Herpes Simplex Virus Type 1 Ribonucleotide Reductase Large Subunit: Regions of the Protein Essential for Subunit Interaction and Dimerization†

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ABSTRACT: We have constructed a series of random N-terminal deletions of the large subunit (R1) of the herpes simplex virus type 1 ribonucleotide reductase. Deletions extended throughout the R1 gene open reading frame and, in total, 31 different truncated polypeptides were expressed in *Escherichia coli* using the T7 expression system. N-Terminal truncations were analyzed for their interaction with the small subunit (R2) of ribonucleotide reductase using a sensitive enzyme-linked immunosorbent assay (ELISA) method and for their ability to complement R2 in ribonucleotide reductase assays. Truncated proteins were also tested for homodimerization using gel-filtration chromatography. The results identified a region of R1 between amino acids 349 and 373 which was essential for subunit interaction. Proteins lacking up to 348 amino-terminal residues associated with R2 and complemented R2 in ribonucleotide reductase assays. Proteins commencing at amino acid 373 and beyond did not interact with R2 and were inactive in enzyme assays. Using a plasmid which expressed an N-terminal deleted protein commencing at amino acid 247, we constructed two defined C-terminal deletions to give proteins comprising amino acids 247–434 and 247–996 of R1. Neither of these truncated proteins bound R2 and we concluded that a second region between amino acids 996 and 1137 (the C-terminus) is required for interaction with R2. Gel-filtration studies indicated that deletion of the first 420 amino acids from R1 did not affect dimerization. However, deletions of 457 amino acids and larger gave proteins which existed as monomers. Both of the C-terminal deleted proteins formed homodimers and we conclude that residues essential for large subunit dimerization are located between amino acids 421 and 434.

The *de novo* synthesis of DNA is dependent upon a supply of the four deoxyribonucleotides which are provided by the enzyme ribonucleotide reductase (EC 1.17.4.1) by reduction of the corresponding ribonucleoside diphosphates (Thelander & Reichard, 1979). The prototype for eukaryotic RR[1] is the enzyme from *Escherichia coli*, which consists of R1 and R2 subunits, and in the active form of the enzyme, homodimers of R1 and R2 associate in an $\alpha_2\beta_2$ configuration. Each R1 subunit contributes a substrate binding site and redox-active thiols and the R2 subunits provide a stable tyrosyl radical(s) associated with two binuclear iron centers (Stubbe, 1990; Nordlund *et al.*, 1990). A feature common to many herpes viruses in the presence within their genomes of open reading frames which encode R1 and R2 subunits. DNA sequencing of herpes simplex virus (HSV) types 1 and 2 (McLauchlan & Clements, 1983; Nikas *et al.*, 1986; McGeoch *et al.*, 1988; Swain & Galloway, 1986), Epstein–Barr virus (Baer *et al.*, 1984; Gibson *et al.*, 1984), varicella zoster virus (Davison & Scott, 1986), equine herpesvirus type 1 (Telford *et al.*, 1992) and herpesvirus saimiri (Albrecht *et al.*, 1992) has identified genes encoding R1 and R2. Pseudorabies virus has been shown to induce a novel RR activity following infection (Lankinen *et al.*, 1982; Cohen *et al.*, 1987) and the open reading frame for the R1 subunit has recently been identified (de Wind *et al.*, 1993). Amino acid sequence comparisons of herpesvirus R1 and R2 subunits and the enzymes from *E. coli*, vaccinia virus, bacteriophage T4, and mouse reveal distinct homologies related to functionally important regions of the proteins (Eriksson & Sjöberg, 1989). A feature of the HSV R1 subunit is the presence of a unique N-terminal extension of approximately 300 amino acids (Nikas *et al.*, 1986) which is not involved in ribonucleotide reduction (Ingemarson & Lankinen, 1987; Lankinen *et al.*, 1989; Conner *et al.*, 1992a) and has a distinct protein kinase activity (Ali *et al.*, 1992; Chung *et al.*, 1989; Conner *et al.*, 1992b; Paradis *et al.*, 1991).

Although HSV RR is dispensible for viral replication in actively dividing cells *in vitro* (Goldstein & Weller, 1988a,b; Preston *et al.*, 1988), mutants which fail to produce any active RR are impaired for growth in serum-starved cells or cells grown at 39 °C (Preston *et al.*, 1988; Goldstein & Weller, 1988b). Experiments with mouse models of pathogenicity indicate that RR is essential for virulence and reactivation from latency (Brandt *et al.*, 1991; Cameron *et al.*, 1988; Jacobsen *et al.*, 1989) and may be required for viral pathogenesis in humans. Several authors have advised caution when extrapolating results from species to species (Jacobsen *et al.*, 1989; Turk *et al.*, 1989), and a requirement for viral RR in HSV infection of humans has yet to be demonstrated. However, Brandt *et al.* (1991) demonstrated poor growth of an RR null mutant in primary cultures in human corneal fibroblasts. Furthermore, RR-deficient mutants of PRV were found to be avirulent for pigs (de Wind *et al.*, 1993). Thus the balance of evidence suggests an essential role for herpesvirus RR *in vivo* and indicates that the protein is a potential target for antiviral chemotherapy.

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[1] Abbreviations: RR, ribonucleotide reductase; R1, large subunit of RR; R2, small subunit of RR; HSV, herpes simplex virus; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; IPTG, isopropyl thio-β-D-galactoside; M Ab, monoclonal antibody; SDS sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; PEG, poly(ethylene glycol); ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

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The nonapeptide YAGAVVNDL is an effective and specific inhibitor of HSV RR (Dutia *et al.*, 1986; Cohen *et al.*, 1986) and corresponds to the C-terminus of the R2 subunit. The nonapeptide functions by binding to R1 (Paradis *et al.*, 1988) and so prevents normal R1/R2 subunit association (McClements *et al.*, 1988; Paradis *et al.*, 1988; Darling *et al.*, 1990). The interaction of R1 with the C-terminus of R2 appears to be a feature common to the class of RR enzymes defined by having two nonidentical subunits. Peptides corresponding to the C-termini of HSV, *E. coli*, and mammalian R2 inhibit the homologous enzyme but have no effect on the heterologous enzymes (Consetino *et al.*, 1991), and recent results have shown that the C-terminus of *E. coli* (Climent *et al.*, 1991) and HSV (Filatov *et al.*, 1992) R2 have all the determinants for interaction with R1. The nonapeptide YAGAVVNDL has no effect on HSV replication in tissue culture, probably because it fails to penetrate cells (Dutia *et al.*, 1986), but rational drug design based on the peptide may result in an effective antiviral agent. Smaller and more potent analogues of YAGAVVNDL have been described (Biomega, 1990; Gaudreau *et al.*, 1987).

Despite the identification of the region of R2 which interacts with R1, there is a paucity of information on R1 amino acids which associate with R2. Nikas *et al.* (1990) identified a point mutation in the HSV temperature-sensitive mutant, *ts*1207 (Preston *et al.*, 1984), in which a serine at residue 961 is substituted by an asparagine and this prevents subunit association. This site does not appear to be at the R1/R2 interface as peptides from this region, and antibodies made against those peptides, failed to affect subunit association. The serine residue is highly conserved in R1 proteins from a number of species and substitution by asparagine probably distorts R1 conformation (Nikas *et al.*, 1990).

We wish to investigate, by detailed structural analysis, the interaction of HSV R1 with the inhibitory peptide YAGAVVNDL, the results of which will be beneficial to rational drug design. In the three-dimensional structure of *E. coli* R2 the C-terminus was not resolved (Nordlund *et al.*, 1990; Atta *et al.*, 1992), possibly because the presence of R1 is required for its ordered configuration. We intend to analyze, by X-ray crystallography, the 3-D structure of HSV R1 complexed to YAGAVVNDL to identify regions and residues which are in contact with the peptide. HSV R1 has been overexpressed in *E. coli* using the T7 system and purified in high yields (Furlong *et al.*, 1991). The R1 protein from this source, however, is unsuitable for protein crystallization as it forms insoluble aggregates at concentrations above 0.5 mg/mL (unpublished data), and significantly higher concentrations will probably be required to promote crystal formation. In this study we describe the construction and expression in *E. coli* of a number of truncated HSV R1 proteins and compare their properties to those of intact R1. We report the identification of suitable replacements for full-length R1 in structural studies.

MATERIALS & METHODS

Construction of N-Terminal Deletions. The parent recombinant plasmid "10/2" containing R1 from HSV-1 (Furlong *et al.*, 1991) was linearized at a unique *Xba*I site within the T7 promoter region. Exonuclease III digestion for different times was used to produce a range of deletions in both directions from the *Xba*I site, and the ends were rendered blunt by digestion with mung bean nuclease. The products were then digested to completion with *Pst*I, generating truncated R1 fragments, blunt at the end within the R1 coding region and with half a *Pst*I site at the other end within the ampicillin resistance gene. These fragments were isolated from agarose gels and purified with Geneclean (Stratech). Simultaneously, the vector pAET8C, a vector derived from pET8C (Studier *et al.*, 1989) in which the pBR backbone of pET8C had been replaced by pAT153, was linearized at the *Nco*I site, end-filled with Klenow, and then digested with *Pst*I. The vector fragment including the T7 promoter was isolated from an agarose gel. This promoter fragment was ligated to the range of deletion fragments described above, the mixture was transformed into *E. coli* DH5 cells, and the resulting colonies were isolated on agar plates with selection for ampicillin resistance enabled by reconstitution of the β-lactamase gene by ligation at the *Pst*I site. The DNA isolated from these colonies was analyzed for truncated R1 DNA by combined restriction digestion with *Xba*I and *Xho*I and also with *Xba*I and *Eco*RI, followed by electrophoresis on 1% agarose gels. An estimation of the extent of deletion was made from these gels and the precise point of deletion was determined by DNA sequence analysis (Sanger *et al.*, 1977). This cloning procedure is shown in Figure 1. The N-terminally truncated proteins were named according to the location of the new N-terminus within the R1 primary sequence. Thus the truncated protein, dN245R1, has 243 amino acids deleted (residues 2–244), and after the R1 initiator methionine, which is retained in the expressing plasmid, commences at amino acid 245 of R1. Expression of protein was achieved in *E. coli* BL21 cells using induction with IPTG as described in Furlong *et al.* (1992) and was detected by Western blotting using R1 specific antisera (106 and Mab 11,453, see below).

The plasmid which expressed the truncated protein dN247R1 was used to prepare two defined C-terminal deletions. The first of these was prepared by insertion of a translation stop codon in all three frames by ligation of a 21-base-pair blunt-ended duplex into the unique R1 *Bam*HI site present in dN247R1 DNA, rendered blunt by end-filling with Klenow. The expressed protein, dN247/dC434R1, consisted of R1 amino acids 247–434. The second C-terminal deletion was achieved by insertion, into the unique *Bst*EII site of dN247R1 DNA, of a 19-base-pair duplex containing translation stop codons in all three frames and with both ends complementary to the *Bst*EII restriction site. The resulting protein, dN247/dC996R1, when expressed in *E. coli* consisted of R1 amino acids 247–996.

Production of R1 Specific Antibodies. The truncated protein, dN245R1, purified as described for intact R1 (Furlong *et al.*, 1991), was used as immunogen for the production of an R1 monoclonal and R1 polyclonal antibodies. Prior to injection into mice dN245R1 was treated with 0.4 μg/mL trypsin (Conner *et al.*, 1992a). This removed the remainder of the unique N-terminus of HSV R1 and destroyed an immunodominant epitope at the junction of the unique N-terminus and reductase domains of the protein (Lankinen *et al.*, 1993). Donor mice were immunized with three intraperitoneal injections of trypsinized dN245R1 in complete Freund's adjuvant at weekly intervals, followed by three boosts of protein in PBS intraperitoneally over a period of 250 days. A final intraperitoneal injection was given and, 4 days later, spleen cells were fused with P3/X63-Ag8 myeloma cells using PEG1000. Hybrid cells were selected in HAT medium and specific antibodies were identified by ELISA using trypsinized dN245R1 as antigen. Undiluted culture supernatants were used for all experiments. The monoclonal antibody used in this study was designated MAb 11,453.

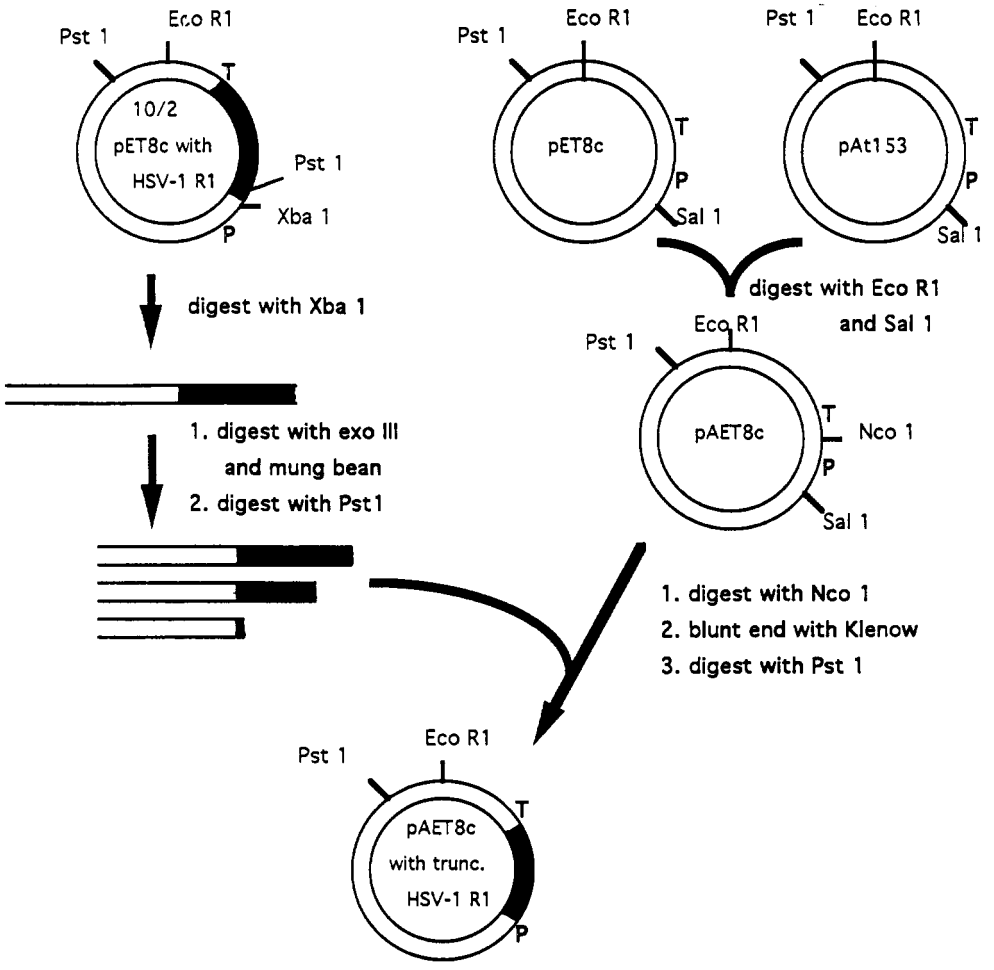

FIGURE 1: Construction of plasmids for expression of R1 N-terminal deletions. R1 coding sequences are shaded black. The letters P and T indicate the location of the T7 promoter and terminator, respectively.

The purified truncation dN245R1 was also used to raise a polyclonal antiserum (106) in a rabbit. The rabbit was injected 4 times at biweekly intervals with 50 μg of purified intact protein. Serum was obtained 1 month after the fourth injection and, for Western blots, was used at a dilution of 1:200.

Polyclonal antiserum F3, raised against R1 amino acids 282–436, was used in ELISA assays at a dilution of 1:100 (Conner *et al.*, 1992a).

R2 Binding ELISA with MAb 11,453. The method for determination of R2 binding using an ELISA assay was based on that of Conner *et al.* (1992a). Briefly, 96-well plates were coated with purified HSV R2 at a concentration of 0.3 μg/well and incubated overnight at 4 °C. Nonspecific protein interactions were blocked by incubation of the plates with 1% (w/v) skimmed milk powder in PBS for 1 h, after which 100 μL of partially purified extracts of intact R1 or the truncations were added and incubated at 20 °C for 1 h. This partial purification was achieved using ammonium sulfate fractionation of proteins released by lysozyme treatment of *E. coli* (Furlong *et al.*, 1991). Truncated proteins were optimally precipitated by 33% ammonium sulfate (data not shown) and the pellet was resuspended in a minimum volume of 25 mM HEPES, pH 7.6, with 2 mM DTT.

Plates were then washed 6 times with PBS containing 0.1% (v/v) NP40, 100 μL of MAb 11,453 supernatant was added, and after a 1-h incubation, plates were again washed 6 times in PBS with 0.1% NP40. Sheep anti-mouse IgG/HRP conjugate (100 μL) was then added, and after a further 1-h incubation and washing cycle, color 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% (v/v) H_2O_2]. The absorbance at 405 nm was determined after 30–60 min using a Multiskan plate reader (Titertek).

Binding of the two defined C-terminal truncated proteins was determined, using the F3 antiserum raised as described in Conner *et al.* (1992a). Competition experiments with the peptide YAGAVVNDL were performed for truncated proteins which bound R2 to determine the concentration of peptide required to reduce the OD 405 nm by 50% compared to controls in the absence of peptide (IC_{50} value). Dilutions of truncated proteins were chosen which gave OD readings of between 0.5 and 1.0 absorbance units over a 15–30-min incubation period. Aliquots (50 μL) of peptide solution at various concentrations were added to the wells prior to the addition of 50 μL of partially purified extract. Color development of the ELISA was linear over a 1-h incubation.

The concentration of truncated proteins which bound R2 was determined using the R2 binding ELISA by interpolation of a standard curve prepared with a known concentration of purified R1. The concentration of other truncated proteins was estimated by comparing limiting dilutions on Western blots with known amounts of R1. For the N-terminally truncated proteins the blots were probed with MAb 11,453, and for the two C-terminally-truncated proteins antiserum F3 was used.

Enzyme Activity of R1 Truncated Proteins. The ability of R1 truncated proteins to complement R2 in RR assays was assessed using the method of Darling *et al.* (1987). Ammonium sulfate-fractionated extracts were used in assays with 3 μg of purified HSV-1 R2 (Lankinen *et al.*, 1991).

Homodimerization of R1 Truncated Proteins. Dimerization of R1 truncated proteins was analyzed using FPLC Superose 12 and Superdex 75 gel-filtration columns. Extracts from cultures expressing truncated proteins were prepared by an alternative method as follows. Bacterial pellets from 300-mL cultures were resuspended in 2 mL of 25 mM HEPES buffer, pH 7.6, with 2 mM DTT and 4 mM CHAPS and stored overnight at −70 °C. Proteins were extracted by sonication and particulate material was removed by centrifugation at 13000*g* for 10 min. Supernatants were then applied directly to the gel-filtration column equilibrated with the same buffer. The flow rate for both columns was 0.2 mL/min and twenty 1-mL fractions were collected per run. Fractions 6–13 were analyzed by SDS–PAGE and Western blotting and R1 truncated proteins were detected using either MAb 11,453 or polyclonal antiserum 106. Molecular mass calibration for each column was performed using the following protein standards: carbonic anhydrase (29 kDa), ovalbumin (44 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (440 kDa).

Purification of Truncated Proteins by Heparin Affi-Gel Chromatography. The three truncated proteins, dN237R1, dN245R1, and dN247R1, were purified from 10 L of bacterial culture by heparin Affi-Gel chromatography (Furlong *et al.*, 1991). Minor modifications to the chromatographic procedures are as follows. Bound proteins were eluted from the column with a 0–1 M gradient of NaCl developed over 5 column volumes and R1 protein containing fractions were desalted into 25 mM HEPES buffer with 2 mM DTT and 4 mM CHAPS.

RESULTS

Sequence analysis of the random truncations described above defined 294 constructs containing truncated R1 genes. Of these, 86 were in frame with the T7 promoter, but only a subset of 34 of these expressed a truncated polypeptide after induction with IPTG—three of the 34 R1 N-terminal deletions were duplicates. Protein expression of a selection of these was demonstrated by Western blots, probed with MAb 11,-453 (Figure 2A, lanes 1–22). Figure 2B is a line diagram, drawn to scale, representing intact R1 and the 31 different N-terminally truncated proteins. Examination of Figure 2A (lanes 1–22) reveals a wide range of concentrations of the N-terminal deletions in the partially purified extracts, and we have previously reported an analysis of the variation in levels of expression (Furlong *et al.*, 1992). Of particular note are three truncations, dN245R1 (lane 2), dN237R1 (lane 3), and dN247R1 (lane 13), which are expressed at 5–10-fold greater levels than intact R1 (lane 1). The plasmid used to express one of these truncated proteins, dN247R1, was the parent plasmid for the two defined C-terminally truncated proteins, which, followed induction with IPTG, were also expressed at high levels (Figure 2A, lanes 25 and 26). These double deletions are also shown in Figure 2B and are named according to the location of the N-terminus and the last amino acid at the new C-terminus (dN247/dC434R1 and dN247/dC996R1).

Ammonium sulfate fractions from 300-mL cultures of each truncated protein were prepared and tested in an R2 binding ELISA. The effectiveness of MAb 11,453 in this ELISA was assessed using increasing concentrations of purified R1 as shown in Figure 3A. The OD at 405 nm increased over an R1 concentration range of 0–16 μg/well. Figure 3B demonstrates the ability of the nonapeptide YAGAVVNDL to prevent the interaction of R1 and dN245R1 with R2 using

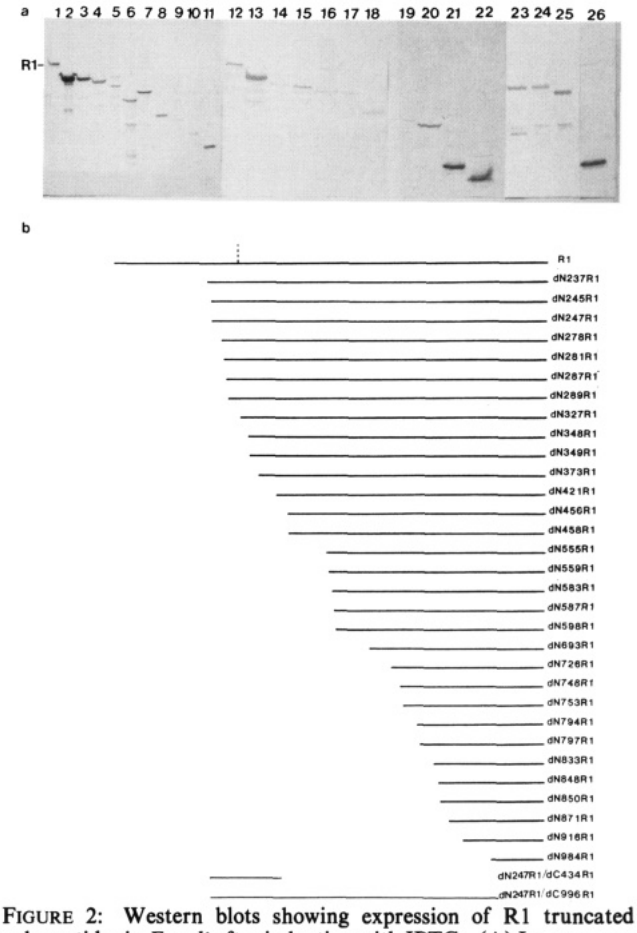

FIGURE 2: Western blots showing expression of R1 truncated polypeptides in *E. coli* after induction with IPTG. (A) Lanes are as follows: (1 and 12) intact R1, (2) dN245R1, (3) dN237R1, (4) dN281R1, (5) dN327R1, (6) dN555R1, (7) dN458R1, (8) dN693R1, (9) dN748R1, (10) dN794R1, (11) dN871R1, (13) dN247R1, (14) dN289R1, (15) dN349R1, (16) dN373R1, (17) dN421R1, (18) dN583R1, (19) dN598R1, (20) dN726R1, (21) dN916R1, (22) dN984R1, (23) dN245R1, (24) dN247R1, (25) dN247/dC996R1, and (26) dN257/dC434R1. Lanes 1–22 were developed using MAb 11,453 and lanes 23–26 were developed using F3. (B) Line diagram, drawn to scale, of all the truncations used in this study. The dotted line on R1 indicates the junction between the unique N-terminus and the conserved reductase domains of the protein.

the ELISA. The IC_{50} value interpolated from the curve for R1 is 27 μM, which falls within the ranges of values obtained from previously published data (Dutia *et al.*, 1986; Gaudreau *et al.*, 1987; Conner *et al.*, 1992a). The IC_{50} value for dN245R1 is 30 μM, which indicates that the truncated protein binds R2 with the same affinity as intact R1.

The Western blots in Figure 2A show that MAb 11,453 reacts with all N-terminally truncated proteins, demonstrating that the ELISA could be used to test their ability to interact with R2. Limiting dilutions of partially purified extracts of each truncated protein were performed and this readily identified those which bound R2. Examples of truncated proteins which interacted with R2 or showed no affinity for R2 are shown in Figure 3C(i) and (ii). Of particular note are those shown in Figure 3C(ii) which define a region required for subunit interaction. DN327R1 and dN349R1 bind R2, whereas dN373R1 and dN421R1 show no affinity for R2. Since all truncated proteins commencing downstream of amino acid 373 showed no affinity for R2, the region between amino acids 349 and 373 is therefore mandatory for subunit interaction.

The strength of interaction of truncated proteins with R2 was quantified using the peptide YAGAVVNDL. Examples

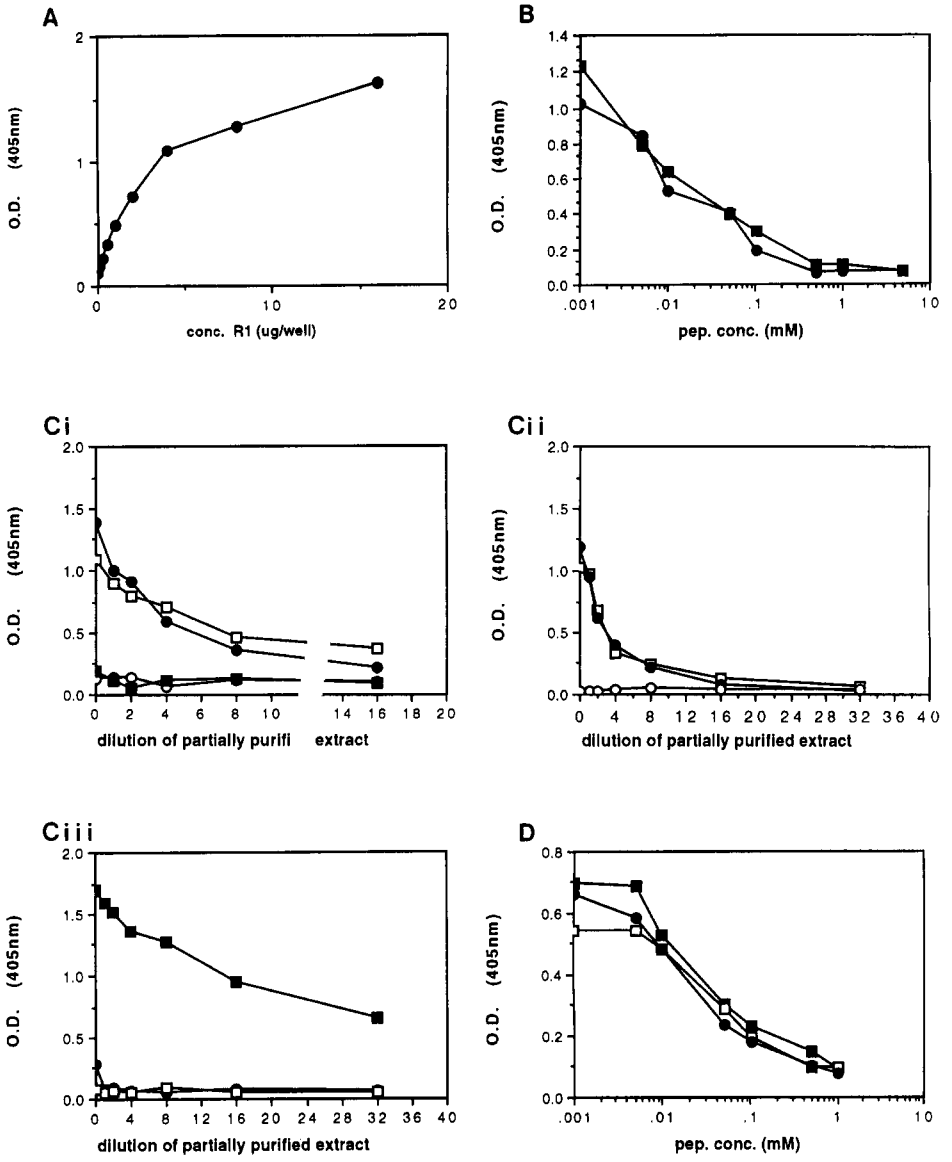

FIGURE 3: Analysis by ELISA of R2 binding properties of R1 truncations. (A) Effects of increasing R1 concentration on OD 405 nm using MAb 11,453 in R2 binding ELISA. (B) Inhibition of binding of R1 and dN245R1 to R2 by the peptide YAGAVVNDL as detected using MAb 11,453 in the R2 binding ELISA. (C) Doubling dilution curves for partially purified extracts of a number of truncations tested in the R2 binding ELISA. (i) R1 (●), dN281R1 (□), dN555R1 (■), and dN871R1 (○); (ii) dN327R1 (●), dN349R1 (□), and dN373R1 and dN421R1 (○); (iii) dN247R1 (■), dN247/dC996R1 (□), and dN247/dC434R1 (●). (D) Inhibition of interaction of dN247R1 (■), dN327R1 (●), and dN349R1 (□) with R2 using the peptide YAGAVVNDL.

of inhibition curves are shown in Figure 3D for dN247R1, dN327R1, and dN349R1, which gave IC_{50} values of 35, 30, and 40 μM, respectively, all similar to the values obtained for R1 and dN245R1. These data indicate that truncation up to amino acid 349 does not affect affinity for R2.

The double deletions dN247/dC434R1 and dN247/dC996R1 were also tested for R2 binding by ELISA. As neither of these proteins reacted with MAb 11,453, the fusion protein antiserum F3 was used to detect binding. The data, Figure 3C(iii), indicate that these two proteins, in contrast to dN247R1, do not interact with R2. Both dN247/dC434R1 and dN247/dC996R1 contain R1 amino acids 349–373, demonstrated above to be essential for subunit interaction, but neither protein binds R2. We conclude that a second region, downstream of amino acid 996, is also required for subunit interaction.

Extracts fractionated by amonium sulfate precipitation were utilized to determine the ability of R1 truncated proteins to complement R2 in RR assays, and the results for a number of these are shown in Table I. All truncated proteins which bound R2 in the ELISA showed no appreciable loss of RR activity, whereas all those which showed no interaction with R2 were inactive.

Table I: RR Activity of Intact R1 and Some N-Terminal Truncated Proteins

protein	amount of R1 protein/assay (μg)	% turnover[a]	sp act. [nmol of dCDP min^{-1} (mg of protein)$^{-1}$]
intact R1	2	13.6	11.3
dN245R1	3	18.2	10.2
dN327R1	2	11.8	10.4
dN349R1	3	16.0	9.7
dN373R1	5	0	0
dN421R1	5	0	0
dN247R1/dC996R1	5	0	0

[a] Percent turnover indicates the amount of substrate (CDP) converted to product (dCDP) during the reaction.

Analysis by gel filtration of the partially purified extracts prepared as described above indicated that ammonium sulfate precipitation promoted nonspecific protein aggregation and the extracts were unsuitable for dimerization studies. Using

Table II: Analysis of Truncations by Gel Filtration on Superose 12[a]

(A) Superose 12 Column

truncation	6	7	440K ▼ 8	200K ▼ 9	150K ▼	66K ▼ 10	11	29K ▼ 12	13
R1	–	–	++	++		–	–	–	–
dN245R1	–	–	++	++		–	–	–	–
dN287R1	–	–	++	++		–	–	–	–
dN349R1	–	–	++	++		–	–	–	–
dN373R1	–	–	++	++		–	–	–	–
dN421R1	–	–	+	++		+	–	–	–
dN458R1	–	–	–	+		++	–	–	–
dN555R1	–	–	–	+		++	+	–	–

(B) Superdex 75 Column

truncation	6	7	200K ▼ 8	67K ▼	44K 9 ▼	29K 10 ▼	11	12	13
R1	–	–	+++		–	–	–	–	–
dN245R1	–	–	+++		–	–	–	–	–
dN349R1	–	–	+++		–	–	–	–	–
dN373R1	–	–	+++		–	–	–	–	–
dN421R1	–	–	+++		–	–	–	–	–
dN458R1	–	–	++		++	–	–	–	–
dN555R1	–	–	+		++	+	–	–	–
dN693R1	–	–	–		++	++	–	–	–
dN726R1	–	–	–		++	++	–	–	–
dN748R1	–	–	–		+	++	–	–	–
dN794R1	–	–	–		+	++	–	–	–
dN916R1	–	–	–		–	++	++	–	–
dN247/dC434R1	–	–	+		++	+	–	–	–
dN247/dC996R1	–	–	+++		–	–	–	–	–

[a] Numbers 6–13 refer to the gel-filtration fractions which were analyzed by SDS–PAGE and Western blotting. Twenty 1-mL fractions were collected per truncated protein analyzed. The elution positions of the molecular mass standards are indicated at the top of the table. The symbols in the columns refer to whether the protein was detected by Western blotting in the fraction (+/–) and also give an estimate of the intensity of the band if detected (+ low, ++ intermediate, +++ high intensity).

dN245R1 and a variety of extraction procedures and reagents, a modified procedure was determined. Proteins were extracted from *E. coli* by sonication into a HEPES buffer with 4 mM CHAPS, and under these conditions dN245R1 behaved as a dimer (Table IIA). Similar extracts for most truncated proteins were prepared, and the elution profiles for some of these from the Superose 12 column are summarized in Table IIA. A noticeable shift in elution volume occurs with dN421R1 and dN458R1. The elution volume of the standards is also indicated in Table IIA and the estimated molecular masses of dN421R1 and dN458R1, 130 and 70 kDa, respectively, are consistent with the shift from dimeric to monomeric R1. A second FPLC gel-filtration column, Superdex 75 (Table IIB), was employed to confirm this initial observation. The molecular mass exclusion limit of this column (70 kDa) is such that R1 and, if the above observation is correct, truncated proteins forming dimers will elute in the void volume, whereas monomeric truncated proteins will penetrate the matrix and elute in larger volumes. The results shown in Table IIB, demonstrate that dN245/dN421R1 elute exclusively in the void volume and are dimeric. The next largest truncated protein tested, dN458R1, and all subsequent truncated proteins analyzed eluted in volumes greater than the void volume and are therefore monomeric. The molecular mass of dN458R1, as estimated from the standards shown in Table IIB, was 66 kDa, which is similar to the predicted monomeric molecular mass. Furthermore, the elution volumes of the smaller truncated proteins analyzed also approximates their predicted monomeric molecular masses (data not shown). Interestingly, both the double deletions behave as dimers, with the protein dN247/dC434R1 eluting with an approximate molecular mass of 50 kDa. The smallest N-terminally truncated protein tested, dN916R1, which has a similar number of amino acids to dN247/dC434R1, eluted with a molecular mass of 30 kDa. These results demonstrate that R1 amino acids between residues 421 and 434 are necessary for the formation of R1 homodimers.

Table III: Summary of Properties of R1 and Truncated Proteins

protein	concn[a] (μg/mL)	binds R2 in ELISA[b]	IC_{50}, YAGAVVNDL (μM)	active in RR assays	forms homodimers
intact R1	100	+	27	+	+
dN237R1	400	+	35	+	+
dN245R1	1000	+	30	+	+
dN247R1	600	+	45	+	+
dN278R1	100	nd	–	nd	nd
dN281R1	100	+	38	+	+
dN287R1	100	+	30	+	+
dN289R1	50	+	40	+	+
dN327R1	50	+	30	+	+
dN348R1	6	+	nd	nd	nd
dN349R1	50	+	35	+	+
dN373R1	50	–	–	–	+
dN421R1	50	–	–	–	+
dN456R1	6	–	–	nd	nd
dN458R1	50	–	–	–	–
dN555R1	50	–	–	–	–
dN559R1	25	–	–	–	nd
dN583R1	50	–	–	–	–
dN587R1	25	–	–	–	nd
dN598R1	25	–	–	–	nd
dN693R1	100	–	–	–	–
dN726R1	100	–	–	–	–
dN748R1	50	–	–	–	–
dN753R1	200	–	–	–	–
dN794R1	50	–	–	–	–
dN797R1	12.5	–	–	–	nd
dN833R1	6	–	–	nd	nd
dN848R1	25	–	–	–	–
dN850R1	6	–	–	nd	nd
dN871R1	200	–	–	–	nd
dN916R1	100	–	–	–	–
dN984R1	100	–	–	–	nd
dN247/dC434R1	400	–	–	–	+
dN247/dC996R1	400	–	–	–	+

[a] Concentration was calculated for R1 proteins in partially purified extracts prepared under identical conditions. [b] +, yes; –, no; nd, not done.

Table III is a summary of results for all of the truncated proteins and shows that most truncations were expressed at levels either equivalent to or significantly lower than the intact protein. However, three truncated proteins, with properties identical to those of intact R1 (in terms of RR activity and affinity for R2), were expressed at high levels. These three truncated proteins were purified to apparent homogeneity using heparin Affi-Gel chromatography. Figure 4 shows a Coomassie-stained SDS–polyacrylamide gel of R1, dN237R1, dN245R1, and dN247R1; each band represents the yield of protein from approximately equal numbers of bacterial cells. The yields and properties of these three truncated proteins, prepared under identical conditions, are compared with those of intact R1 in Table IV. The IC_{50} values given in Table IV show some discrepancy with those given in Table III but they are still within the range obtained from previously published data (Dutia *et al.*, 1986; Gaudreau *et al.*, 1987; Conner *et al.*, 1992a) and differences are probably due to experimental variation.

DISCUSSION

The nonapeptide YAGAVVNDL corresponding to the C-terminus of the HSV R2 subunit inhibits HSV RR by binding to R1 and disrupting normal subunit association. The

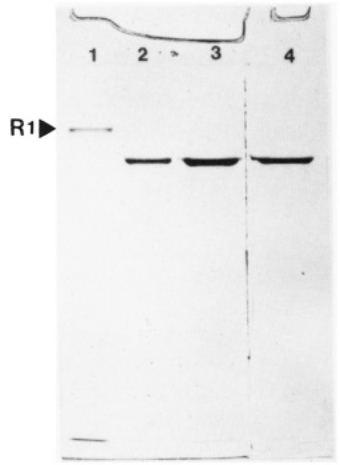

FIGURE 4: Coomassie-stained SDS–polyacrylamide gel showing the four purified proteins intact R1 (1), dN237R1 (2), dN245R1 (3), and dN247R1 (4). The amount of protein loaded per well represents the yield from approximately equal numbers of *E. coli* cells.

Table IV: Properties of Purified R1 and N-Terminal Truncated Proteins[a]

	% purity[b]	yield[c] (μg/L)	sp act. [nmol of dCDP min^{-1} (mg of protein)$^{-1}$]	IC_{50}, YAGAVVNDL[d] (μM)
intact R1	70	100	14.1	35
dN237R1	80	250	17.2	50
dN245R1	90	1000	13.6	37
dN247R1	90	700	11.7	45

[a] Values in this table were calculated using purified proteins and may differ from results given in Table III. [b] Estimated from Coomassie Blue stained SDS–polyacrylamide gels. [c] Yield is the amount of protein purified from 1 L of bacterial culture. [d] Calculated from R2 binding ELISA.

residues or regions of HSV R1 which interact directly with YAGAVVNDL and R2 are not known. Investigation of the R1/YAGAVVNDL complex by X-ray crystallography requires milligram amounts of purified protein at high concentrations. We have overexpressed HSV R1 in *E. coli* using the T7 expression system and purified the protein in high yields (Furlong *et al.*, 1991). Although the protein is fully active in RR assays at low concentrations, attempts to concentrate R1 result in loss of activity due to R1 precipitation (unpublished data).

We initiated this study to define regions of R1 essential for functional subunit interaction and, accordingly, constructed a series of random N-terminal deletions and two defined C-terminal deletions. The truncated proteins were used to identify two regions which are essential for interaction with R2 and must therefore be retained in any truncated proteins used for structural studies of R1/YAGAVVNDL interactions.

The first region, corresponding to R1 amino acids 349–373, was identified using the random N-terminally truncated proteins in an R2 binding ELISA. Analysis was facilitated by production of a monoclonal antibody which recognized all the N-terminally truncated proteins, incidentally indicating that the epitope is downstream of R1 amino acid 984. IC_{50} values with the peptide YAGAVVNDL in the ELISA demonstrated that the antibody did not interfere with subunit interaction. Analysis of Figure 3 shows that the ELISA readily detected polypeptides which interacted with R2, and Figure 3C(ii) clearly demonstrates the requirement of R1 amino acids 349–373 for R2 binding.

The second region of R1 essential for subunit interaction was demonstrated using the C-terminal truncated proteins derived from the N-terminal deletion dN247R1 and a previously described R2 binding ELISA (Conner *et al.*, 1992a) in which binding is monitored with an antibody made against residues 282–436 of R1. Since dN247/dC996R1 did not interact with R2, we deduce the requirement for a second region, between amino acids 996 and 1137. Ribonucleotide reductase activity assays confirmed the absolute requirement for both these regions of the protein.

We propose several models which may account for the requirement for both regions. First, residues in both regions may be in direct contact with YAGAVVNDL and the tertiary structure juxtaposes these regions to form an R2 binding pocket or groove. If either region is absent from a polypeptide, as in dN373R1 or dN247/dC996R1, then the conformation of the binding pocket is perturbed and the polypeptide can no longer interact with the C-terminus of R2. Alternatively, only one of the regions has residues in direct contact with R2 but the correct conformation of this region is dependent upon the presence of the other region. We also consider it possible that neither of these regions is in direct contact with R2 but their deletion affects R1 conformation such that R2 binding is prevented.

The active form of ribonucleotide reductase results from the association of homodimeric R1 and R2 subunits, and it is likely that dimerization of R1 and R2 is essential for activity. Gel-filtration studies of the N- and C-terminally truncated proteins identified a region of R1 essential for dimer formation. It was observed that N-terminally truncated proteins lacking up to 420 amino acids exist as dimers. Protein dN458R1 and those with deletions beyond this residue behaved as monomers. Both C-terminal truncated proteins were dimeric. We conclude that residues between amino acids 421 and 434 are required for dimerization. However, as we were unable to quantify the strength of interaction between R1 polypeptides for dimer formation, we cannot exclude the possibility that other regions or residues are involved in R1 dimerization.

Chung *et al.* (1991) reported that a peptide corresponding to HSV-2 R1 amino acids 419–432 inhibited HSV RR activity with an IC_{50} value of 300 μM and functioned by disruption of RR complex formation. Comparison with R1 proteins from other species shows this region is conserved only in HSV-1 R1 and occurs within a series of five leucine repeats between residues 409 and 437. Although our results do not identify the specific residues involved in dimer formation, they suggest that the ability of this peptide to inhibit HSV RR activity arises from disruption of R1 dimers. This possibility remains to be tested. It will also be necessary to identify the residues directly involved in the dimerization of HSV R1 and R1 proteins from a variety of species before it can be concluded that HSV R1 dimerization motifs are unique. It is also possible that this region is not directly involved in R1 dimerization but that deletion affects R1 conformation such that homodimerization is no longer possible.

The sequence at the C-terminus of other herpesvirus R2 subunits, including those of varicella zoster virus, Epstein–Barr virus, equine herpes virus 1, and herpesvirus saimiri, is highly conserved, suggesting some degree of conservation of the R1 contact residues. Numerous residues in the region 996–1137 of HSV-1 R1 are conserved in R1 of other herpesviruses, although the sequence between residues 349 and 373 appears to be unique to HSV R1. Identification of the R1 contact residues and elucidation of the mechanism of subunit interaction will require more detailed analysis. This may be achieved by site-directed mutagenesis of individual residues within the essential region or by using synthetic peptides from these regions to inhibit subunit interaction.

However, both approaches have limitations and may produce equivocal results. First, as Nikas *et al.*, (1990) demonstrated, a point mutation at R1 amino acid 961 was sufficient to abrogate subunit interaction although this region is outside the R1/R2 interface. Second, as two distinct regions are involved, synthetic peptides from a single region may not disrupt the interaction. The alternative approach of X-ray crystallography of a truncated protein which retains the properties of R1 essential for RR activity and remains soluble at high concentrations may facilitate elucidation of the mechanism of subunit interaction. Fortunately, three truncated proteins generated in this study retain RR functions and are expressed at significantly greater amounts than intact R1. These truncated proteins have been purified in high yields and are soluble in low-salt buffers at concentrations in excess of 5 mg/mL. They can therefore be used in place of intact R1 in protein crystallization trials.

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