PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 280-284 (1976)

Deoxycytidine Kinase Activity from Rabbit Kidney Cells Infected with Herpes Simplex Virus Type 1 or 2[1] (39192)

TAKEO OGINO[2] AND FRED RAPP

Department of Microbiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, College of Medicine, Hershey, Pennsylvania 17033

Within the past decade, two subtypes of herpes simplex virus (HSV) have been distinguished. Herpes simplex virus type 1 (HSV-1), commonly isolated from nongenital regions, and herpes simplex virus type 2 (HSV-2), generally found in genital areas, differ in a variety of biochemical and biophysical properties (1). Cells infected with HSV express increased levels of deoxythymidine kinase (TdR kinase) activity and contain an enzyme that appears to be virus-coded and unique for each virus subtype (2–5). Likewise, deoxycytidine kinase (CdR kinase) activity has been shown to increase after infection with polyoma virus or HSV (6, 7). In fact, data from one study with virus mutants (8) and from a biochemical study (9) suggest that the TdR and CdR kinase activities expressed in HSV-infected cells are due to the same protein. Of additional interest is the possibility that CdR kinase is important to the metabolism and action of 1-β-D-arabinofuranosylcytosine (ara-C), a drug used frequently in virus studies and as an antitumor agent (10–12). For these reasons, we investigated the CdR kinase activity in cells infected with HSV-1 and HSV-2.

Materials and methods. Cells and virus. Primary rabbit kidney (RK) cells and virus stocks were prepared as previously described (2). Experiments were conducted with HSV-1 (strain Seibert) and HSV-2 (strain 316D), kindly provided by Dr. William Rawls, Baylor College of Medicine, Houston, Texas.

Extracts of virus-infected cells. Primary RK cells were infected and extracts prepared as described (2). In these studies the input multiplicity for HSV-1 infection was one plaque-forming unit (PFU) per cell and for HSV-2 infection was 0.5 PFU per cell. At designated times, the culture medium was removed, and the cells were washed three times with Tris-buffered saline (pH 7.4), dislodged with the aid of a rubber policeman, pelleted by centrifugation, and resuspended in approximately 0.5 ml of 0.15 *M* KCl in 0.05 *M* Tris-HCl buffer (pH 8.0) containing 0.003 *M* 2-mercaptoethanol. The suspensions were disrupted by ultrasonic disintegration and centrifuged at 15,000 rpm for 60 min at 4° in the SS-34 rotor of a Sorval centrifuge. The supernatant fractions were used as the source of enzyme. Protein determinations were carried out by the method of Lowry *et al.* (13).

Enzyme assay. The CdR kinase assay was the same as that described by Ives *et al.* (14). The reaction mixture, in a total volume of 0.25 ml, contained 0.05 *M* Tris-HCl (pH 8.0), 10 m*M* ATP, 10 m*M* $MgCl_2$, 15 m*M* NaF, 0.1 μCi of [2-^{14}C]deoxycytidine (CdR, specific activity 60 mCi/mmole, Schwarz/Mann), and the enzyme extract (50 to 250 μg of protein). Assay tubes were incubated for 15 min at the temperatures indicated in Results, and reactions were terminated by immersing the tubes in boiling water for 2 min. The amount of phosphorylated CdR was determined by the DEAE-cellulose disk method. In some experiments the products were examined by descending paper chromatography on Whatman No. 1 paper in a solvent of isobutyric acid:water:ammonia (66:33:1.5) (15). At all temperatures under these conditions enzyme activity was linearly related to both the amount of cellular protein and to the time of incuba-

[1] Conducted under Contract No. N01 CP53516 within the Virus Cancer Program of the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

[2] Present address: Department of Microbiology, Hamamatsu University, School of Medicine, Hamamatsu, Shizuoka, Japan.

tion, and less than 3% of the available substrate (CdR) was utilized. TdR kinase activity was assayed as previously described (5) with 2.86 nmole of [^{14}C]TdR for enzyme from uninfected cells and 10.86 nmole of [^{14}C]TdR for enzymes induced by HSV-1 and HSV-2.

Thermal inactivation of CdR kinase activity. Each enzyme extract (0.5 ml of uninfected, HSV-1 infected, and HSV-2 infected) was incubated at the temperatures and for the periods indicated in Results. At each time, 100 μl of extract were removed and assayed for CdR kinase activity as described above. Residual activity was shown as the percentage activity at zero time.

Sephadex G-100 chromatography. Gel filtration was performed at 4° as previously described (5). A sample of enzyme extract (1.5 ml) was applied to a Sephadex G-100 superfine column (3.14 cm^2 × 58 cm) and eluted with 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.15 *M* KCl and 1 m*M* dithiothreitol. Elution proceeded at approximately 10 ml/hr, with 96 drops (about 2.3 ml) collected per fraction. A sample (100 μl) of each fraction was assayed for CdR kinase activity at 38° using the reaction mixture described above. Extracts from uninfected cells were incubated for 3 hr with 0.1 μCi of [^{14}C]CdR (sp act 60 mCi/mmole) while extracts containing HSV-1 and HSV-2 induced enzymes were incubated for 1 hr with 1.0 μCi of [^{3}H]CdR (sp act 28 Ci/mmole). TdR kinase activity was assayed as described (5) with 2.86 nmole of [^{14}C]TdR for uninfected cell extracts and 10.86 nmole of [^{14}C]TdR for virus-induced enzymes. Molecular weights were estimated from the calibrated column as described (5).

Results. CdR kinase activity in virus-infected RK cells. When RK cells were infected with HSV-1 (Seibert) and HSV-2 (316D), the CdR kinase activity assayed at 38° increased 5- to 15-fold relative to uninfected controls (Table I). However, when the HSV-1 enzyme was assayed at 16°, a 30- to 80-fold increase in activity was measured. Similar results were obtained with extracts prepared from hamster embryo fibroblasts infected with these same virus strains (unpublished observations).

Figure 1 shows that the HSV-1 induced enzyme had a temperature optimum of 16° while the activities of the host and HSV-2 induced enzymes were maximal at 38°. In contrast, the TdR kinase activities measured

TABLE I. CdR Kinase Activity in Rabbit Kidney Cells after Infection with HSV-1 (Seibert) or HSV-2 (316D).

Hours after infection	Uninfected		HSV-1 infected		HSV-2 infected	
	16° [a]	38° [a]	16°	38°	16°	38°
12	0.04	0.31	1.59	0.84	1.21	2.01
18	0.03	0.20	2.50	1.10	1.18	2.00
24	0.07	0.19	2.06	1.01	1.90	2.91
30	0.03	0.22	1.64	0.94	1.55	2.62

[a] CdR kinase activity was assayed at 16 or 38°. CdR kinase activity is shown as nmole. [^{14}C]CdR phosphorylated per milligram protein per 15 min at 16 or 38°.

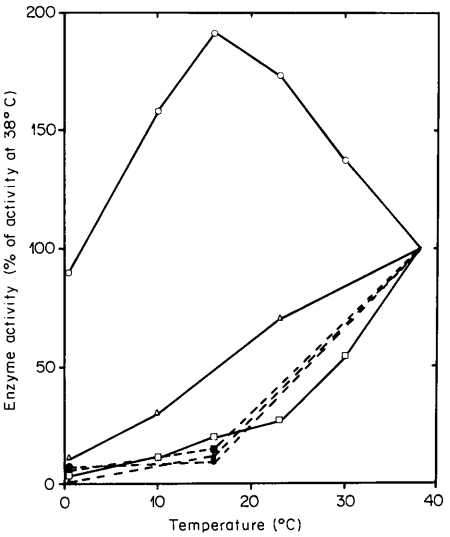

FIG. 1. Effect of temperature on CdR kinase and TdR kinase activities. Enzyme activity is shown as the percentage of activity at 38°. ○——○, HSV-1 (Seibert) induced CdR kinase. △——△, HSV-2 (316D) induced CdR kinase. □——□, CdR kinase from uninfected cells. ●- - -●, HSV-1 (Seibert) induced TdR kinase. ▲- - -▲, HSV-2 (316D) induced TdR kinase. ■- - -■, TdR kinase from uninfected cells. CdR kinase activities at 38° (nmole [^{14}C]CdR phosphorylated per milligram protein per 15 min at 38°) are 0.33, 0.62, and 1.2 for uninfected, HSV-1, and -2 induced enzymes, respectively. TdR kinase activities (nmole [^{14}C]CdR phosphorylated per milligram protein per 15 min at 38°) are 2.1, 63.3, and 80.0 for uninfected, HSV-1 and -2 induced enzymes.

in similarly prepared extracts of normal, HSV-1, and HSV-2 infected cells were highest at 38°.

To be certain that the CdR used in these assays was not converted to deoxyuridine or deoxyuridine monophosphate (11, 16) when incubated with these crude cellular extracts, the products were examined by paper chromatography as described in Materials and Methods. The only product detected by this technique was the CdR kinase dependent deoxycytidine monophosphate.

Thermostability of CdR kinase activity. Data on thermostability of CdR kinase activity in extracts of uninfected and HSV-infected RK cells are presented in Table II. All three extracts were incubated at 0.5 and 38° for varying periods prior to assay of residual enzyme activity at 38°. At both temperatures the HSV-2 induced enzyme was less stable than either the host or HSV-1 enzyme. Unexpectedly, the HSV-2 enzyme lost about 40% of its activity after incubation at 0.5° for 90 min. Similar results were obtained when the activities were assayed at 16°.

Sephadex G-100 column chromatography. Elution patterns of CdR and TdR kinase activities are presented in Fig. 2. Both enzyme activities extracted from HSV-infected RK cells eluted at the same position (Figs. 2A, B), an observation consistent with the claim that CdR and TdR kinases are the same protein. The estimated molecular weights for HSV-1 and HSV-2 induced enzymes are 67,000 and 60,000, respectively. CdR kinase activity from uninfected cells eluted at the same position as HSV-1 induced enzyme activity (Fig. 2C, estimated mol wt of 67,000). Host cell TdR kinase activity eluted as a heterogeneous peak and at a different position from CdR kinase activity (Fig. 2C). Approximately one-third of the input enzyme activity was recovered in each case, and recovery did not coincide with thermostability.

Discussion. CdR kinase activity induced by HSV-1 (Seibert) in RK cells exceeded the enzymatic activity of uninfected cells by 30- to 80-fold. Further, the temperature optimum for this activity was 16°. In contrast, both cellular and HSV-2 (316D) induced CdR kinases operated more effectively at 38°. When the same extracts were assayed for TdR kinase activity, however, enzyme activity was greater at 38° than at 16°.

While the physiological meaning of these observations is not clear, the fact that low temperature favors the induction and replication of the frog herpesvirus permits speculation (17). It may be of value to examine the character of enzymes induced by the frog herpesvirus in infected or tumor cells. It is possible that the low temperature optimum of the HSV-1 induced CdR kinase may represent an evolutionary phenomenon. If true, this might suggest that HSV-2 is derived from HSV-1.

Marked thermolability of the HSV-2 induced CdR kinase was evidenced even when the preparations were diligently maintained in an ice-water bath (0.5°). Various investigators have similarly reported a difference in the thermostability of both TdR kinase

TABLE II. STABILITY OF CDR KINASE ACTIVITY FROM RABBIT KIDNEY CELLS INFECTED WITH HSV-1 (SEIBERT) OR HSV-2 (316D).[a]

Incubation		Residual activity (%)		
Temperature (°)	Time (min)	Uninfected (1.5 mg/ml)[b]	HSV-1 infected (1.66 mg/ml)[b]	HSV-2 infected (2.22 mg/ml)[b]
0.5	0	100 (0.34)[c]	100 (0.96)[c]	100 (0.79)[c]
	30	101	101	87
	60	96	101	70
	90	90	108	61
38	0	100 (0.31)[c]	100 (0.84)[c]	100 (0.79)[c]
	15	88	70	14
	30	74	65	12

[a] Enzyme assay was done at 38° for 15 min.

[b] Figure in parenthesis represents the amount of protein.

[c] Figure in parenthesis represents the specific activity of CdR kinase at zero time (nmole [^{14}C]CdR phosphorylated per milligram protein per 15 min at 38°).

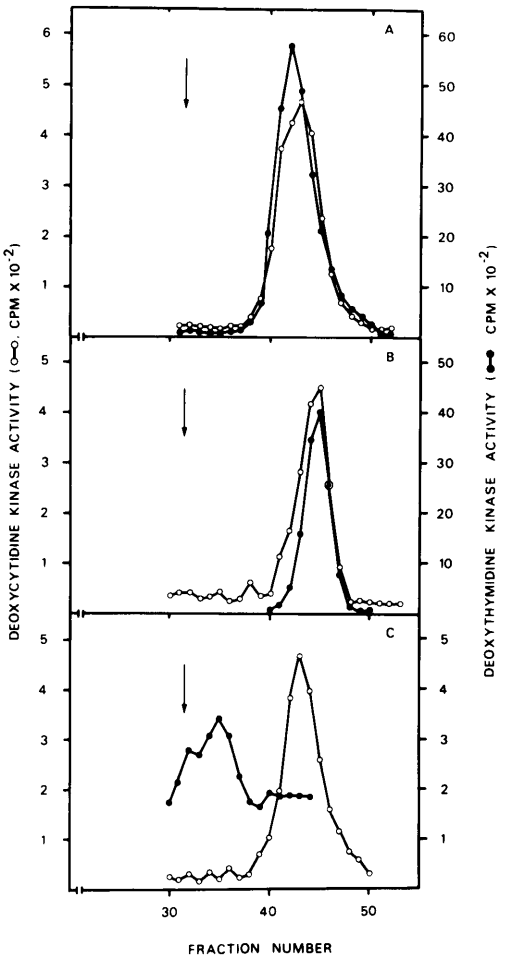

FIG. 2. Gel filtration of CdR kinase and TdR kinase on Sephadex G-100 (superfine). (A) HSV-1 (Seibert) infected cell extract. (B) HSV-2 (316D) infected cell extract. (C) Uninfected cell extract. ○——○, CdR kinase activity. ●——●, TdR kinase activity. ↓ shows the position of totally excluded proteins. The enzyme activity was assayed as described in Materials and Methods. One tenth microcurie of [^{14}C]CdR was used as the substrate for uninfected CdR kinase, and 1.0 μCi of [^{3}H]CdR was used to assay HSV-1 and -2 induced CdR kinases. One tenth microcurie of [^{14}C]TdR was used as substrate for all three TdR kinases.

and DNA polymerase activities extracted from HSV-1 and HSV-2 infected cells (2, 3, 18). Since neither the cellular nor the HSV-1 induced enzyme displayed this lability, the HSV-2 induced CdR kinase may represent a genetic variant of a virus-coded or modified cell enzyme. As CdR kinase appears to be critical to the action of ara-C (10, 12), this thermolability may account for the recent observation that HSV-2 is less sensitive than HSV-1 to inhibition by ara-C (19). Direct comparison of the thermolability of TdR and CdR kinase were not carried out but are similar at 38°.

The gel filtration data are consistent with the proposal that one protein in HSV-infected cells (deoxypyrimidine kinase) may possess both CdR and TdR kinase activities (8, 9). The data presented are similar to those obtained by electrophoresis (9). However, the data do not exclude the possibility that these two enzymes are unique entities with similar molecular sizes. Because HSV-1 induced TdR kinase can change its molecular size under varying conditions (5), additional gel filtration studies with a variety of buffers should establish whether or not CdR kinase activity behaves in a parallel manner. Also, in HSV-infected cells, the induced CdR kinase activity precedes the appearance of TdR kinase activity (unpublished observations).

If, however, both enzyme activities are contained in one molecule, it seems reasonable to speculate that their expression involves a temperature-dependent conformational change. In the case of the HSV-1 induced activities, the temperature optimum for CdR kinase was 16° while maximum TdR kinase activity was obtained at 38°. Future experiments should substantiate or negate the existence of the proposed deoxypyrimidine kinase.

Summary. When rabbit kidney cells were infected with herpes simplex virus type 1 (strain Seibert) or herpes simplex virus type 2 (strain 316D), deoxycytidine kinase (CdR kinase) activity, assayed at 38°, increased 5- to 15-fold relative to controls. The CdR kinase activity induced by type 2 virus was more thermolabile than the enzyme activity induced by type 1 virus. When CdR kinase activity was assayed at various temperatures between 0.5 and 38°, maximum activity for type 1 enzyme was obtained at 16° while maximum activities for host and type 2 enzymes were obtained at 38°. Both type 1 and type 2 induced CdR kinase activities eluted at the same positions as deoxythymidine kinase activities on a Sephadex G-100 column. The estimated mol wt for HSV-1 (Sei-

bert) and HSV-2 (316D) induced CdR kinases are 67,000 and 60,000, respectively.

1. Nahmias, A. J., and Dowdle, W. R., Progr. Med. Virol. **10,** 110 (1968).
2. Ogino, T., and Rapp, F., Virology **46,** 953 (1971).
3. Thouless, M. E., and Skinner, G. R. B., J. Gen. Virol. **12,** 195 (1971).
4. Thouless, M. E., J. Gen. Virol. **17,** 307 (1972).
5. Ogino, T., Shiman, R., and Rapp, R., Intervirology **1,** 80 (1973).
6. Kára, J., and Weil, R., Proc. Nat. Acad. Sci. USA **57,** 63 (1967).
7. Perera, P. A. J., and Morrison, J. M., Biochem. J. **117,** 21P (1970).
8. Hay, J., Perera, P. A. J., Morrison, J. M., Gentry, G. A., and Subak-Sharpe, J. H., *in* "CIBA Foundation Symposium on Strategy of the Viral Genome" (G. E. W. Wolstenholme and M. O'Connor, eds.), p. 355–376. Churchill Livingstone, Edinburgh and London (1971).
9. Jamieson, A. T., and Subak-Sharpe, J. H., J. Gen. Virol. **24,** 481 (1974).
10. Momparler, R. L., and Fischer, G. A., J. Biol. Chem. **243,** 4298 (1968).
11. Durham, J. P., and Ives, D. H., Mol. Pharmacol. **5,** 358 (1969).
12. Graham, F. L., and Whitmore, G. F., Cancer Res. **30,** 2636 (1970).
13. Lowry, O. H., Rosensebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
14. Ives, D. H., Durham, J. P., and Tucker, V. S., Anal. Biochem. **28,** 192 (1969).
15. Zimmerman, Jr., J. E., Raska, Jr., K., and Strohl, W. A., Virology **42,** 1147 (1970).
16. Maley, G. F., and Maley, F., Arch. Biochem. Biophys. **101,** 342 (1963).
17. Granoff, A., Fed. Proc. **31,** 1626 (1972).
18. Hay, J., Moss, H., and Halliburton, I. W., Biochem. J. **124,** 64P (1971).
19. Nutter, R. L., and Rapp, F., Cancer Res. **33,** 166 (1973).

Received April 21, 1975. P. S. E. B. M. 1976, Vol. 151.