The ribonucleotide reductase domain of the R1 subunit of herpes simplex virus type 2 ribonucleotide reductase is essential for R1 antiapoptotic function

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The R1 subunit (ICP10) of herpes simplex virus type 2 (HSV-2) ribonucleotide reductase (RR), which in addition to its C-terminal reductase domain possesses a unique N-terminal domain of about 400 aa, protects cells against apoptosis. As the NH₂ domain on its own is not antiapoptotic, it has been postulated that both domains of R1 or part(s) of them could be necessary for this function. Here, N- and C-terminal deletions were introduced in HSV-2 R1 to map the domain(s) involved in its antiapoptotic potential. The results showed that, whereas most of the NH₂ domain including part of the recently described putative α-crystallin domain is dispensable for antiapoptotic activity, it is the integrity of the structured RR domain that is required for protection. As the α-crystallin domain appears to play an important role in protein folding and oligomerization, the N-terminal boundary of the antiapoptotic domain could not be defined precisely. In addition, this study provided evidence that overexpression of HSV-2 R2 at levels up to 30-fold more than HSV-2 R1 did not decrease protection from tumour necrosis factor alpha, indicating that the R1 surface where R2 binds is not involved in antiapoptotic activity. Importantly, this result suggests that the co-expression of both RR subunits during the lytic cycle should not affect protection from this cytokine.

INTRODUCTION

Herpesviruses of the alpha and gamma subfamilies encode a DNA polymerase, as well as enzymes involved in generating deoxyribonucleotides, notably ribonucleotide reductase (RR), an enzyme consisting of two subunits, R1 and R2. Therefore, they can replicate without the need for these cellular S-phase enzymes. The alphaherpesviruses herpes simplex virus (HSV) and varicella-zoster virus establish latency in non-dividing neuronal cells. As these neurones have shut down the synthesis of their own RR, the virally encoded R1 protein, through association with its complementary viral R2 subunit, is essential for reactivation by providing deoxyribonucleotides for viral DNA replication (Jacobson et al., 1989).

The R1 subunits of human HSV-1 and HSV-2 (also named ICP6 and ICP10) possess, in addition to a C-terminal RR domain of ~745 aa, an N-terminal domain of ~400 aa that is not found in HSV R1 proteins of species other than primates. As the unique NH₂ domain is dispensable for RR activity (Conner et al., 1992; Massie et al., 1998) and HSV-1 R1 begins to be expressed earlier in the lytic cycle than its R2 partner (Clements et al., 1977; Preston et al., 1978), it has been suggested that the protein could have additional function(s) through its NH₂ domain (Conner et al., 1994).
Two additional activities have been associated with HSV-2 R1 (Chabaud et al., 2003; Langelier et al., 2002; Perkins et al., 2002a). First, our observation that R1(Δ2–357), an HSV-2 R1 derivative with its first 357 residues deleted, was proapoptotic (Massie et al., 1998) led to the finding that full-length HSV-2 R1 has an antiapoptotic function that can protect cells from apoptosis induced either by expression of R1(Δ2–357) or by death receptor triggering by tumour necrosis factor alpha (TNF) or Fas ligand (FasL) (Langelier et al., 2002). The demonstration that the NH₂ domain on its own was not antiapoptotic ruled out the hypothesis that this new function could be attributable solely to the ablated domain, and it was postulated that both domains of R1 or part(s) of them could be necessary for the function (Langelier et al., 2002). Evidence was also provided from infection experiments with two HSV-1 R1 deletion mutants (hrR3 and ICP6A) showing that, in addition to its RR function essential for viral reactivation, the HSV-1 R1 could contribute to viral propagation by preventing apoptosis induced by TNF (Langelier et al., 2002).

Secondly, the presence in HSV R1 proteins of a stretch exhibiting weak similarity to the α-crystallin domain of the small heat-shock proteins (sHsps) led to the observation that HSV-2 R1 displays a chaperone activity as efficient as Hsp27. As mammalian R1, which does not exhibit similarity to an α-crystallin domain, does not have either chaperone or antiapoptotic activity, it was proposed that the α-crystallin domain of HSV-2 R1 could play an important role in the antiapoptotic function of the protein (Chabaud et al., 2003).

Genome sequencing of betaherpesviruses including human, chimpanzee, rhesus, mouse, rat and tupua cyclomegalovirus (CMV) as well as human herpesviruses 6 and 7 identified an open reading frame with homology to R1 but not one for R2. Sequence alignments indicated that essential RR catalytic residues were missing in these betaherpesvirus R1 proteins, and biochemical characterization of three of these demonstrated that they did not exhibit RR activity (Lembo et al., 2004; Patrone et al., 2003; Sun & Conner, 1999). Random transposon mutagenesis of the murine CMV genome identified the R1 gene as blocking apoptosis induced by the virus itself in endothelial cells (Brune et al., 2001). Evidence has also been provided that human CMV R1 can contribute towards protection from Fas-induced apoptosis (Patrone et al., 2003).

Studies to assess the role of the R1 antiapoptotic function in HSV-2 pathogenesis, including reactivation from latency, will require an HSV-2 mutant defective in R1 antiapoptotic activity. However, as RR activity is required for viral DNA replication during reactivation in animal ganglion neurones, this mutant must be functional in RR activity without being proapoptotic, as is R1(Δ2–357). Here, by constructing N- and C-terminal deletions in HSV-2 R1, we mapped the domain of HSV-2 R1 involved in its antiapoptotic potential. In addition, we have provided evidence that the R2-binding surface is not involved in protection from TNF-induced apoptosis.

**METHODS**

**Cell lines, plasmids and viruses.** Conditions for the culture of HeLa, A549-tTA and 293-tTA cells were as described previously (Massie et al., 1998). Details of the construction of plasmids and viruses expressing HSV-2 R1 (strain 333) and its deletion mutants are available as supplementary material (available in IGV Online).

To isolate A549 cell lines inducibly expressing HSV-2 R1 fused to green fluorescent protein (R1-GFP), A549-tTA cells were co-transfected with pAdTR5-R1–GFP and pTK-Hygro using the polyethylenimine method (Durocher et al., 2002) in the presence of anhydrotetracycline (15 ng ml⁻¹) to repress R1-GFP expression. After hygromycin selection at 200 μg ml⁻¹, resistant colonies were pooled. After induction of R1-GFP expression following overnight removal of the anhydrotetracycline, several single fluorescent cells were isolated with a micromanipulator (Caron et al., 2000). Cell lines were derived by growing these cells in the presence of hygromycin (100 μg ml⁻¹) and anhydrotetracycline (15 ng ml⁻¹).

For R2 co-infection experiments, an HSV-2 R2-expressing virus, AdCR5-R2, was used in combination with a virus expressing a new transactivator, AdCMV-cTA, derived from the cuncate inducible system (Mullick et al., 2006). AdCMV-cTA was constructed using standard recombination in 293 cells (Massie et al., 1998), whereas AdCR5-R2 containing the HSV-2 R2 gene derived from pSVRkBHSV-16 (Lamarche et al., 1990) was constructed using a positive selection method based on the adenovirus (Ad) protease (Elahi et al., 2002).

**Transfection.** HeLa cells were seeded 1 day before transfection in 60 mm dishes at a concentration of 5 × 10⁵ cells per dish or in six-well plates at 1.5 × 10⁶ cells per well. The calcium phosphate technique was used to transfect 25 μg pAdCMV5-R1 or pAdCMV5-R1(Δ2–249) plasmid ml⁻¹, 50 μg pAdCMV5 empty vector used as control, pAdCMV5-R1(Δ2–312) or pAdCMV5-R1(Δ2–357) ml⁻¹ and 100 μg pAdCMV5-R1(1–1113) or pAdCMV5-R1(1–1123) ml⁻¹. After 24 h, cells were washed twice with sterile PBS and 1 ml (per well) or 2.5 ml (per dish) of fresh medium was added with an appropriate concentration of Tet system-approved fetal calf serum (TSA-FCS; Clontech).

**Infection.** A549-tTA cells were plated at a concentration of 3 × 10⁴ cells per well (six-well plate) or 1 × 10⁶ cells per 60 mm dish 1 day before the experiment. Cells were infected using conditions to enhance viral adsorption (Langelier et al., 2002). The m.o.i. of AdTR5-R1 (5 p.f.u. per cell), AdTR5-R1–GFP (25 p.f.u. per cell), AdCMV5-GFP-R1 (50 p.f.u. per cell), AdCMV5-GFP (50 p.f.u. per cell), AdTR5-R1(Δ2–357) (50 p.f.u. per cell), AdTR5-R1(Δ2–398) (250 p.f.u. per cell), AdTR5-R1(Δ2–496) (250 p.f.u. per cell), AdTR5-R1(1–983) (50 p.f.u. per cell) and AdTR5-R1(1–1081) (100 p.f.u. per cell) were chosen to obtain roughly similar levels of expression of the different recombinant proteins.

To evaluate the effect of R2 overexpression on R1 antiapoptotic potential, A549-tTA cells were co-infected with increasing m.o.i. of AdCR5-R2 and 50 p.f.u. per cell of AdCMV5-cTA. The medium was removed 24 h later and the cells were reinoculated for 7 h with 5 p.f.u. per cell of AdTR5-R1 or mock infected. When used, the peptidomimetic inhibitor of HSV RR, BILD1633 (Boehringer Ingelheim) (Moss et al., 1996), was added 6 h before infection and maintained throughout the assay.

**Apoptosis assays.** To determine the antiapoptotic potential of the proteins, apoptosis was induced by adding either cycloheximide (CHX) (15 μg ml⁻¹; Sigma) alone as a control or CHX (15 μg ml⁻¹) plus human recombinant TNF (2.5 ng ml⁻¹; Sigma). Fc:FasL (a recombinant hexameric form of human FasL) (Holler et al., 2003), kindly provided by P. Schneider (University of Lausanne,
Switzerland), was used at 25 ng ml⁻¹ with CHX at 15 μg ml⁻¹. Generally after 7 h of treatment, the percentage of apoptotic cells was scored by microscopic observation in at least ten randomly selected fields, as described previously (Langelier et al., 2002). Cells were then harvested for caspase-3 activity measurements, as described previously (Langelier et al., 2002). The percentages of TNF-specific apoptosis and TNF-specific caspase-3 activity were evaluated using formulas that eliminated the intrinsic toxicity of some proteins and CHX as follows: (i) % TNF-specific apoptosis = [(% apoptosis in CHX+TNF-treated cells – % apoptosis in CHX-treated cells)/(100 – % apoptosis in CHX-treated cells)] × 100 (Baumler et al., 2003); (ii) TNF-specific caspase-3 activity = caspase-3 activity in CHX+TNF-treated cells – caspase-3 activity in CHX-treated cells. To determine the proapoptotic activity of the recombinant proteins, cells were incubated with 1 ml (per well) or 2.5 ml (per dish) of fresh medium containing 0.5 % TSA-FCS for 8 h after infection or 30 h after transfection.

**Immunoblot analysis and protein solubility.** Crude total protein extracts were obtained by sonication in 80 mM Tris/HCl (pH 6.8), 2 % SDS and 6 M urea. SDS-PAGE and immunoblotting were performed with 10 μg protein per well, as described previously (Lamarche et al., 1996). For HSV-2 R1 detection, the rabbit polyclonal anti-HSV-2 R1 antiserum 168R1 (Langelier et al., 1998) or anti-HSV R1 mouse monoclonal antibody (mAb) 932 (Ingemarson & Lankinen, 1987) was used. For HSV-2 R2 detection, the polyclonal antibody (pAb) P9 was used (Cohen et al., 1986a). A rabbit polyclonal antibody generated using bacterially expressed GFP was used for GFP detection. A SuperSignal detection kit (Pierce) was used according to the manufacturer’s instructions. To determine protein solubility, cell extracts in HD buffer [50 mM HEPES (pH 8.1), 2 mM DTT] were sonicated and centrifuged at 100 000 g for 1 h at 4 °C. Cell extracts in RIPA buffer [20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % SDS, 1 % NP-40, 1 % sodium deoxycholate, 1 mM PMSF] were incubated for 15 min at 4 °C before sonication, followed by centrifugation at 16 000 g for 30 min at 4 °C. The supernatant fraction was removed and stored at −80 °C. The pellet fraction was resuspended in the same buffer as the supernatant and homogenized by sonication before storage. The percentage solubility was evaluated by immunoblotting appropriate amounts of both fractions with the antiseraum 168R1, as described previously (Langelier et al., 2002).

**RR assay.** To determine the RR activity of the different R1 proteins, infected A549-tTA cells or transfected HeLa cells were used to prepare crude protein extracts, as described previously (Lamarche et al., 1996). R1 activity was determined by adding excess amounts of HSV-2 R2, partially purified as described previously (specific activity, 200 U mg⁻¹), to limiting amounts of R1 (Lamarche et al., 1996). One unit of RR was defined as the amount of enzyme that generated 1 nmol dCDP min⁻¹.

**RESULTS**

**Free N and C termini of full-length R1 are not necessary for antiapoptotic activity**

In a first step towards mapping the R1 domain(s) important for its antiapoptotic activity, we examined the importance of free N and C termini of the full-length HSV-2 R1, a protein of 1144 aa, by creating GFP chimeras as illustrated in Fig. 1 and by infecting A549-tTA cells with Ad recombinants expressing the chimeric proteins. When observed by fluorescence microscopy, both chimeras exhibited a cytoplasmic localization, in sharp contrast to GFP, which was observed equally in both the cytoplasm and the nucleus (Fig. 2a). The diffuse fluorescence of both chimeras, which was similar to the immunofluorescence staining observed for the native R1, suggested that both proteins were soluble. This was confirmed by analysis of protein solubility after fractionation by centrifugation in HD buffer (Table 1).

Protection from TNF by recombinants expressing either the chimeras, native R1 or GFP was evaluated by measuring apoptosis by microscopic observation and an *in vitro* caspase-3 activity assay (Fig. 2b). The results showed that both chimeras were as effective as native R1 in protecting cells, whereas, as previously observed by us (Langelier et al., 2002), GFP was not protective. The chimeras also protected A549-tTA cells from apoptosis triggered by R1(A2–357) expression (data not shown). The data indicated that free termini are not required for the R1 antiapoptotic function. Interestingly, the addition of GFP at the C terminus completely eliminated RR activity, whereas fusion at the N terminus did not affect this activity (Fig. 1).

**Inducible HSV-2 R1–GFP expression leads to TNF resistance**

The observation that the R1–GFP chimeric protein was as effective as native R1 in protecting cells from TNF-induced apoptosis led us to establish inducible A549-tTA cell lines, where expression of the chimeric protein was driven by TR5, a tetracycline-responsive promoter (Massie et al., 1998). Six independent single-cell clones were isolated by micromanipulation of fluorescent cells after briefly turning on chimeric protein expression and thereafter growing them in the presence of anhydrotetracycline. One inducible line, A549-tTA-HSV-R1–GFP, was selected from two undetectable-background/high-inducer lines for detailed study.

When chimeric protein expression was kept in the OFF state in the presence of anhydrotetracycline, A549-tTA-HSV-R1–GFP cells did not express detectable levels of R1–GFP, as shown by immunoblotting (Fig. 3a), and were destroyed by CHX+TNF treatment as efficiently as their parental counterparts. As expected, switching on R1–GFP expression following anhydrotetracycline removal induced TNF resistance with a time course paralleling the detection of GFP fluorescence by microscopy (Fig. 3b). At the 36 h time point, TNF resistance (>95 %) was equivalent to the level previously described as being maximal in A549-tTA cells infected with Ad recombinants expressing HSV-2 R1 (Langelier et al., 2002). At this time, the R1–GFP concentration quantified by immunoblotting represented 0.1 % of the total cell protein (data not shown). As this concentration was equivalent to the minimal level of HSV-2 R1 necessary for maximal protection (Langelier et al., 2002), it could be concluded that the addition of GFP at the R1 C terminus did not affect its antiapoptotic potential. Moreover, we observed that, at 36 h after induction of R1–GFP, A549-tTA-HSV-R1–GFP cells were also resistant to Fc: FasL-induced apoptosis (data not shown). These results showed that, in addition to Ad recombinant infection and
A major part of the HSV-2 R1 NH2 domain is not essential for protection from TNF-induced apoptosis

The functional importance of the NH2 domain of HSV-2 R1 in the control of cell death was first suggested by the cytotoxicity of R1(A2–357), an HSV-2 R1 subunit with a deletion of its first 357 aa (Massie et al., 1998). Subsequently, the observation that the NH2 domain per se was insufficient for protection from TNF-induced apoptosis suggested that both domains of R1 or part(s) of them were necessary for antiapoptotic activity (Langelier et al., 2002). To assess the importance of different segments of the NH2 domain, including the recently described ɑ-crystallin-like domain (Chabaud et al., 2003), a series of deletion mutants was constructed either in CMV5 promoter-driven expression plasmids or in TR5-inducible Ad recombinants as illustrated in Fig. 1. The concentration of plasmids transfected into HeLa cells and the m.o.i of Ad recombinants used to infect A549-tTA cells were adjusted to obtain roughly equivalent accumulation of recombinant proteins (Fig. 4a). By measuring apoptosis by microscopic observation and an in vitro caspase-3 activity assay, the proapoptotic activity (Fig. 4b) and protection from TNF (Fig. 4c) of the different truncated proteins were evaluated. The formulas used to calculate the percentages of TNF-specific apoptosis and the TNF-specific caspase-3 activity eliminated the proapoptotic activity of CHX and of some truncated proteins. As our previous work had shown that R1(A2–357) was mostly insoluble (Massie et al., 1998), protein solubility was assessed by immunoblot analysis after fractionation by centrifugation in HD or RIPA buffer (Table 1). The deletion A2–249, which removes a large part of a hydrophilic region located between aa 130 and 294, gave a non-cytotoxic soluble protein with full antiapoptotic...
The deletion Δ2–312, which removes all of the amino acids preceding the a-crystallin domain, also gave a non-cytotoxic soluble protein, but this protein showed a slight decrease in antiapoptotic activity. Included as a negative control in our analysis of the antiapoptotic potential, the strongly cytotoxic and barely soluble R1(D2–357) gave an unexpected result in that most of the cells resisting its cytotoxicity were protected from TNF (see photomicrographs in Fig. 4d). Other deletions where the a-crystallin domain was removed either completely as in R1(D2–398), R1(D2–496) and R1(Δ107–446) or in part as in R1(D378–445) gave fully insoluble products. None of these four truncated proteins was either proapoptotic or protective against TNF-induced apoptosis [data not shown for R1(D107–446) and R1(Δ378–445)]. Taken together, these results indicated that nearly all of the NH₂ domain including the first two-thirds of the a-crystallin domain is dispensable for the antiapoptotic function. The data also suggested that part of the a-crystallin domain, which appears to play a role in protein folding, is important for this function. Finally, the fact that four NH₂ deletion mutants showed no mutant-specific phenotype in spite of the aggregation of their protein products suggested that neither the cytotoxicity nor the protection induced by

### Table 1. Solubility of R1 and mutant R1 proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Solubility (%)</th>
<th>HD buffer</th>
<th>RIPA buffer</th>
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</thead>
<tbody>
<tr>
<td>R1</td>
<td>56</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>R1(Δ2–249)</td>
<td>44</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>R1(Δ2–312)</td>
<td>ND</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>R1(Δ2–357)</td>
<td>3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>R1(Δ2–398)</td>
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<tr>
<td>R1(Δ2–496)</td>
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<tr>
<td>R1(Δ107–446)</td>
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<td>R1(Δ378–445)</td>
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<td>ND</td>
<td></td>
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<tr>
<td>R1(1–496)</td>
<td>35</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>R1(1–834)–GFP</td>
<td>35</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>R1(1–983)</td>
<td>92</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>R1(1–1081)</td>
<td>ND</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>R1(1–1113)</td>
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<td>4</td>
<td></td>
</tr>
<tr>
<td>R1(1–1123)</td>
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<td></td>
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<tr>
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</tr>
<tr>
<td>GFP–R1</td>
<td>72</td>
<td>ND</td>
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*Values are the means of at least two independent determinations.
all of the structured parts of R1, exhibited protection from TNF (Fig. 5). The ~2-fold reduction in protection of R1(1–1123) compared with full-length R1 was probably due to lower solubility of the truncated protein (Table 1). These data demonstrated that the flexible C-terminal tail is dispensable for protection and suggested that C-terminal amino acids beyond the flexible tail are important for this activity.

**The R2-binding face of HSV-2 R1 is not important for antiapoptotic function**

As a second approach towards mapping the R1 surface(s) important for antiapoptotic function, we tested the effect of R1-binding molecules. On binding to R1, R2 covers a large surface known as the R2-binding face of R1; thus, it could compete with binding to R1 of cellular protein(s) controlling apoptosis. In order to accumulate R2 in large excess over R1, A549-tTA cells were first co-infected with the Ad recombinants AdCR5-R2 (at two different m.o.i.) and AdCMV-cTA for 24 h before being infected for 7 h with AdTR5-R1. Quantification of each protein by comparison with purified R1 and R2 standards showed that the ratio of R2 : R1 ratio was >30 at the higher m.o.i of AdCR5-R2 tested. This large R2 overproduction did not produce any decrease in protection from TNF (Fig. 6a). HSV-2 R2 expressed alone in parallel controls was not protective (data not shown). This result indicated that the R2-binding surface is not involved in R1 antiapoptotic activity. Subsequently, as it has been suggested that the R2 C terminus binds to R1 in a crevice formed between zl and other parts of the protein (Bonneau et al., 1996; Cohen et al., 1986b), we tested the effect of a peptidomimetic inhibitor of HSV RR that binds within this crevice (Liuzzi et al., 1994; Moss et al., 1996). This compound, which exhibits an EC$_{50}$ of 0.3 µM against HSV in cell culture (Liuzzi et al., 1994; Moss et al., 1996), added at concentrations up to 10 µM did not alter the R1 antiapoptotic activity against TNF (Fig. 6b). This result suggested that the R1 surface interacting with the R2 C terminus is not involved in protection.

**DISCUSSION**

One of the major findings of the present work was that a large part of the NH$_2$ domain of HSV-2 R1 (the first 312 aa) could be deleted without significantly affecting protection against TNF and R1(Δ2–357). The lack of importance of the N terminus of R1 was also supported by results showing that adding GFP at this extremity altered neither RR activity nor the antiapoptotic function of the protein. This conclusion was rather unexpected as we had initially thought that the proapoptotic activity of the R1(Δ2–357) truncated protein could be due to antiapoptotic properties of the missing NH$_2$ domain (Langelier et al., 2002). In addition, others had reported evidence that the protection afforded by HSV-2 R1 to 293 cells from staurosporine involved residues contained in its first 300 aa (Perkins et al., 2002a, b, 2003). It is possible that the domain(s) of R1 implicated

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**Fig. 3.** Inducible expression of HSV R1–GFP protects cells from TNF-induced apoptosis. Before being seeded in six-well plates, A549-tTA-HSV-R1–GFP cells were washed twice with medium containing (OFF) or not (ON) anhydrotetracycline (15 ng ml$^{-1}$), which represses R1–GFP expression. At the indicated times, cells were treated with CHX or CHX+TNF for 8 h. (a) The level of recombinant protein expression in total extracts of CHX-treated cells was evaluated by immunoblotting with an anti-GFP polyclonal antibody. (b) The percentage of TNF-specific apoptosis (■, □) was determined under microscopic observation 7 h after treatment. The percentage of GFP-positive cells (●, ○) was determined by microscopic observation of fluorescence in CHX-treated cells. Closed symbols, ON state; open symbols, OFF state. Values represent the mean±SD of a representative experiment, which was repeated three times.
in the control of apoptosis could differ regarding the proapoptotic stimuli or the cell types used. However, we have been unable to test this hypothesis directly as our attempts to demonstrate protection by HSV-2 R1 from staurosporine or proapoptotic stimuli other than TNF or FasL and R1(D2–357) were unsuccessful (Langelier et al., 2002). It is noteworthy that Perkins et al. (2002a, 2003) have ascribed the HSV-2 R1 antiapoptotic potential to a putative intrinsic protein kinase activity associated with its NH2 domain, in spite of the fact that extensive biochemical studies have demonstrated that both HSV R1 proteins are devoid of intrinsic protein kinase activity (Conner, 1999; Langelier et al., 1998). The role of the first 312 aa remains to be determined. However, as these residues are removed by proteolysis in vivo (Ingemarson & Lankinen, 1987) and as they are not highly conserved between HSV-1 and HSV-2 (Nikas et al., 1986), this segment could be of minor importance. Recent sequencing of three HSVs of Old World monkeys (Perelygina et al., 2003; Tyler & Severini, 2006; Tyler et al., 2005) revealed that the R1 of these viruses possessed a shorter NH2 domain than that of human HSVs, as they lack the poorly conserved segment of aa ~ 140–310 of human HSVs. Interestingly, strong homology of the R1 protein of these cercopithecine herpesviruses with R1 of the human viruses is observed from aa 319 of HSV-2 R1. Moreover, it is from this position, near the beginning of the

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**Fig. 4.** Proapoptotic and antiapoptotic activities of N-terminally truncated HSV-2 R1. Cells were transfected with the plasmid pAdCMV5 (Mock), pAdCMV5-R1 (R1), pAdCMV5-R1(D2–249) (249), pAdCMV5-R1(D2–312) (312) or pAdCMV5-R1(D2–357) (357), or mock-infected (Mock), or infected with the Ad recombinants AdTR5-R1 (R1), AdTR5-R1(D2–357) (357), AdTR5-R1(D2–398) (398) or AdTR5-R1(D2–496) (496) as described in Methods. (a) The level of recombinant protein expression was evaluated 30 h after transfection or 8 h after infection by immunoblotting with the anti-R1 polyclonal antibody 168R1. Quantification of the amount of R1 revealed a 5-fold higher level in transfected cells than in infected cells (not shown). (b) Proapoptotic activity was evaluated 30 h after transfection or 8 h after infection by scoring the percentage of apoptosis under microscopic observation in ten randomly chosen fields (shaded bars). Cells were then harvested for caspase-3 activity measurement (filled bars; pmol min⁻¹ mg⁻¹). (c) The percentages of TNF-specific apoptosis (shaded bars) and TNF-specific caspase-3 activity (filled bars) were evaluated 30 h after transfection or 7 h after infection as described in Methods. In (b) and (c), values represent the mean ± SD of two experiments performed in duplicate. (d) Phase-contrast photomicrographs of cells infected or not for 12 h and subsequently treated for 12 h with CHX or CHX+TNF.
putative α-crystallin domain, that the two human HSV R1 proteins begin to be highly similar.

Our second main observation was that overexpression of HSV-2 R2 did not decrease HSV-2 R1 protection from TNF-induced apoptosis. As our previous experiments have shown that saturation is attained with a 5-fold excess of R2 over R1 (Lamarche et al., 1994), this result strongly suggested that the HSV-2 R1 antiapoptotic property does not involve its R2-binding surface (~30% of the total surface of R1). The fact that the R2-binding surface may not be involved in protection from TNF indicates not only that other faces of R1 are implicated in this activity but also that co-expression of both RR subunits during the HSV lytic cycle should not affect R1 antiapoptotic activity. Therefore, as R1 is expressed during a long period of the lytic cycle, its antiapoptotic activity may play an important role not only during the short period where R1 is expressed alone at the beginning of the infection but also throughout the lytic cycle.

Results obtained with our other deletion mutants showed that the reductase domain is involved in the antiapoptotic function, but it has been difficult to define precisely the boundaries of the antiapoptotic domain. With the exception of R1(D2–312), all of the other deletions on the N-terminal...
side, which removed the putative α-crystallin domain either completely or in part, drastically altered protein solubility. Four of these, R1(Δ2–398), R1(Δ2–496), R1(Δ107–446) and R1(Δ378–446), were completely insoluble products without proapoptotic or antiapoptotic activity. The slightly soluble R1(Δ2–357) was proapoptotic for a high percentage of cells, but it could also be considered to be antiapoptotic, as cells resistant to its toxicity were resistant to TNF. From the fact that all of these truncated proteins were lacking the putative α-crystallin domain either totally or in part, it can be hypothesized that this domain is important for protein folding and/or oligomerization. Such a role has been demonstrated for the α-crystallin domain of αHsps (reviewed by MacRae, 2000; Studer et al., 2002) and suggested for the homologous domain present in p23, a co-chaperone of Hsp70 (Garcia-Ranea et al., 2002). The wild-type RR activity of the small fraction of R1(Δ2–357) recovered in soluble form in HD buffer, which does not contain detergent, suggested that this protein could fold properly, whereas the complete insolubility of R1(Δ2–398), R1(Δ2–496), R1(Δ107–446) and R1(Δ378–445) indicated that these other truncated proteins could be misfolded. As adding 1 M NaCl to the HD buffer used for protein solubilization increased the solubility of R1(Δ2–357) to ~50%, whereas it did not alter the insolubility of R1(Δ2–398) and R1(Δ2–496) proteins, it can be further hypothesized that the slightly soluble R1(Δ2–357) forms aggregates of aberrant oligomers, whereas the completely insoluble truncated proteins form aggregates of misfolded molecules (S. Chabaud & Y. Langelier, unpublished observations). The difference in folding of these proteins is the most likely explanation for the differences in their anti/proapoptotic potential. The mechanism underlying the paradoxical anti/proapoptotic properties of R1(Δ2–357) is currently unknown. One could imagine that, as has been described for TNF (reviewed by Gupta, 2002), it could induce both a death process and a survival response. Depending on the balance between these two signals, cells will engage either in apoptosis or in survival. The anti/proapoptotic properties of R1(Δ2–357) and the fact that the putative α-crystallin domain is absent in cellular R1, which does not protect against TNF, suggest that part of this domain could be important for antiapoptotic activity.

Results obtained with C-terminal deletions demonstrated that the R1 flexible COOH tail is not necessary for its antiapoptotic function. Thus, it can be concluded that the C-terminal cysteine pair (Cys1140, Cys1142) involved in the electron transfer with thioredoxin is not implicated in this activity. The inactivity of R1(1–1113) was most probably due to its very poor solubility. Based on the crystallographic structure of Escherichia coli R1 and a three-dimensional model of the HSV-1 R1 domain (kindly provided by H. Eklund, Swedish University of Agricultural Sciences, Sweden) (Eklund et al., 2001; Uhlin & Eklund, 1994) and referring to the numbering of the structural elements of E. coli R1, the R1(1–1113) ends after the helix zl localized on the exterior of the molecule, whereas the active R1(1–1123) with its additional 10 aa includes a small loop and βJ. As most of these 10 aa are localized inside the molecule in a deep pocket on the R2-binding face, they are not likely to be involved directly in antiapoptotic activity. Most importantly, as several residues in βJ should interact on one side with αl and on the other side with residues in αD, βE and notably with the catalytic Cys439, it is likely that the most important role of βJ in the antiapoptotic function is through stabilization of the R1 structure. Our longer C-terminal deletions suggested that a region between aa 1081 and 1123 could be involved in antiapoptotic activity. Hence, R1(1–1081) and shorter proteins were not altered in solubility but were all inactive in protection. However, it must be noted once again that these deletions could affect the overall structure of the protein; hence, site-specific mutagenesis would be required to ascertain the importance of this region.

Interestingly, a large part of the domain defined here as being essential for protection corresponds to the domain common to all of the eight known betaherpesviruses R1 proteins. Murine and human CMV R1 proteins have been shown to be implicated in the control of apoptosis (Brune et al., 2001; Patrone et al., 2003). A multiple sequence alignment of the two HSV R1 proteins with the eight known betaherpesvirus R1 proteins, which are RR inactive, revealed that the most conserved part (globally >30% similarity) of the protein is located towards the C terminus between HSV-2 R1 aa 834 and 971. Modelling of the surface-exposed residues in this segment using the HSV-1 R1 model showed that most of the conserved residues are located on the face opposite the R2-binding face (T. Sulea & Y. Langelier, unpublished data). Given its conservation, the segment aa 834–971 could be important for antiapoptotic activity. Importantly, data indicating that human CMV R1 could contribute to protection from Fas-induced apoptosis have been obtained with a knock-out viral mutant and not in experiments where the human CMV R1 was expressed alone. Unfortunately, human CMV R1 when expressed by transfection in 293 cells (Patrone et al., 2003) or in HeLa cells (S. Chabaud & Y. Langelier, unpublished results) is insoluble. Further site-specific mutagenesis will be required to determine the putative role of these residues in apoptosis protection.

The addition of GFP at the C terminus did not alter the antiapoptotic function but completely eliminated RR activity. The most probable explanation for the effect on the latter activity is that GFP creates steric hindrance to R2 binding. Interestingly, the R1–GFP chimeric protein, with these attributes, could be an important tool for complementation studies aimed at determining the role of the antiapoptotic function. Moreover, owing to the properties of this chimera, we isolated the inducible cell line A549-tTA-HSV-R1–GFP. Upon switching on R1–GFP expression, these cells showed resistance to TNF-induced apoptosis as efficiently as cells transiently expressing R1–GFP either from transfection or Ad infection. As they were selected in the
absence of continuous R1–GFP expression, they represent a better model to study the mechanism of action of R1 than cells constitutively expressing the protein for several generations (Perkins et al., 2002a) that could have undergone several modifications through adaptation to R1 expression.

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