Ribonucleotide Reductase Induced by Herpes Simplex Virus has a Virus-specified Constituent

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(Accepted 1 October 1982)

SUMMARY

Ribonucleotide reductase, an enzyme found in all prokaryotic and eukaryotic cells that synthesize DNA, is induced by herpes simplex virus (HSV). In this study the effect of anti-HSV antiserum on the induced ribonucleotide reductase has been examined and the ability of different temperature-sensitive (ts) mutants of HSV-1 to induce the enzyme has been investigated. The HSV-1-induced ribonucleotide reductase was inhibited by antiserum raised against infected cell lysates but not by preimmune serum. The wild-type (ts+ ) virus induced similar levels of ribonucleotide reductase at 31 °C and 38.5 °C (the permissive and non-permissive temperatures respectively for the ts mutants). All ts mutants induced approximately wild-type levels of the enzyme at 31 °C. At 38.5 °C, two of the four ts mutants studied also induced wild-type levels of enzyme but ts G failed to induce any activity while ts K induced variable but low levels. The enzyme activity induced by ts G at 31 °C was thermolabile both in vivo and in vitro. These results provide the first strong evidence that the induced ribonucleotide reductase activity is at least partially virus-coded.

INTRODUCTION

Herpes simplex virus (HSV) induces a number of enzymes involved in DNA metabolism including a DNA polymerase (Keir & Gold, 1963), deoxynucleoside pyrimidine kinase (Kit & Dubbs, 1963; Jamieson et al., 1974), exonuclease (Keir & Gold, 1963) and ribonucleotide reductase (Cohen, 1972). Considerable changes in the dNTP concentration in HSV-infected cells have been reported (Jamieson & Bjursell, 1976a, b) and it is likely that these reflect an altered intracellular environment where the presence of HSV-induced enzymes enables virus DNA synthesis to take place under conditions where host DNA synthesis is inhibited.

Ribonucleotide reductase occupies a central role in DNA synthesis. It catalyses synthesis of all four deoxyribonucleotides by direct reduction of the corresponding ribonucleotides. The enzyme is found in all cells synthesizing DNA and the levels of activity suggest that it is involved in regulation of DNA synthesis. Nucleotides act as positive and negative effectors in a complex allosteric control of the enzyme's substrate specificity and activity (Thelander & Reichard, 1979). The mammalian enzyme consists of two non-identical subunits which are inactive alone (Thelander et al., 1980; Eriksson & Martin, 1981).

The HSV-induced CDP reductase activity differs from the mock-infected cell enzyme activity in a number of biochemical properties. It is refractory to concentrations of dTTP and dATP which completely inhibit the mock-infected activity and, unlike the cellular enzyme, has no absolute requirement for Mg2+ (Ponce de Leon et al., 1977; Langelier et al., 1978; Huszar & Bacchetti, 1981). Induction of the enzyme is correlated with multiplicity of infection, requires RNA and protein synthesis and can be prevented by u.v. irradiation of the virus stock (Langelier & Buttin, 1981). However, although the data support virus involvement in induction of the enzyme activity, there is no compelling evidence that it is virus-coded.

Temperature-sensitive (ts) mutants have proved to be a potent tool in providing genetic evidence that the HSV DNA polymerase and alkaline exonuclease are virus-coded (Crombie, 1975; Hay et al., 1976; Francke et al., 1978). The association of a thermolabile enzyme with a ts
mutation is strong evidence that at least one constitutive polypeptide of the enzyme is virus-coded. In the experiments reported here, ts mutants of HSV-1 have been used to study the induction of ribonucleotide reductase and a mutant which induces a thermolabile enzyme has been used to show that the enzyme activity is at least partially coded by HSV.

METHODS

Cells and virus. BHK-21 clone 13 cells (Macpherson & Stoker, 1962) were maintained in Eagle’s medium supplemented with 10% tryptose-phosphate broth and 10% calf serum. HSV-1 strain 17 wild-type (ts+) and ts mutants derived from strain 17 have been described previously (Brown et al., 1973; Marsden et al., 1976). With the exception of ts 1, all mutants were of the non-syncytial (syn−) phenotype. Spontaneous revertants of ts G syn+ (G+ rev 1 and G+ rev 2) were isolated by D. Dargan. These revertants may be clonally related. G+ rev 2 is a syncytial revertant of ts G syn+.

For assay of ribonucleotide reductase activity, cells grown to 75% confluency on 90-mm plastic dishes or in 80-oz roller bottles in Eagle’s medium supplemented with 10% calf serum (EC10) were infected at an m.o.i. of 5 or mock-infected. After 1 h adsorption at the appropriate temperature, the inoculum was replaced with pre-warmed EC10.

Antiserum. Antiserum to high salt extracts of HSV-1 strain 17-infected 3T12-3 cells was raised in rabbits and was a gift from Mr R. G. Dalziel.

Preparation of extracts for ribonucleotide reductase assays. Infected or mock-infected cells were harvested at specified times by scraping into the medium. The cells were pelleted by low-speed centrifugation, washed twice with ice-cold phosphate-buffered saline (136 mM-NaCl, 2.8 mM-KCl, 8.0 mM-Na2HPO4, 1.44 mM-KH2PO4, pH 7.2, 0.68 mM-CaCl2, 0.49 mM-MgCl2), and frozen in 50 mM-Tris-HCl pH 7.8, 1 mM-dithiothreitol at −70 °C. After thawing, the cells were disrupted by sonication and centrifuged either at 1800 g for 10 min or 43500 g for 30 min. Extracts were stored at −70 °C. Protein concentration was determined as described by Lowry et al. (1951).

Ribonucleotide reductase assay. The assay was carried out essentially as described by Langelier et al. (1978) except that magnesium was routinely omitted. The standard reaction mixture contained 5-4 mM-Tris-HCl pH 7.0, 0.054 mM-FeCl3, 4.3 mM-NaF, 10 mM-dithiothreitol, 2.7 mM-ATP, 0.054-CDP, 11 μCi/ml [3H]CDP (11 Ci/mmol) and 1 mg enzyme extract. When optimal conditions for the mock-infected cell ribonucleotide reductase activity were required, 2 mM-magnesium acetate was added as indicated. The total volume was 90 μl or, when the concentration of protein in the extract was low, multiples of this. After incubation for 60 min at 31 °C, the assay was stopped by addition of 10 M-perchloric acid to a final concentration of 1 M. The precipitated protein was removed by centrifugation and the nucleotides in the supernatant were converted to their monophosphates by incubation in a boiling water bath for 15 min. After cooling, the supernatants were neutralized with KOH and the precipitated potassium perchlorate removed by centrifugation. Aliquots of the supernatant to which unlabelled CMP and dCMP had been added as markers were spotted on Whatman grade 1 chromatography paper and the nucleotides were separated by descending chromatography in saturated sodium tetraborate:5 M-ammonium acetate:0.5 M-EDTA :ethanol (80:20:0.5:220, by vol.) for 40 h (Reichard, 1958). After drying, spots were visualized by u.v. cut out and the radioactivity counted in toluene/PPO.

Effect of antiserum on ribonucleotide reductase activity. Aliquots of crude extract containing 1 mg protein were incubated with preimmune or immune rabbit serum at 37 °C for 30 min, cooled on ice and assayed for ribonucleotide reductase activity. Mock-infected assays contained 2 mM-magnesium acetate.

Thermostability in vitro. Aliquots of crude extract containing 1 mg protein were incubated for appropriate times in a water bath at 38.5 °C or 45 °C. After cooling on ice, the extract was assayed for ribonucleotide reductase activity.

Thermostability in vivo. Infected cells were incubated for 15 h at 31 °C, and then the medium was replaced with pre-warmed EC10 with or without 50 μg/ml cycloheximide and incubation continued at 38.5 °C. Cells were harvested at appropriate times after shift up and assayed for ribonucleotide reductase.

Materials. [3H]CDP was purchased from Amersham International. All other chemicals were obtained from Sigma or BDH (Analar grade).

RESULTS

Induction of ribonucleotide reductase

Fig. 1 shows that infection of BHK-21 C13 cells with HSV-1 strain 17 ts+ results in a five- to tenfold elevation of the CDP reductase activity over that found in mock-infected cells. The maximum levels of activity induced were the same at both 31 °C and 38.5 °C. At 31 °C the activity peaked at 15 h post-infection while at 38.5 °C the peak activity was reached at 6 h. In the experiments described below, virus-infected cells were harvested after 6 h at 38.5 °C or 15 h at 31 °C.
Fig. 1. Time course of ribonucleotide reductase induction by HSV-1 strain 17 in BHK-21 cells at 31 °C and at 38.5 °C. △, Mock-infected at 31 °C; ▽, mock-infected at 38.5 °C; ●, HSV-1-infected at 31 °C; ○, HSV-1-infected at 38.5 °C. 25000 ct/min = 1 nmol dCMP.

Fig. 2. Effect of antiserum against HSV-1 strain 17-infected 3T12-3 cells and of preimmune serum on ribonucleotide reductase activity induced in HSV-1 strain 17-infected or mock-infected BHK-21 cells. Extracts containing 1 mg protein were preincubated with serum for 30 min at 37 °C and then assayed for ribonucleotide reductase activity. ●, HSV-1-infected + preimmune serum; ○, HSV-1-infected + antiserum; ■, mock-infected + preimmune serum; □, mock-infected + antiserum.

Effect of antiserum on HSV-1-induced ribonucleotide reductase

Fig. 2 shows that preincubation with rabbit antiserum raised against HSV-1 strain 17-infected 3T12-3 cells inhibited the ribonucleotide reductase activity induced in HSV-1-infected BHK-21 cells. Preimmune serum enhanced the enzyme activity. A similar effect of preimmune serum has been observed with the HSV-1-induced DNA polymerase (Keir et al., 1966) and it may be the result of a protective effect of serum proteins on the lability of the enzyme during preincubation at 37 °C. The mock-infected BHK-21 cell ribonucleotide reductase activity was inhibited by both sera.

Ribonucleotide reductase induced by ts mutants of HSV-1

A number of ts mutants of HSV-1 strain 17 were screened for induction of ribonucleotide reductase at permissive (31 °C) and non-permissive (38.5 °C) temperature. Fig. 3 shows the activity in mock-infected cells and induced in cells infected with wild-type virus and four ts mutants. The activity is expressed as percentage of wild-type activity. The mutants induced significant ribonucleotide reductase activity at permissive temperature (PT) although ts G and ts H induced less activity than the others. At non-permissive temperature (NPT) ts H and ts B induced wild-type levels and these mutants are classed as ribonucleotide reductase-positive. Some variability in induction at 38.5 °C was observed with ts K: the mutant extract was frequently ribonucleotide reductase-negative, but on several occasions small but variable amounts of activity were detected at NPT. These invariably were considerably reduced from what was obtained at PT. The ts G mutant consistently failed to induce any detectable activity at non-permissive temperature. Mutant ts G is DNA-positive, while ts K, ts H and ts B are all DNA-negative. Five other DNA-positive mutants (ts A, ts C, ts F, ts M, ts I syn) were also screened and all induced ribonucleotide reductase at 38.5 °C (data not shown).
To investigate the possibility that ts G had an altered time course of induction of the enzyme such that activity was not detectable at 6 h post-infection (Brown et al., 1973), ts G-infected cell extracts were assayed at various times after infection. Fig. 4 shows that no activity could be detected up to 15 h post-infection at NPT whereas at 31 °C a time course similar to wild-type was found.
Mutant HSV ribonucleotide reductase

Fig. 6. Effect of temperature shift-up on ribonucleotide reductase activity induced at permissive temperature by wild-type virus, ts G and ts K. After 15 h infection at 31 °C, infected cells were shifted to 38.5 °C, incubated for appropriate times with or without 50 μg/ml cycloheximide, then harvested and assayed for ribonucleotide reductase activity. O, Wild-type; •, wild-type + cycloheximide; □, ts G; ▪, ts G + cycloheximide; ▲, ts K; △, ts K + cycloheximide. 100% activity was 14,700 cpm/mg/h for wild-type virus, 7,950 cpm/mg/h for ts G and 13,000 cpm/mg/h for ts K. Mock-infected activity was 2,400 cpm/mg/h 15 h post-infection.

Enzyme activity of ts G revertants

If the failure of ts G to induce ribonucleotide reductase is a consequence of the ts lesion, revertants of the mutant would be expected to show normal induction of the enzyme. If, however, failure is unrelated to the ts lesion, ts + revertants would continue to exhibit abnormal ribonucleotide reductase induction. The ribonucleotide reductase activity in cells infected with spontaneous revertants of ts G at PT and NPT is shown in Fig. 5. As can be seen, both revertants induce normal amounts of ribonucleotide reductase activity at NPT and thus behave like wild-type.

Heat stability of mutant ribonucleotide reductase

The failure of ts G to induce ribonucleotide reductase at NPT could be due either to a temperature-sensitive control function which prevented synthesis of the enzyme at NPT, or to the synthesis of a temperature-sensitive ribonucleotide reductase which is non-functional at NPT.

To examine these possibilities, temperature shift-up experiments were carried out. Sets of five 90-mm plates were infected with virus or mock-infected. After incubation for 12 to 15 h at 31 °C, one plate from each set was harvested and the remainder were shifted to 38.5 °C and half incubated without addition and half in the presence of 50 μg/ml cycloheximide to prevent further protein synthesis. At appropriate times, the plates were harvested and assayed. The effect of shift-up on ribonucleotide reductase activity induced by wild-type, ts K and ts G virus is shown in Fig. 6. The ts G-infected cells rapidly lost their activity and 2 h after shift-up the enzyme activity was consistently the same as that found in mock-infected cells. Similar decreases in the ribonucleotide reductase activity in wild-type or ts K-infected cells were not seen. Addition of cycloheximide on shift-up of wild-type or ts K-infected cells led to a decrease in ribonucleotide reductase activity but the decrease was consistently less than that observed in ts G-infected cells. Hence, the loss of activity in ts G-infected cells cannot be explained by a lesion in de novo synthesis of the enzyme.

Fig. 7 shows the results of an experiment in which cells infected with the revertants G+ rev 1 and G+ rev 2 were shifted up 13 h post-infection in parallel with ts G and wild-type virus-infected cells. Both revertants have regained wild-type virus ribonucleotide reductase heat stability. In this experiment, the wild-type-induced enzyme activity showed a considerable increase in activity on shift-up. This indicates that the ribonucleotide reductase activity had not
Fig. 7. Effect of temperature shift-up on ribonucleotide reductase activity induced at permissive temperature by wild-type virus, ts G, G+ rev 1 and G+ rev 2. After 13 h infection at 31 °C, infected cells were shifted to 38.5 °C, incubated for appropriate times with or without 50 μg/ml cycloheximide, then harvested and assayed for ribonucleotide reductase activity. O, Wild-type; I, wild-type + cycloheximide; □, ts G; ■, ts G + cycloheximide; △, G+ rev 1; ▲, G+ rev 1 + cycloheximide; ◇, G+ rev 2; ●, G+ rev 2 + cycloheximide. 100% activity was 7570 ct/min/mg/h for wild-type virus, 4760 ct/min/mg/h for ts G, 11630 ct/min/mg/h for G+ rev 1 and 12820 ct/min/mg/h for G+ rev 2. Mock-infected activity was 2260 ct/min/mg/h 15 h post-infection.

Fig. 8. Effect of incubation at (a) 38.5 °C or (b) 45 °C on ribonucleotide reductase activity induced by wild-type virus (O), ts K (■), ts G (▲), G+ rev 1 (○) and G+ rev 2 (△). Extracts were prepared from infected cells after 15 h at 31 °C and aliquots containing 1 mg protein were incubated for appropriate times and then assayed for ribonucleotide reductase. 100% activity was: (a) 14330 ct/min/mg/h for wild-type, 15680 ct/min/mg/h for ts K, 7220 ct/min/mg/h for ts G, 16210 ct/min/mg/h for G+ rev 1 and 22520 ct/min/mg/h for G+ rev 2; (b) 6700 ct/min/mg/h for wild-type, 5940 ct/min/mg/h for ts K and 6390 ct/min/mg/h for ts G.

reached a maximum before shift-up. Increases in activity on shift-up to NPT were observed in other experiments with ts K, G+ rev 1 and G+ rev 2 but never with ts G even when the ts G-induced ribonucleotide reductase could be shown to be still increasing at PT.

The thermostability of the ribonucleotide reductase induced by the wild-type virus, ts K, ts G, G+ rev 1 and G+ rev 2 viruses was further examined in vitro. Crude extracts were prepared from infected cells 15 h after infection at 31 °C. Aliquots of these extracts each containing 1 mg
Mutant HSV ribonucleotide reductase protein were incubated at 38.5 °C for 0 to 90 min and then assayed for reductase activity. Fig. 8(a) shows that the activity induced by ts G was less stable than that induced by the wild-type, ts K, G* rev 1 or G* rev 2 viruses. The thermolability of the ts G-induced ribonucleotide reductase could be even more clearly demonstrated at 45 °C (Fig. 8(b)).

These results show that the ribonucleotide reductase induced by ts G at permissive temperature is thermolabile in vivo and in vitro. Thus, the failure of ts G to induce ribonucleotide reductase at NPT is due to induction of a polypeptide which is non-functional at this temperature rather than to an altered control of the enzyme's synthesis. The ts K mutant, on the other hand, controls the synthesis of a ribonucleotide reductase activity which has an unaltered thermostability in vivo and in vitro.

DISCUSSION

The induction of a novel ribonucleotide reductase in cells infected with HSV has been well-documented (Cohen, 1972; Huszar & Bacchetti, 1981; Langelier & Buttin, 1981). The biochemical differences between the host cell enzyme and the virus-induced enzyme, the kinetics of induction and the correlation of induction with multiplicity of infection are compatible with the hypothesis that the enzyme is virus-coded. The failure, reported here, of the ts G mutant of HSV to induce the enzyme and the demonstration that antiserum raised against HSV-1-infected 3T12-3 cells inhibits the ribonucleotide reductase activity induced by HSV-1 in BHK-21 cells support the hypothesis. The above quoted results do not, however, eliminate the possibility that the altered enzyme is a virus-induced cellular enzyme which has common antigenic determinants in the two cell lines. Strong evidence that the enzyme activity is at least partially virus-coded is provided by the demonstration that ts G induces an enzyme which is thermolabile at NPT in vivo and is less stable than the wild-type in vitro. At NPT, the mutant ts K sometimes induces low levels of ribonucleotide reductase. The ts K gene product controls the switch from immediate-early to early transcription (Preston, 1979; Watson & Clements, 1978) and a likely explanation for the result is that ribonucleotide reductase is encoded by a very early gene which at NPT is transcribed to a low and variable extent in ts K-infected BHK-21 cells. Thus, the ribonucleotide reductase may be encoded by the minor mRNA species sometimes found under immediate-early conditions in BHK-21 cells (Watson & Clements, 1978; McLauchlan & Clements, 1982).

Two distinct classes of ribonucleotide reductase have been described. The class found in bacteria and most eukaryotes is composed of two non-identical subunits, requires iron for the activity, and is inhibited by hydroxyurea (Thelander & Reichard, 1979). The second class is the monomeric Lactobacillus leichmannii type which uses adenosyl cobalamin as a cofactor and is not inhibited by hydroxyurea (Elford, 1968). Although no data are available on the HSV-induced ribonucleotide reductase, the fact that it is inhibited by hydroxyurea (Langelier & Buttin, 1981; B. M. Dutia, unpublished results) makes it likely that it falls into the first class of enzymes composed of two non-identical subunits. Evidence presented here shows that at least one subunit of the virus-induced reductase is virus-coded. Whether there are one or two subunits and whether a host cell subunit is also involved in the activity induced following HSV infection remains to be determined.

The HSV-induced thymidine kinase is not essential for HSV replication in exponentially growing tissue culture cells (Dubbs & Kit, 1964) but becomes essential in serum-starved cells (Jamieson et al., 1974). In contrast, HSV-induced DNA polymerase is absolutely essential for HSV replication in tissue culture (Hay & Subak-Sharpe, 1976).

The role of the reductase in virus replication remains to be determined. Ribonucleotide reductase is responsible for the de novo synthesis of dNTPs and might be necessary for effective virus DNA synthesis and virus replication. The plaque-forming ability of ts G is reduced by more than 10^6-fold at NPT yet ts G synthesizes approximately 20% of the virus DNA synthesized by wild-type virus at NPT in exponentially growing cells (B. M. Dutia, unpublished results). Thus, ts G is apparently able to synthesize some virus DNA in the absence of the virus-induced ribonucleotide reductase activity. It is not known, however, whether this DNA is normal and infectious. Furthermore, the possibility that the cellular ribonucleotide reductase...
provides dNTPs for virus DNA synthesis or that virus ribonucleotide reductase activity, below the limits of sensitivity of the assay, is responsible for synthesis of some dNTPs cannot be ruled out. Hence, clear conclusions regarding the essential nature of the ribonucleotide reductase cannot be drawn from the present study.

Definitive evidence for an essential role for ribonucleotide reductase would be provided by the demonstration that the temperature-sensitive lesion in ts G and the lesion in the reductase are due to the same impaired gene function. The successful isolation of two spontaneous ts+ revertants of ts G which have simultaneously regained the ability to induce wild-type levels of ribonucleotide reductase at the non-permissive temperature and which have reverted to wild-type heat stabilities suggests that the reductase lesion is the temperature-sensitive mutation. The data support the hypothesis that the ts G mutation and the virus ribonucleotide reductase are in the same gene and that the reductase, in some way not yet clear, is essential for HSV replication.

The ts G lesion has been mapped in the Hind III fragment of HSV-1 within the DNA encoding a mRNA of 5.0 kilobases (kb) and close to or within the DNA sequence encoding a 1.2 kb mRNA (V. G. Preston, data presented at the Herpesvirus Workshop, Bologna, Italy, 1981). These RNAs which encode 136,000 mol. wt. and 38,000 mol. wt. polypeptides respectively (Anderson et al., 1981) have been detected in small amounts under immediate-early conditions in BHK-21 cells (McLauchlan & Clements, 1982) and at least the 136,000 mol. wt. polypeptide has been observed under immediate-early conditions in BHK-21 cells (MacDonald, 1980). Experiments are now underway to investigate whether this map location defines the reductase gene and to determine the role of the ribonucleotide in virus replication. Demonstration of an essential role for the reductase would be of major importance because it would raise the possibility of this enzyme being amenable to specific anti-metabolite treatment and a means of controlling HSV infections.

I am grateful to Professor J. H. Subak-Sharpe and Dr H. S. Marsden for encouragement and valuable criticism of the manuscript. My thanks are due to Dr V. G. Preston for permission to quote unpublished results. I would also like to thank Mrs F. E. Jamieson and Mrs A. Orr for skilled technical assistance.

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Mutant HSV ribonucleotide reductase


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(Received 30 April 1982)