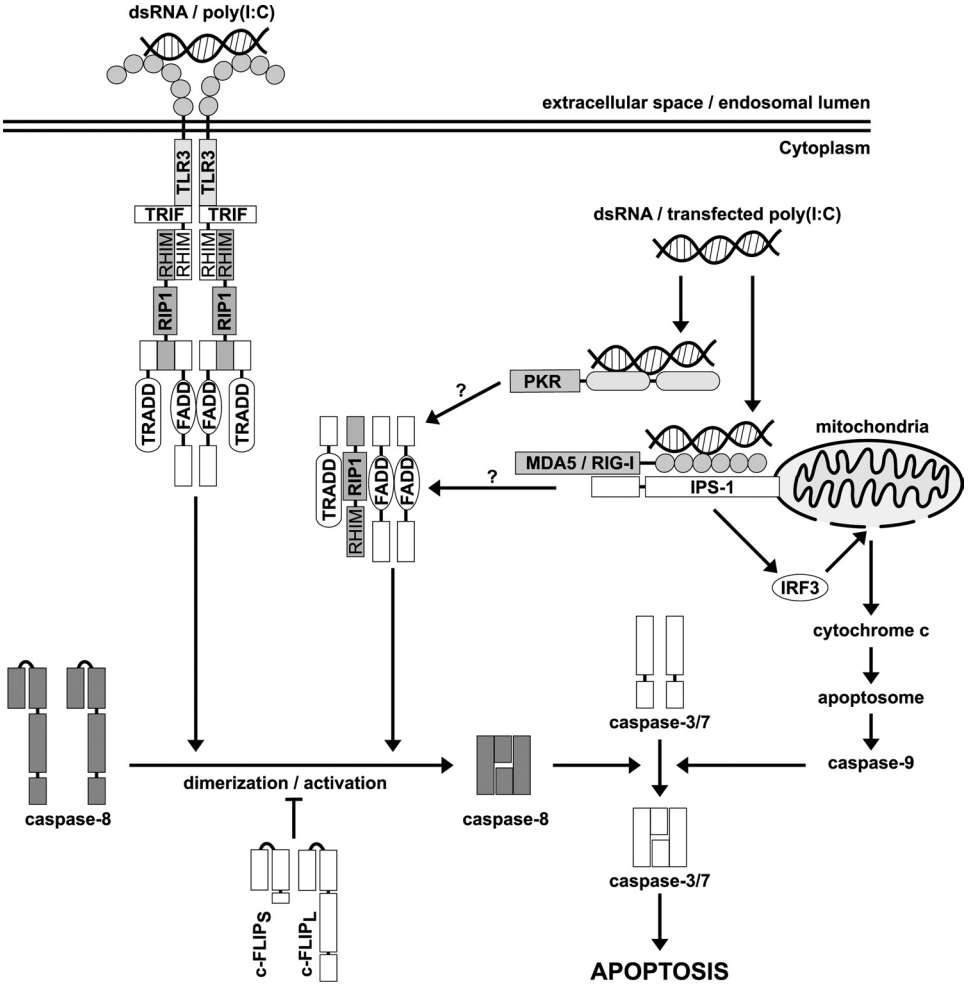

FIG. 1. Schematic representation of signaling pathways involved in dsRNA/poly(I · C)-induced apoptosis. See the text for details and abbreviations.

medium (69), whereas cytosolic dsRNA, mimicked by transfection of poly(I · C), is detected by intracellular sensors (21, 65). Once activated by extracellular dsRNA, TLR3 interacts with TRIF, a Toll/interleukin receptor domain-containing adaptor, which on one hand mediates IFN production through nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF-3) activation and, on the other hand, triggers apoptosis (26, 58, 75). TRIF proapoptotic potential maps within its C-terminal RIP homotypic interaction motif (RHIM) and involves direct interaction with RIP1, a RHIM- and death domain-containing serine/threonine protein kinase regulating multiple cellular processes, including cell death and inflammation (35, 53). Downstream of RIP1, TRIF-induced apoptosis requires the activation of caspases through a complex containing TNF-α receptor 1-associated protein with death domain (TRADD), Fas-associated death domain (FADD), and caspase 8 (also called FLICE) (16, 26). Once activated, caspase 8 can cleave and activate the effector caspase 3/7. Cellular FLICE-inhibitory proteins (c-FLIPs) negatively regulate caspase 8 activation in both death receptor and RLR signaling cascades via formation of catalytically inactive procaspase 8/c-FLIP heterodimers (54, 77).

Several pathways activated by the cytosolic dsRNA sensor PKR or RLRs can participate in apoptosis induction by poly(I · C) transfection. In numerous cell systems, including HeLa cells, PKR plays a major role in the death process, mainly through TRADD/FADD-mediated activation of caspase 8 (5, 21, 22, 29, 52, 56). Binding of long dsRNAs, such as poly(I · C), to the cytosolic RLR MDA5 also activates the transcription factors NF-κB and IRF-3 through interaction with the adapter protein IFN-β promoter stimulator 1 (IPS-1), which is anchored in the outer mitochondrial membrane (39). Apoptosis triggering through IRF-3 can occur via two pathways: (i) stimulation of transcription of two BH3-only proteins, Noxa and Puma, which by antagonizing prosurvival Bcl-2 proteins leads to Bax activation, loss of mitochondrial membrane integrity, and cytochrome *c* release in the cytosol (7), and (ii) direct binding of activated IRF-3 to cytosolic Bax through a BH3-like domain, which drives loss of mitochondrial membrane integrity and release of cytochrome *c* (10, 76). With apoptosis protease-activating factor 1, cytochrome *c* forms a multimeric protein structure called apoptosome, a platform for successive activation of caspase 9 and caspase 3/7 (61). IPS-1 can also induce apop-

tosis independently of IRF-3 (45) via caspase 8 activation triggered by a complex formed with TRADD, RIP1, and FADD (47, 54).

In a large variety of cell types, apoptosis induction by dsRNA is a rather slow and inefficient process. In contrast, rapid engagement of the apoptotic machinery has been observed in several immortalized or tumor cell lines, including HeLa and HaCaT cells, in response to intracellular poly(I · C) or after treatment with extracellular poly(I · C) in the presence of either cycloheximide (CHX) or a second mitochondrion-derived activator of caspase mimetics (29, 30, 33, 72). Recent reports have stressed the importance of caspase 8 activation via TLR3 and its adaptor TRIF in apoptosis induced by extracellular poly(I · C) in some of these immortalized or cancer cells (33, 72).

HSV ribonucleotide reductase consists of two homodimeric subunits, HSV R1 and HSV R2, which associate to form the holoenzyme. By providing deoxyribonucleotides essential for viral DNA replication, this enzyme plays an essential role in virus multiplication in quiescent cells, notably in neurons (24). In addition to being the catalytic subunit for ribonucleotide reduction, HSV R1 possesses several non-ribonucleotide reductase-related activities, including (i) chaperone activity similar to that of small heat shock proteins (8), (ii) the ability to stimulate translation in quiescent cells by promoting eIF4F translation complex assembly (71), and (iii) antiapoptotic properties (23, 44). The extensively studied role of HSV type 1 (HSV-1) R1 and HSV-2 R1 in the antiapoptotic response extends from the impairment of apoptosis induced by the mitochondrial pathway through activation of the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 and the phosphatidylinositol-3-kinase/Akt axes by HSV-2 R1 (23) to the protection of epithelial cells by both HSV R1s from TNF-α- and FasL-induced apoptosis through constitutive interaction with caspase 8 (15, 44).

In light of previous reports showing the key role of caspase 8 activation in poly(I · C)-induced apoptosis (29, 72), the present study was undertaken to validate the hypothesis that HSV R1s could prevent poly(I · C)-induced apoptosis by virtue of their ability to inhibit caspase 8 activation (15). Our demonstration that HSV R1s impair the apoptotic host defense mechanism triggered by dsRNA adds a new facet to the ability of HSVs to manipulate antiviral responses.

MATERIALS AND METHODS

Cell culture and infection. HeLa and A549-tTA cells were cultured as reported elsewhere (8, 44). The previously described recombinant adenoviruses (Ad) AdCMV5-R1, AdTR5-GFP, AdTR5-R1-GFP, AdTR5-R1(1-834)-GFP, and AdTR5-R1Δ(2-249)-GFP express HSV-2 R1, green fluorescent protein (GFP), HSV-2 R1-GFP, HSV-2 R1(1-834)-GFP, and HSV-2 R1Δ(2-249)-GFP, respectively (9, 44). One day after plating, HeLa and A549tTa cells were infected with recombinant adenoviruses at multiplicities of infection chosen to obtain roughly similar expression levels of recombinant R1 proteins: AdCMV5-R1, 10 PFU/cell; AdTR5-CuO (empty vector), 10 PFU/cell; AdTR5-GFP, 25 PFU/cell; AdTR5-R1-GFP, 25 PFU/cell; AdTR5-R1(1-834)-GFP, 50 PFU/cell; and AdTR5-R1Δ(2-249)-GFP, 5 PFU/cell (9, 15, 44). HeLa cells were infected with the HSV-2 strain HG-52, the R1-null mutant HSV-1 ICP6Δ (24), or its parental strain, KOS, at 10 PFU/cell for the indicated periods.

Transfection. One day after plating, HeLa cells were transfected by the calcium phosphate method with plasmid pAdCMV5 (empty vector), pAdCMV5-R1 (HSV-2 R1), pEGFP C1 (empty vector; Clontech), pLBPf1-GST (glutathione *S*-transferase [GST]), or pLBPf1-GST-R1 (GST-HSV-1 R1) (74). To generate the plasmid pLBPfl-GST, the GST open reading frame was amplified by PCR from the vector pGEX-6P-3 (GE) with primers GST-N-Pfl (5′ CGT ACG CCA TGT CCC CTA TAC TAG GTT) and GST-C-Pac (5′ TTA ATT AAA TCC GAT TTT GGA GGA TGG TC) (Integrated DNA Technologies). The resulting fragment was digested with the restriction enzymes Pfl23II (Fermentas) and PacI (New England BioLabs) and inserted in the previously described plasmid pLBPfl (6). The open reading frame for HSV-1 R1 was amplified from HSV-1 KOS genomic DNA with the primers R1-N-Pac (5′ TTA ATT AAG GCC AGC CGC CCA GCC G) and R1-C-Pme (5′ GTT TAA ACT CAC AGC GCG CAG CTC ATG). The product was digested with PacI and Pme1 (New England BioLabs), and inserted into pLBPfl-GST, forming the plasmid pLBPfl-GST-R1.

pCMV14-3XFLAG (empty vector), pCMV14-3XFLAG-TRIF (TRIF-FLAG), pCMV14-3XFLAG-TRIFΔC(1-541) (TRIFΔC-FLAG), pcDNA3.1 (empty vector), and pcDNA3-6myc-RIP1 (myc-RIP1) were transfected using Lipofectamine 2000 according to the manufacturer's (Invitrogen) protocol (35). Where indicated, 50 μM Z-VAD-fmk (Bachem) was added to the culture medium 6 h after transfection to block cell death induced by recombinant protein expression.

Apoptosis determination. Forty-eight hours after transfection or 8 h after infection, the cells were treated with CHX (15 μg/ml; Sigma), CHX (15 μg/ml) plus human recombinant TNF-α (2.5 ng/ml; Sigma), poly(I · C) (10 μg/ml; Calbiochem), or CHX (15 μg/ml) plus poly(I · C) (10 μg/ml) or transfected with poly(I · C) (10 μg/ml) using Lipofectin (Invitrogen) (29). After 6 h of treatment, the percentage of apoptotic cells was scored by microscopic observation in at least 5 randomly selected fields (15, 44). Attached and detached cells were then collected, washed twice in phosphate-buffered saline (PBS), and lysed with buffer appropriate for subsequent assays. Caspase 3/7 activity was evaluated by measuring DEVD-AFC cleavage (44). To evaluate apoptosis triggered by recombinant TRIF or RIP1 expression, HeLa cells were infected for 8 h with recombinant adenoviruses and then cotransfected with appropriate expression vectors mixed with pEGFP C1. Twenty-four hours after transfection, GFP-positive adherent and nonadherent cells were scored in 5 randomly selected fields, and the percentage of adherent cells was calculated (adherent GFP-positive cells/total GFP-positive cells × 100). They were then harvested with buffer appropriate for subsequent assays.

Gene silencing. The sequence of TRIF-specific small interfering RNA (siRNA) was as follows (the 19-nucleotide portion of the sense strand of the targeted mRNA is depicted): 5′-UUAGAGUUGUCAUCGAAUC-3′. Allstars negative-control siRNA from Qiagen served as a nonsilencing control siRNA. HeLa cells seeded in 6-well plates were transfected with siRNA (40 nM) using Lipofectamine 2000.

Immunoprecipitation. After infection or transfection, the cells were collected, washed twice with PBS, and lysed at 4°C for 15 min in immunoprecipitation (IP) 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). The lysates were precleared by incubation with protein G Sepharose 4B beads (Amersham Biosciences) for 2 h at 4°C, followed by centrifugation. Anti-GFP antiserum (9), anti-FLAG (M2; Sigma), anti-myc (clone 9E10; Upstate), or anti-RIP1 (clone 38; BD Biosciences) monoclonal antibodies (MAb) were incubated with protein G Sepharose 4B beads for 2 h at 4°C. Precleared lysates were incubated with antibody-coated beads for 2 h at 4°C. As a control, precleared lysates were incubated for 2 h at 4°C with protein G Sepharose beads. The beads were recovered by centrifugation and washed 5 times with IP buffer, and the immunoprecipitated proteins were eluted in sodium dodecyl sulfate (SDS) sample-loading buffer for subsequent immunoblot analysis.

Glutathione *S*-transferase (GST) pulldown. After transfection, cells were collected and lysed with IP buffer as described above. The lysates were precleared by incubation with protein G Sepharose 4B beads for 2 h at 4°C, followed by centrifugation. Precleared lysates were incubated with glutathione Sepharose 4B beads (Amersham) for 2 h at 4°C. The beads were recovered by centrifugation and washed 5 times with IP buffer, and the bound proteins were eluted by boiling in SDS sample-loading buffer.

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. Cytoplasmic extracts were obtained by lysis in IP buffer. The protein content was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting (43). Primary antibodies for immunoblotting included anti-FADD (A66-2; BD Biosciences), anti-β-actin (AC15; Abcam), anti-RIP1 (G322-2; BD Biosciences), anti-FLAG (M2; Sigma), anti-myc (9E10; Upstate), anti-HSV-1 infected cell polypeptide 0 (ICP0) (5H7; Abcam), anti-c-FLIP (NF6; Alexis Biochemicals), and anti-TRIF (AL227; Alexis Biochemicals). GFP and GFP-tagged proteins were detected with the MAb anti-GFP B-2 (Santa

Cruz Biotechnologies) or a polyclonal antibody against GFP (9). Caspase 8 was detected with the MAb anti-caspase 8 1C12 (Cell Signaling) or the MAb 1C15 (kindly provided by Marcus Peter) (64). Polyclonal anti-R1 serum 168R1 and polyclonal anti-R2 serum P9 were used for HSV R1 and HSV R2 detection, respectively (13, 44).

Statistical analyses. The data are expressed as mean and standard error of the mean (SEM). Statistical differences between groups were determined by 2-way analysis of variance, followed by a Bonferroni posttest with Prism 4 software (GraphPad Software).

RESULTS

HSV-1 and HSV-2 infections prevent poly(I · C)-induced apoptosis. To determine whether HSV-infected cells could be protected from poly(I · C)-induced apoptosis, we chose HeLa cells, because they rapidly undergo apoptosis upon treatment with either extracellular poly(I · C) in the presence of CHX, to shut down the synthesis of short-lived apoptosis inhibitors [CHX+poly(I · C)], or intracellular poly(I · C) achieved through transfection with Lipofectin [lipo+poly(I · C)] (33, 52). As shown in Fig. 2A and B, treatment of HeLa cells with CHX+poly(I · C) for 6 h produced morphological changes typical of apoptosis, including membrane blebbing, cell body condensation, and detachment from the substratum in more than 95% of cells, whereas lipo+poly(I · C) was slightly less efficient, with 60% of cells exhibiting apoptotic morphology. Caspase 3/7 activity, measured with DEVD-AFC as a substrate, correlated fairly well with the percentages of apoptotic cells (Fig. 2C). To first assess the effect of HSV infection on poly(I · C)-induced apoptosis, cells were infected with HSV-1 strain KOS or HSV-2 strain HG-52 for 8 h before being treated with CHX+poly(I · C) or lipo+poly(I · C), an infection period known to be sufficient for maximal protection from CHX-plus-TNF-α-induced apoptosis (44). Both HSV-1 and HSV-2 infections almost completely prevented the appearance of cells exhibiting apoptotic morphology (Fig. 2B) and caspase 3/7 activation (Fig. 2C) by either CHX+poly(I · C) or lipo+poly(I · C). These results demonstrate that HSV-1 and HSV-2 infections protect cells from apoptosis induced by both CHX+poly(I · C) and lipo+poly(I · C).

To determine the time required for HSV-mediated protection, HeLa cells were mock infected or infected with HSV-1 strain KOS or HSV-2 strain HG-52 for increasing periods before exposure to CHX+poly(I · C), the most effective of the two poly(I · C) treatments. Protection from CHX+poly(I · C)-induced apoptosis, as scored by apoptotic morphology, became detectable between 2 and 4 h after HSV-2 infection and about 1 h earlier with HSV-1 (Fig. 2D). However, for both HSVs, maximal protection was reached within 6 to 8 h, a time at which the level of apoptosis induced by CHX+poly(I · C) became comparable to the level observed in HSV-infected cells treated with CHX only. Altogether, these results indicate that the protective effect of HSVs is mediated by the synthesis of an immediate-early/early viral protein(s), for which R1 is a likely candidate.

Cells infected with the HSV-1 R1-null mutant ICP6Δ are sensitized to poly(I · C)-induced apoptosis. Studies showing that apoptosis induced by CHX+poly(I · C) or lipo+poly(I · C) necessitates caspase 8 activation (29, 30, 72), together with evidence that HSV R1 impairs caspase 8 activation (15, 44), suggest that the viral protein could contribute to the protection of HSV-infected cells against poly(I · C)-induced apoptosis. To test this hypothesis, HeLa cells were infected for 8 h with the HSV-1 R1 deletion mutant ICP6Δ or its wild-type (WT) parent, KOS, and further treated with poly(I · C), CHX+poly(I · C), or lipo+poly(I · C). In these experiments, the extent of apoptosis was evaluated by assessing caspase 8 activation by immunoblotting and caspase 3/7 activity by *in vitro* enzymatic assay. As expected from the data presented in Fig. 2, KOS infection efficiently impaired caspase 8 proteolytic processing (Fig. 3A, lanes 11 and 14) and caspase 3/7 activation (Fig. 3B) by either CHX+poly(I · C) or lipo+poly(I · C) (Fig. 3A, lanes 10 and 13, and Fig. 2B). In sharp contrast, ICP6Δ infection failed to prevent full-length caspase 8 (p55/53) cleavage (Fig. 3A, lanes 12 and 15) and only slightly reduced caspase 3/7 activation resulting from both treatments (Fig. 3B). Most strikingly, ICP6Δ-infected cells treated with poly(I · C) without CHX showed strong activation of caspase 8 (Fig. 3A, compare lane 9 to lanes 7 and 8) and caspase 3/7 (Fig. 3B), whereas activation of these caspases was either barely detected in mock infection or absent in KOS infection. The increased sensitivity of ICP6Δ-infected cells to poly(I · C)-induced apoptosis was even more evident when the percentage of apoptotic cells was scored. Indeed, the percentage climbed from <10% in mock- and KOS-infected cells to >95% in ICP6Δ-infected cells (data not shown). These results indicate that HSV-1 R1 is crucial in the protection of HSV-infected cells against poly(I · C)-induced apoptosis and that ICP6Δ infection sensitizes HeLa cells to poly(I · C)-induced apoptosis.

HSV infection affects the accumulation of known regulators of caspase 8 activation. c-FLIPs and cellular inhibitor of apoptosis proteins (c-IAPs) regulate caspase 8 activation in both the death receptor and TLR3 signaling pathways (18, 68, 70, 77). A decline in the c-FLIP protein level, starting around 10 h postinfection, has been implicated in the sensitivity of immature dendritic cells to HSV-induced apoptosis (38). As downregulation of these cellular inhibitors of caspase 8 activation could be responsible for the sensitization of ICP6Δ-infected HeLa cells to poly(I · C)-induced apoptosis, we measured the protein levels of c-FLIPs and c-IAPs during the onset of HSV infection. Figure 3C illustrates that infection with KOS or ICP6Δ led to a large decrease in the levels of long (c-$FLIP_L$) and short (c-$FLIP_S$) isoforms of c-FLIP, which started between 2 and 4 h postinfection. At 8 h, both isoforms were barely detectable (Fig. 3C). The c-IAP1 level remained stable until 4 h postinfection and gradually declined thereafter, whereas c-IAP2 decreased only slightly at the 8 h time point. Importantly, the rates of decrease of c-$FLIP_L$, c-$FLIP_S$, and c-IAP1/2 proteins were similar in infection with both viruses, showing that HSV-1 R1 does not mediate the observed effects on the accumulation of these antiapoptotic proteins. For all ICP6Δ infections, exponentially growing cells were used to prevent the inhibition of translation of viral mRNAs, which would occur if the cells were growth arrested (71). Evidence that the synthesis of viral proteins was not impaired in our ICP6Δ-infected cells was obtained from the observation that HSV R2 and ICP0 accumulated at similar rates in cells infected with both viruses (Fig. 3C). This renders unlikely the possibility that the antiapoptotic defect of ICP6Δ against poly(I · C) is related to a lower ability of the cells to support the synthesis of viral polypeptides. Taken together, these results indicate that during HSV-1 in-

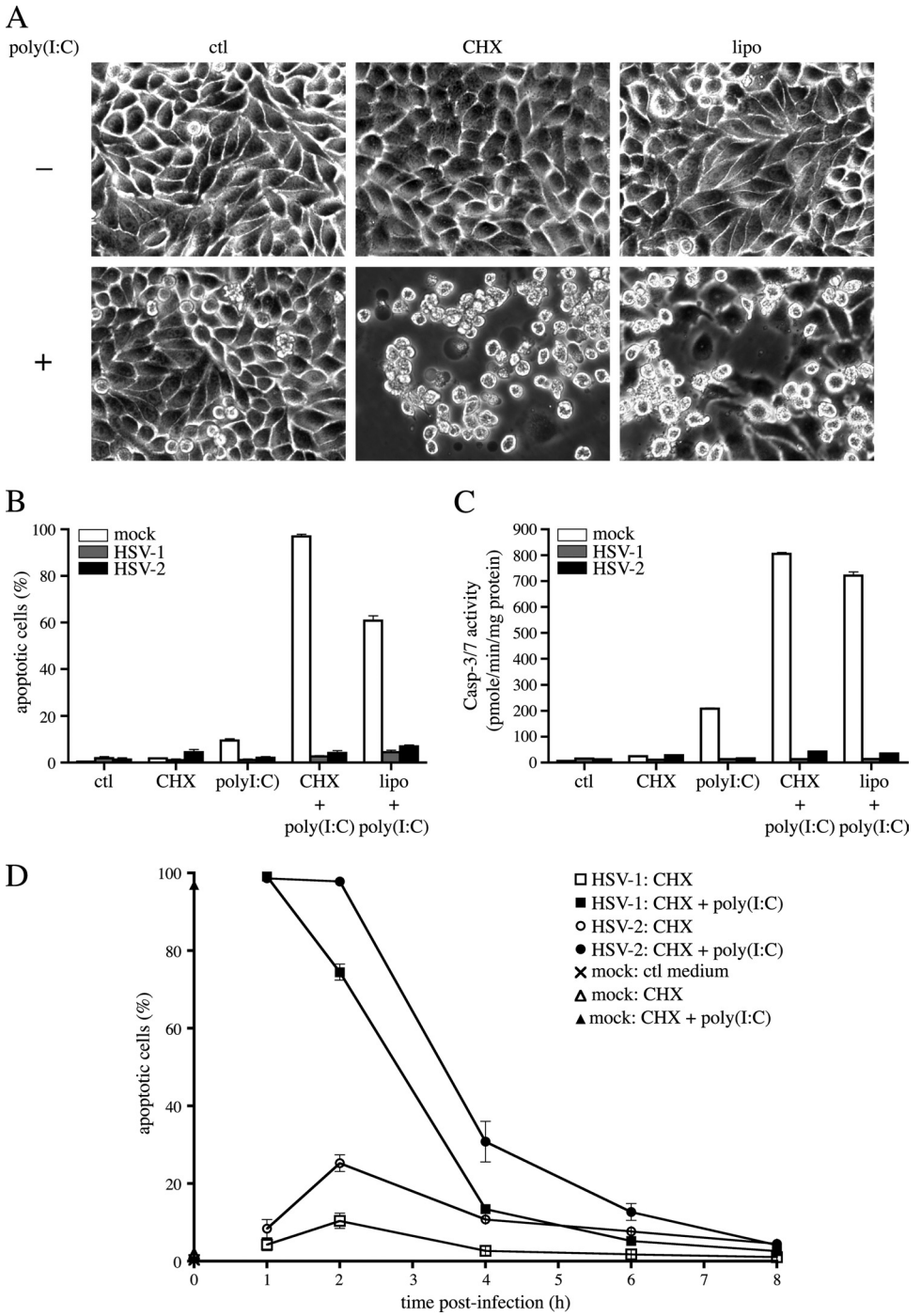

FIG. 2. HSV-1 and HSV-2 infections protect HeLa cells against poly(I · C)-induced apoptosis. (A) One day after plating, HeLa cells were treated with control medium (ctl), CHX, poly(I · C), CHX+poly(I · C), or lipo+poly(I · C). Phase-contrast microphotographs were taken 6 h later. (B) HeLa cells were mock infected or infected with either HSV-1 strain KOS (HSV-1) or HSV-2 strain HG-52 for 8 h before the addition of control medium (ctl), CHX, poly(I · C), CHX+poly(I · C), or lipo+poly(I · C). The percentages of apoptotic cells were scored after 6 h (mean plus SEM). (C) Cells prepared for panel B were harvested, and the cytoplasmic lysates were tested for caspase 3/7 (Casp-3/7) activity (mean plus SEM). (D) HeLa cells were mock infected or infected with HSV-1 strain KOS (HSV-1) or HSV-2 strain HG-52 (HSV-2) for increasing periods before the addition of CHX, CHX+poly(I · C), or control medium (ctl medium). The percentages of apoptotic cells were scored 6 h after treatment (mean ± SEM).

fection, HSV-1 R1, owing to its property as a viral inhibitor of caspase 8 activation (15), could serve to functionally replace the declining cellular inhibitors c-FLIP$_L$, c-FLIP$_S$, and c-IAP1.

HSV-1 R1 and HSV-2 R1 inhibit poly(I · C)-induced apoptosis. As individually expressed HSV-1 R1 and HSV-2 R1 inhibit TNF-α-mediated apoptosis by impairing caspase 8 activation (15, 44), we hypothesized that both proteins could prevent apoptosis induced by the dsRNA mimetic via a similar mechanism. To confirm this possibility, HeLa cells were transfected with plasmids encoding HSV-1 R1 fused to GST (Fig.

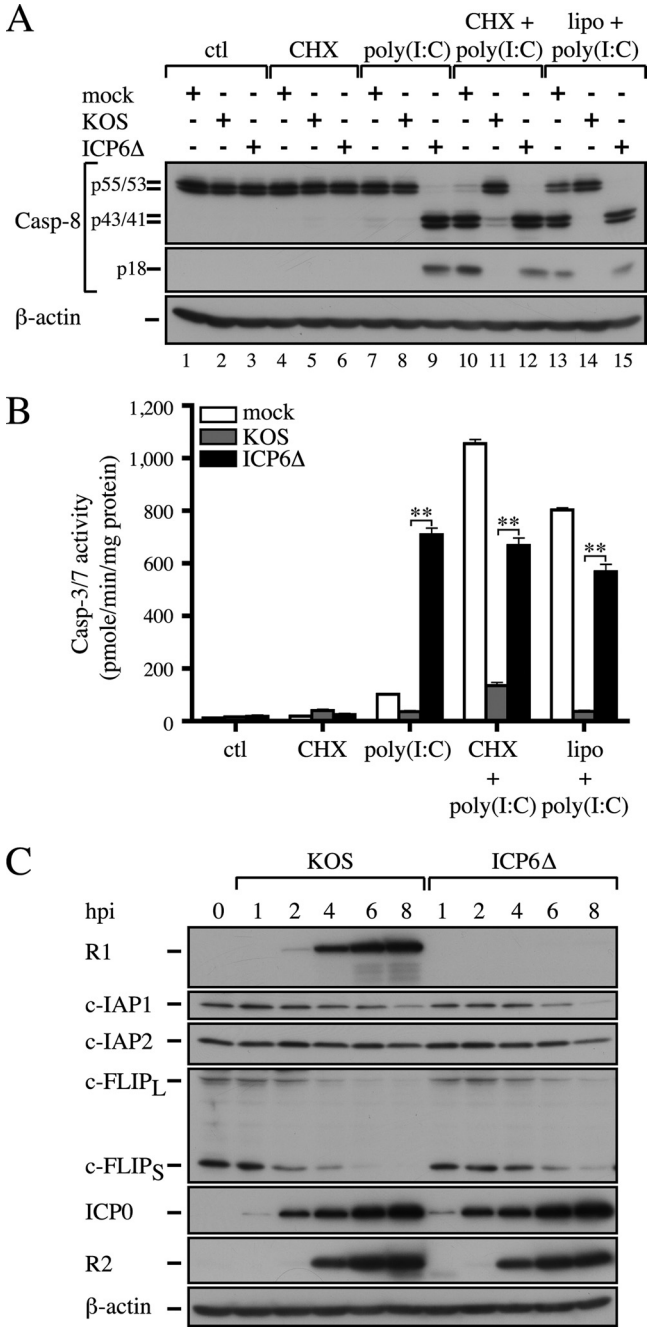

FIG. 3. The HSV-1 R1-null mutant ICP6Δ is defective in blocking apoptosis induced by poly(I · C). (A and B) HeLa cells were mock infected or infected with HSV-1 KOS (KOS) or HSV-1 ICP6Δ (ICP6Δ) for 8 h before the addition of control medium (ctl), CHX, poly(I · C), CHX+poly(I · C), or lipo+poly(I · C). The cells were collected after 6 h. Cytoplasmic cell lysates were analyzed by immunoblotting for caspase 8 (Casp-8) (MAb 1C12) and β-actin protein content (A) and tested for caspase 3/7 (Casp-3/7) activity (B). The immunoblots and caspase activities (mean plus SEM; $n = 6$; **, $P < 0.01$) are representative of 3 independent experiments performed in duplicate. (C) Levels of c-FLIP$_L$ and c-FLIP$_S$ isoforms and c-IAP1/2 decrease after HSV infection. HeLa cells were infected with either HSV-1 KOS (KOS) or HSV-1 ICP6Δ (ICP6Δ). They were collected at the indicated times postinfection (hpi), and total protein extracts were immunoblotted for HSV R1, c-IAP1, c-IAP2, c-FLIP, HSV R2, ICP0, and β-actin protein content determination. The immunoblots are representative of 2 independent experiments performed in duplicate.

4A) or HSV-2 R1 (Fig. 4B). At 48 h posttransfection, the cells were treated with poly(I · C), CHX+poly(I · C), lipo+poly(I · C), or CHX plus TNF-α as a positive control. As observed in previous experiments, apoptosis scored either by apoptotic morphology (top) or activation of caspase 8 (middle, lanes 9, 11, and 13) and caspase 3/7 (bottom) was efficiently triggered in cells transfected with control plasmids upon treatment with CHX+poly(I · C), lipo+poly(I · C), or CHX plus TNF-α. In sharp contrast, the percentage of apoptotic cells (top), proteolytic processing of caspase 8 (middle, lanes 10, 12, and 14), and caspase 3/7 activation (bottom) were highly reduced when these treatments were applied to cells expressing GST-HSV-1 R1 or HSV-2 R1. The results suggest that both HSV-1 R1 and HSV-2 R1, expressed individually, protect HeLa cells from poly(I · C)-induced apoptosis by preventing caspase 8 cleavage and caspase 3/7 activation.

HSV-2 R1 inhibits TRIF- and RIP1-dependent apoptosis. Extracellular poly(I · C) is detected by TLR3, which, in turn, triggers apoptosis through a pathway involving TRIF, RIP1, FADD, and caspase 8 (26, 28, 35, 72). It has been reported that TRIF or RIP1 overexpression efficiently activates caspase 8 and apoptosis in HeLa cells (35). As HSV-2 R1 inhibits apoptosis mediated by extracellular poly(I · C), we sought to determine whether HSV-2 R1 could prevent apoptosis induced by TRIF or RIP1 overexpression. As our previous studies on protection afforded by HSV-2 R1 against the proapoptotic HSV-2 R1(Δ2-357) had shown that full protection could be achieved by first infecting cells with a recombinant adenovirus encoding HSV-2 R1 (Ad HSV-2 R1) for 8 h before starting the expression of the proapoptotic protein, a similar procedure was adopted here (9, 44). HeLa cells that had been either mock infected or infected for 8 h with Ad HSV-2 R1 or a control recombinant adenovirus with an empty cassette (Ad ctl) were further transfected with plasmids encoding FLAG-tagged TRIF or myc-tagged RIP1. TRIFΔC-FLAG, a TRIF deletion mutant lacking a C-terminal region (171 amino acids) encompassing the RHIM motif required for interaction with RIP1, served as a negative control (35). To identify transfected cells, a GFP expression vector was cotransfected with TRIF and RIP1 constructs. At 24 h posttransfection, TRIF- and RIP1-induced apoptosis was evaluated by quantifying the percentage of GFP-expressing cells that remained adherent with a healthy morphology. When transfection of TRIF-FLAG or myc-RIP1 was performed after mock or Ad ctl infection, the majority of GFP-positive cells were seen floating in the medium with apoptotic morphology; only 25% of TRIF-transfected cells and 40% of RIP1-transfected cells remained adherent (Fig. 5A). In sharp contrast, when cells had been infected with Ad HSV-2 R1 prior to TRIF-FLAG or myc-RIP1 transfection, more than 90% of GFP-positive cells maintained their adherence (Fig. 5A). Analyses of caspase 8 processing revealed that TRIF-FLAG and myc-RIP1 induced caspase 8 activation, as demonstrated by the accumulation of its fully processed p18 large subunit in mock- and Ad ctl-infected cell lysates (Fig. 5B, lanes 4, 5, 10, and 11), whereas corresponding lysates from cells expressing HSV-2 R1 exhibited barely detectable amounts of the p18 cleaved fragment (Fig. 5B, lanes 6 and 12). As expected, TRIFΔC-FLAG overexpression did not significantly induce cell detachment (Fig. 5A) or caspase 8 activation (Fig.

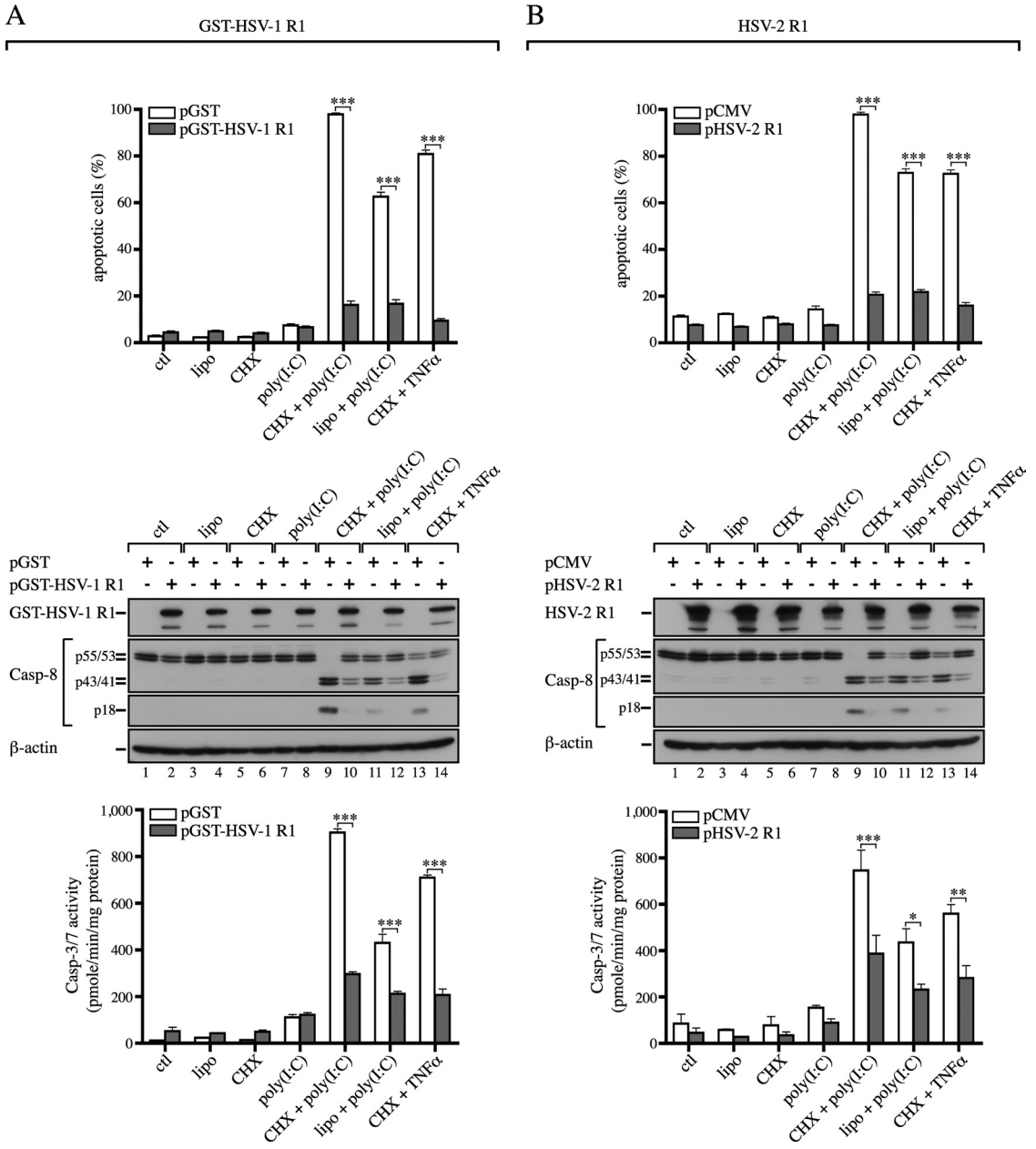

FIG. 4. HSV-1 R1 and HSV-2 R1 inhibit caspase activation induced by poly(I · C). HeLa cells were transfected with pLBPf1-GST (pGST) or pLBPf1-GST-R1 (pGST-HSV-1 R1) (A) and pAdCMV5 (pCMV) or pAdCMV5-R1 (pHSV-2 R1) (B). After 48 h, the cells were left untreated (ctl) or treated with CHX, poly(I · C), CHX+poly(I · C), or lipo+poly(I · C). They were treated with CHX plus TNF-α as a positive control for HSV R1 protection. The percentages of apoptotic cells were scored after 6 h (top). Cytoplasmic lysates were immunoblotted for HSV R1, caspase 8 (Casp-8) (MAb 1C12), and β-actin protein content determination (middle) and tested for caspase 3/7 (Casp-3/7) activity (bottom). The percentages of GFP-positive cells evaluated on parallel dishes cotransfected with pEGFP C1 gave transfection efficiency estimates of >80%. The immunoblots, percentages of apoptotic cells, and caspase activities (mean plus SEM; $n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are representative of 3 independent experiments.

5B, lanes 7 to 9), confirming that interaction between TRIF and RIP1 is required to signal apoptosis (35).

We also observed that HSV-2 R1 had a significant impact on overexpressed recombinant proteins (Fig. 5B). First, HSV-2 R1-expressing cells accumulated a higher level of TRIF-FLAG protein than control cells (compare lanes 4 and 5 to lane 6). As TRIF-FLAG protein accumulation in HSV-2 R1-expressing cells was comparable to that in mock-infected cells transfected with TRIF-FLAG in the presence of the pancaspase inhibitor Z-VAD-fmk (lanes 6 and 13), it suggests that the higher TRIF-FLAG accumulation seen in HSV-2 R1-expressing cells results from apoptosis inhibition by HSV-2 R1. Second, as expected from previous studies reporting RIP1 cleavage by caspase 8 during FasL- and TNF-α-induced apoptosis (40, 48, 51), a processed form of myc-RIP1 (myc-RIP1$_C$) was noted upon myc-RIP1 overexpression in the absence of protection against

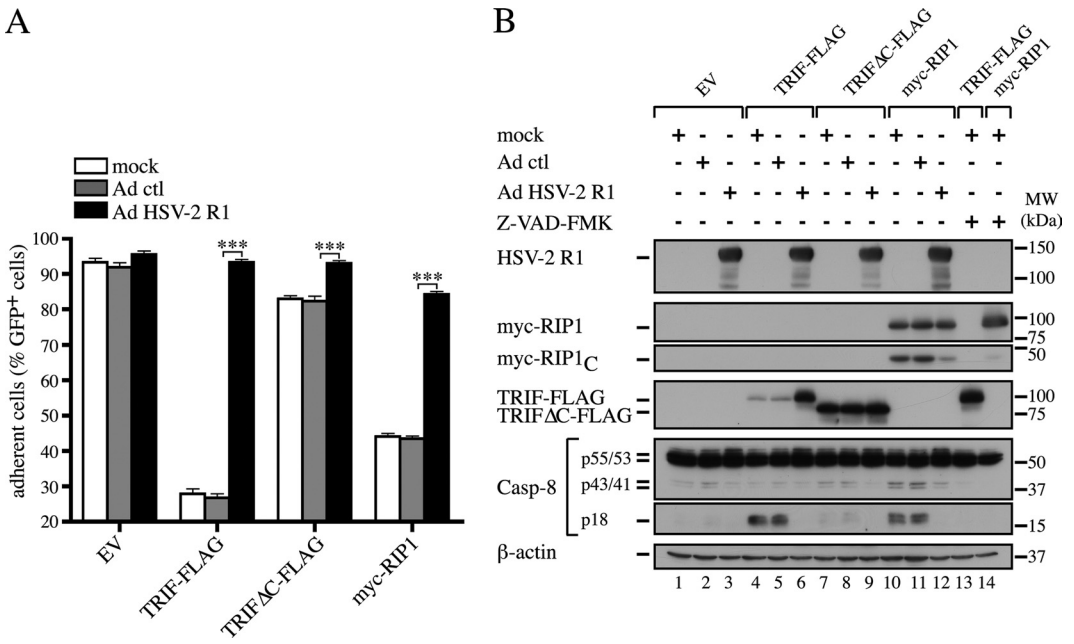

FIG. 5. HSV-2 R1 inhibits apoptosis induced by TRIF or RIP1 overexpression. HeLa cells were mock infected or infected with AdTR5CuO (Ad ctl) or AdCMV5R1 (Ad HSV-2 R1). After 8 h, the cells were cotransfected with pEGFP C1 mixed with empty vector (EV) or expression plasmids encoding TRIF-FLAG, TRIFΔC-FLAG, or myc-RIP in the presence or absence of Z-VAD-fmk. (A) The percentages of GFP-positive adherent cells with healthy morphology were scored after 24 h. (B) Cytoplasmic cell lysates were immunoblotted for HSV-2 R1, caspase 8 (Casp8) (MAb 1C12), β-actin, and myc-tagged and FLAG-tagged protein content determination. Myc-RIP1$_C$ indicates a processed form of myc-RIP1. The percentages of GFP-positive adherent cells and immunoblots (mean plus SEM; $n = 3$; ***, $P < 0.001$) are representative of 3 independent experiments.

apoptosis in mock- and Ad ctl-infected cells (lanes 10 and 11). myc-RIP1$_C$ levels in the controls (lanes 10 and 11) were decreased either by Z-VAD-fmk (lane 14) or HSV-2 R1 expression (lane 12), which both diminished caspase 8 activation. Altogether, these results demonstrate that HSV-2 R1 efficiently impairs apoptosis and caspase 8 activation induced by ectopic expression of TRIF and RIP1.

HSV R1s interact with RIP1. As recent reports indicated that M45, a catalytically inactive homologue of HSV R1 from murine cytomegalovirus (MCMV), interacts with RIP1 in a way that prevents TRIF-induced apoptosis (49, 66), we examined whether HSV-1 R1 and HSV-2 R1 could interact with RIP1. For HSV-1 R1, HeLa cells were transfected with expression plasmids encoding GST-tagged HSV-1 R1 or GST, and GST pulldown assays were conducted (Fig. 6A). The detection of both full-length caspase 8 (p55/53) and endogenous RIP1 in the GST-tagged HSV-1 R1 complex (lane 2), but not in the GST control (lane 1), revealed that HSV-1 R1 interacts not only with full-length caspase 8 (p55/53), but also with RIP1. To assess the interaction between HSV-2 R1 and RIP1, we first immunoprecipitated myc-RIP1 with an anti-myc antibody from extracts derived from HeLa cells infected with Ad ctl or Ad HSV-2 R1 and subsequently transfected with a myc-RIP1 expression vector in the presence of Z-VAD-fmk to prevent myc-RIP1 cleavage (Fig. 6B). As seen in lane 2, HSV-2 R1 coimmunoprecipitated with myc-RIP1. Interestingly, this interaction did not affect the binding of myc-RIP1 to endogenous FADD, which was equivalently coimmunoprecipitated in the presence or absence of HSV-2 R1 (lanes 1 and 2).

Two studies on M45 domains required for interaction with RIP1 arrived at divergent conclusions. One report proposed that a putative RHIM motif localized in the N-terminal portion of M45 is involved in the interaction with RIP1 (66), whereas the other concluded that the C-terminal region of the R1 homology domain of M45 is required for interaction with RIP1, with the N-terminal end encompassing the RHIM motif being dispensable (49). Interestingly, a RHIM-like sequence was also identified by sequence alignment in the unique N-terminal domain of HSV-2 R1 (46). Therefore, it was interesting to analyze which HSV-2 R1 domains could be involved in the RIP1 interaction. To this end, A549tTA cells were infected with recombinant adenoviruses encoding HSV-2 R1-GFP, HSV-2 R1(1-834)-GFP (a C-terminal deletion mutant devoid of antiapoptotic activity against TNF-α or FasL), or R1Δ(2-249)-GFP (an N-terminal truncation mutant showing antiapoptotic properties against TNF-α but lacking the putative RHIM-like motif) (9). GFP-tagged proteins were immunoprecipitated from lysates of infected cells with anti-GFP antiserum (Fig. 6C). Both caspase 8 and RIP1 were detected in immunoprecipitates from cells expressing HSV-2 R1-GFP (lane 4) or HSV-2 R1Δ(2-249)-GFP (lane 3) but not in those from cells expressing GFP (lane 1) or HSV-2 R1(1-834)-GFP (lane 2). These results show that (i) HSV-2 R1 can form a complex, not only with overexpressed myc-RIP1, but also with endogenous RIP1, and (ii) the reductase domain of HSV-2 R1, but not its N-terminal domain containing the putative RHIM sequence, is essential for interaction with full-length caspase 8 (p55/53), as well as with RIP1.

To assess whether HSV R1s could interact with RIP1 in the context of HSV infection, HeLa cells were infected with either

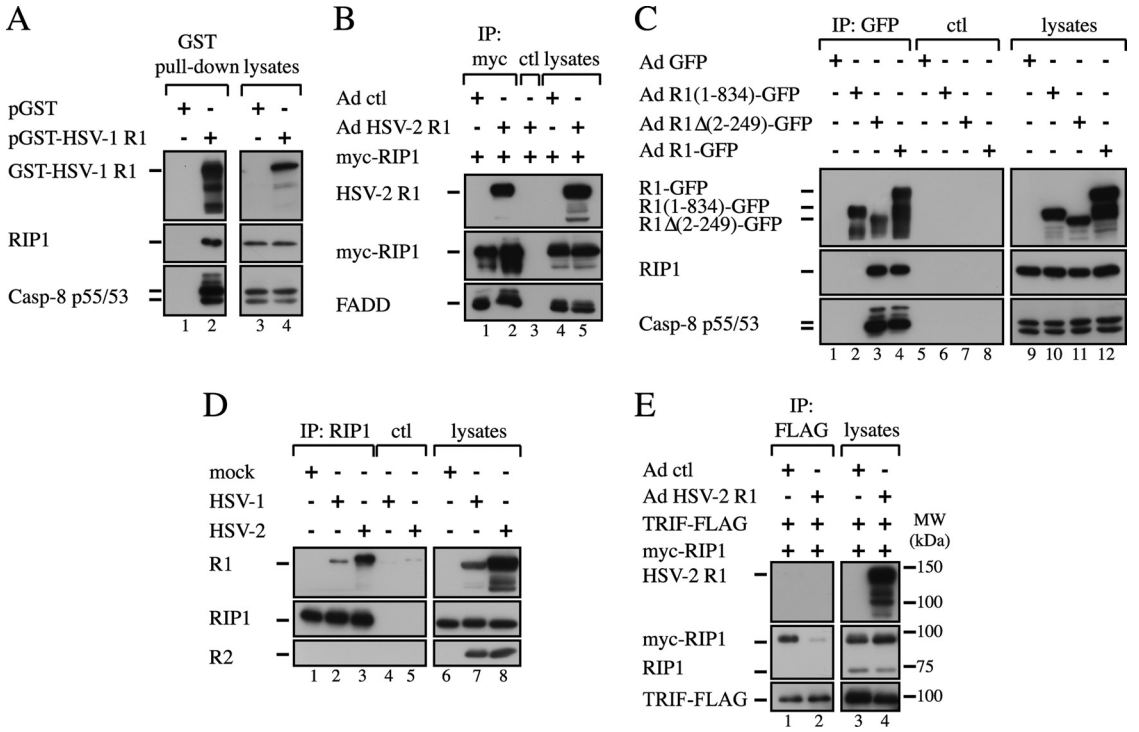

FIG. 6. HSV R1s interact with RIP1. (A) HSV-1 R1 coprecipitates with RIP1. HeLa cells were transfected with pLBPf1-GST (pGST) or pLBPf1-GST-R1 (pGST-HSV-1 R1) for 48 h. GST-tagged proteins were precipitated by GST pulldown. Precipitates (GST pulldown) and whole-cell lysates were immunoblotted for HSV-1 R1, caspase 8 (Casp-8) (MAb 1C15), and RIP1 protein content determination. (B) HSV-2 R1 coimmunoprecipitates with RIP1. HeLa cells were infected with AdTR5CuO (Ad ctl) or AdCMV5R1 (Ad HSV-2 R1). After 8 h, the infected cells were transfected with pcDNA3-6myc-RIP1 (myc-RIP1) for 24 h in the presence of Z-VAD-fmk to maintain cell viability. Myc-tagged proteins were immunoprecipitated with anti-myc antibody. Immunoprecipitates (IP: myc) and whole-cell lysates (lysates) were immunoblotted for HSV-2 R1, FADD, and myc-tagged protein content determination. (C) A large part of the N-terminal, but not the C-terminal, domain of HSV-2 R1 is dispensable for interaction with RIP1 and caspase 8. A549-tTA cells were infected with AdTR5-R1-GFP (Ad R1-GFP), AdTR5-R1(1-834)-GFP (Ad R1(1-834)-GFP), or AdTR5-GFP (Ad GFP) for 24 h. The GFP-tagged proteins were immunoprecipitated with polyclonal anti-GFP antiserum. Immunoprecipitates (IP: GFP) and whole-cell lysates (lysates) were immunoblotted for caspase 8 (MAb 1C15), RIP1, and GFP-tagged protein content determination. (D) HSV-1 R1 and HSV-2 R1 coimmunoprecipitate with RIP1 in the context of HSV infection. HeLa cells were infected with HSV-1 (strain KOS) or HSV-2 (strain HG-52) for 8 h. RIP1 was immunoprecipitated using anti-RIP1 (clone 38) MAb. Immunoprecipitates (IP: RIP1) and whole-cell lysates (lysates) were immunoblotted for HSV R1s, RIP1, and caspase 8 protein content determination. (E) HSV-2 R1 inhibits the interaction between RIP1 and TRIF. HeLa cells were infected with AdTR5CuO (Ad ctl) or AdCMV5R1 (HSV-2 R1) and 8 h later cotransfected with a plasmid encoding myc-RIP1 and a plasmid expressing TRIF-FLAG in the presence of Z-VAD-fmk. The FLAG-tagged proteins were immunoprecipitated with anti-FLAG (M2) MAb. The immunoprecipitate (IP: FLAG) and whole-cell lysate (lysates) protein contents were analyzed by immunoblotting with the polyclonal anti-R1 serum 168R1 and with anti-RIP1 (G322.2) and anti-FLAG (M2) MAbs. As a precipitation control (ctl), precleared lysates were incubated with protein G Sepharose beads without antibody. The immunoblots are representative of at least 2 experiments performed in duplicate.

HSV-1 KOS or HSV-2 HG-52 for 8 h, and RIP1 was immunoprecipitated with an anti-RIP1 antibody. Immunoblot analyses performed with polyclonal anti-R1 serum 168R1 revealed that both HSV-1 R1 and HSV-2 R1 coimmunoprecipitated with RIP1 (Fig. 6D, lanes 2 and 3). Note that 168R1 serum, which was raised with purified HSV-2 R1, recognized HSV-1 R1 less efficiently than HSV-2 R1 (C. Gilbault and Y. Langelier, unpublished results). These data demonstrate that both HSV R1s interact physiologically with RIP1 during infection.

HSV-2 R1 inhibits RHIM-dependent interaction between TRIF and RIP1. To assess the impact of HSV-2 R1 interaction with RIP1, we tested whether HSV-2 R1 could affect binding between TRIF and RIP1, which is essential for TRIF-induced apoptosis (26, 35). To this end, HeLa cells were infected with Ad ctl or Ad HSV-2 R1 and then cotransfected with myc-RIP1 plus TRIF-FLAG in the presence of Z-VAD-fmk. Immunoprecipitation of FLAG-tagged proteins showed that myc-RIP1 coimmunoprecipitated with TRIF-FLAG in Ad ctl-infected cells (Fig. 6E, lane 1). When the cells had been infected with Ad HSV-2 R1 prior to transfection, the interaction between myc-RIP1 and TRIF-FLAG was greatly reduced (Fig. 6E, lane 2). In addition, FLAG immunoprecipitates revealed that HSV-2 R1 did not interact with TRIF-FLAG (Fig. 6E, lane 2). These results suggest that the binding of HSV-2 R1 to RIP1 decreases the interaction between TRIF and RIP1.

Poly(I · C) induces caspase 8 activation through a TRIF-dependent signaling pathway in mock- and ICP6Δ-infected cells. As TRIF is known to play a key role in the signaling pathway involved in apoptosis induction by extracellular poly(I · C) (26, 28, 35, 72), we next sought to determine whether it was involved in the caspase 8 activation seen in cells sensitized to poly(I · C) upon infection with the HSV-1 R1

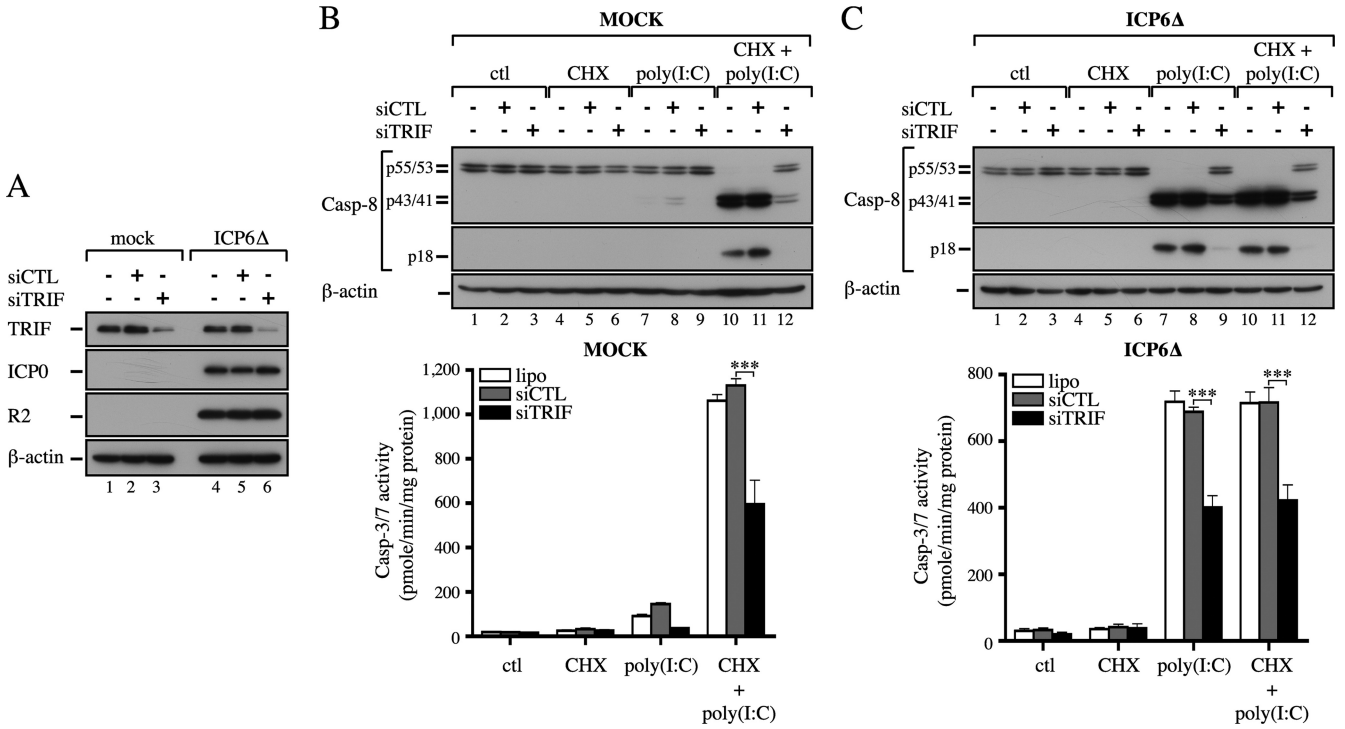

FIG. 7. TRIF silencing inhibits caspase activation induced by extracellular poly(I · C) in mock- and ICP6Δ-infected cells. (A) TRIF siRNA efficiently decreases TRIF expression. HeLa cells were transfected with control siRNA (siCTL) or TRIF siRNA (siTRIF) using Lipofectamine 2000. After 48 h, the cells were mock infected or infected with HSV-1 ICP6Δ for 8 h. Total protein extracts were immunoblotted for HSV R1, HSV R2, ICP0, TRIF, and β-actin protein content determination. (B and C) Effect of TRIF silencing on caspase activation. HeLa cells were transfected with siRNAs and subsequently mock infected (B) or ICP6Δ infected (C) for 8 h before the addition of control medium (ctl), CHX, poly(I · C), or CHX+poly(I · C). The cells were collected after 6 h. Cytoplasmic cell lysates were immunoblotted for caspase 8 (Casp-8) (MAb 1C12) and β-actin protein content determination (top) and tested for caspase 3/7 (Casp-3/7) activity (bottom). The immunoblots and caspase activities (mean plus SEM; $n = 6$; ***, $P < 0.001$) are representative of 3 independent experiments performed in duplicate.

deletion mutant ICP6Δ. To this end, TRIF silencing was attempted by siRNA transfection for 48 h in HeLa cells before mock infection or infection with ICP6Δ for 8 h, followed or not by poly(I · C) treatment. As shown by immunoblotting (Fig. 7A), efficient knockdown of TRIF expression was achieved by TRIF siRNA (lanes 3 and 6), but not control siRNA (lane 2 and 5). The results also revealed that ICP6Δ infection did not alter TRIF levels (Fig. 7A, compare lanes 1 to 3 to lanes 4 to 6). As expected from previous reports, TRIF silencing in mock-infected cells inhibited the proteolytic processing of caspase 8 induced by CHX+poly(I · C) (Fig. 7B, top, lanes 9 and 12) and reduced caspase 3/7 activity by about 50% (Fig. 7B, bottom). The slight activation of caspases triggered by poly(I · C) alone was also decreased. In ICP6Δ-infected cells, where caspases were activated to similar extents by poly(I · C) and CHX+poly(I · C) (Fig. 7C), the effect of TRIF silencing on caspase 8 (Fig. 7C, top, lanes 9 and 12) and caspase 3/7 (bottom) activation was similar to that seen in mock infection. Moreover, TRIF silencing did not affect the resistance of KOS-infected cells to poly(I · C)-induced apoptosis (data not shown). These results demonstrate that caspase 8 and caspase 3/7 activation by poly(I · C), in the absence or presence of CHX, involves, at least in part, a TRIF-dependent signaling pathway in both mock- and ICP6Δ-infected HeLa cells.

DISCUSSION

HSV infection confers protection against apoptosis induced by the virus itself and a variety of exogenous proapoptotic stimuli, including the death receptor ligands TNF-α and FasL (3, 20, 41, 44). We previously found that the HSV-1 and HSV-2 ribonucleotide reductase R1 subunits play an important role in protecting HSV-infected epithelial cells from death receptor-induced apoptosis by inhibiting caspase 8 activation through direct interaction with the caspase 8 prodomain (9, 15, 44). The present work shows that (i) infection by either HSV-1 or HSV-2 protects cells against poly(I · C)-induced apoptosis, (ii) HSV R1s are essential in the inhibition of poly(I · C)-induced apoptosis; and (iii) interaction between HSV R1s and both caspase 8 and RIP1 could be involved in the antiapoptotic effect.

A first indication of HSV R1 involvement in impairing poly(I · C)-induced apoptosis came from our data revealing that the protective effect appears in HSV-1- and HSV-2-infected cells at a time compatible with synthesis of the protein. Two observations subsequently substantiated the key role of HSV R1s in this antiapoptotic outcome: (i) deleting the HSV-1 R1 gene produced a mutant virus, ICP6Δ, unable to protect cells, and (ii) individually expressed HSV-1 R1 and HSV-2 R1 efficiently inhibited the apoptotic process. The extents of pro-

tection observed after WT virus infection or HSV R1 transfection were essentially similar whether apoptosis was induced by extracellular poly(I · C) in the presence of CHX or by transfected poly(I · C), albeit the latter treatment was slightly less proapoptotic. This indicates that HSV R1s could inhibit apoptosis induced by viral dsRNA, which accumulates either intracellularly in the course of HSV infection or extracellularly after the lysis of infected cells.

The hypothesis that HSV R1 could inhibit poly(I · C)-induced apoptosis was based on our previous demonstration that HSV R1s and full-length caspase 8 interact constitutively and directly in a way that suppresses caspase 8 dimerization/activation. Evidence that this interaction is required for protection against death receptor ligands was provided by the observation that the HSV-2 R1 deletion mutant R1(1-834)-GFP and Epstein-Barr virus R1, which do not protect against apoptosis induced by death receptor ligands, do not interact with caspase 8 (15). The present findings that HSV-2 R1 efficiently blocks apoptosis and caspase 8 activation triggered by ectopic expression of TRIF and RIP1 led us to demonstrate that HSV-1 R1 and HSV-2 R1 can also constitutively interact with RIP1 when expressed either alone or in combination with other viral genes during HSV infection. The present work did not delineate the relative importance of the two interactions (HSV R1/caspase 8 versus HSV R1/RIP1) in protection against poly(I · C)-induced apoptosis. On one hand, the C-terminal deletion mutant HSV-2 R1(1-834)-GFP, which is devoid of antiapoptotic activity against TNF-α and FasL (9) and poly(I · C) (F. Dufour and Y. Langelier, unpublished data), does not interact with caspase 8 and RIP1. On the other hand, the N-terminal deletion mutant R1Δ(2-249)-GFP, lacking the putative RHIM motif, interacts with both proteins and protects against both types of proapoptotic stimulus.

As caspase 8 activation has been shown to be essential for apoptosis induction by extracellular poly(I · C) in several cell lines, including HeLa cells (33, 72), it is likely that HSV R1/caspase 8 binding is central to protection against extracellular dsRNA. Whether the same interaction is involved in protection against transfected poly(I · C) is more difficult to assess, as several pathways have been implicated in apoptosis initiation by intracellular dsRNA (Fig. 1). Considering that HSV-2 R1 does not interfere with cell death mediated by a variety of agents triggering the mitochondrial pathway (Bax overexpression, etoposide, staurosporine, and menadione) (44), together with data showing that caspase 8 activation is an apical event of the caspase cascade when HeLa cells are transfected with poly(I · C) (30), it is tempting to speculate that HSV R1/caspase 8 binding also plays a key role in the protective effect against intracellular dsRNA. The importance of HSV R1/RIP1 interaction in preventing poly(I · C)-induced apoptosis is substantiated by our data showing that HSV-2 R1 inhibits interaction between the TLR3 adaptor TRIF and RIP1, an interaction known to be essential for apoptosis induction by either extracellular poly(I · C) or TRIF overexpression (35). Therefore, it is reasonable to suggest that both interactions (HSV R1/caspase 8 and HSV R1/RIP1) contribute to the protection of infected cells from apoptosis triggered by dsRNA sensors, the activation of which elicits the assembly of signaling complexes that share common proteins, such as RIP1 and caspase 8 (21, 39, 69).

In their ability to interact with RIP1, HSV R1s are similar to MCMV M45, a distant homologue of the ribonucleotide reductase R1 subunit, which has been shown to interact with RIP1 and to inhibit TNF-α- and TRIF-induced apoptosis (49, 66). In agreement with the data of Mack et al. on M45 (49), the N-terminal end of HSV-2 R1 encompassing the putative RHIM motif is dispensable for RIP1 binding. In view of M45's ability to inhibit TNF-α-mediated apoptosis, it would be interesting to determine whether M45 shares with HSV R1s the ability to interact with caspase 8. It also remains to be determined whether, similarly to M45, HSV R1s could interfere with other RIP1 functions implicated in dsRNA sensors and death receptor signaling pathways (46), and more specifically, whether they could, like M45, interact with RIP3 to control virus-associated necrosis (67).

The physiological role of HSV R1 inhibition of dsRNA-induced apoptosis remains unclear. The massive induction of apoptosis seen in ICP6Δ-infected cells upon treatment with poly(I · C) in the absence of CHX is a strong demonstration of the importance of HSV R1s in protection against dsRNA. Our search for an explanation of this impressive sensitization to poly(I · C) revealed a new physiological role for HSV R1s. Indeed, our finding that infection of HeLa cells with HSV-1 (KOS and ICP6Δ) (Fig. 3) and HSV-2 (Dufour and Langelier, unpublished) produces a rapid decrease in c-FLIP$_S$ and c-FLIP$_L$ protein levels, and a slower decline of c-IAP1, indicated that HSV R1s could serve to functionally replace these cellular inhibitors of caspase 8 activation during HSV infection. The need to replace cellular caspase 8 inhibitors that are fading out could be particularly important when newly infected cells encounter large amounts of extracellular dsRNAs and/or death receptor ligands. For dsRNAs, reports showing that stable viral dsRNAs released by infected cells (50) and cellular RNA liberated by dying cells are potent host-derived activators of TLR3 (37), together with our data showing that the TLR3/TRIF pathway signals apoptosis in ICP6Δ-infected cells treated with poly(I · C), support the need for antiapoptotic molecules, such as HSV R1s, to protect infected cells against TLR3-induced apoptosis triggered by molecules released by neighboring infected cells dying at the end of the viral replication cycle. For death receptor ligands, numerous studies have shown their abundance at the sites of viral replication and latency (reviewed in reference 15). In this regard, it is interesting to mention that, since our recent report that ICP6Δ infection does not protect HeLa cells against Fc:FasL (15), our preliminary experiments have shown that ICP6Δ infection sensitizes HeLa cells, not only to dsRNAs, but also to Fc:FasL. By their ability to act at a converging step of diverse apoptosis signaling pathways, HSV R1s can efficiently counteract death signals coming simultaneously from death receptor ligands and dsRNAs.

Viral dsRNAs accumulating intracellularly and, more especially, dsRNA structures present in HSV-1 ICP0 mRNAs have been proposed to be key triggers in apoptosis induction at the beginning of HSV infection (57, 63). Based on this assumption, one would expect, from our finding that HSV R1s inhibit apoptosis induced by transfected poly(I · C), that cells infected with an HSV R1-null mutant would be killed by apoptosis. This did not occur when HeLa cells were infected with ICP6Δ (Fig. 3A and B), but our previous finding that in A549 cells apop-

tosis was higher upon ICP6Δ infection than upon WT KOS infection (44) suggests that the importance of HSV-1 R1 in counteracting apoptosis induced by the virus itself could vary in a cell line-specific manner. This variation could be related to cell-specific changes in the expression of other HSV anti-apoptotic molecules. In this respect, the latency-associated transcript (LAT) products, which are, along with HSV R1, the only HSV products known to date to have the potential to inhibit caspase 8 activation induced by both death receptor ligands and caspase 8 overexpression, are likely candidates (1, 27). Interestingly, the lower expression of LATs in immature dendritic cells than in epithelial cell lines, including HeLa cells, was recently related to the higher sensitivity of the former cells to apoptosis induced by HSV infection (38).

Results indicating that HSV-1-dependent apoptosis could be mainly triggered through mitochondrial activation of caspase 9 without significant contribution of caspase 8 stress the importance of other antiapoptotic proteins in controlling apoptosis induced by the virus itself (4). Among them, US3, gJ, and Us11, ICP34.5, and vhs may act at different key points (32, 57) in the multiple pathways that may be activated concomitantly by intracellular dsRNAs (Fig. 1) and/or by other viral molecules, such as dsDNA (11). However, it must be kept in mind that these viral proteins with known antiapoptotic properties are not sufficient to inhibit apoptosis induced by transfected poly(I · C) during infection with the HSV R1-null mutant ICP6Δ. This could be due to the fact that the apoptotic signal is much stronger upon poly(I · C) transfection than upon HSV infection. Additional studies are warranted to better define the importance of HSV R1s in inhibiting dsRNA-induced apoptosis. We can expect that clearer conclusions will be obtained from studies on cells more susceptible to HSV-induced apoptosis, such as immature dendritic cells (38) or primary glial cells (2).

HSVs have developed a large number of strategies to prevent both alarming and suicidal components of the antiviral response inimical to viral replication (25, 55). HSV R1s are a good example of multifunctional proteins, as they not only supply deoxyribonucleotides necessary for viral DNA replication, but also exert antiapoptotic activity that protects host cells from death receptor-induced apoptosis. This study reveals that, in addition, HSV R1s protect HSV-infected cells against poly(I · C)-induced caspase 8 activation, possibly through their interaction with RIP1 and caspase 8. Interestingly, this type of viral protection is not restricted to HSV, since a recent study showed that infection with the classical swine fever virus, a positive-sense RNA virus, protects endothelial cells from extracellular poly(I · C)-mediated apoptosis, notably by inhibiting caspase 8 activation (34). Thus, viral inhibition of dsRNA-mediated apoptosis could be a strategy used by several viruses to ensure host cell survival and, therefore, efficient replication and spreading.

Finally, our results might contribute to a better comprehension of the mechanisms involved in the synergy observed recently between oncolytic HSV vectors containing HSV R1 deletion and proapoptotic chemotherapy treatments (36).

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We declare that we have no conflict of interest.

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