The R1 subunit of herpes simplex virus ribonucleotide reductase has chaperone-like activity similar to Hsp27

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Abstract HSV-2 R1, the R1 subunit of herpes simplex virus (HSV) ribonucleotide reductase, protects cells against apoptosis. Here, we report the presence in HSV-2 R1 of a stretch exhibiting similarity to the α-crystallin domain of the small heat shock proteins, a domain known to be important for oligomerization and cytoprotective activities of these proteins. Also, the HSV-2 R1 protein, which forms multimeric structures in the absence of nucleotide, displayed chaperone ability as good as Hsp27 in a thermal denaturation assay using citrate synthase. In contrast, mammalian R1, which does not contain an α-crystallin domain, has neither chaperone nor anti-apoptotic activity. Thus, we propose that the chaperone activity of HSV-2 R1 could play an important role in viral pathogenesis.

Key words: Herpes simplex virus; Ribonucleotide reductase R1; Anti-apoptotic function; α-Crystallin domain; Chaperone activity; Hsp27

1. Introduction

The herpes simplex virus type 1 and 2 (HSV-1, -2) ribonucleotide reductase (RR), which converts ribonucleoside diphosphates to the corresponding deoxyribonucleotides, plays a key role in the synthesis of viral DNA in resting cells (reviewed in [1]). The association of two subunits, denoted R1 and R2, the former of which contains the active site, forms the holoenzyme. Based on studies of *Escherichia coli* RR, the HSV holoenzyme is typically depicted as an $R1_2R2_2$ heterotetramer. The HSV RRs differ from their mammalian homologues by being produced in excess over their requirement for viral DNA synthesis (N. Lamarche and Y. Langelier, unpublished results) and by being unresponsive to allosteric nucleotide effectors [1]. Moreover, whereas mammalian R1 is monomeric in the absence of added ligands [2–4], HSV R1 like *E. coli* R1 [5] is believed to be dimeric. For *E. coli* R1, two α-helices located in the first third of the protein provide essential contacts for dimerization [6].

Two observations suggest that the HSV R1s could be proteins with more than one function: (i) they possess a unique NH_2 domain of ∼400 amino acids that is dispensable for ribonucleotide reduction [7], and (ii) their synthesis begins before that of their R2 partners [8]. The long-standing view that their unique NH_2 domain could possess an intrinsic protein kinase activity [9–12] was ruled out by the demonstration that both HSV-1 R1 and HSV-2 R1 do not possess such an activity [13,14]. We previously found that the HSV-2 R1 protects the cells from death receptor-induced apoptosis, suggesting that it could contribute to viral propagation by preventing apoptosis induced by the immune system. The R1 NH_2 domain expressed on its own does not exhibit anti-apoptotic activity, suggesting that both domains of R1 or part(s) of them are necessary for this new function [15].

The reports of homology between HSV-2 R1 and the small heat shock protein (sHsp) HspB8 [16,17] led us to observe in HSV R1 a stretch exhibiting similarity to the α-crystallin domain of sHsps. In these proteins, the C-terminal α-crystallin domain is flanked by a short flexible tail and by a variable N-terminal domain. The 3D structures of Hsp16.5 from *Methanococcus jannaschii* and of Hsp16.9 from wheat show that, despite a low level of identity (∼20%), the α-crystallin domains of these evolutionarily distantly related proteins adopt a similar immunoglobulin G-like fold [18,19]. A group of 25 proteins of the *Arabidopsis* genome were reported to contain one or more α-crystallin domains at variable positions in their polypeptide chains [20]. The α-crystallin domain acts as a flexible structural building block for the formation by sHsps of high molecular mass structures. sHsps exhibit the in vitro ability to bind unfolded proteins and to prevent their aggregation in an ATP-independent manner (reviewed in [21]). Recent genetic data demonstrated a correlation between sHsp function in vivo and chaperone activity in vitro [22]. Also, the chaperone as well as other more specific activities of several sHsps are likely responsible for their cytoprotective and anti-apoptotic activity against several cytotoxic treatments including activation of the death receptors [23,24].

It was recently found that the α-crystallin domain and the cochaperone of Hsp90, p23, which has the same 3D folding as the α-crystallin domain, show a pattern of conserved residues suggesting a common evolutionary origin for both domains.

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p23-like domains were detected in several families of proteins fused with other domains in several multidomain proteins [25].

Here, we show that the anti-apoptotic HSV-2 R1 with its α-crystallin domain forms a hexameric structure in the absence of nucleotide and has a chaperone activity as potent as Hsp27. Devoid of such a domain, the mammalian R1 does not have chaperone activity and does not protect cells from apoptosis induced by death receptor activation.

2. Materials and methods

2.1. Materials

The conditions for the culture of human HeLa and A549-tTA cells were as reported [26]. The plasmids, pAdCMV5-HSV-2 R1 for the expression of the HSV-2 R1 and pAdCMV5-GFP for the expression of a mutated green fluorescent protein, have been described [26,27]. Plasmid pAdCMV5-HuR1 for the expression of the human R1 (HuR1) was constructed by inserting in pAdCMV5 the HuR1 coding sequence taken from plasmid pJVETLZ-HuR1. Recombinant HSV-2 R1 was purified by peptide affinity as described [13]. Recombinant mouse R1 (mR1), purified by peptide affinity [28], was kindly provided by Barry Cooperman. Recombinant Chinese hamster Hsp27 and Δ5–23Hsp27, a chaperone inactive mutant of Hsp27 (H. Lambert and J. Landry, unpublished results), were obtained from glutathione *S*-transferase–Hsp27 fusion proteins. The fusion proteins were affinity purified on glutathione-Sepharose beads, treated with thrombin to release the Hsp27 proteins and further purified to homogeneity by anion exchange chromatography [29].

2.2. Gel filtration

For the experiment with 0.7 μM HSV-2 R1, the purified protein was slightly labeled with [γ-^{32}P]ATP taking advantage of traces of contaminating protein kinase(s) as described [13]. Samples (100 μl) were loaded on a Superdex 200 hr10/10 column with 50 mM HEPES (pH 7.9), 2 mM dithiothreitol containing either 150 mM NaCl (standard buffer) or 1 M NaCl at a flow rate of 0.5 ml/min. The radioactivity was measured by Cerenkov counting. Blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase, (158 kDa) and bovine serum albumin (65 kDa) were used as molecular mass standards.

2.3. Chaperone activity

The kinetics of thermal denaturation of citrate synthase (CS) were measured using a Varian spectrophotometer (model Cary 1 Bio) equipped with a temperature-controlled cell holder. CS was diluted to 75 nM (dimer) in 50 mM HEPES (pH 7.5) in the absence or presence of Hsp27, HSV-2 R1 or mR1. Light scattering was measured at 320 nm at 43°C.

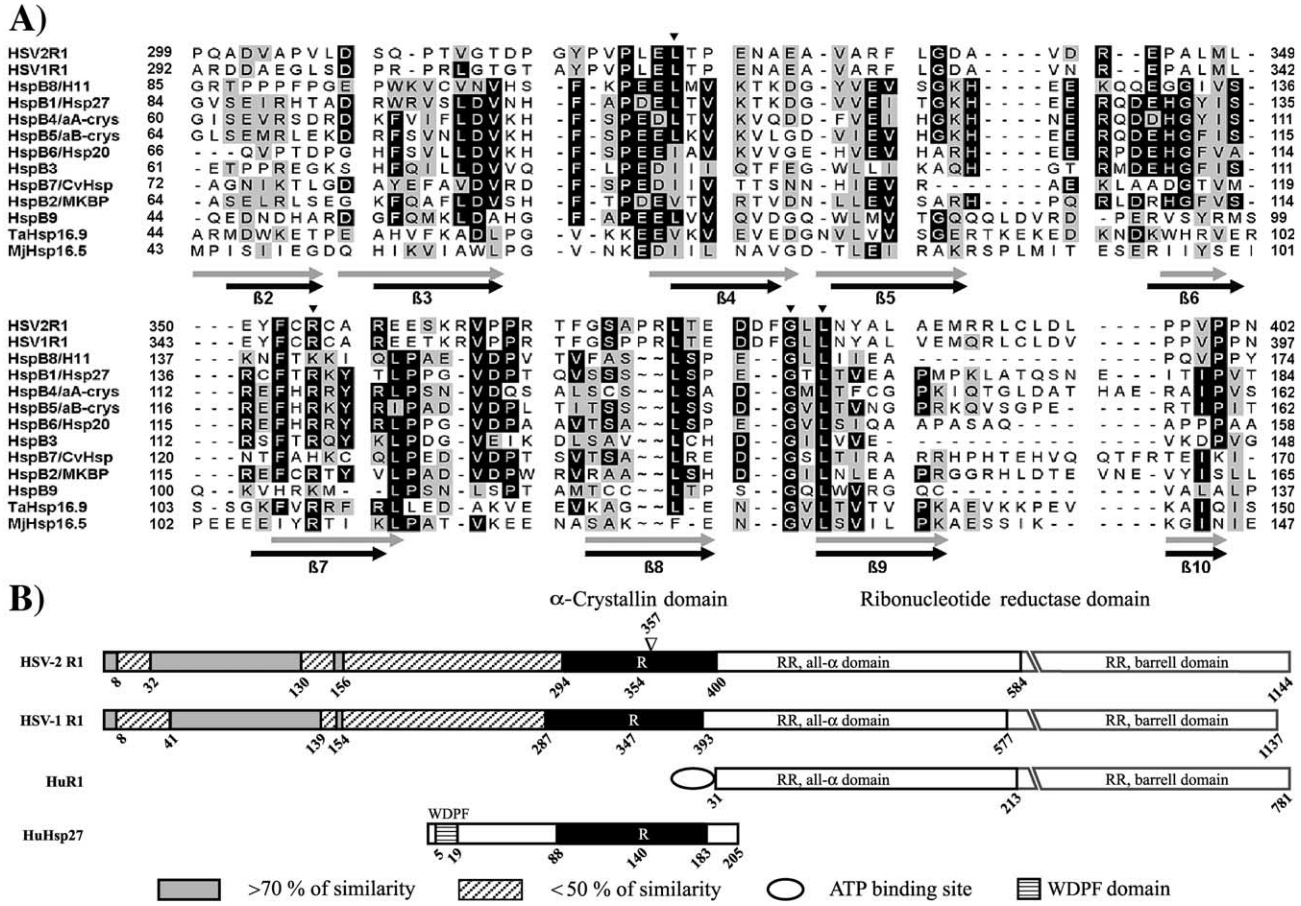

Fig. 1. HSV R1s exhibit similarity to the α-crystallin domain of sHsps. A: Alignment of the α-crystallin domains of the two HSV R1s, nine of the known human sHsps, wheat Hsp16.9 and *M. jannaschii* Hsp16.5. Residues conserved in at least six of the 13 sequences are in black. The bottom lines show the β-strands present in the secondary structures of TaHsp16.9 and MjHsp16.5, respectively [18,19]. The human sHsps are here designated 'HspB 1–9' as proposed by Kappé et al. [17] and by their corresponding old nomenclature. B: Schematic representation of the domains present in HSV R1s, HuR1 and Hsp27. For HSV R1s, regions exhibiting >70% similarity between the two HSV types are depicted by dark gray boxes and <50% by hatched boxes. The reductase domain contains two sub-domains termed all alpha domain (Pfam, ribonuc_red_lg) and barrel domain (Pfam, ribonuc_red_lgC). The α-crystallin domain shown in black is absent in HuR1 where the amino-terminal part forms a nucleotide binding site. The WDPF domain in Hsp27 is responsible for intermolecular Hsp27 interactions [29] and essential for chaperone activity (H. Lambert and J. Landry, unpublished results).

2.4. Induction and analysis of apoptosis

HeLa cells were seeded in 6-well plates (1.5×10^5 cells/well) 24 h before transfection with increasing amounts of plasmids using the calcium phosphate technique. After 36 h, the cells received new medium containing either 15 μg/ml cycloheximide (CHX) or 15 μg/ml CHX plus 2.5 ng/ml tumor necrosis factor (TNF) or no additives for the control cultures. After 7 h, the percentage of apoptotic cells was determined under microscopic observation in 10 randomly selected fields as recently detailed [15]. Briefly, percent apoptosis was calculated by dividing the number of cells with apoptotic morphology (exhibiting membrane blebbing and/or cell body condensation) by the total number of cells. In control experiments, Hoechst staining had indicated that cells scored as apoptotic by morphology also exhibited nuclear condensation and fragmentation. Caspase 3 and caspase 8 activities were evaluated using the ApoAlert™ (Clontech) fluorescent assay kits as described [15].

2.5. Protein extraction and immunoblot analyses

Protein extracts were prepared as described [13]. For HSV R1 and HuR1 detection, 168R1, a rabbit polyclonal anti-HSV-2 R1 antiserum, and a monoclonal antibody specific for HuR1 kindly provided by Mike Cordingley were used, respectively. Quantification of the percent recombinant protein in total protein extracts was done by immunoblotting using as standards purified HSV-2 R1 or extracts of 293 cells overexpressing HuR1.

3. Results

3.1. HSV R1 possesses a domain with similarity with the α-crystallin domain of sHsps

Recently, a protein, first described as having homology with the amino-terminal domain of the HSV-2 R1 [16], was later demonstrated to be a member of the sHsp family and named HspB8 [17]. These observations prompted us to scrutinize R1 for the presence of an α-crystallin domain. A Clustal W alignment of the α-crystallin domains of nine human sHsps was used to generate a profile that was compared with the HSV-1 and HSV-2 R1 sequences. The best similarity detected spans from amino acids 300 to 400. The alignment presented in Fig. 1A, which results from improvement by manual editing, shows that many consensus residues are conserved in the R1 sequences. Noteworthy are four residues (indicated by triangles): L325, R354, G380 and L382 in HSV-2 R1. The Leu residue corresponding to L325, when changed by even a relatively conservative Ala mutation in *Synechocystis* Hsp16.6, caused severe loss of Hsp16.6 function in vivo [22]. The R354 residue corresponds to a mutation site in two human genetic disorders: the R116C mutation in αA-crystallin and the R120G mutation in αB-crystallin respectively cause autosomal dominant cataract [30] and desmin-related myopathy [31]. The G380 and L382 residues present in the HSV-2 R1 hydrophobic motif, GLLNY, are the most conserved amino acids among all known sHsps [20,32]. However, some other consensus residues are not conserved in the R1 sequences explaining why the R1 α-crystallin domain is not detected by standard profiles such as the 'heat shock hsp20 protein family profile' of Prosite. These replacements could result from constraints imposed by the presence on one side of the unique NH_2 domain exhibiting a low level of similarity between the two HSV R1s ($<50\%$) and on the other side of the large highly homologous RR domain (Fig. 1B).

3.2. HSV-2 R1 forms high molecular weight complexes

The α-crystallin domain in HSV-2 R1 suggested a chaperone function. As the chaperone activity of sHsps is often related to their capacity to oligomerize [18,22], we first determined the molecular mass of HSV-2 R1 by gel filtration on a Superdex 200 column in physiological salt conditions without adding any nucleotide. For an R1 concentration of 0.7 μM, we observed with either unlabeled R1 detected by optical density (data not shown) or ^{32}P-labeled R1 (Fig. 2A) a major broad peak corresponding to molecular masses $\geq$750 kDa, the value expected for an R1 hexamer, and a minor one at ~250 kDa, the value expected for an R1 dimer. At a five-fold higher R1 concentration, the peak corresponding to the dimer disappeared, indicating that the formation of multimers is dependent on the concentration of the protein (Fig. 2B). Adding 1 M NaCl to the protein and the eluent produced a drastic shift of the peak to a position slightly higher than for the dimer (Fig. 2B), suggesting that oligomerization involved complementary ionic charges.

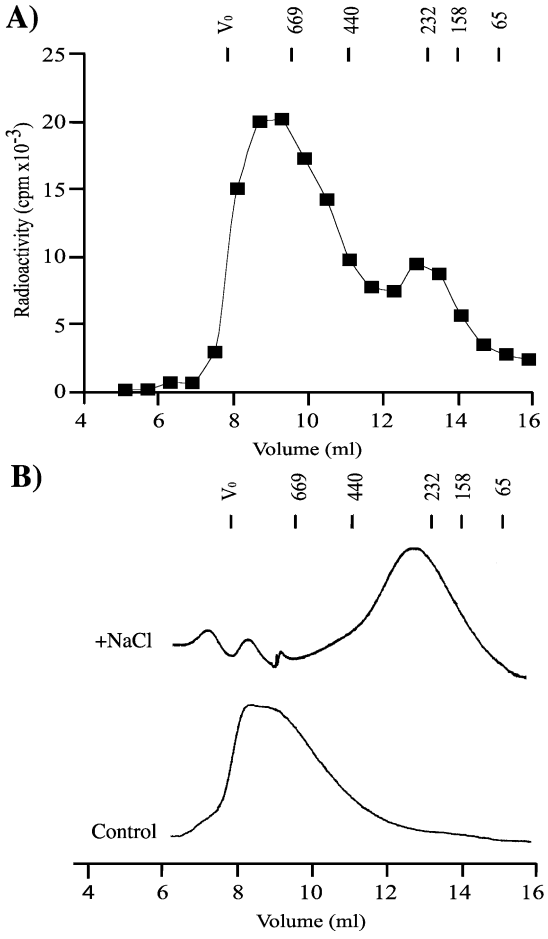

Fig. 2. Gel filtration chromatography shows that HSV-2 R1 forms hexameric structure. A: Purified ^{32}P-labeled HSV-2 R1 (0.7 μM) was loaded on a Superdex 200 column and radioactivity in each fraction was measured. B: HSV-2 R1 (3.5 μM) was loaded in standard buffer (Control) or in the presence of 1 M NaCl (NaCl) and the absorbance at 280 nm was recorded. The elution volume of protein standards and blue dextran (V_0) are shown above.

3.3. HSV-2 R1 suppresses the thermal aggregation of CS

We next investigated the HSV-2 R1 chaperone activity by measuring the effect of adding it in increasing concentrations on the kinetics of denaturation of CS at 43°C. The α-crystallin domain containing protein Hsp27 and an inactive mutant of Hsp27, Δ5–23Hsp27, were used as positive and negative controls, respectively. At concentrations higher than 4 μg/

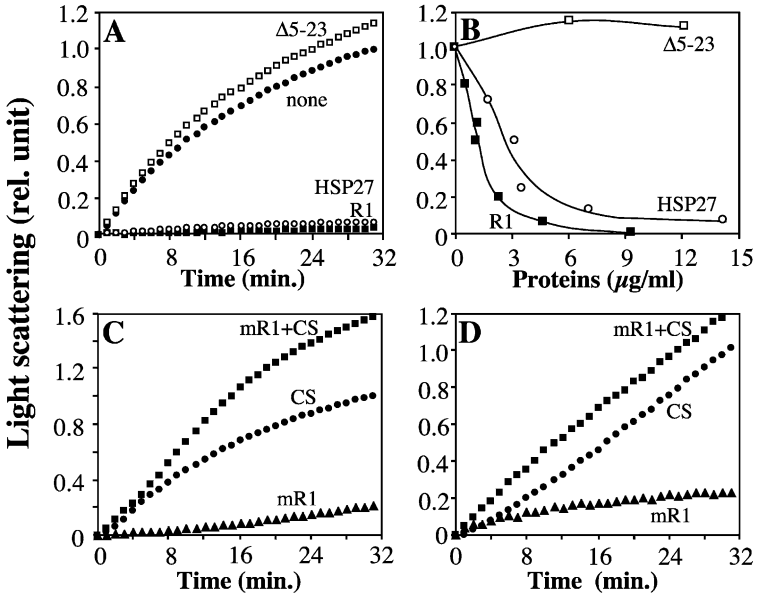

Fig. 3. HSV-2 R1 but not mammalian R1 suppresses the thermal aggregation of CS. A: Kinetics of denaturation of CS. Solutions containing CS alone (●), HSV-2 R1 (9.4 μg/ml)+CS (■), Hsp27 (14.2 μg/ml)+CS (○) or Δ5–23Hsp27 (12.4 μg/ml)+CS (□) were heated at 43°C for up to 30 min. Light scattering at 320 nm is indicated in relative arbitrary units. B: Dose-dependent protection of denaturation as shown by the relative light scattering at 30 min as a function of the concentration of the chaperones. Symbols are as in A. C: Kinetics of denaturation of CS. Solutions containing CS alone (●), mR1 (13.5 μg/ml)+CS (■) or mR1 alone (▲) were processed as described in A. D: The experiment described in C was repeated in the presence of 4 mM ATP plus 10 mM $MgCl_2$.

ml, HSV-2 R1 totally inhibited the denaturation and aggregation of CS at 43°C as measured from the light scattering of the CS solution (Fig. 3A) or the amount of CS pelletable at 17 000 × *g* after 30 min of heating (data not shown). The IC_{50} (the concentration required to inhibit 50% of CS denaturation) value was 1.1 μg/ml for HSV-2 R1 as compared to 3.2 μg/ml for Hsp27. Δ5–23Hsp27 had no effect at concentrations up to 25 μg/ml (Fig. 3B). Considering that at the low effective concentration of HSV-2 R1 it was present as a mixture of hexamer and dimer with masses between 750 kDa and 250 kDa whereas Hsp27 forms a 24-mer of 600 kDa, it can be estimated that R1 (IC_{50} = 1.5–4.5 nM) has a chaperone activity as good as if not better than Hsp27 (IC_{50} = 5.3 nM).

3.4. *Cellular R1 does not protect against apoptosis and does not possess chaperone activity*

Attempts to determine the functional importance of the HSV R1 α-crystallin domain in the HSV-2 R1 anti-apoptotic activity by deleting it completely or in part have been hampered by obtaining either pro-apoptotic proteins such as R1(Δ2–357) [13] or fully insoluble products such as R1(Δ2–398), R1(Δ2–496), R1(Δ378–445) and R1(Δ107–446) (unpublished data). Thus, to get a first insight into the importance of the α-crystallin domain in both anti-apoptotic and chaperone activities of HSV R1, we asked whether mammalian R1, an R1 without an α-crystallin domain, would exhibit such activities. To study the anti-apoptotic activity, HeLa cells were transfected with plasmids expressing either the HSV-2 R1 or HuR1 and the extent of apoptosis was measured by microscopic observation (Fig. 4A) and determination of caspase 3 and caspase 8 activities (Fig. 4B). Whereas the HSV-2 R1 efficiently protected cells against apoptosis induced by TNF+CHX as previously observed [15], HuR1 was not protective at all. Protein quantification performed using appropriate standards for the samples transfected with 50 μg/ml of plasmids showed that both R1 proteins were produced in roughly similar amounts (Fig. 4C).

The ability of purified mR1 to suppress the thermal aggregation of CS was studied in the conditions used to demonstrate the chaperone activity of HSV-2 R1 (Fig. 3C). These experiments showed that mR1 (13.5 μg/ml) not only did not impair the aggregation of CS but also that it was self-aggregating when heated at 43°C. Similar results were obtained in

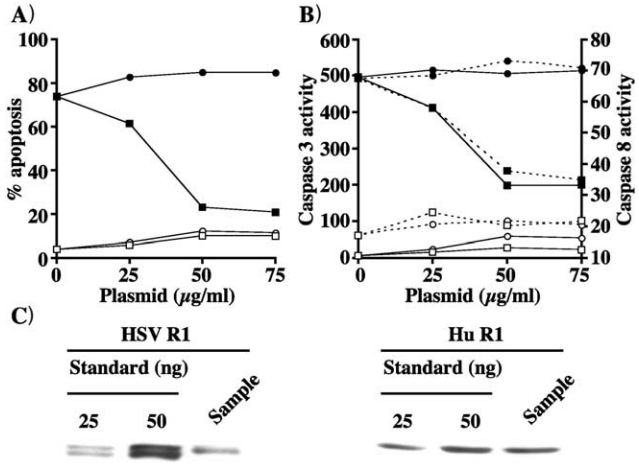

Fig. 4. HuR1 does not protect against apoptosis. HeLa cells were transfected with increasing amounts of the plasmids pAdCMV5-HSV-2R1 (■, □) or pAdCMV5-HuR1 (●, ○). After 36 h, CHX (□, ○) or CHX+TNF (■, ●) were added for 7 h. A: The percent apoptotic cells was evaluated under microscopic observation. Values represent the means of 10 randomly selected fields. B: Caspase 3 (solid lines) and caspase 8 (dashed lines) activities are expressed in pmol/min/mg. C: The amounts of HSV-2 R1 and HuR1 accumulated in cells transfected with 50 μg/ml of plasmid were evaluated with an immunoblot analysis by comparison with standards.

the presence of 4 mM ATP plus $MgCl_2$ (Fig. 3D). These R1 ligands are necessary for mammalian RR activity [33] and it has been shown that at these concentrations they cause R1 oligomerization [2,3]. These data showed that mammalian R1, an R1 without an α-crystallin domain, has neither chaperone nor anti-apoptotic activity.

4. Discussion

Here, we have shown that the HSV-2 R1, which contains a domain exhibiting similarity to the α-crystallin domain of sHsps, has a chaperone activity similar to Hsp27, a sHsp. To our knowledge, HSV R1 is the first protein produced by human cells identified as containing one α-crystallin domain followed by another functional domain, herein a RR domain. Only plant proteins have been reported to contain an α-crystallin domain at variable positions in their polypeptide chains. For none of these plant proteins, which are unrelated to sHsps, has a function been ascribed to this structural motif [20]. Interestingly, for some of the proteins containing p23-like domains such as B5+B5R flavo-hemo cytochrome NAD(P)H oxidoreductase, this motif is present as a hinge between the cytochrome and the reductase domains. From what is known about the function of the p23-like domain in the different protein families in which it is present, it was postulated that the p23 domain is involved in stabilizing folding structure and/or in mediating protein–protein interaction [25].

The position of the α-crystallin domain in HSV R1, where it replaces the allosteric regulatory site able to bind ATP or dATP present in non-viral R1 (Fig. 1B), suggests that it could be important for multimerization that occurs in the absence of nucleotide. mR1, which is devoid of an α-crystallin domain, sediments mainly as a monomer in the absence of nucleotide [2–4], and requires ATP concentrations above 1 mM to form hexamers [3]. By comparison with what is known for sHsps and p23 domain-containing proteins, we propose as a model for HSV R1 that the α-crystallin domain could contribute to the protein quaternary structure by providing additional point(s) of interaction between the two monomers, thus stabilizing the dimer. Additionally or alternatively the domain could provide point(s) of contact essential for multimerization. Our observation that the HSV-2 R1 forms a multimeric structure at relatively low concentration in the absence of any added nucleotide is in favor of this model.

The chaperone mechanism of sHsp appears to involve temperature-regulated exposure of hydrophobic binding sites present mainly in their α-crystallin domains [18,19]. As we have shown that the HSV R1 but not mR1 exhibited chaperone activity, it can be suggested that the presence of the α-crystallin domain is important for the chaperone activity. In addition, α-crystallin domains are thought to play an important role in preventing sHsp self-aggregation during heat stress [18]. Hence, we have observed that HSV R1 did not aggregate during heating at 43°C whereas mR1 readily formed insoluble precipitate. Also noteworthy is the observation that the RR activity of HSV R1 is far more resistant to heat inactivation than that of mammalian R1, a 20 min heat treatment at 50°C inactivating only 10% of the former and more than 99% of the latter [34]. Direct evidence for the importance of the α-crystallin domain in the newly discovered HSV R1 activities has so far proven difficult to obtain because partial or total deletion of the domain alters the solubility of the protein. More specific mutations such as substitution of conserved residues R354 and L382 could be helpful as similar substitutions in sHsp have been shown to reduce their chaperone activity [35,36]. The deleterious effect of deleting the α-crystallin domain on R1 protein solubility suggests that this domain could also play an important role in the polypeptide folding.

Since viruses are obligate intracellular parasites, the folding of viral proteins is generally accommodated by host chaperones that are often induced upon viral infection. An increasing number of viruses have been described to encode chaperones of diverse families including homologues of Hsp70, DnaJ and GroES [37]. However, HSV is the first virus described to encode a protein having a structural domain and chaperone activity similar to sHsp. The importance of this new function of HSV R1 in viral pathogenesis remains to be studied. One possibility is the protection of viral polypeptides during HSV reactivation triggered by fever. Thus, by assisting cellular chaperones in protecting viral proteins during heat stress, HSV R1 could contribute to the efficiency of virus production. In favor of that are observations made with the HSV-1 R1 null mutant ICP6Δ. When tested in exponentially growing Vero cells where the cellular RR complemented the defect in viral reductase, this mutant grew as well as the wild type at 33°C. In contrast, at 39.5°C the growth of the mutant was severely compromised whereas that of the wild type was only slightly affected [38]. Another possibility is that even at 37°C HSV R1, which accumulates at a high level (2% of total proteins), could be necessary to prevent protein aggregation during the high rate of viral protein synthesis.

In summary, the anti-apoptotic HSV-2 R1, which possesses a domain distantly related to the α-crystallin domain of sHsps, has also a chaperone activity similar to that of sHsps. In contrast, without an α-crystallin domain mammalian R1 does not have chaperone activity. As we have also observed that HuR1 could not impair apoptosis induced by death receptor activation, it is tempting to speculate that the α-crystallin domain is important for the HSV R1 anti-apoptotic activity. It could act either directly via its role for chaperone activity or oligomer formation or indirectly by being necessary for an efficient folding of the protein. Formation of large oligomers involving the α-crystallin domain has been shown to be important for the anti-apoptotic activity of some sHsps [39,40].

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