The unique N terminus of the herpes simplex virus type 1 large subunit is not required for ribonucleotide reductase activity

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Using purified bacterially expressed herpes simplex virus type 1 ribonucleotide reductase large subunit (R1) and the proteolytic enzymes chymotrypsin and trypsin, we have generated stable N-terminal truncations. Chymotrypsin removes 246 amino acids from the amino terminus to produce a fragment (dN246R1) which retains full enzymic activity and affinity for the small subunit (R2). Treatment of R1 with trypsin produces a 120K protein and a cleavage at amino acid residue 305 to produce a fragment (dN305R1) which remains associated with a 33K N-terminal polypeptide. Although this 33K–dN305R1 complex retains full binding affinity for R2 its reductase activity is reduced by approximately 50%. Increasing the concentration of trypsin removes the 33K N-terminal polypeptide resulting in dN305R1 which, when bound to R2, has full ribonucleotide reductase activity. Like R1, dN246R1 and dN305R1 each exist as dimers showing that the first 305 amino acids of R1 are not necessary for dimer formation. These results indicate that, in structural studies of subunit interaction, dN246R1 or dN305R1 can be considered as suitable replacements for intact R1.

Introduction

DNA synthesis is dependent on the formation of deoxyribonucleotides from the four corresponding ribonucleoside diphosphates, a reaction which is catalysed by the enzyme ribonucleotide reductase (EC 1.17.4.1) (Thelander & Reichard, 1979; Reichard, 1988). Herpes simplex virus (HSV) encodes its own ribonucleotide reductase (RR) (Cohen, 1972; Cohen et al., 1974; Dutia, 1983; Preston et al., 1984; Frame et al., 1985; Bacchetti et al., 1986; Ingemarson & Lankinen, 1987), a feature which is common to several other herpesviruses including Epstein–Barr virus (EBV) (Henry et al., 1978; Baer et al., 1984; Gibson et al., 1984), varicella-zoster virus (VZV) (Davidson & Scott, 1986; Dutia et al., 1986; Spector et al., 1987), pseudorabies virus (Lankinen et al., 1982; Cohen et al., 1987) and equine herpesvirus (Cohen et al., 1977; Telford et al., 1990).

In HSV-1, the RR enzyme consists of a large subunit, R1, and a small subunit, R2, which have Mr values of 136K and 38K respectively (Frame et al., 1985; Bacchetti et al., 1986; Darling et al., 1988). The subunits exist as a tight complex in an (x2fl 2 structure similar to the RR enzymes of Escherichia coli and mammalian cells (Ingemarson & Lankinen, 1987).

Amino acid sequence analysis has revealed distinct homologies between HSV R1 and the R1 subunits from EBV, vaccinia virus, VZV, E. coli, T4 bacteriophage and mouse (Eriksson & Sjöberg, 1989) which relate to the functionally important regions of R1. However, in the case of HSV R1 the homologies are restricted to the C-terminal two-thirds of the molecule with HSV-1 and -2 R1 possessing, in addition, unique N-terminal domains of 311 and 318 amino acids respectively (Nikas et al., 1986). The unique N-terminal domain in HSV R1 accounts for the substantially greater Mr of this subunit (136K) when compared with the relatively uniform Mr values of R1 from other species (80K to 90K).

The precise relationship of the HSV R1 unique N-terminal domain to the activity of the enzyme is unknown but it has been proposed that this region is not directly involved in ribonucleotide reduction (Ingemarson & Lankinen, 1987; Lankinen et al., 1989; Wymer et al., 1989). Ingemarson & Lankinen (1987) demonstrated that HSV-1 R1 is degraded to polypeptides of Mr 110K, 93K and 81K in extracts of HSV-1-infected Vero cells, and indicated that the 93K protein retains reductase activity. Using antipeptide antibodies it was shown that this degradation was from the N terminus with retention of the C-terminal reductase domain, and that proteolytic cleavage also occurred during the course of HSV-1 infection in BHK cells (Lankinen et al., 1989).

Comparison of the amino acid sequences of HSV-1 and -2 R1 has shown that despite 80% identity in the C-
terminal reductase domain they have only 38% identity in the N-terminal domain (Nikas et al., 1986). Recently it has been demonstrated that the N-terminal domain of HSV-2 R1 can function as a protein kinase. Sequence analysis identified conserved protein kinase domains within the N terminus of HSV-2 R1 and both the intact protein as well as an expressed 57K N-terminal fragment were capable of autophosphorylation (Chung et al., 1989). Further studies suggested that the HSV-2 R1 N-terminal domain functions as a membrane-associated protein kinase (Chung et al., 1990). Although the N terminus of HSV-1 R1 contains some of the conserved protein kinase domains, HSV-1 R1 did not autophosphorylate (Chung et al., 1989).

HSV RR has been shown to be essential for virus growth in non-dividing cells in vitro at 37°C, in dividing cells grown at elevated temperatures (39°C) (Preston et al., 1984, 1988; Goldstein & Weller, 1988a, b; Jacobson et al., 1989) and for pathogenicity in mice (Cameron et al., 1988; Jacobson et al., 1989). A nonapeptide corresponding to the C terminus of R2 has been demonstrated to be an effective and specific inhibitor of HSV RR (Dutia et al., 1986; Cohen et al., 1986). This peptide, YAGAVVNDL, represents a site of interaction on R2 with R1 and inhibits enzyme activity by interfering with subunit association (McClements et al., 1988; Paradis et al., 1988; Darling et al., 1990). The nonapeptide can serve as a lead compound for an antiviral drug; development would be greatly assisted by structural studies of R1–R2 and R1–peptide interactions. To this end we have recently described the overexpression and purification of enzymically active R1 and R2 in E. coli (Furlong et al., 1991; Lankinen et al., 1991). However, full-length R1 is likely to be too large for some structural studies to characterize in detail the R1–R2 and R1–peptide interactions. We now describe the production of two stable truncated forms of R1, generated by cleavage within the unique N-terminal domain, and compare their enzyme activities, as well as their R2- and their peptide-binding with these properties of full-length R1.

**Methods**

**HSV R1 and R2.** HSV-1 R1 and R2 were overexpressed separately in E. coli BL21 DE3 cells as described previously (Furlong et al., 1991; Lankinen et al., 1991). R2 was purified as previously described (Lankinen et al., 1991). The source of R1 was either the 33% ammonium sulphate fraction or the heparin–Affi-Gel purified subunit (Furlong et al., 1991).

Proteolytic digestion and identification of cleavage sites. Initial experiments were performed using an R2 affinity matrix to extract R1 from ammonium sulphate-precipitated bacterial extracts. Purified R2 was coupled to CNBr-activated Sepharose at a concentration of 1 mg/ml. One hundred μl of matrix was mixed with 200 μl of R1 extract for 20 min in an Eppendorf tube, the supernatant was then removed and the matrix washed three times with 1 ml of 25 mM-HEPES buffer pH 7.6, with 2 mM-DTT, 0.5 mM-NaCl and 0.05% (w/v) NP40. After a final wash with 25 mM-HEPES buffer pH 7.6, containing 2 mM-DTT, the proteolytic enzymes trypsin or chymotrypsin were introduced to the matrix at a final concentration of 0.4 μg/ml in 200 μl of HEPES-DTT buffer. Samples were mixed continuously at room temperature for up to 1 h, the supernatant was then removed and the remaining matrix prepared for SDS-PAGE by the addition of 100 μl SDS-PAGE denaturing buffer (Laemmli, 1970). SDS-PAGE was performed on 5 to 12% gradient gels. Control R1 was bound to the R2 matrix and incubated at room temperature for up to 1 h in the absence of proteases.

For N-terminal sequencing, proteins bound to the R2 matrix after 1 h incubation with trypsin or chymotrypsin were separated by SDS-PAGE in 5 to 12% gradient gels using 0.1 M-Tris–0.1 M-Tricine pH 8.25 with 0.1% SDS as the cathode buffer and 0.2 M-Tris–HCl pH 8.9 as the anode buffer. Proteins were transferred by electroblotting to PVDF Problott membranes in a 10 mM-CAPS buffer pH 11.5 with 10% ethanol for 2 h at 250 mA and, after extensive washing in distilled water the PVDF membrane was stained for 1 min in 0.1% (w/v) Coomassie blue R250 in 50% (v/v) water/methanol. After destaining in 50% (v/v) water/methanol, bands were excised and sequenced on an Applied Biosystems 477A protein sequencer (at the Department of Geology, University of Glasgow, U.K.) for between five and 10 cycles.

**M. analysis.** Ms were estimated from SDS-PAGE using protein standards of known Ms including the R1 subunit from E. coli which was a gift from JoAnne Stubbe, Department of Chemistry, M.I.T., Cambridge, Mass., U.S.A. Estimation of Ms was also performed on an FPLC Superose 12 column calibrated with gel filtration standards (Sigma).

**Western blotting.** Western blotting was essentially as described by Towbin et al. (1987). Antiserum C3, raised against an R1 peptide corresponding to amino acid numbers 1117 to 1131 (Lankinen et al., 1989), was used at a dilution of 1:20. Fusion protein from the N-terminus of HSV-1 R1 corresponding to amino acids 2 to 179 (F1), 167 to 343 (F2) and 282 to 434 (F3), and the antisera raised against these fusion proteins will be described elsewhere. The R1 amino acids were fused to β-galactosidase (H. Lankinen, unpublished results). Prior to use, the antisera were absorbed with proteins from a 50% ammonium sulphate-precipitated fraction prepared from an extract of non-induced E. coli BL21 DE3 cells. Proteins of this extract were immobilized at a concentration of 5 mg/ml to CNBr-activated Sepharose and were mixed in a ratio of 1:1 (v/v) with the antisera for 2 h before use. Fusion protein antisera were used at a dilution of 1:40. Bound antibodies were detected with Protein A conjugated to horseradish peroxidase (Sigma) diluted 1000-fold in PBS and the chromogenic substrate 4-chloro-1-naphthol.

**Enzyme activity of proteolytic fragments.** R1 purified by heparin–Affi-Gel chromatography was treated with trypsin or chymotrypsin in solution at a protease concentration of 0.4 μg/ml. Before proteolysis, R1 at a protein concentration of 30 μg/ml was desalted using an FPLC fast desalt column into 25 mM-HEPES buffer pH 7.6 with 2 mM-DTT and 0.05% (v/v) NP40. To determine the optimum time for the complete conversion of intact R1 to the stable truncated fragments, R1 was incubated with the proteases at room temperature for up to 4 h. Aliquots were removed at various time intervals, frozen immediately on dry ice and then analysed by SDS-PAGE. RR activity was determined in the presence of 2 μg purified R2 according to the method of Darling et al. (1987). R1 incubated in the absence of proteases was used as a control and protein determinations following incubation were performed using the BCA reagent (Pierce). Trypsin, chymotrypsin and α2 macroglobulin were obtained from Boehringer Mannheim and soybean trypsin inhibitor, aprotinin, TLCK and TPCK were obtained from Sigma.
Synthesis of oligopeptides. Peptides of the sequences STSya-GAVVNDL, YAGAVVNDL and AVVNDL from the C terminus of R2 were synthesized by continuous flow F-moc chemistry (reviewed by Atherton et al., 1979; Sheppard, 1983) on an LKB Biolyx peptide synthesizer. Following synthesis, peptides were cleaved from the resin and side chain-protecting groups were removed by standard protocols. The Mr values of peptides were determined by mass spectrometry (M-Scan) and gave values identical to those expected. The purity of the peptides, as estimated by reverse phase HPLC, monitored at 225 nm, was greater than 70%.

Comparison of the R2-binding properties of intact R1 with trypsin and chymotrypsin-treated R1. Two different methods were developed to compare the R2-binding properties of the truncated R1 products with those of intact R1. An ELISA-based method was used for the chymotrypsin-treated R1. Ninety-six-well ELISA plates (Dynatech Immulon II) were coated with R2 at a concentration of 0.3 μg/well and left overnight at 4 °C. Unreacted sites were blocked with 0.5% (w/v) milk powder and 0.05% v/v NP40 of purified R1 and chymotrypsin-treated R1, both of initial protein concentration 140 μg/ml, were added to the wells (100 μl/well) and incubated for 2 h at room temperature. Plates were than washed six times with PBS containing 0.5% (v/v) milk powder and 0.05% v/v NP40. Fusion protein antisera F3, described in the Western blotting section, was used at a dilution of 1:1000 (100 μl/well). After 1 h the plates were washed, 100 μl of a 1:100 dilution of Protein A–horseradish peroxidase conjugate (Sigma) was added and incubated for a further 1 h. Colour development was achieved using 100 μl of substrate solution (ABTS at 0.5 mg/ml in 0.1 M-citrate-phosphate buffer pH 4.0, with 0.03% H2O2). The absorbance (at 405 nm) was determined after 30 min using a Multiskan (Titertek).

Results

HSV-1 R2 immobilized on CNBr-activated Sepharose was capable of binding the R1 present in the ammonium sulphate fraction of an extract from E. coli overexpressing HSV-1 R1. After washing, R1 was the major protein associated with the matrix (Fig. 1a, lane 3). Incubation of the R1 bound to the immobilized R2 with either trypsin or chymotrypsin for 1 h resulted in two major novel polypeptide products (lanes 4 and 5) of apparent Mr 87K and 96K respectively. Mr, standards (lanes 1 and 2) included subunit R1 from E. coli (lane 1, M, 86K) (Thelander & Reichard, 1979; Carlson et al., 1984). R2 from the matrix can also be seen in lanes 3 and 5. The minor 33K band (lane 4) in trypsin-treated R1 will be discussed later.

By using R1 bound to the R2 matrix and the two proteases, sufficient amounts of the 96K and 87K bands were produced to allow N-terminal sequencing. The 87K fragment generated by a 1 h incubation with trypsin had an N-terminal sequence of LGTGTAYPVP which corresponds to cleavage between arginine 305 and leucine 306. The 96K truncated R1 produced by chymotrypsin had an N-terminal sequence of SNDTA which corresponds to cleavage between tryptophan 246 and serine 247. The 87K trypsic and 96K chymotryptic fragments were designated dN305R1 and dN246R1 respectively and are depicted with R1, in the scale drawing of Fig. 1(b).

Identical proteolytic products were obtained by digestion of purified R1 in solution with trypsin and chymotrypsin. Fig. 2 shows the proteolytic fragments obtained by treatment of heparin–Affi-Gel-purified R1 with either trypsin (2a) or chymotrypsin (2b) for various times up to 4 h. Proteolysis of R1 is rapid and complete within 1 h (lane 5). Further digestion causes both dN305R1 and dN246R1 to decrease slightly in intensity, although this effect is more pronounced in the case of trypsin. Moreover, in the case of trypsin (Fig. 2a), a novel polypeptide of apparent Mr, 33K (estimated from Fig. 1a) can be observed after 15 min. By increasing the concentration of trypsin to 0.8 μg/ml, dN305R1, which lacked this 33K polypeptide, was produced (Fig. 2c, lane 3).

To determine the sizes of the native proteins, R1, dN246R1 and dN305R1 were analysed by gel filtration on an FPLC Superose 12 column. The gel filtration column was calibrated with proteins of known Mr, as shown in Fig. 3. The R1 proteins were applied to the column separately; 0.5 ml fractions were collected and analysed by SDS–PAGE. All three R1 proteins eluted with fraction volumes between 9 and 10.5 ml. This corresponds to an Mr, range of 150K to 300K indicating that the proteins exist as dimers. Interestingly the 33K
Fig. 1. (a) SDS-PAGE of R1 bound to R2 affinity matrix, treated with either trypsin or chymotrypsin. Lane 1, subunit R1 from E. coli RR; 2, Mr markers; 3, R1 incubated without proteases; 4, R1 incubated with trypsin; 5, R1 incubated with chymotrypsin. Protein standard Mr values are indicated on the left of the gel and R1, R2 and sizes of proteolytic fragments are given on the right. (b) A scale drawing representing intact R1 and the proteolytic truncations dN246R1 and dN305R1.

polypeptide produced by digestion of R1 with trypsin at 0.4 μg/ml co-eluted with dN305R1 suggesting that it remains associated with dN305R1.

The origin of this 33K band was investigated using Western blotting. Three R1–β-galactosidase fusion protein antisera were raised against R1 amino acids 2 to 179 (F1), 167 to 343 (F2) and 282 to 434 (F3); these were then reacted with R1 treated with 0.4 μg/ml trypsin for 30 min. The results are given in Fig. 4 in which lane 1 represents the reaction with antiserum F1, lane 2 antiserum F2 and lane 3 antiserum F3. Mr values were estimated using rainbow markers (Amersham). Two intermediate bands of approximately 130K and 120K were observed during the tryptic digestion of R1. The 130K band was observed with all three antisera whereas the 120K band was visible with only F2 and F3. Trypsin then appears to act directly on the 120K protein by cleaving at amino acid 305 to produce dN305R1 (87K) and a 33K band, both of which react with antiserum F2 and F3. The 33K polypeptide did not react with F1.

Both dN305R1 and dN246R1 were detectable (results not shown) when reacted in Western blots with antiserum C3 raised against a peptide corresponding to R1 amino acids 1117 to 1131 (Lankinen et al., 1989), indicating that they probably have the intact C terminus of R1 and could not have lost more than 10 to 20 amino acids from this end.

The RR activities of dN246R1 and dN305R1 were compared with that of intact R1 and the results are given in Table 1. As a control for the action of the proteases during RR assay, a number of protease inhibitors were screened to identify those which blocked digestion by trypsin or chymotrypsin but had no effect on RR activity. TLCK, soybean trypsin inhibitor, aprotinin and α2 macroglobulin all prevented tryptic digestion of R1, while chymotryptic digestion was prevented by TPCK, aprotinin and α2 macroglobulin. However, of these, only α2 macroglobulin, at a concentration of 0.1 units/100 μl, was found to have no effect on RR. Accordingly α2 macroglobulin was added to the assay mix before the RR assay. R1 was incubated in either the presence or the absence of 0.8 μg/ml trypsin or 0.4 μg/ml chymotrypsin for 1 h and the resulting RR specific activities were determined (Table 1). Treatment of R1 with either 0.8 μg/ml trypsin or 0.4 μg/ml chymotrypsin results in a 25% loss of total protein and the resulting specific activities of dN246R1 and dN305R1 are indistinguishable from that of untreated R1. When R1 was treated with 0.4 μg/ml trypsin which results in the retention of a 33K N-
terminal polypeptide there was a negligible loss of total protein but a 50% loss of activity (Table 1). This loss of activity was prevented by the presence of α₂ macroglobulin during the 1 h incubation with 0.4 μg/ml trypsin prior to RR assay (data not shown).

To compare the relative binding affinities of R1 and dN246R1, an R1/R2-binding ELISA was developed. Fig. 5 indicates the effects of increasing R1 protein concentration on absorbance at 405 nm and establishes calibration curves. Equimolar concentrations of R1 and dN246R1 (1.4 μg/well and 1 μg/well respectively) were then used to obtain IC₅₀ values for the three peptides STSYAGAVVNDL, YAGAVVNDL and AVVNDL previously shown to interfere with RR enzyme activity (Dutia et al., 1986; Cohen et al., 1986; Gaudreau et al., 1987) and the results are shown in Fig. 6(a) and (b). In order to obtain an absorbance of 0.5 in the absence of added peptide, the plate with dN246R1 was read after 15 min, whereas that with intact R1 was read after 30 min. Over a 30 min incubation period horseradish peroxidase colour development was linear (data not shown). Each assay was performed in duplicate; Table 2 lists the IC₅₀ values for inhibition of binding of the three peptides, calculated from the curves in Fig. 6(a) and (b).

The dN305R1 protein could not be detected in this ELISA presumably because of loss of the epitope(s)
Table 1. The effects of trypsin or chymotrypsin treatment on the specific activity of R1*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Treatment</th>
<th>Protein concentration (µg/ml)</th>
<th>Turnover %</th>
<th>Specific activity</th>
<th>Remaining Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>None</td>
<td>32</td>
<td>19.7</td>
<td>13.3</td>
<td>100 (± 8)</td>
</tr>
<tr>
<td>dN305R1</td>
<td>0.8 µg/ml trypsin</td>
<td>24</td>
<td>15.5</td>
<td>13.0</td>
<td>98 (± 8)</td>
</tr>
<tr>
<td>dN246R1</td>
<td>0.4 µg/ml chymotrypsin</td>
<td>25</td>
<td>16.2</td>
<td>14.1</td>
<td>106 (± 10)</td>
</tr>
<tr>
<td>dN305R1/33K</td>
<td>0.4 µg/ml trypsin</td>
<td>30</td>
<td>10.3</td>
<td>7.3</td>
<td>55 (± 5)</td>
</tr>
</tbody>
</table>

* This experiment was performed four times to obtain the mean remaining activity (percentage ± S.D.).

Table 2. Comparison of IC₅₀ values for the inhibition of R1, dN246R1 and dN305R1 binding to R2 for three peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC₅₀ values (µM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
</tr>
<tr>
<td>STSYAGAVVNDL</td>
<td>16</td>
</tr>
<tr>
<td>YAGAVVNDL</td>
<td>48</td>
</tr>
<tr>
<td>AVVNDL</td>
<td>180</td>
</tr>
</tbody>
</table>

* The IC₅₀ values of the peptides are for inhibition of binding of R1, dN246R1 and dN305R1 to R2, calculated from the curves given in Fig. 6(a), 6(b) and 8. The IC₅₀ values for the three peptides for inhibition of RR activity were also determined in RR activity assays using the bacterially expressed pure subunits.

+ ND, Not determined.

**Fig. 4. Western blot analysis of purified R1 treated with 0.4 µg/ml trypsin for 30 min. Lane 1 was probed with fusion protein antiserum F1; lane 2 with fusion protein F2; lane 3 with fusion protein antiserum F3; lane 4, intact R1 probed with fusion protein antiserum F1. M₀ standards as indicated on the left were obtained using rainbow markets. Arrows on the right side indicate proteolytic products mentioned in the text.**

**Fig. 5. Effects of increasing concentrations of either intact R1 (■) or dN246R1 (■■) on colour development in the R1/R2-binding ELISA.**

**Fig. 6. Graph of bound vs unbound R1 to R2 in the R1/R2-binding ELISA.**

**Fig. 7. Graph of bound vs unbound R1 or dN305R1 to the R2 affinity matrix in the presence of decreasing amounts of YAGAVVNDL.**

**Fig. 8. Graph of bound vs unbound R1 or dN305R1 to the R2 affinity matrix in the presence of decreasing amounts of STSYAGAVVNDL.**

**Fig. 9. Graph of bound vs unbound R1 or dN305R1 to the R2 affinity matrix in the presence of decreasing amounts of AVVNDL.**

recognized by the detecting antibody. Accordingly a different approach was used to determine IC₅₀ values for the two peptides STSYAGAVVNDL and YAGAVVNDL, which involved binding of R1 or dN305R1 to an R2 matrix in the presence of various concentrations of peptides. Fig. 7 shows the results obtained when R1 or dN305R1 were incubated with the R2 affinity matrix in the presence of decreasing amounts of YAGAVVNDL. A similar experiment was performed with STSYAGAVVNDL and the R1, dN305R1 and R2 bands were
Properties of two HSV R1 truncations

Fig. 6. Effects of increasing concentrations of the peptides STSYAGAVVNDL (■), YAGAVVNDL (○) and AVVNDL (▲) on the binding of intact (a) R1 or (b) dN246R1 to R2 as determined by the R2-binding ELISA.

quantified by densitometry. R1 or dN305R1 bands were expressed as a fraction of the amount of R2 in the same lane (to compensate for variations in the amount of matrix used for each point) and these values were used to calculate percentage protein bound (percentage band intensity). R1 and dN305R1 bound to R2 in the absence of peptide (lane 6) was taken to represent 100% binding and the starting material for these assays is shown in lanes 7 and 8. The IC₅₀ values of the two peptides for inhibition of binding, calculated from Fig. 8 are given in Table 2 together with IC₅₀ values of the peptides for inhibition of HSV-1 RR activity.

Discussion

We have previously shown that HSV-1 R1 expressed in bacteria has the same specific activity and apparent Mr as R1 in partially purified extracts from HSV-1-infected BHK cells (Furlong et al., 1991). Here two proteolytic enzymes, trypsin or chymotrypsin, were used to produce two stable truncations of the bacterially expressed HSV-1 R1. Both enzymes cleaved within the unique N-terminal domain of the intact R1. Chymotrypsin cleaves at amino acid 246 to produce a 96K polypeptide (dN246R1) which retains R2-binding affinity and is fully active. At a similar concentration, trypsin cleaves at residue 305 to produce an 87K polypeptide (dN305R1) and a 33K N-terminal polypeptide which remain associated during gel filtration and binding to an R2 affinity matrix (data not shown). The association appears to reduce RR activity by 50%, since increasing the trypsin concentration completely removes the 33K N-terminal polypeptide resulting in dN305R1 with full RR enzyme activity. Retention of this 33K polypeptide probably affects RR activity by interfering with substrate binding or inducing conformational changes in dN305R1 which affect the active site. As with dN246R1, dN305R1 retains full R2 affinity and both proteins exist as dimers.

Proteins dN246R1 and dN305R1 retain homology with the large subunit of RR proteins from other species (Nikas et al., 1986; Erikson & Sjöberg, 1989) which, in the case of HSV-1 R1, commences at amino acid 339. The results presented in this paper clearly indicate that the first 305 amino acids of the unique N terminus of HSV-1 R1 are not required for enzyme activity, R2-binding or dimerization.

Degradation products of R1 with Mr values of 110K, 93K and 81K have been detected in infected cell extracts (Frame et al., 1985; Ingemarson & Lankinen, 1987; Lankinen et al., 1989) and the 93K polypeptide is enzymically active (Ingemarson & Lankinen, 1987). Using a range of antipeptide antibodies, this degradation was shown to be N-terminal (Lankinen et al., 1989). Western blotting performed on infected cell extracts detected bands equivalent to dN246R1 and dN305R1 (data not shown) and we believe that these two polypeptides are equivalent to the 93K and 81K species observed by others both in vitro and in vivo. However, to confirm this it would be necessary to perform N-terminal sequencing on these two polypeptides derived from infected cell extracts.

No noticeable proteolysis of dN246R1 and dN305R1 was observed with chymotrypsin or trypsin, suggesting that these carboxy regions of R1 have a well defined structure, resistant to proteolysis. In contrast, the N-terminal domain is susceptible to proteolysis by both trypsin and chymotrypsin. Only with 0.4 µg/ml trypsin was a polypeptide corresponding to the N-terminal cleavage product (33K) observed by SDS-PAGE and this was removed by increasing the concentration of protease. It is probable that N-terminal polypeptides produced by protease treatment of R1 were further digested to undetectable smaller peptides. Western blotting with an R1 antiserum raised against residues 1117 to 1131 (C3) indicated that this epitope was still present in dN264R1 and dN305R1, suggesting that the C terminus in both these truncations had remained intact.
Fig. 7. SDS-polyacrylamide gels showing the effects of peptide YAGAVVNDL on the binding of (a) dN305R1 or (b) R1 to the R2 affinity matrix. Peptide concentrations were as follows: lane 1, 800 μM; 2, 400 μM; 3, 80 μM; 4, 40 μM; 5, 8 μM; 6, 0 μM. Lanes 7 and 8 represent the starting material prior to mixing with the R2 affinity matrix.

Using purified R2 to coat ELISA plates we have developed an ELISA method for quantifying R1. Known peptide inhibitors of R1–R2 interaction gave IC_{50} values in ELISA comparable to those obtained using enzyme assays (Dutia et al., 1986; Cohen et al., 1986; Gaudreau et al., 1987). Binding of dN246R1 to R2 was also assayed by ELISA and peptide inhibitors blocked its binding with similar IC_{50} values. However, as can be seen from the calibration curve of protein concentration against absorbance, up to twofold less dN246R1 than intact R1 is required to produce a given A_{405}. This probably reflects a greater affinity of the detecting F3 antiserum for the dN246R1. Alternatively more dN246R1 may bind than intact R1 per ELISA well because the greater size of R1 may impose greater steric hindrance. The 33K/dN305R1 protein was not detected by antiserum F3 in ELISA indicating that the recognized epitope(s) is (are) located within the junction region between the unique N-terminal domain and the reductase domain.

Fig. 8. Effects of increasing concentrations of the peptides STSYAGAVVNDL (△, ■) and YAGAVVNDL (○, □) on the binding of R1 (△, ○) and dN305R1 (■, □) to the R2 affinity matrix. Results are expressed as percentage band intensity determined by densitometry of SDS–polyacrylamide gels stained with Coomassie blue. The band intensity in the absence of peptide was 100%.
Use of the R2 matrix facilitated measurement of IC_{50} values for the dN305R1/33KR1 complex.

HSV RR is essential for virus growth in vitro in non-dividing cells and in dividing cells grown above 37 °C since growth of mutants producing no active RR is severely compromised under these conditions (Preston et al., 1984; Preston et al., 1988; Cameron et al., 1988; Goldstein & Weller, 1988a, b; Jacobson et al., 1989). In mice, virulence of the temperature-sensitive mutants ts1222 (in R2) and ts1207 (in R1), neither of which produces an active RR, was reduced \( \geq 10^6 \)-fold when compared to the parental strain (Cameron et al., 1988). Further, a virus from which most of the R1 gene was deleted was impaired in its ability to produce acute and reactivatable latent infections in the mouse eye model (Jacobson et al., 1989). These studies indicate that HSV RR could be a potential target for antiviral chemotherapy although studies in humans will be required to confirm this.

The nonapeptide, YAGAVVNDL, corresponding to the C terminus of the HSV RR small subunit is a potent and specific inhibitor of this RR (Dutia et al., 1986; coven et al., 1986) and is presently under study as a lead compound for drug development. Structural studies on R1–R2 interactions will be of immense benefit in achieving this goal. Although both subunits have been overexpressed in E. coli and purified to homogeneity (Furlong et al., 1991; Lankinen et al., 1991) truncated versions of R1 may be of more use in structural studies by virtue of their smaller size. We have produced two stable truncated R1 polypeptides by proteolysis and have shown that they are identical to intact R1 in terms of activity and R2-binding affinity. These truncated proteins are likely to be useful for studies on R1–R2 interactions and protein crystallization.

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References


INGEMARSON, R. & LANKINEN, H. (1987). The herpes simplex virus type 1 ribonucleotide reductase is a tight complex of the type \(a_2\beta_2\) composed of 40K and 140K proteins, of which the latter shows multiple forms due to proteolysis. *Virology* 156, 417–422.


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