Identification of a Herpes Simplex Virus Type 1 Polypeptide Which Is a Component of the Virus-induced Ribonucleotide Reductase

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SUMMARY

We have characterized a temperature-sensitive mutant of herpes simplex virus type 1 (HSV-1), 17tsVP1207, that induces a thermolabile ribonucleotide reductase activity. This mutant was derived from the multiple mutant tsG. Fine-structure mapping studies showed that the defect in 17tsVP1207 lies within an 800 bp sequence between genome map coordinates 0.580 and 0.585 in the gene encoding a polypeptide of 140000 mol. wt. (Vmw136, ICP6). Since the mutation in this polypeptide produced a temperature-sensitive ribonucleotide reductase activity, Vmw136 must be a component of the herpes simplex virus-induced ribonucleotide reductase. The mRNA of Vmw136 has a common 3' terminus with an mRNA encoding a 38000 mol. wt. polypeptide (Vmw38). Although the polypeptide-coding sequences of these mRNAs do not overlap, monoclonal antibodies against Vmw136 immunoprecipitated Vmw38 as well as Vmw136 from wild-type HSV-1-infected cells. Our data do not exclude the possibility that Vmw38 is part of the ribonucleotide reductase complex but suggest that this species on its own is not responsible for the HSV-induced enzyme activity.

INTRODUCTION

The mammalian ribonucleotide reductase, a key enzyme in DNA synthesis, catalyses the reduction of the four ribonucleotides to the corresponding deoxyribonucleotides. In contrast to its cellular counterpart, the herpes simplex virus (HSV)-induced enzyme does not have an absolute requirement for Mg\(^{2+}\) and ATP, and is insensitive to allosteric regulation by dTTP and dATP (Cohen, 1972; Ponce de Leon et al., 1977; Langelier et al., 1978; Huszar & Bacchetti, 1981; Averett et al., 1983). Antiserum against the partially purified HSV-2 enzyme has been shown to immunoprecipitate several virus-infected cell-specific polypeptides, the major species of which has a mol. wt. of approximately 144000 (Huszar & Bacchetti, 1983; Huszar et al., 1983). Monoclonal antibodies which immunoprecipitate two polypeptides of approximate mol. wt. 144000 and 38000 from HSV-1 and HSV-2-infected cell extracts, and react with the virus-induced ribonucleotide reductase have also been described (Huszar & Bacchetti, 1983; Bacchetti et al., 1984).

Recently, an HSV-1 mutant, tsGsyn\(^+\), has been reported which induces at the permissive temperature (PT) a ribonucleotide reductase activity that was thermostable both in vivo and in vitro (Dutia, 1983). It was clear, however, from genetic experiments that tsGsyn\(^+\) contained more than one defect. For this reason, the mutated sequence affecting the ribonucleotide reductase activity was cloned into a bacterial plasmid vector and recombined into HSV-1 ts\(^+\) strain 17 DNA. This paper reports the isolation and characterization of the mutant 17tsVP1207 (ts1207) and identification of a virus-specified constituent of the induced ribonucleotide reductase.

METHODS

Cells. BHK-21 clone 13 cells (Macpherson & Stoker, 1962) were grown in Eagle's medium containing twice the standard concentration of vitamins and amino acids, 10% tryptose phosphate broth and 10% calf serum. Human foetal lung cells (Flow 2002) were propagated in Eagle's medium containing foetal calf serum instead of calf serum.
Virus. The wild-type HSV-1 was strain 17. The mutant tsGsyn+ was isolated from a bromodeoxyuridine-mutagenized stock of this strain (Brown et al., 1973). Since this mutant contained several ts lesions, the BamH1 o fragment, which contained one of the mutations, was cloned into the bacterial plasmid vector pAT153 and subsequently recombined into HSV-1 ts+ strain 17 DNA. From this transfection experiment the mutant 17tsVP1207 was isolated. For brevity, in this paper the mutant is referred to as ts1207. The HSV-1 KOS mutants tsT36, tsV37 (Chu et al., 1979) and tsX45 (Machtiger et al., 1980), which have lesions within genome map units 0.49 to 0.64 (Parris et al., 1980; Pancake et al., 1983), were kindly provided by P. A. Schaffer.

Ribonucleotide reductase assay. Crude extracts were obtained from virus- or mock-infected cells as described by Dutia (1983). Partially purified cellular and virus-induced ribonucleotide reductase was prepared according to the protocol of Huszar & Bacchetti (1981). Radioactive ribonucleotide reductase was obtained from virus- or mock-infected cells labelled from 1 to 14 h post-infection with 100 μCi/ml [35S]methionine (1030 Ci/mmol) in Eagle's medium containing one-fifth the normal concentration of methionine. The assay was carried out as described by Dutia (1983) except that Tris–HCl buffer in the standard reaction mix was replaced by 50 mM-HEPES pH 7.6.

Marker rescue. Marker rescue was performed as described by Stow et al. (1978) with the modification of Preston (1981). Restriction endonuclease fragments used in marker rescue were separated by agarose gel electrophoresis in the presence of ethidium bromide (Wilkie & Cortini, 1976). The DNA was visualized under long-wave u.v. light and excised from the gels. The DNA was eluted from the gel slices by high voltage electrophoresis and concentrated by ethanol precipitation.

Complementation test and single-step growth of virus. The yield test was carried out as described by Brown et al. (1973) except that cell monolayers of human foetal lung cells (Flow 2002) were used instead of BHK-21 clone 13 cells in suspension. Single-step growth of virus in BHK cells was performed in the same way as the yield complementation test. In these experiments an m.o.i. of 5 p.f.u./cell was used.

Synthesis of virus DNA in exponentially growing and serum-starved cells. Serum-starved resting cells were obtained essentially as described by Jamieson et al. (1974) except that cells were grown in medium containing 0-5%, instead of 1% calf serum. BHK cells were infected at an m.o.i. of 5 p.f.u./cell and incubated at 31 °C (PT) or 39.5 °C (non-permissive temperature, NPT). At 18 h post-infection, virus- and mock-infected cells were scraped into the medium, pelleted by low-speed centrifugation, washed once in 1 × SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate) and resuspended in 1 × SSC containing 2% (w/v) SDS and 25 µg/ml proteinase K. After incubation for 3 h at 37 °C, the DNA was phenol-extracted and concentrated by ethanol precipitation. The deproteinized DNA was treated with RNase A (100 µg/ml), re-extracted with phenol and chloroform and again concentrated by ethanol precipitation. The DNA was denatured with NaOH and immobilized on nitrocellulose filters essentially as described by Kafatos et al. (1979) using a manifold (Bethesda Research Laboratories).

The plasmid containing HSV-1 EcoRI sequences (pGX38) was labelled in vitro with [32P]dCTP and [32P]dGTP by nick translation (Rigby et al., 1977) to a specific activity of about 108 c.p.m. per µg DNA. This probe was denatured with NaOH and hybridized to the denatured virus-infected cell DNA as described by Park et al. (1983).

Monoclonal antibodies. Monoclonal antibodies 48S and 2S were kindly provided by M. Zweig. The other monoclonal antibody, MA1026, was prepared essentially as described by Palfreyman et al. (1983) except that the SP2/0-Ag14 cell line (Schulman et al., 1978) was used as the parental myeloma cell in fusion reactions.

Immunoprecipitation of ribonucleotide reductase. Samples containing 300 µg of partially purified ribonucleotide reductase were diluted 1:1 with 0.2 M-Tris–HCl pH 8.0, 0.20% (v/v) glycerol, 1% (v/v) NP40, 1% (w/v) sodium deoxycholate (2 × extraction buffer; Showalter et al., 1981). The diluted enzyme was incubated for 3 h on ice with antibody, then 40 µl of a 50% (v/v) suspension of Protein A-Sepharose beads in 1× extraction buffer was added and incubation was continued for a further 1 h. The Protein A-Sepharose-antigen antibody complexes were pelleted by centrifugation and the supernatants assayed for ribonucleotide reductase activity. The pellet was treated for SDS-PAGE as described by Zweig et al. (1979) and the polypeptides subsequently separated on a 7-5% polyacrylamide gel (Marsden et al., 1978).

Polypeptide nomenclature. Virus-infected cell polypeptides are labelled according to their estimated molecular weights on SDS-polyacrylamide gels (Marsden et al., 1978). Thus, the virus-induced polypeptides of mol. wt. 136000 and 38000 are described as Vmw136 and Vmw38. Mapping data (Marsden et al., 1978) suggest that Vmw136 is equivalent to the 140000 mol. wt. polypeptide of Anderson et al. (1981) and ICP6 (Honess & Roizman 1973, 1974; Wilcox et al., 1980).

RESULTS

Identification of the gene involved in ribonucleotide reductase activity

A lesion in tsGsyn+, which induces a thermolabile ribonucleotide reductase activity (Dutia, 1983), was located by marker rescue within BamHI o sequences (map units 0.574 to 0.600). It was clear, however, from marker rescue experiments that tsGsyn+ contained more than one defect since the 'rescued' ts+ virus had a lower relative efficiency of plating (e.o.p.; NPT/PT)
**HSV-1 ribonucleotide reductase**

![Diagram of HSV-1 BamHI o restriction map]

**Fig. 1.** Map location of the ts1207 mutation. The solid part of BamHI o represents the region in which the ts lesion maps. The positions of transcripts are taken from Anderson *et al.* (1981) and McLauchlan & Clements (1983a). The mutation was rescued with restriction endonuclease fragments from pGX36 (contains the HSV-1 ts + strain 17 BamHI o fragment). The relative efficiency of plating was calculated from the yield of progeny virus at the PT and NPT from the transfected cells.

**Table 1.** Ribonucleotide reductase activity induced by HSV-1 strain 17 and ts1207 at 31 and 39.5 °C

<table>
<thead>
<tr>
<th>Virus</th>
<th>Temperature (°C)</th>
<th>Ribonucleotide reductase activity (% HSV-1 wild-type activity at 31 °C)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 strain 17</td>
<td>31.0</td>
<td>100.0</td>
</tr>
<tr>
<td>ts1207</td>
<td>31.0</td>
<td>14.7</td>
</tr>
<tr>
<td>HSV-1 strain 17</td>
<td>39.5</td>
<td>85.2</td>
</tr>
<tr>
<td>ts1207</td>
<td>39.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* The enzyme activity in mock-infected cells has been subtracted from all values.

than wild-type virus. For this reason, the BamHI o fragment from the mutant was cloned into a plasmid vector and subsequently recombined into HSV-1 ts + strain 17 DNA. From this transfection experiment the mutant ts1207 was isolated. The failure of ts1207 to induce ribonucleotide reductase activity at the restrictive temperature (Table 1) strongly suggested that the gene encoding part or all of the virus-induced ribonucleotide reductase activity mapped within BamHI o. The mutation was mapped more finely by DNA transfection experiments in which separated KpnI plus XhoI, and BglII plus EcoRI double-digest fragments from pGX36, which contains the HSV-1 ts + BamHI o fragment, were used in marker rescue of the mutant. The lesion mapped within an 800 bp fragment in the region common to XhoI plus KpnI b, and BglII plus EcoRI a (Fig. 1). Two mRNAs, 5.0 kb and 6.9 kb in size, have been located in this part of the genome. The 5 kb mRNA, which encodes a polypeptide of mol. wt. 140000 (Vmw136), is synthesized early in infection whereas the 6.9 kb mRNA, which specifies a 54000 mol. wt. polypeptide, is a late message (Anderson *et al.*, 1981).

Nucleotide sequence and S1 nuclease mapping data strongly suggest that the coding sequences for the 54000 mol. wt. polypeptide lie upstream from the translational start signal of Vmw136 (Anderson *et al.*, 1981; J. McLaughlan & J. B. Clements, personal communication). The ts1207 lesion has therefore resulted in an amino acid change in Vmw136. Since the mutation in this polypeptide produces a ts ribonucleotide reductase activity, Vmw136 must be a component of the HSV-1-induced enzyme.
Table 2. Complementation of ts1207 with mutants from defined complementation groups

<table>
<thead>
<tr>
<th>Cistron</th>
<th>Mutant</th>
<th>ts1207</th>
<th>tsT</th>
<th>tsV</th>
<th>tsX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-27</td>
<td>tsT</td>
<td>1*</td>
<td>56-1</td>
<td>26-9</td>
<td>9-0</td>
</tr>
<tr>
<td>1-29</td>
<td>tsV</td>
<td>1</td>
<td>201.0</td>
<td>13-2</td>
<td></td>
</tr>
<tr>
<td>1-31</td>
<td>tsX</td>
<td>1</td>
<td>10-3</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* Values represent complementation indices calculated from the formula given in Brown et al. (1973) for the yield complementation test.

Complementation studies with ts1207

Previous work by Schaffer et al. (1978) assigned tsGsyn+, the multiple mutant containing the ts1207 lesion, to the same complementation group as tsN20. Since, however, the tsN20 mutation mapped in a different part of the genome from the ts1207 defect, complementation tests were carried out using defined mutants which contained lesions close to or within the BamHI o region of HSV-1. The three mutants tested, tsT, tsV and tsX, all gave positive complementation indices with ts1207 (Table 2). Crosses with tsX gave lower indices than the other genetic crosses because this mutant is leaky, even at a high NPT of 39.5 °C. From the results, ts1207 appears to be in a different cistron from tsT, tsV and tsX. This conclusion, however, should be considered tentative until more precise map locations of the tsT, tsV and tsX lesions are known. The gene encoding Vmw136 is large and false positive complementation values could be obtained as a consequence of intragenic recombination (Preston, 1981).

Mutant virus growth and DNA synthesis

As mentioned earlier, ts1207 failed to induce detectable amounts of virus-specific ribonucleotide reductase activity at 39-5 °C (Table 1). Interestingly, at the PT of 31 °C, this
mutant induced considerably lower amounts of this enzyme than wild-type virus. To find out whether the reduced levels of ribonucleotide reductase activity affected growth of ts1207 at 31 °C, single-step growth of the mutant and wild-type virus were compared. Fig. 2 shows that although ts1207 grew slightly more slowly than ts+ strain 17 at 31 °C, the final yield of mutant virus was similar to wild-type virus. At NPT, ts1207 was leaky. It should be noted that at this temperature lower amounts of ts+ strain 17 were produced compared with the yield of virus at 31 °C.

Initially, the DNA phenotype of ts1207 at the NPT was determined by isopycnic density gradient centrifugation of 3H-labelled virus-infected cell DNA. These experiments showed that the HSV ribonucleotide reductase was required for virus DNA replication since the mutant synthesized considerably reduced amounts of virus DNA at the NPT compared with wild-type virus. There are several explanations for the small amount of virus DNA present in ts1207-infected cells at 39.5 °C. The mutant could be leaky and induce sufficient functional ribonucleotide reductase, below the limits of detection of the enzyme, for limited DNA synthesis to occur. Alternatively, there could be sufficient deoxyribonucleotide pools supplied by the host ribonucleotide reductase to permit some virus replication. In order to try to distinguish between these possibilities, the DNA phenotype of ts1207 in exponentially growing and serum-starved cells was determined by dot-blot hybridization, using cloned HSV-1 EcoRI f as a probe (Fig. 3).

The amount of virus DNA present in each sample was estimated from densitometer tracings of the autoradiograph. In exponentially growing cells, ts1207 synthesized approximately tenfold less DNA than did wild-type virus at 39.5 °C. In resting cells at 39.5 °C, the mutant made considerably lower amounts of DNA, the levels of which were barely above background. At this temperature and at 31 °C, however, wild-type virus DNA replication in serum-starved cells was about three- to fourfold lower than in exponentially growing cells. For this reason, it is difficult to assess the role of the host ribonucleotide reductase at the NPT. It is, nevertheless, clear that

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**Fig. 3.** Spot hybridization of pGX38 (contains HSV-1 EcoRI f) to mock- and virus-infected cell DNA extracted from exponentially growing (E) and resting (R) cells or mock-infected (MI) cells. DNA (0.5 μg) was diluted serially twofold and spotted on to nitrocellulose paper. Control HSV-1 strain 17 DNA represents in lane 1, 1000 copies (10 ng); 2, 200 copies; 3, 100 copies; 4, 20 copies; 5, 10 copies; 6, 2 copies; 7, 1 copy; 8, 0.2 copy of HSV-1 DNA per cell.
Fig. 4. Immunoprecipitation of polypeptides from partially purified ribonucleotide reductase extracts by monoclonal antibodies. Polypeptides labelled with [35S]methionine were eluted from Protein A-Sepharose-antigen-antibody complexes, separated on a 7.5% polyacrylamide gel and detected by fluorography. Lanes 1 and 2 show the partially purified extracts from which the immunoprecipitations were made. The other lanes show the HSV-1-infected and mock-infected (MI) cell polypeptides immunoprecipitated by control ascitic fluid (C) and the indicated monoclonal antibodies. No polypeptides were immunoprecipitated from mock-infected cell extracts.

either the cellular ribonucleotide reductase cannot supply sufficient amounts of deoxyribonucleotides for virus DNA synthesis or that the host enzyme functions very inefficiently in HSV-infected cells because it is sensitive to allosteric regulation. Similar conclusions have been drawn by Leary et al. (1983) who found that HSV was sensitive to hydroxyurea even when grown on hydroxyurea-resistant cells. It is of interest, however, to note that ts1207 synthesized similar amounts of DNA to wild-type virus at 31 °C both in exponentially growing and resting cells, even though this mutant induced considerably lower amounts of ribonucleotide reductase than wild-type virus.
HSV-1 ribonucleotide reductase

Immunoprecipitation of the ribonucleotide reductase

Earlier work by Huszar & Bacchetti (1983) showed that the HSV-2-induced ribonucleotide reductase was inhibited by a monoclonal antibody that precipitated, in addition to HSV-2 Vmw38, small amounts of a 144000 mol. wt. polypeptide from virus-infected cell extracts. We were interested in confirming their results with HSV-2 and finding out whether there was an association between Vmw38 and Vmw136 in HSV-1-infected cells. Three monoclonal antibodies, 48S, 2S and MA1026, against HSV-1 Vmw136 were tested. These antibodies did not directly inhibit HSV-1 wild-type-induced ribonucleotide reductase activity but when antibody–antigen complexes were immunoprecipitated with Protein A-Sepharose, the enzyme was removed from the supernatant and could be recovered from the pelleted material (data not shown). Fig. 4 shows the polypeptides immunoprecipitated by the monoclonal antibodies from partially purified enzyme preparations of HSV-1 ts ÷ strain 17-infected cells. The antibodies precipitated two polypeptides with approximate mol. wt. 140000 and 38000 from all the enzyme extracts tested. Interestingly, the amount of Vmw38 that immunoprecipitated along with Vmw 136 of HSV-1 strain 17 was consistently less than the amount of the HSV-2 counterpart that immunoprecipitated along with the Vmw136 of HSV-2 strain HG52 (B. M. Dutia & J. W. Palfreyman, unpublished results). The reason for the presence of minor polypeptides in the immunoprecipitates is not known; they may represent degradation products of Vmw136.

DISCUSSION

We have characterized a mutant of HSV-1 that has a ts ribonucleotide reductase activity and have identified a polypeptide of 140000 mol. wt. (Vmw136; ICP6) which is an essential component of the virus-induced enzyme. The mRNA of Vmw136 is a major early species although small amounts can be detected in virus-infected cells when cycloheximide has been added from the time of virus infection (Holland et al., 1979, 1980; Watson et al., 1979; McLauchlan & Clements, 1982). The polypeptide Vmw136 is phosphorylated (Marsden et al., 1978; Wilcox et al., 1980) and is predominantly cytoplasmic in its location (Wilcox et al., 1980; Showalter et al., 1981). In HSV-1 and HSV-2 monoclonal antibodies have been isolated which bind to the ribonucleotide reductase and immunoprecipitate two virus-specific polypeptides of mol. wt. 144000 and 38000 (Huszar & Bacchetti, 1983; Bacchetti et al., 1984). We have confirmed these results in HSV-1 using monoclonal antibodies against HSV-1 Vmw136. The three monoclonal antibodies examined all precipitated a 38000 mol. wt. polypeptide (Vmw38) as well as Vmw136 from ts ÷ strain 17-infected cells. Although the mRNA of Vmw136 has a common 3' terminus with the mRNA of Vmw38 (McLauchlan & Clements, 1982) the polypeptide-coding sequences do not overlap (Draper et al., 1982, McLauchlan & Clements, 1983a). It is, however, still possible that these two polypeptides have common antigenic sites. Alternatively, Vmw136 and Vmw38 could be tightly bound together in an enzyme complex which is precipitated by antibody to a single component of the complex. Since small amounts of Vmw38 were present in partially purified HSV-1 and HSV-2 ribonucleotide reductase preparations (Huszar & Bacchetti, 1983; Huszar et al., 1983; B. Dutia, unpublished results), it is likely that Vmw136 and Vmw38 are present in a complex.

The mammalian ribonucleotide reductase consists of two non-identical subunits, M1 and M2 (Thelander et al., 1980) the monomers of which have molecular weights of 84000 and 55000 respectively. Although the HSV and pseudorabies virus-induced enzymes have similar biochemical properties to the host cell enzyme, for example sensitivity to hydroxyurea (Langelier & Buttin, 1981; Lankinen et al., 1982), and for pseudorabies virus at least, a stable tyrosine-free radical (Lankinen et al., 1982), there are differences in Mg2+ and ATP requirements and in allosteric controls (Cohen, 1972; Ponce de Leon et al., 1977; Langelier et al., 1978; Huszar & Bacchetti, 1981; Averett et al., 1983). In these latter properties the HSV ribonucleotide reductase resembles the bacteriophage T4-specific enzyme (Berglund, 1972a,b).

Two regions of the HSV-2 genome which morphologically transform cells in vitro have been identified (Reyes et al., 1979; Jariwalla et al., 1980; Galloway & McDougall, 1981). One region
lies within the BglII n fragment (map coordinates 0.58 to 0.63) and the other is within the adjacent fragment BglII c (map coordinates 0.41 to 0.58). The HSV-2 equivalent of HSV-1 Vmw38 is a major species encoded entirely within BglII n whereas the HSV-2 Vmw136 is partly encoded by BglII c and partially by BglII n (Galloway et al., 1982; McLauchlan & Clements, 1983b). There has been speculation that the HSV-2 polypeptide Vmw38 might encode the virus-specific ribonucleotide reductase and thereby trigger morphological transformation by causing an imbalance in deoxyribonucleotide pools in the cell which would enhance mutagenesis (Huszar & Bacchetti, 1983). Our results, together with information on nucleotide sequence homology between HSV-1 and HSV-2 (McLauchlan & Clements, 1983b) do not exclude the possibility that this polypeptide (or the equivalent Vmw38 of HSV-1) is part of the ribonucleotide reductase complex but suggest that the polypeptide on its own is not responsible for the enzyme activity. Recent results of Galloway & McDougall (1983), who used subfragments of BglII n to morphologically transform cells, suggest that Vmw38 is not required for the initiation of this process. It is also unlikely that Vmw143 of HSV-2 is involved in transformation because the coding sequences of this polypeptide are not completely contained within BglII n or c.

Correlation of the failure of the mutant to induce the enzyme activity at the restrictive temperature with the inability of the virus to grow at that temperature indicates that the HSV ribonucleotide reductase is essential for virus replication. Our results provide strong evidence that at least part of the virus ribonucleotide reductase is encoded by the gene of Vmw136. Since the enzyme is required for virus growth it is potentially a very useful target for antiviral drugs. Knowledge of the specific virus polypeptide required for enzyme activity should greatly aid this field of research.

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