A single amino acid substitution in the large subunit of herpes simplex virus type 1 ribonucleotide reductase which prevents subunit association

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The herpes simplex virus type 1 temperature-sensitive (ts) mutant ts1207 does not induce detectable levels of ribonucleotide reductase activity at the non-permissive temperature (NPT, 39.5 °C). The ts lesion prevents the association of the enzyme's large (RR1) and small (RR2) subunits to give an active holoenzyme and maps within the gene specifying RR1. Here, it is shown that ribonucleotide reductase activity at the non-permissive temperature (NPT, 39.5 °C). The ts lesion prevents the association of the enzyme's large (RR1) and small (RR2) subunits to give an active holoenzyme and maps within the gene specifying RR1. Here, it is shown that the ts mutant phenotype is due to the substitution of an asparagine for the wild-type (wt) serine at RR1 position 961, which is located within a region highly conserved between herpesviral and cellular RR1 subunit polypeptides. This ts1207 asparagine is predicted to alter a wt α-helix to a β-strand. We have used synthetic oligopeptides, corresponding to the wt amino acid sequence of the mutation site, and antisera raised against them to determine whether this region is involved in subunit association. Neither the oligopeptides nor the antisera inhibit the enzyme activity, or the reconstituted activity formed by mixing intact RR2 and RR1 subunits present in partially purified extracts of cells infected at the NPT with ts1207 or ts1222 (an HSV-1 mutant with a lesion in the RR2 subunit), respectively. We infer from these results that the site of the mutation is unlikely to be positioned at the surface of RR1 and hence is probably not directly involved in subunit association. We suggest that the mutation site identifies an important RR1 region whose alteration in ts1207 changes the structure of a contact region(s) positioned at the RR1/RR2 interface.

Introduction

Herpes simplex virus type 1 (HSV-1) encodes its own ribonucleotide reductase (RR; Dutia, 1983) which catalyses reduction of all four ribonucleoside diphosphates (NDPs) to the corresponding deoxyribonucleoside diphosphates (Averett et al., 1983). The active HSV-1 holoenzyme is composed of two non-identical subunits (RR1 and RR2) (Dutia, 1983; Frame et al., 1985; Preston et al., 1984; Bacchetti et al., 1986), which are homodimers of the Vmw136 (RR1) and Vmw38 (RR2) polypeptides (Ingemarson & Lankinen, 1987). The RR1 and RR2 polypeptides are the products of genes UL39 and UL40 of the HSV-1 genome (McGeoch et al., 1988), which map in the unique long region (Fig. 1) and their nucleotide and predicted amino acid sequences have been published (McLauchlan & Clements, 1983a, b; Nikas et al., 1986; McGeoch et al., 1988).

The primary structure of the HSV-1 enzyme has been extensively studied (Caras et al., 1985; Standart et al., 1985; Nikas et al., 1986; Swain & Galloway, 1986) and the main conclusions are as follows. Firstly, the HSV-1 RR1 and RR2 polypeptides share 21 blocks of conserved amino acids with cellular and other herpesviral RRs, the function of which may be related both to enzyme structure and activity. Notably, one of the RR1 blocks, with a consensus sequence of GXGXXG (G, glycine) and a predicted secondary structure of β-sheet/turn/α-helix, most probably represents the enzyme's substrate binding site (Nikas et al., 1986). Secondly, the HSV-1 RR1 polypeptide has a unique N-terminal region which is absent from other RR1 polypeptides (Nikas et al., 1986). It has been proposed that this region is not directly involved in ribonucleotide reduction (Nikas, 1989; Lankinen et al., 1989; Wymer et al., 1989). Recent experiments have shown directly that at least the first 149 amino acids of this domain are not necessary for enzyme activity (Lankinen et al., 1989).

RR is an essential function for HSV-1 growth and DNA replication (Dutia, 1983; Preston et al., 1984, 1988; Goldstein & Weller, 1988a, b). Studies with the temperature-sensitive (ts) HSV-1 mutant ts1207, which has a lesion in RR1, demonstrated that, at the non-permissive temperature (NPT), virus with this lesion was unable to induce detectable levels of RR in infected cells and its replication was severely restricted (Preston et al., 1984). Interestingly, although at the NPT ts1207 expresses both RR1 and RR2 polypeptides, the lesion

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prevents their association to form a functional complex (Frame et al., 1985). Other studies with a second HSV-1 ts mutant, ts1222, which has a lesion in RR2, have shown that the enzyme is required for virus replication in non-dividing tissue culture cells (Preston et al., 1988). Finally, functional RR has been shown to be essential for virus pathogenicity in mice (Cameron et al., 1988; Jacobson et al., 1989).

Recent interest has focused on the enzyme as a target for antiviral compounds, because the disruption of subunit interaction may itself be exploitable (Frame et al., 1985). This approach resulted from the observation that a synthetic oligopeptide corresponding to the nine C-terminal amino acids of RR2 specifically inhibits viral RR (Dutia et al., 1986; Cohen et al., 1986) by preventing subunit association (McClements et al., 1988, Darling et al., 1988). Clearly, the identification and mapping of the RR1 and RR2 contact regions is of importance.

This communication examines whether the region in RR1 where the ts1207 lesion is located is involved in subunit association. We show that a single amino acid change from a serine to an asparagine at position 961 in RR1 is responsible for the ts1207 phenotype. Analyses of the site of the mutation by biochemical means demonstrate that although this region is positioned at the surface of RR1, it appears not to be directly involved in subunit interaction.

**Methods**

**Tissue culture cells.** BHK-21 clone 13 cells (Macpherson & Stoker, 1962) were grown in Eagle's medium supplemented with 10% tryptose phosphate and 10% calf serum.

**Viruses.** The wild-type (wt) virus used throughout this study was HSV-1 strain 17+ (Brown et al., 1973). ts1207 was derived from the HSV-1 mutant tsG, isolated by J. Subak Sharpe and described by Brown et al. (1973), by recombinating the *tsG* BamHI o fragment (map units 0.574 to 0.600; Fig. 1) into wt HSV-1 DNA (Preston et al., 1984). The HSV-1 mutant ts1222 has been characterized (Preston et al., 1988; Darling et al., 1988). Infections with ts1207 and ts1222 were carried out at the NPT of 39.5°C.

**Recombinant plasmids and nucleotide sequence analysis.** The XhoI/BgII subfragment of the *tsG* BamHI o fragment contains the lesion responsible for the ts1207 phenotype (Preston et al., 1984; Fig. 1). To identify the nucleotide change(s) within the XhoI/BgII fragment, this fragment was isolated from plasmid pBamHI o which consists of *tsG* BamHI o cloned into the bacterial plasmid vector pAT153, and was kindly provided by Dr V. G. Preston. The XhoI/BgII fragment was end-repaired using T4 DNA polymerase and then cloned into a *SmaI*-digested M13mp8 vector. Sequence analysis of the mutament fragment was performed using the dideoxynucleotide chain termination method (Sanger et al., 1980). Synthetic pentadecamer oligonucleotides, used as primers in sequencing reactions, were kindly provided by Dr J. McLauchlan using a Biosearch 8600 DNA synthesizer.

**Computer analysis of sequences.** Computing was performed with a DEC PDP 11/44 computer running under the RSX 11M operating system. Sequencing data were handled as detailed elsewhere (Nikas et al., 1986). Predicted amino acid sequences were derived from nucleotide sequences with the PTRANS program (Taylor, P. 1986). Optimal alignment of nucleotide and amino acid sequences was performed with the HOMOL programme of Taylor (1984) and secondary structure predictions were performed with the program of Chou & Fasman (1978).

**Ribonucleotide reductase assay.** Partially purified extracts from cells infected with wt HSV-1 or with the HSV-1 mutants ts1207 and ts1222 were made as described previously (Darling et al., 1988). All assays were performed at 37°C. RR activity was measured as described by Darling et al. (1987), except that bacitracin (1 mM; Sigma, Gaudreau et al., 1987) and α-macroglobulin (0.2 units) were included in the reaction mixture to prevent proteolysis of peptides (Telford et al., 1990).

Inhibition of reconstitution of ribonucleotide reductase activity by antibodies and synthetic peptides. IgG was first purified from mouse ascites fluid or rabbit serum by ion-exchange chromatography using an 8 ml Mono Q column (Pharmacia) with 50 mM-Tris–HCl pH 8.0 and a linear gradient of NaCl from 0 to 400 mM-NaCl. Fractions containing IgG were pooled and concentrated using an Amicon ultrafiltration cell fitted with a YM-5 membrane (M, 5000 cut-off) under nitrogen. The concentration of IgG was adjusted to 2 mg/ml.

The effect of the various IgG and synthetic peptides on RR activity was investigated by preincubating the holoenzyme (500 μg) with the immunoglobulin or peptide (90 μg) for 15 min at 37°C prior to addition of the remaining reagents. To assess their effect on the reconstitution of RR activity, antibodies or oligopeptides (90 μg) were incubated for 15 min at 37°C with the appropriate subunit (280 μg) prior to the addition of the complementary subunit (280 μg) and the reaction mixture.

**Synthetic oligopeptides and generation of antipeptide sera.** Peptide 1 (RNSQFVALMPTA, RR1 positions 939 to 970) was purchased from Peptide and Protein Research. Peptide 3 (YAGAYVNDL; RR2 positions 332 to 340) and peptide 4 (EDVEKDKPNRPY, corresponding to positions 116 to 126 of the 25K protein encoded by the HSV-1 gene US4; McGeoch et al., 1985), were purchased from Cambridge Research Biologicals. Peptide 2 (MKHGLRNSQFVALMPT, RR1 positions 332 to 340) and peptide 4 (EDVEKDKPNRPY, corresponding to positions 116 to 126 of the 25K protein encoded by the HSV-1 gene US4; McGeoch et al., 1985), were purchased from Cambridge Research Biologicals.
Results and Discussion

Sequence analysis of the nucleotide changes in the HSV-1 mutant ts1207

The lesion in ts 1207, which fails to induce any detectable RR activity at the NPT, was located by marker rescue experiments in the Xhol/BglII fragment contained within the coding sequence of RR1 (Preston et al., 1984; Fig. 1). To determine the alteration(s) responsible for the ts mutant phenotype, the entire sequence of the mutant Xhol/BglII fragment was obtained in both orientations using the dideoxynucleotide chain termination method (Sanger et al., 1980) as described in Methods. The nucleotide sequence of the mutant fragment was then aligned with that of the equivalent wt fragment (Fig. 2) obtained previously (Nikas et al., 1986). This analysis resulted in the identification of two nucleotide substitutions in ts1207, both of a guanine with an adenine residue, at HSV-1 nucleotide positions 2439 and 2915. These changes are consistent with the way that the initial multiple mutant tsG, which was used to give rise to ts1207, was generated (Brown et al., 1973), namely using 5'-bromodeoxyuridine, a mutagen which in the enol state induces guanine:cytosine to adenine:thymine transitions (reviewed in Freese, 1963).

Translation of the wt and mutant nucleotide sequences resulted in the identification of two nucleotide substitutions in ts1207, both of a guanine with an adenine residue, at HSV-1 nucleotide positions 2439 and 2915. These changes are consistent with the way that the initial multiple mutant tsG, which was used to give rise to ts1207, was generated (Brown et al., 1973), namely using 5'-bromodeoxyuridine, a mutagen which in the enol state induces guanine:cytosine to adenine:thymine transitions (reviewed in Freese, 1963).

Translation of the wt and mutant nucleotide sequences with the PTRANS program (Taylor, P., 1986) and subsequent comparison of the predicted amino acid sequences with the HOMOL program (Taylor, 1984), demonstrated that the first nucleotide change did not alter the encoded amino acid whereas the second changed the RR1 serine residue at position 961 to an asparagine (Fig. 2).

Structural analysis of the wild-type and mutant RR1 polypeptides

The amino acid sequences of the wt and mutant RR1 proteins were analysed using the secondary structure prediction algorithm of Chou & Fasman (1978), and portions of the program outputs are displayed in Table 1.
Table 1. Secondary structure predictions for the HSV-1 RR1 and HSV-1 ts1207 mutant RR1 polypeptides

<table>
<thead>
<tr>
<th>Amino acid (aa) number</th>
<th>HSV-1 RR1</th>
<th>HSV-1 ts1207 RR1</th>
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</thead>
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<tr>
<td></td>
<td>aa</td>
<td>Hy*</td>
</tr>
<tr>
<td>950</td>
<td>R</td>
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</tr>
<tr>
<td>951</td>
<td>Q</td>
<td>−0.314</td>
</tr>
<tr>
<td>952</td>
<td>S</td>
<td>0.300</td>
</tr>
<tr>
<td>953</td>
<td>M</td>
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</tr>
<tr>
<td>954</td>
<td>M</td>
<td>0.057</td>
</tr>
<tr>
<td>955</td>
<td>K</td>
<td>−0.229</td>
</tr>
<tr>
<td>956</td>
<td>H</td>
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</tr>
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<td>L</td>
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</tr>
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<tr>
<td>970</td>
<td>A</td>
<td>−0.414</td>
</tr>
</tbody>
</table>

* Hy, Hydropathicity value.
† SS, Secondary structure prediction.
‡ H, Strong α-helical conformation.
§ t, Turn.
∥ B, β-Strand conformation.
¶ h, α-Helical conformation.

The program predicted that the wt serine 961 is located in an unstructured region between an α-helix and a β-strand. This prediction is in good agreement with that obtained with the GARNIER program (Garnier et al., 1978; data not shown). In contrast, the asparagine residue of the ts1207 RR1 polypeptide, which replaced the wt serine 961, was predicted to adopt a turn conformation. Further, the predicted β-strand following the wt serine 961 was altered to an α-helix in ts1207 (ts1207 positions 962 to 967; Table 1). These were the only changes predicted in the secondary structure of the mutant RR1 as compared to that of the wt RR1.

The amino acid change in the ts1207 RR1 polypeptide is located within a previously identified highly conserved block of four amino acids present in several RR1 herpesviral polypeptides (termed block 10 by Nikas et al., 1986; Fig. 3). These are RR1 of herpes simplex virus type 2 (HSV-2; Swain & Galloway, 1986), Vmw87 of varicella-zoster virus (VZV; Davison & Scott, 1986) and Vmw93 of Epstein-Barr virus (EBV; Baer et al., 1984). Further, two of these amino acids, the wt serine 961 and the adjacent asparagine, also are conserved in the M1 polypeptide of mouse RR (Caras et al., 1985) and the B1 polypeptide of Escherichia coli RR (Carlson et al., 1984; Nilsson et al., 1988). These residues are polar according to the Venn diagram of amino acid classification (Taylor, W. R., 1986), and as such they have the propensity to be localized in hydrophilic regions of the protein which tend to be the least conserved amino acids between related proteins (Creighton, 1983). Indeed, both residues are located close to the end of a hydrophilic RR1 region (Fig. 3), which was predicted to be the largest hydrophilic region of the RR1 polypeptide (Nikas, 1989). However, both amino acids are highly conserved and this would suggest that they may have a specific function.

Is the site of the ts1207 lesion located at the interface between RR1 and RR2?

As mentioned previously, Frame et al. (1985) demonstrated that, although both the RR1 and RR2 polypeptides are present in ts1207-infected cells, the mutation in RR1 inhibits subunit association at the NPT. In addition, we know by means of gel filtration that the size of the ts1207-encoded RR1 subunit is identical to that of the wt RR1 (our unpublished data), which indicates that the lesion in ts1207 does not prevent the association of RR1 polypeptides to form an RR1 subunit homodimer. These observations, in conjunction with the obtained secondary structure predictions, suggested that the ts1207 mutation may reside within an RR1 region...
HSV-1 RR1 and RR2 subunit association

373

+20

-20

HSV-1 RR1 MKHGLRNSQFVALMPTA (954 to 970)
HSV-2 RR1 MKHGLRNSQFVALMPTA (961 to 976)
VZV Vmw87 CAYGLNSQFVALMPTV (592 to 608)
EBV Vmw93 VRQGLFNSQFIALMPT (580 to 596)
Mouse M1 AKYGERNELLIALAPMPA (580 to 596)
E. coli B1 KTHGRLNSRFLALMPSA (607 to 623)

Block 10

Fig. 3. Alignment of RR1 polypeptide sequences from various organisms from the region in which the ts1207 mutation is located. The amino acid positions of the shown sequences are given in parenthesis. Boxed sequences indicate a stretch of highly conserved amino acids and correspond to block 10, as described elsewhere (Nikas et al., 1986). The diagram above the alignment shows the hydropathicity plot corresponding to the aligned HSV-1 RR1 region. Hydrophobic regions lie between 0 and +20 and hydrophilic regions between 0 and -20.

directly involved in subunit association with RR2. Possibly, the predicted change of the wt x-helix to a b-strand in ts1207 alters the secondary structure of this RR1 region at the NPT and so the two subunits are unable to associate. Alternatively, this region of RR1 may not be directly involved in subunit association but instead be a structural entity important for full integrity of the molecule. The alteration(s) to the secondary structure around amino acid 961 in ts1207 RR1 may affect the structure of actual contact regions located elsewhere in the polypeptide backbone which, at the NPT, generates inactive enzyme.

To differentiate between these two possibilities, we used synthetic oligopeptides which correspond to the wt sequence of the mutation site. This approach followed from the earlier observations of Dutia et al. (1986) and Cohen et al. (1986) that a synthetic nonapeptide corresponding to the C terminus of RR2 can specifically inhibit enzyme activity. For this reason, peptides 1 (959 to 970) and 2 (954 to 969), which correspond to wt RR1 amino acid sequences that flank serine 961, were tested as described in Methods. These experiments also included peptide 3 (RR2, 332 to 340), which is the inhibitory nonapeptide, and peptide 4 (US4, 116 to 126), which corresponds to an amino acid sequence of the HSV-1 25K protein (McGeoch et al., 1985) as controls.

The peptides were tested in vitro for their effect on the wt activity of RR from partially purified HSV-1-infected cell extracts. Further, because an active holoenzyme can be formed by the intact RR1 and RR2 subunits, each contained in partially purified extracts of ts1222- and ts1207-infected cells respectively (Darling et al., 1988; Huang et al., 1988), the oligopeptides were also tested in vitro for their effect on the reconstituted activity. The latter approach was followed because a possible RR1 site involved in subunit interaction may be buried deep in the

Fig. 4. Effect of synthetic oligopeptides on HSV-1 RR activity. (a) The holoenzyme was preincubated with peptide for 15 min prior to the addition of the reaction mixture as described in Methods, except that to increase the sensitivity of the assay the final concentration of CDP was reduced to 20 µM and that of [3H]CDP increased to 2 µCi. (b) Reconstituted enzyme. The peptides used were preincubated for 15 min with the ts1207-infected cell extract (280 µg) prior to the addition of the ts1222-infected cell extract (280 µg) and the reaction mixture. Peptide 3 was assayed by preincubation with the ts1222-infected cell extract first followed by the addition of the ts1207-infected cell extract and the reaction mixture. (●) Peptide 1 (RNSQFVALMPA, corresponding to HSV-1 RR1 amino acids 959 to 970); (▲) peptide 2 (MKHGLRNSQFVALMPA, corresponding to HSV-1 RR1 amino acids 954 to 970); (◇) peptide 3 (YAGAVVNDL, corresponding to the nine C-terminal amino acids of HSV-1 RR2); (■) peptide 4 (EDVEKDKNRPY, corresponding to amino acids 116 to 126 of the HSV-1 25K protein product of gene US4 plus a C-terminal tyrosine).
subunit interface once the complex has been formed and thus be inaccessible to the peptides, although recent experiments indicate that this may not be a problem as the two subunits exist in equilibrium between associated and dissociated states (Darling et al., 1990).

As shown in Fig. 4(a), peptides 1 and 2 did not inhibit RR activity, although a slight reduction in enzyme activity was seen at high concentrations of peptide 3. At low peptide concentrations, peptide 1 was stimulatory. In contrast, peptide 3 completely inhibited activity whereas peptide 4 had no effect. This experiment was repeated to assess the effect of the peptides on the reconstituted activity. For this purpose, peptides were pre-incubated with the RR2 subunit (except for peptide 3, which was pre-incubated with RR1) followed by the addition of the complementary subunit prior to assay (Fig. 4b). Again, similar results were obtained: peptides 1 and 4 stimulated RR activity at low concentrations, peptide 2 was slightly inhibitory and peptide 3 was highly inhibitory.

It is not possible to assess the significance of the slight inhibition seen with the peptide 2 although the observation is quite reproducible. The peptide contains five amino acids (MKHGL) at its amino terminus which are absent from the non-inhibitory peptide 1. Perhaps these amino acids constitute part of a site of subunit interaction and mediate the slight inhibitory effect. Another possibility would be that peptide 2 is able to assume a conformation more inhibitory of subunit association. In spite of these possibilities, the simplest interpretation of the observation that peptides 1 and 2 do not markedly inhibit enzyme activity is that the site of the mutation is not directly involved in subunit association.

To investigate this interpretation further, we tested antiserum C2, directed against a peptide encompassing the site of the ts1207 lesion, and a number of control antisera for their ability to inhibit enzyme activity (Table 1). We reasoned that, if the site were at the interface between RR1 and RR2, the attachment of an antibody to this site would sterically hinder subunit association and hence enzyme activity.

As shown in Table 2, antiserum C2 had no effect either on the native enzyme activity or on the reconstituted activity. In contrast, antiserum 14995 (directed against the nonapeptide YAGAVVNDL) inhibited the reconstituted activity by almost 79%, though it did not inhibit the wt activity. Others (Cohen et al., 1986) have reported an antiserum, P9, made against the same nonapeptide which weakly neutralized (50%) native enzyme. We assume their serum to have higher avidity or additional specificities.

One of the monoclonal antibodies, MAb 7602, completely neutralized both native and reconstituted enzyme activity. It will be informative to identify the epitope recognized by this antibody. Most likely it will be a discontinuous one because MAb 7602 does not react on Western blots (data not shown) and could be directed against the active site. The MAbs 932, 1026 and 535 had no effect on native enzyme activity but inhibited reconstituted activity by approximately 50%. These antibodies recognize the native holoenzyme (our unpublished data) so they are probably not directed against epitopes which are directly involved in subunit association. It is more likely that they are directed against epitopes which lie close to a site of RR1/RR2 subunit interaction and that the observed inhibitory effect may be due to steric hindrance by the antibody, preventing efficient subunit reassociation. Finally, MAb 1100, 1033 and 7689 did not inhibit either native or reconstituted enzyme activity. We infer that they are not directed towards sites on RR1 needed for association with RR2 or catalytic activity.

In conclusion, we have identified a point mutation in RR1 which renders the enzyme inactive. The site of this mutation is important for the functional conformation of RR1 because, at the NPT in cells infected with ts1207, association of RR1 and RR2 is inhibited (Frame et al., 1985). However, neither peptides corresponding to the wt sequence and encompassing this site nor antiserum C2 (raised against peptide 1 of RR1) markedly inhibited enzyme activity or the functional reassociation of the subunits. The lack of inhibition by the C2 antiserum together with the fact that it strongly immunoprecipitates the active holoenzyme (data not shown) would suggest that the ts1207 mutation is not located at the subunit interface, but at a distant site on the surface of the molecule.
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