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Use of the PBS2 uracil-DNA glycosylase inhibitor to differentiate the uracil-DNA glycosylase activities encoded by herpes simplex virus types 1 and 2

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Summary

The bacteriophage PBS2 encoded uracil-DNA glycosylase (UNG) inhibitor was examined for its effect upon the nuclear UNG activities of KB, HeLa, and Vero cells infected with herpes simplex virus (HSV) type 1 or 2 and mock-infected cells. UNG activity from HSV-1 infected cells exhibited the greatest sensitivity to inhibition by the inhibitor, while UNG activity from cells infected with HSV-2 exhibited the greatest resistance. This differential effect was dependent upon the virus, cell line, and buffer system used in the reaction. Furthermore, the PBS2 UNG inhibitor's differential effect, provides a means of distinguishing the herpesvirus UNG activities from one another, and from the cellular UNG activity. Therefore, this method of identification should prove to be useful for the purification and characterization of the viral enzymes from infected cell nuclear extracts.

Uracil-DNA glycosylase; Herpes simplex virus; PBS2 inhibitor

Introduction

Uracil residues may be introduced into DNA either by the incorporation of dUTP by DNA polymerase (Bessman et al., 1958; Grafstrom et al., 1978), or by the deamination of cytosine residues that are present in DNA (Lindahl and Nyberg, 1974; Duncan and Miller, 1980). Regardless of the method by which uracil residues

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arise in DNA they may be removed through the action of the enzyme uracil-DNA glycosylase (UNG) (Lindahl, 1979). UNG cleaves the N-glycosyl bond between the uracil base and the deoxyribose phosphate moiety within the DNA resulting in the release of uracil and the formation of DNA containing an apyrimidinic (AP) site (Lindahl et al., 1977; Lindahl, 1982). The AP site is then repaired by a base-excision repair system (Lindahl, 1979). With the exception of some *Mycoplasma* (Williams and Pollack, 1988), all prokaryotic and eukaryotic organisms that have been examined possess UNG. Thus most organisms make a concerted effort to exclude uracil from their DNA.

In contrast, bacteriophage PBS2 is unique among organisms in that it contains uracil instead of thymine residues in its DNA (Takahashi and Marmur, 1963). Following infection of *Bacillus subtilis*, PBS2 induces several proteins which allow PBS2 to exclude dTMP from its DNA and incorporate dUMP in its place (Kahn, 1963; Tomita and Takahashi, 1969; Price and Cook 1972; Price and Fogt, 1973; Price and Frato, 1975). Furthermore, to insure that the newly synthesized phage DNA is not hydrolyzed by the *B. subtilis* UNG, the virus also encodes a protein that inhibits the activity of the host UNG (Friedberg et al., 1975; Cone et al., 1980).

Several studies have demonstrated that the PBS2 UNG inhibitor's activity is specific for UNG (Karran et al., 1981; Wang and Mosbaugh, 1989). In addition, it has been demonstrated that the inhibitor inhibits the activity of UNGs from several prokaryotic and eukaryotic organisms (Corrie and Friedberg, 1980; Karran et al., 1981; Wang and Mosbaugh, 1989). Recently the gene encoding the PBS2 UNG inhibitor was cloned and sequenced (Wang and Mosbaugh, 1988). This gene was demonstrated to code for an acidic polypeptide with a molecular weight of 9477.

Infection of cells with HSV results in the induction of UNG activity (Caradonna and Cheng, 1981) and the structural gene encoding this HSV enzyme has been identified (Caradonna et al., 1987; Mullaney et al., 1989). Putative genes encoding for UNG have also been identified for Epstein-Barr virus (Baer et al., 1984) and Varicella-Zoster virus (Davison and Scott, 1986; Davison and Taylor, 1987). However, attempts to purify these viral induced UNGs have not been successful. This is due in part to the inability to distinguish between cellular and viral UNGs. Recently we demonstrated that the PBS2 inhibitor could be used to distinguish the UNG of *Mycoplasma lactucae* from those of *B. subtilis* and *Escherichia coli* (Williams and Pollack, 1990). In the present study we demonstrate that the PBS2 UNG inhibitor can be used to distinguish not only the UNG activities found in nuclei of mock-infected cells but also the HSV encoded UNG activities.

Materials and Methods

Materials

Dulbecco's Modified Eagles Medium, non-radioactive nucleotides, calf thymus DNA, DEAE-Sephacel, and Sephadex-G 50, were purchased from Sigma Chemical Co., St. Louis, MO. Endonuclease-free *Escherichia coli* DNA polymerase I

was purchased from Boehringer Mannheim, Indianapolis, IN. [5-^{3}H] dUTP (18 Ci/mmole) was purchased from Moravek Biochemicals Inc., Brea, CA. *Bacillus subtilis* strains SB19E and SB5 and bacteriophage PBS2 were obtained from I. Takahashi, McMaster University, Hamilton, Ontario and R.B. Guyer, Pennsylvania State University, University Park, PA. Calf serum was obtained from Hyclone, Logan, Utah.

Cell growth conditions and viral infections

KB cells were grown as monolayers in Dulbecco's Modified Eagles Medium containing 5% (v/v) calf serum, 0.38% (w/v) sodium bicarbonate, 1% (v/v) non-essential amino acids and gentamicin sulfate (50 μg/ml) at 37°C in a 5% CO_2 atmosphere. Vero cells were grown under the same conditions as KB cells except calf serum was replaced by 5% (v/v) fetal calf serum. HeLa cells were grown as monolayers in RPMI 1640 medium containing 5% (v/v) calf serum, 0.19% (w/v) sodium bicarbonate, 1% (v/v) non-essential amino acids and gentamicin sulfate (50 μg/ml) at 37°C in a 5% CO_2 atmosphere. Approximately 8×10^7 cells were infected with HSV type 1 (strain KOS) or type 2 (strain HG-52) at a multiplicity of infection of 10 pfu/cell. Infected and mock-infected cells were harvested 18 h post-infection and pelleted by centrifugation at $1100 \times g$. The freshly harvested cells were used for the preparation of nuclear extracts.

Cell extractions

The cell pellets were resuspended in 2 ml of cell extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose) and allowed to swell on ice for 5 min. The cells were then lysed by dounce homogenization in a pre-cooled 10 ml dounce homogenizer. Cell breakage (≥95%) was monitored by Trypan blue dye exclusion. Nuclei were isolated from the extracts by centrifugation ($800 \times g$) at 4°C for 20 min. The supernatants were discarded. The nuclear pellets were washed once by resuspension in 2 ml of cell extraction buffer and collected by centrifugation. The nuclear pellets were resuspended in 5 ml of nuclear extraction buffer (10 mM Tris-HCl pH 7.5, 2 mM DTT, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 20% (v/v) glycerol) and lysed by sonication on ice using a Branson Sonifier Model 350 (microtip, setting 4) for 1 min in a series of four 15 s pulsed bursts separated by 30 s cooling periods. The nuclear extracts were clarified by centrifugation at $20\,000 \times g$ for 15 min at 4°C. The supernatants, which were designated as the crude nuclear extracts, were decanted and aliquoted into 0.5 ml portions and stored at −20°C until used for enzyme assays.

Purification of PBS2 UNG inhibitor

The PBS2 UNG inhibitor was purified from PBS2 (Cone et al., 1980) infected *B. subtilis* using a modification of the procedure. Following gel filtration chromatography, the inhibitor was heated to 100°C for 10 min and centrifuged for 30

min at 20000 $\times g$. The supernatant was aliquoted into 0.5 ml portions and stored at −20°C until use. A unit of PBS2 UNG inhibitor activity was defined as the amount of inhibitor required to neutralize a unit of *B. subtilis* UNG activity under our assay conditions.

Enzyme assays

UNG Enzyme activity was determined in each of two reaction mixtures. The Tris buffered assay system (TRI) contained in a final volume of 0.2 ml, 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM EDTA, 0.01% (w/v) BSA, 4 μg [^{3}H]-uracil calf thymus DNA (6000 cpms/μg), and the enzyme sample. The Hepes buffered assay system (HEP) contained in a final volume of 0.2 ml, 70 mM Hepes-KOH, pH 7.8, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) BSA, 4 μg [^{3}H]-uracil calf thymus DNA (6000 cpms/μg), and the enzyme sample. Calf thymus DNA containing [^{3}H]-uracil residues was prepared with [^{3}H]-dUTP using the nick-translation procedure described by Rigby et al. (1977).

Assays were performed by pre-incubating the enzymes at 37°C for 15 min in a 0.1 ml total volume containing the above reaction mixture without [^{3}H]-uracil calf thymus DNA. Reactions were initiated by the addition of 0.1 ml of the appropriate reaction mixture containing 4 μg [^{3}H]-uracil calf thymus DNA. The reactions were stopped by placing on ice and immediately adding 25 μl of sheared calf thymus DNA (1 mg/ml) and 25 μl cold 50% (w/v) trichloroacetic acid (TCA). After 20 min at 0°C, samples were centrifuged (1100 $\times$ g for 5 min), and radioactivity was determined by placing 0.2 ml of the supernatant in Beckman Ready Solve scintillation cocktail (4 ml) neutralized by the addition of 75 μl 1.2 M KOH. Samples were counted in a Beckman LS-8100 scintillation spectrophotometer. A unit of uracil-DNA glycosylase activity was defined as the amount of enzyme required to release 1 nmole of [^{3}H]-uracil as acid soluble material per minute at 37°C.

Assay of PBS2 UNG inhibitor Inhibitor activity was determined in each of the two reaction systems. In the standard inhibitor assay, 0.001–0.002 units of uracil-DNA glycosylase (diluted immediately before use) and varying amounts of inhibitor (as indicated in the figures) were combined in a final volume of 0.1 ml of either TRI or HEP without [^{3}H]-uracil calf thymus DNA. After incubation at 37°C for 15 min, the reactions were supplemented with 0.1 ml of TRI or HEP containing 4 μg [^{3}H]-uracil calf thymus DNA. The reactions were incubated an additional 60 min at 37°C. Acid soluble radioactivity was determined in the same manner described above.

Inhibitor assays were conducted as described above for enzymes from all the sources studied, and an approximately equivalent number of units of each enzyme were used. The PBS2 UNG inhibitor was determined to have 6.67 and 5.00 units of inhibitory activity per mg of protein respectively in the HEP and TRI reaction mixtures when tested against *B. subtilis* UNG activity.

Protein determination

Protein concentrations were determined using the Coomassie blue dye binding method as described by Bio-Rad Laboratories using BSA as the standard.

Results

To determine whether the PBS2 UNG inhibitor could be used for distinguishing the HSV 1 and 2 UNG activities from each other and from nuclear UNG activity present in mock-infected cells, we determined the effect of the PBS2 UNG inhibitor on the UNG activities present in both mock-infected and HSV-infected cells. The data are shown in Figs. 1–3 and are summarized in Table 1. All the UNG activities were inhibited in a dose dependent manner by the PBS2 inhibitor. However, the amount of inhibitor required for equivalent levels of inhibition differed with respect to the source of the enzyme and also with the buffer composition of the reaction mixture. In general, higher amounts of inhibitor were required in the HEP system than in the TRI system to cause 50% inhibition (I_{50}) of enzyme activity. This difference was not due to an effect of the buffer composition on enzyme activity, since in the absence of inhibitor similar levels of activity were observed (data not shown). The UNG activities from mock-infected human cells (Figs. 1,2) were more resistant to inhibition by the PBS2 UNG inhibitor than the UNG activity from VERO cells (Fig. 3). Using the TRI buffer system, approximately twice as much inhibitor was required to inhibit the UNG activities from KB and HeLa cells to an equivalent level (I_{50}) as what was observed for the UNG activity from VERO cells. Similarly in the HEP system, 1.3- and 2.6-fold greater amounts of inhibitor were required to inhibit the UNG activities from HeLa and KB cells respectively.

TABLE 1
Effect of PBS2 UNG inhibitor on various UNG activities

Source of UNG[a]	Buffer system	
	TRI	HEP
	I_{50}[b]	
KB	0.55	0.60
HSV-1 infected KB	0.43	0.36
HSV-2 infected KB	0.87	1.15
HeLa	0.52	0.30
HSV-1 infected HeLa	0.30	0.50
HSV-2 infected HeLa	0.89	1.11
VERO	0.25	0.23
HSV-1 infected VERO	0.37	0.23
HSV-2 infected VERO	0.44	0.98

[a] Nuclei from mock-infected and infected cells (8×10^7) were used as the source of UNG. Assays were performed using the TRI and HEP systems as described in Materials and Methods.

[b] I_{50}: amount of inhibitor in μg that is required to inhibit 50% of the UNG activity under our assay conditions.

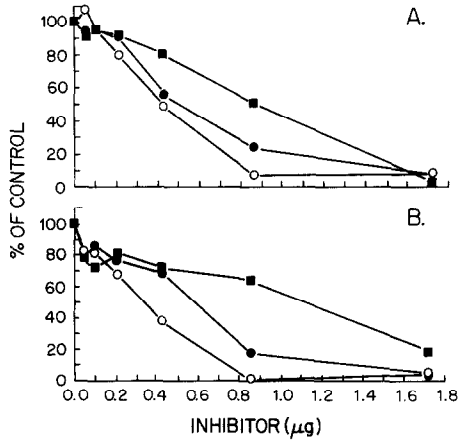

Fig. 1. Inhibition of KB nuclear and HSV-encoded UNG activities by PBS2 UNG inhibitor. Reactions were performed as described in Materials and Methods. Reaction mixtures contained either 0, 0.054, 0.108, 0.216, 0.433, 0.865, or 1.73 μg of PBS2 UNG inhibitor. A. TRI system, 0.0014 unit UNG. B. HEP system, 0.0017 unit UNG. Symbols: (•), mock-infected KB nuclear extract; (○), HSV-1-infected KB nuclear extract; (■), HSV-2-infected KB nuclear extract.

The UNG activities from cells infected with HSV-2 exhibited the greatest level of resistance to the inhibitor (Figs. 1–3). Depending upon the cell line and the buffer system employed, it required 1.6-fold (KB, HeLa, TRI system) to 4.0-fold (VERO, HEP system) greater amounts of inhibitor to inhibit the UNG activity present in HSV-2-infected cells when compared to mock-infected cells. Conversely,

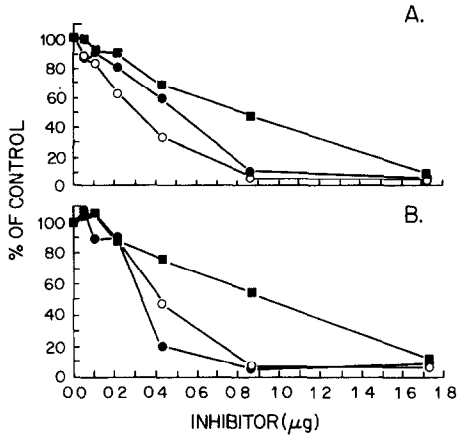

Fig. 2. Inhibition of HeLa nuclear and HSV-encoded UNG activities by PBS2 UNG inhibitor. Reactions were performed as described in Materials and Methods. Reaction mixtures contained either 0, 0.054, 0.108, 0.216, 0.433, 0.865, or 1.73 μg PBS2 UNG inhibitor. (A) TRI system, 0.0014 unit UNG. (B) HEP system, 0.0017 unit UNG. Symbols: (•), mock-infected HeLa nuclear extract; (○), HSV-1-infected HeLa nuclear extract; (■), HSV-2-infected HeLa nuclear extract.

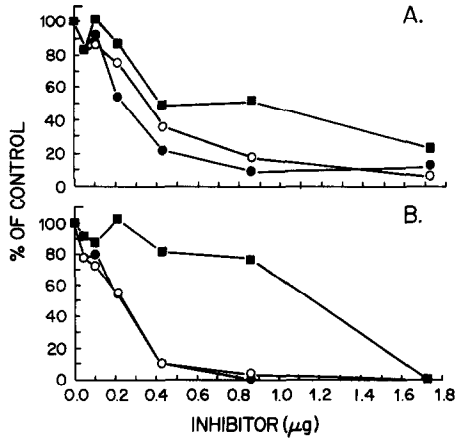

Fig. 3. Inhibition of VERO nuclear and HSV-encoded UNG activities by PBS2 UNG inhibitor. Reactions were performed as described in Materials and Methods. Reaction mixtures contained either 0, 0.054, 0.108, 0.216, 0.433, 0.865, or 1.73 μg PBS2 UNG inhibitor. (A) TRI system, 0.0014 unit UNG. (B) HEP system, 0.0017 unit UNG. Symbols: (•), mock-infected VERO nuclear extract; (○), HSV-1-infected VERO nuclear extract; (■), HSV-2-infected VERO nuclear extract.

UNG activities from cells infected with HSV-1 exhibited levels of sensitivity to the inhibitor which were either similar to that observed with mock-infected cells (VERO, HEP system) or which were more sensitive to inhibition by the inhibitor (KB, both buffer systems, and HeLa, TRI system). However, there were two exceptions to this in which greater amounts of inhibitor were required for inhibition of UNG activity (I_{50}) when compared to the mock-infected cells (VERO, TRI system and HeLa, HEP system).

Discussion

With the exception of some *Mycoplasma* (Williams and Pollack, 1988) all organisms that have been examined possess UNG activity. UNGs have been purified and characterized from several prokaryotic (Lindahl, 1974; Lindahl et al., 1977; Cone et al., 1977; Leblanc et al., 1982; Kaboev et al., 1985) and eukaryotic organisms (Talpaert-Borle et al., 1979; Caradonna and Cheng, 1980; Crosby et al., 1981; Domena and Mosbaugh, 1985; Domena et al., 1988). Regardless of the source of the enzyme they are all very similar both physically and biochemically. Recent studies have also demonstrated that there is a high degree of homology between the genes encoding for UNG from *Escherichia coli* (Varshney et al., 1988), *Saccharomyces cerevisiae* (Percival et al., 1989) and HSV (Mullaney et al., 1989). These results support the hypothesis that UNG is a highly conserved protein even among unrelated organisms.

While the structural genes encoding for the HSV-1 and HSV-2 encoded UNGs have been identified (Caradonna et al., 1987; Worrad and Caradonna, 1988; Mul-

laney et al., 1989), attempts to purify these enzymes from HSV-infected cells have not been successful. This is due in part to the lack of specific agents that can be used to distinguish these enzymes from that of the host. The data presented in this study demonstrate that the PBS2 encoded UNG inhibitor can be useful not only for distinguishing the HSV-1- and -2-encoded UNGs from each other, but also from cellular nuclear UNGs. The differential effect of the PBS2 UNG inhibitor was dependent not only on the cell line and virus used, but also upon the buffer system used in the assay.

Except for a recent study concerning the UNG from *Mycoplasma lactucae* (Williams and Pollack, 1990) previous studies with the PBS2 inhibitor did not demonstrate that the inhibitor could be used to differentiate UNG activity from various organisms (Corrie and Freidberg, 1980; Karran et al., 1981; Wang and Mosbaugh, 1989). This could be due to differences in the amounts of inhibitor used in these studies or it may reflect minor structural differences in the various UNGs that affect the ability of the PBS2 UNG inhibitor to bind to the UNG. Regardless, our results demonstrate that the PBS2 UNG inhibitor can be used to distinguish the HSV encoded UNGs. Furthermore, these results suggest that the UNG inhibitor could be a useful agent when purifying UNGs and when examining structural features of UNGs from various organisms. In addition, the UNG inhibitor may have utility as an UNG antigen specific protein blotting reagent, and/or an UNG specific affinity matrix ligand.

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