Leucine repeats in the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR; ICP10) are involved in RR activity and subunit complex formation

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Computer-assisted comparison of the herpes simplex virus type 2 (HSV-2) ribonucleotide reductase large subunit (RR1) sequence with the known primary structures of other RR1 proteins revealed a motif consisting of five leucines occurring at every seventh residue between positions 409 to 437. This motif is specific to HSV RR1 proteins. A synthetic oligopeptide (LA-4) corresponding to 15 residues in the internal portion of the motif inhibited HSV-2 RR activity. In immunoprecipitation experiments, LA-4 disrupted a complex consisting of RR1, the small RR subunit and a previously uncharacterized 180K protein, apparently of cellular origin. We deduce that the LA-4 sequence represents a critical RR1 site involved in RR complex formation and enzymic activity.

Herpesviruses, including herpes simplex virus type 1 (HSV-1), HSV-2, Epstein–Barr virus, varicella-zoster virus, pseudorabies virus and equine herpesviruses 1 and 3, induce a novel ribonucleotide reductase (RR) which is distinct from the enzyme found in uninfected cells (Averett et al., 1983; Dutia, 1983; Lankinen et al., 1982; Henry et al., 1978; Cohen et al., 1977; Davison & Scott, 1986); the HSV RR is required for virus growth in non-dividing cells (Cameron et al., 1988; Goldstein & Weller, 1988; Preston et al., 1984, 1988; Jacobson et al., 1989). The holoenzyme consists of two subunits (Cohen et al., 1985; Frame et al., 1985; Bacchetti et al., 1986); the large subunit (RR1) is a 140K protein, designated ICP6 for HSV-1 and ICP10 for HSV-2, whereas the small subunit (RR2) is a 38K protein encoded by a 1.2 kb mRNA overlapping the 3' end of the 5'-0 kb mRNA that encodes RR1 (McLauchlan & Clements, 1982; Swain & Galloway, 1986). A nonapeptide (YAGAVVNDL) representing the C terminus of HSV RR2 inhibits enzymic activity in vitro by inducing subunit dissociation (Cohen et al., 1986; Dutia et al., 1986; Paradis et al., 1988; McClements et al., 1988). However, sequences within RR1 that are required for complex formation and their role, if any, in enzymic activity have not been identified. In this study we describe a peptide (LA-4), identical in sequence to residues 419 to 432 of HSV-2 RR1, that inhibits RR activity and causes dissociation of a complex consisting of the two RR subunits and a 180K protein.

The construction of expression vectors pJW17 (ICP10), pJW32 (ICP10-PK) and the frameshift mutant (pJW21) has been described (Chung et al., 1989). The ICP10 C-terminal deletion mutant, pJW33, was constructed from pJW17 after BstEII/XbaI digestion and religation; it lacks 145 C-terminal codons and is expected to express a 123K protein. The 38K expression vector, pJW30, was constructed by replacement of the ICP10 coding region in pJW17 with the coding region of HSV-2 strain 333 RR2 (1-1 kb HaeIII fragment) from the parent construct, pGR101 (obtained from G. Hayward, Johns Hopkins University, Baltimore, Md., U.S.A.). Following the addition of XbaI linkers (New England Biolabs) and subcloning into the multipurpose vector pUC18, the fragment was inserted downstream of the simian cytomegalovirus IE94 promoter, replacing the ICP10 coding region in pJW17. To construct pJHL17, the HpaI/HindIII fragment of pJW17, which contains the coding region for the ICP10 protein kinase (PK) domain, was mutated to introduce a BamHI cleavage site at positions 737 and 738 and digested and religated with
AatII/BamHI to delete a 216 bp fragment encompassing amino acid residues 106 to 178. pJHL17 lacks 72 codons and is expected to express a 136K protein.

Monoclonal antibodies (MAbs) specific for ICP10 (MAb 30) or the HSV-2 major DNA-binding protein ICSP11/12 (MAb 27), peptides LA-1, LA-3, LA-2 and LA-5, which correspond to ICP10 residues 13 to 26, 165 to 179, 355 to 369 and 918 to 927 respectively, and polyclonal anti-LA-1 antibody have been described previously (Chung et al., 1989). Peptides LA4, LA-4A, LA-4B and LA-6, corresponding to ICP10 residues 419 to 432, 413 to 425, 426 to 438 and 998 to 1007, respectively, were synthesized by the Merrifield solid-phase method (Stewart & Young, 1969). MAb 99, specific for RR2, and MAb H3, specific for ICP10 (Fig. 1a, lane 1), were prepared from tertiary clones of hybridomas derived from the same fusion used to obtain MAbs 30 and 27. MAb 30 precipitates ICP10 from cells transfected with pJW17 (Fig. 1a, lane 3) but not from cells transfected with pJHL17 (Fig. 1a, lane 4), suggesting that it recognizes a determinant between amino acid residues 106 and 178 (PK domain). MAb H3 precipitates a 136K protein from cells transfected with pJHL17 (Fig. 1a, lane 2); it does not react with pJW32-transfected cells (Fig. 1a, lane 5), suggesting that it recognizes a determinant in the ICP10 RR domain. MAb 18zA5 (specific for gG-2) was obtained from W. Rawls (McMaster University, Ontario, Canada).

The procedure of Averett et al. (1983) was used to assay RR activity. Human epithelial carcinoma no. 2 (HeP-2) cells were preincubated (24 h) in medium 199 with 0.5% newborn calf serum to reduce cellular RR activity (Langelier & Buttin, 1981) and infected (20 p.f.u./cell) with HSV-2 strain G. Extracts (2 x 10^7 cell equivalents/ml) obtained 8 h post-infection (p.i.) in 100 mM-HD buffer (HEPES buffer pH 7.6, 2 mM-DTT) were clarified by centrifugation (100000 g, 1 h, 4 °C) and the HSV RR was precipitated with crystalline ammonium sulphate (45% saturation, 0.258 g/ml). Activity is expressed in RR units (U), where 1 U converts 1 nmol [3H]CDP to dCDP per min.

Both RR subunits and a 180K protein, which does not comigrate with ICP4 or with the major capsid protein ICP5 (Fig. 1b, lane 1), were precipitated from HSV-2-infected HeP-2 cells labelled with [35S]methionine by MAbs 30, H3 and 99 between 0 and 8 h p.i. (Fig. 1b, lanes 2 and 3 for MAbs 30 and H3 respectively; Fig. 1c, lanes 2 and 3 for MAbs 30 and 99 respectively). The 180K protein was also coprecipitated with the two RR subunits by antibody to LA-1 (Fig. 1d, lane 2); however, the 180K protein was not seen in infected cells labelled between 4 and 8 h p.i. (Fig. 1c, lane 1) and it was not precipitated from these extracts by MAbs 30, H3 or 99 (Fig. 1c, lanes 4 to 6). The 180K protein was coprecipitated only when both RR subunits were expressed; it was not observed in MAb 30 precipitates of cells infected under conditions (cycloheximide 0 to 6 h p.i.; [35S]methionine 6 to 7 h p.i.) that support RR1 (Wymer et al., 1989) but not RR2 synthesis (Fig. 1d, lane 4). Its coprecipitation was specific to the RR subunits as it was not precipitated by MAb 27 (Fig. 1d, lane 8), nor by MAb 18zA5 (Fig. 1d, lane 10), which recognize ICSP11/12 and ICSP11/12.
and gG-2 respectively. We deduce that the 180K protein is synthesized only during the first few hours after infection and that it specifically coprecipitates with the two RR subunits. Precipitation of the RR subunits is not due to the fortuitous generation/unmasking (by RR subunit complex formation) of an epitope shared with the 180K protein because the latter is precipitated by antibodies that recognize distinct antigenic determinants (MAbs 30, H3 and 99 and anti-LA-1 serum). Sedimentation analysis was done as described by Ingemarson & Lankinen (1987) except that instead of immunoblotting, the fractions were precipitated with MAb 30 (Fig. 2). RR1, RR2 and the 180K protein cosedimented as described (Ingemarson & Lankinen, 1987).

To characterize the 180K protein further, extracts of 293 cells cotransfected with pJW30 and either pJW17, pJW32, pJW33 or pJW21, and labelled with \[^{35}\text{S}]\text{methionine (100 \mu C/ml) 30 to 40 h post-transfection, were precipitated with MAb 99. As controls, cells were independently transfected with each construct and extracts were precipitated with MAb 30 (pJW17, pJW32, pJW33 and pJW21) or MAb 99 (pJW30). The results are shown in Fig. 3. Consistent with previous findings (Chung et al., 1989), ICP10 was precipitated by MAb 30 from cells transfected with pJW17 (Fig. 3a, b, c, lanes 1) and a 57K protein was precipitated from cells transfected with pJW32 (c, lane 2). Proteins were not precipitated from cells transfected with the frameshift mutant pJW21 (a, lane 2). A 123K protein, consistent with the expected C-terminal truncated RR1, was precipitated by MAb 30 from cells transfected with pJW33 (a, lane 3) and RR2 was precipitated by MAb 99 from cells transfected with pJW30 (b, lane 3). RR1, RR2 and a 180K protein were coprecipitated by MAb 99 from cells cotransfected with pJW30 and pJW17 (b, lane 2). However, RR2 was the only protein precipitated by MAb 99 from cells cotransfected with pJW30 and pJW32, which expresses the 57K amino-terminal PK domain of RR1 (a, lane 4), pJW30 and pJW33, which expresses a 123K RR1 protein lacking the extreme C-terminal 145 codons (a, lane 5), or pJW30 and pJW21, an expression-negative frameshift mutant (data not shown). The 180K protein was not precipitated by MAb 99 from...
Primary structure and inhibitory potential of synthetic oligopeptides on HSV-2 RR activity

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary structure*</th>
<th>Inhibition (%)†</th>
</tr>
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<tbody>
<tr>
<td>LA-1</td>
<td>13/ARSPSERQEPREPE/26</td>
<td>8</td>
</tr>
<tr>
<td>LA-3</td>
<td>165/CARRDARGGAEKDV/179</td>
<td>0</td>
</tr>
<tr>
<td>LA-2</td>
<td>355/CAREESKVRPPRTFG/369</td>
<td>10</td>
</tr>
<tr>
<td>LA-4</td>
<td>419/GFKPLVRRSARLYR/432</td>
<td>62</td>
</tr>
<tr>
<td>LA-5</td>
<td>918/VRGARPFHSH/927</td>
<td>16</td>
</tr>
<tr>
<td>LA-6</td>
<td>998/SKVRTDGETL/1007</td>
<td>15</td>
</tr>
</tbody>
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* Numbers indicate corresponding ICP10 residue numbers.
† Inhibition of RR activity relative to a control without peptide.

Results are the mean of three experiments which varied by less than 10%. Specific activity of the virus enzyme preparation was 21 nmol/h/mg (96.0 lag).

pJW17-transfected cells (c, lane 3). These findings suggest that the 180K protein is coprecipitated only when both intact RR subunits are expressed, and that it is of cellular origin. The levels of protein expressed by pJW32 or pJW33 were somewhat lower than those generally seen with pJW17 or pJW30. However, we do not believe that coprecipitation of the 180K protein depends on the levels of protein expressed by the vectors because these varied in different experiments in which coprecipitation was observed.

A motif consisting of five leucines occurring at every seventh residue was identified by computer-assisted analysis between residues 409 to 437 of ICP10. To investigate the possibility that it may be involved in complex formation, we prepared a synthetic peptide (LA-4) that corresponds to residues 419 to 432 and studied its effect on HSV-2 RR activity. Synthetic peptides corresponding to other regions of the ICP10 protein (LA-1 to LA-6) were used as controls. As summarized in Table 1, for peptides used at a concentration of 0.5 mM only LA-4 caused significant reduction (62%) in RR activity. Minimal, if any, inhibition (0 to 16%) was observed using the other five peptides. Sequences overlapping the left (LA-4A; residues 413 to 425) and right (LA-4B; residues 426 to 438) halves of LA-4 respectively caused 58% and 31% of the inhibition observed with LA-4.

Fig. 4(a) depicts the effect of the dose of LA-4 on inhibition of RR activity. Significant reduction in enzymic activity (43%) was observed in the presence of 100 μM-LA-4 with half of the maximal inhibition occurring at 300 μM; residual RR activity was inhibited at high LA-4 concentrations (1:5 to 3:0 mM). Oligopeptides LA-1 and LA-5 had little, if any, effect on RR activity at all these concentrations. The inhibition of RR activity by LA-4 was paralleled by complex dissociation; addition of LA-4 caused a dose-dependent decrease in the precipitation of RR1 and a 180K protein but had little effect on RR2 precipitation with MAb 99. At the highest LA-4 concentration studied in this series (2:5 mM; Fig. 4b, lane 5), RR2 was the only precipitated protein.

Fig. 4(c) graphs the ratio of the amount of RR1 or 180K protein to that of RR2 immunoprecipitated by MAb 99 at the highest LA-4 concentration studied in this series (2:5 mM; Fig. 4b, lane 5), RR2 was the only precipitated protein.
Thus, the 180K protein was not precipitated from cells transfected with vectors which independently express RR1 respectively, or with pJW21, which expressed only the RR subunits. We believe that the 180K protein to RR2 do not imply stoichiometry in the enzyme complex because the number of methionine residues and, possibly, turnover rates may differ for the three proteins. LA-1, which did not inhibit RR activity, failed to cause subunit dissociation (Fig. 4c) and LA-4 did not decrease the precipitation of ICSP11/12 by MAb 27 (Fig. 4d).

Ingemarson & Lankinen (1987) showed that RR activity has a sedimentation coefficient of 17S (Mr, 370K). Immunoblotting of the gradient fractions with MAbs specific for either RR subunit did not identify proteins other than RR1, its cleavage products and RR2, leading them to conclude that the size of RR corresponds to the interaction of homodimers of RR1 and RR2. However, the stoichiometry in the enzyme complex was not determined and the detection of antigenically unrelated but potentially complexed proteins was precluded by the use of the immunoblotting assay. Indeed, independent experiments indicated that the two RR subunits are the only proteins necessary for RR activity. However, as the enzyme reconstituted in vitro had only 5% of the activity seen in HSV-infected cells, it was suggested that cellular proteins may be involved in enzyme stabilization (Huang et al., 1988). Our findings are consistent with this interpretation; like Ingemarson & Lankinen (1987) we estimate the Mr of the native RR to be 370K. However, unlike them, we used immunoprecipitation and found that a 180K protein cosedimented with the two RR subunits. We believe that the 180K protein is a cellular protein because (i) it was not detected/precipitated in cells labelled between 4 and 8 h.p.i. (after the onset of host shut-off) and (ii) it was precipitated from cells transfected with vectors that express only the RR subunits.

Coproprecipitation of the 180K protein required expression of both intact RR subunits, suggesting that it was not due to the fortuitous presence of shared epitopes. Thus, the 180K protein was not precipitated from cells transfected with vectors which independently express only one of the RR subunits (pJW30 or pJW17), nor from cells in which pJW30 (which expresses RR2) was cotransfected with pJW32 or pJW33, which express the RR1 amino-terminal domain or the C-terminal truncated RR1 respectively, or with pJW21, which is expression-negative. The 180K protein was coprecipitated with RR1 and RR2 from infected cells labelled from 0 h (but not from 4 h) p.i., and by antibodies that recognize different RR1 antigenic determinants (anti-LA-1 serum, MAb 30 and MAb H3) or RR2 determinants (MAb 99); it was not precipitated from cells that were infected under conditions (cycloheximide treatment) that allow expression of RR1 but not RR2, nor was it precipitated by MAbs raised against unrelated proteins. A potentially analogous protein was coprecipitated with the two RR subunits in independent enzyme reconstitution studies (Darling et al., 1988; Frame et al., 1985). We have not excluded the possibility that coprecipitation of the 180K protein is cell type-dependent and occurs only early in infection. Also, we are not sure that the same 180K protein is precipitated from infected and transfected cells. However, we found that the 180K protein is not essential for RR activity and conclude that it may be involved in enzyme stabilization. Indeed, low levels of RR activity were seen in gradient fractions which were free of the 180K protein and in the presence of 1.5 mM-LA-4 when 180K:RR2 was 0.

LA-4 inhibits RR activity and this correlates with the dissociation of the RR1/RR2/180K protein complex. Inhibition is specific in that (i) it was not observed with LA-1 or LA-6, (ii) oligopeptides LA-4A and LA-4B had lower inhibitory activity and (iii) LA-4 did not inhibit the precipitation of ICPS11/12. For heuristic reasons it is particularly interesting that the LA-4 region is located at the junction of the RR and PK domains of ICP10 (Chung et al., 1989), where it might have been generated through the insertion of novel PK sequences into the polypeptide coding region of a precursor HSV RR1 (Smith et al., 1991). While final conclusions on the role of the 180K protein in enzyme stabilization are premature, the generation of an RR-stabilizing domain in the region of PK and RR fusion may provide one interpretation of the conservation of the novel sequences.

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