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Inhibition of Phosphorylation of Cellular dUTP Nucleotidohydrolase as a Consequence of Herpes Simplex Virus Infection

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During an infection with herpes simplex virus, activity of cellular dUTPase decreases as a function of time, post-infection, while virus-encoded dUTPase activity increases. Prelabeling of cells with ^{35}S-methionine and immunoprecipitation analysis, using monoclonal antibodies, indicates that cellular dUTPase protein levels remain the same (with respect to levels in uninfected cells) throughout the infection period. New synthesis of cellular dUTPase does not occur in infected cells as determined by ^{35}S-methionine labeling during infection.

Further characterization of the cellular dUTPase, in uninfected cells, reveals that the protein is post-translationally phosphorylated at serine residues. Pulse labeling of virus-infected cells with ^{32}P-orthophosphate reveals that the phosphorylation rate of the cellular dUTPase protein decreases significantly as a function of time post-infection. In an effort to establish that phosphate turnover was occurring on the cellular dUTPase protein, cells were prelabeled with ^{32}P-orthophosphate and then infected with HSV in the absence of label. Evidence from this experiment indicates that the phosphate moiety is removed from the cellular dUTPase protein during the infection.

A series of viable virus mutants was generated by insertional inactivation of the HSV dUTPase gene. These mutants do not express viral dUTPase activity and HSV dUTPase protein is not detected by western blot analysis. However, in contrast to the wild-type situation, these mutant virus retain significant cellular dUTPase activity throughout infection. Interestingly, phosphorylation of cellular dUTPase protein is now readily detectable in each of the mutant virus-infected cells.

These studies indicate that cellular dUTPase activity is diminished in wild-type HSV-infected cells by a process of dephosphorylation. It also appears that in mutant HSV, lacking the virus dUTPase, the mechanism of dephosphorylation and thus inactivation of cellular dUTPase is not functional. The end result is that the mutant virus can now rely on the cellular activity for its survival.

Key words: dUTPase, monoclonal antibody, insertion mutation, nucleotide metabolism

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dUTPase (deoxyuridine triphosphate nucleotidohydrolase) is an enzyme that catalyzes the hydrolysis of dUTP to dUMP and inorganic pyrophosphate. The prokaryotic dUTPase is a zinc-containing tetramer with a monomer molecular weight of 16,000 [1]. The eukaryotic equivalent is a dimer whose 22,500 dalton monomers associate in the presence of divalent metals such as magnesium or manganese to form the active enzyme [2]. The dUTPase enzyme appears to belong to the class of enzymes involved in nucleotide metabolism which have a "housekeeping" function. The current view is that this enzyme plays a key role in preventing dUTP from entering into the DNA synthetic pathway [3,4]. In addition, the dUTPase enzyme may also provide substrate in the form of dUMP for de novo dTMP synthesis.

The dUTPase function plays an essential role in both prokaryotic and eukaryotic cell proliferation. Attempts to completely inactivate the *E. coli* dUTPase have been unsuccessful [5]. In the eukaryotic system, increased intracellular dUTP levels leading to an increased ratio of dUTP to dTTP may provide an alternate explanation for the mechanism of action of anticancer agents such as fluorodeoxyuridine and methotrexate [6]. These agents appear to produce elevated levels of dUTP via a build-up of dUMP. This leads to increased incorporation of dUMP residues into DNA [7]. Under these conditions, the increased frequency of removal of dUMP residues and reincorporation of dUMP (due to elevated levels of dUTP pools) by an excision-repair process [8] is thought to lead to extensive DNA degradation and eventually cell death.

Given the nature of the dUTPase function, it comes as no surprise to find that HSV (herpes simplex virus)-infected cells contain significant amounts of dUTP-hydrolyzing activity. Previous studies have shown a distinct HSV function with dUTP-hydrolyzing ability [2,9,10]. This work has been corroborated at the genetic level with the location of an HSV gene that encodes a dUTPase function [11].

Evidence presented by Fisher and Preston [12] indicates that HSV mutants containing insertions which inactivate the HSV dUTPase can survive. These results have led to the speculation that the HSV dUTPase gene is not essential for virus growth (at least in proliferating cells in culture). In a more recent study by Williams [13], HSV dUTPase mutants were shown to contain elevated levels of the cellular dUTPase activity.

A major aspect of this study is an attempt to understand the relationship between the cellular and virus counterparts of a similar enzymatic function during infection of cells by HSV. If the uninfected cell is endowed with sufficient quantities of a particular enzymatic activity why, in certain situations, does the virus need to encode a counterpart to this function? Results from the study presented here indicate that the altered environment of the cell, due to HSV infection, leads to a decrease in cellular dUTPase activity. This appears to be due to a specific dephosphorylation of the cellular dUTPase protein. Our observations also indicate that, in mutant virus-infected cells lacking HSV dUTPase, cellular dUTPase is still active and continues to be phosphorylated. It appears that, in contrast to the wild-type situation, the mutant virus can accommodate the phosphorylated state of cellular dUTPase, allowing it to substitute for the viral specific dUTPase.

MATERIALS AND METHODS

Generation of Monoclonal Antibodies to Cellular dUTPase

Procedures for the immunization of mice and spleen cell fusion to P3X63-Ag.653 were those of Siraganian et al. [14]. Briefly, 5 μg of purified dUTPase [2] derived from

HeLa S3 cells (emulsified in an equal volume of Freund's complete adjuvant (Sigma)) was injected into each of 6 BALB/c (Jackson Labs) female mice 8–10 weeks old. Three weeks later the same amount of antigen in Freund's incomplete adjuvant (Sigma) was administered to each mouse. The following week, mice were bled and tested for antibody against dUTPase as described below. Two of the six mice proved positive and their spleens were removed, spleen cell suspensions were made and fused to the mouse myeloma cells and hybrids were selected in HAT (hypoxanthine/aminopterin/thymidine) as described [14].

Screening for antibody from mouse sera and also from growing hybridoma cells was accomplished by the western blotting technique as described by Towbin et al. [15]. Purified HeLa S3 dUTPase (100 μg) was electrophoresed in a blank well and transferred to nitrocellulose. Strips (2 mm) were cut from this nitrocellulose and used to detect specific antibody production with horseradish-peroxidase-conjugated rabbit anti-mouse IgG (Cappel/Worthington) as second antibody and 4-chloro-1-naphthol as substrate [16] or an alkaline phosphatase conjugate with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate (Promega Biotec).

Out of about 300 wells positive for hybridoma growth, 11 produced antibody reactive to HeLa S3 dUTPase by this assay. One particularly good producer (S3dut415) was purified by cell cloning three times by the limiting dilution technique [16]. The rest were banked in liquid nitrogen. Large amounts of this monoclonal antibody were produced in mice by the generation of ascites fluid according to the technique of Parham [17]. Purification was accomplished by ammonium sulfate fractionation and DEAE cellulose chromatography [17]. Purified IgG was judged to be at least 90% pure by SDS polyacrylamide gel electrophoresis.

Radioactive Labeling of Cells

For the labeling of dUTPase protein HeLa S3 cells (at 5×10^6 per 10 cm dish) were incubated for 6 h, before infection, with 200 μCi of ^{35}S-methionine (Amersham) in DMEM (minus methionine). At the time of infection, monolayers were washed twice with PBS containing unlabeled methionine and infected with HSV-1 (strain KOS) [2] in DMEM media containing unlabeled methionine and 1% heat-inactivated fetal bovine serum.

To demonstrate phosphate labeling of the cellular dUTPase protein, HeLa S3 cells (either uninfected or infected with HSV-1) were labeled with 0.5 mCi/ml of ^{32}P-orthophosphate (Amersham) in DMEM (minus phosphate) for the indicated times.

Immunoprecipitation Reactions

At the indicated times, the cell monolayers were harvested after washing with PBS. The cell pellet was then resuspended in 20 volumes of a solution of 1% NP-40, 1 mM phenylmethylsulfonyl fluoride in PBS. The cells were then incubated on ice and centrifuged to remove nuclei [18]. The supernatant was preabsorbed to Sepharose CL-4B and then incubated with an excess of monoclonal antibody (10–20 μg IgG) for 12 h at 4°C. Control immunoprecipitations were performed with nonspecific antisera.

The solution containing the primary antigen-antibody complex was then mixed with 50 μl Protein A–Sepharose (50% w/v) for 1 h at room temperature. The Sepharose was pelleted by low-speed centrifugation and subsequently washed five times with 10 ml each time using RIPA buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, [19]). The Sepharose pellets

were then washed once in 50 mM Tris-Cl pH 8.0 and resuspended in SDS polyacrylamide sample buffer and boiled. Electrophoresis was performed on a 12.6% polyacrylamide gel (SDS-PAGE) as described previously [2]. For detection of ^{35}S-methionine, the gel was impregnated with an autoradiographic enhancer (Fluoro-Hance, Research Products International Corp.), dried, and exposed either to XAR or XRP Kodak x-ray film at −80°C. Fluorography of the gel included the use of intensifying screens (Kodak, X-omatic).

Phosphoamino Acid Analysis

Analysis of phosphorylated amino acid residues of cellular dUTPase was performed on immunopurified and SDS polyacrylamide gel purified protein as described by Hunter and Sefton [20]. Electrophoresis was performed at pH 3.5 for 1 h at 800 volts in glacial acetic acid/pyridine/H_2O 50:5:945 (v/v).

Generation of HSV Mutants Through Insertional Inactivation of the HSV dUTPase

A cDNA which encodes the HSV dUTPase was isolated from a cDNA library. Procedures for the generation of this library as well as the techniques used for screening have been described previously [21]. The probe used for screening was a subfragment of the Eco RI I fragment of the HSV-1 (strain KOS) genome [11].

Inactivation of the dUTPase gene was accomplished by insertion of a foreign fragment of DNA into a unique Kpn I site which exists in the coding region of this gene (see Fig. 6 for map). The foreign DNA insert is a 1.2 Kbp fragment containing the gene for bacterial resistance to chloramphenicol [22]. Recombinant plasmids containing the insert in the HSV dUTPase gene were selected for on the basis of chloramphenicol resistance of the host *E. coli*. The plasmid was isolated and purified by established procedures [23].

The HSV dUTPase cDNA containing the insertion at the Kpn I site was gel purified and cotransfected into CV-1 cells with intact HSV-1 DNA. Procedures used for eukaryotic cell transfection were obtained from Sandri-Goldin et al. [24]; 18 h after transfection, the medium was replaced with DMEM containing 1% heat-inactivated fetal bovine serum and 1% methylcellulose. Three to 4 days after transfection individual plaques were harvested and transferred directly into wells of a 24-well plate containing CV-1 cells. When generalized cytopathic effect was evident the medium (containing virus particles) was removed and analyzed for successful recombinants.

Screening for insertion mutants of the HSV dUTPase gene was accomplished by dot-blot hybridization on nitrocellulose of a 200 μl aliquot of the virus isolate derived from the 24-well plates and probed with a radiolabeled RNA transcript of the chloramphenicol resistance marker gene. Synthesis of the probe was performed as described previously [21].

Four positive isolates were identified out of 100 total virus isolates tested. These isolates were plaque purified three times. Two positives were identified from each of two independent DNA transfections and each positive isolate was derived from a separate plate of transfected cells.

Generation of Antibodies Against HSV dUTPase

The cDNA encoding the HSV dUTPase was cloned into the proper reading frame of a bacterial expression vector (pATH). This plasmid vector consists of a portion of the

TRP E gene of *E. coli* followed by a polylinker that allows one to clone DNA inserts into the proper reading frame. Expression of the fusion protein can be regulated by gratuitous induction of trytophan biosynthesis with indolacrylic acid. Details describing this vector along with the techniques for preparation of the chimeric protein are given by Dieckmann and Tzagoloff [25].

The fusion protein was isolated and purified by SDS polyacrylamide gel electrophoresis. The extract was then injected into rabbits by established procedures. Utilizing western blot analysis, positive serum from one rabbit was shown to react with a 39,000 dalton HSV dUTPase protein in virus-infected cells. In addition, this antiserum will neutralize HSV dUTPase activity but not cellular dUTPase activity. This antiserum will not recognize cellular dUTPase by western blot analysis. The monoclonal antibody generated against the cellular dUTPase does not react with the HSV dUTPase protein.

Miscellaneous Methods

Preparation of cellular extracts for dUTPase activity, enzyme assays, phosphocellulose column chromatography, and protein determinations were as described previously [2]. Western blot analysis was performed according to Towbin et al. [15] using as second antibody alkaline-phosphatase-conjugated goat anti-rabbit IgG. Additional reagents that were used were commercial products of analytical quality. One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 nmole of dUMP from dUTP per minute under the conditions previously established [2]. Enzyme activity was a linear function of time and enzyme concentration for the values reported.

RESULTS

Cellular and Viral dUTPase During an HSV Infection

Owing to the difference in isoelectric points between the cellular and HSV-induced forms of dUTPase, it has been possible to separate the two forms by phosphocellulose column chromatography [2]. Figure 1 illustrates this separation at different times post-infection. As can be seen, there is a decrease in cellular dUTPase activity (flow-through fraction of activity) while the virus-induced species (fraction of activity eluted with 400 mM potassium phosphate) increases in activity as infection progresses.

Inhibition of cellular protein synthesis and/or degradation of cellular dUTPase message coupled with a rapid turnover of the cellular dUTPase protein (as a consequence of HSV infection) could provide a reason for the observations seen in Figure 1. In an attempt to confirm this possibility, HeLa cells were labeled with ^{35}S-methionine and then infected with HSV 1 (KOS) for 12 h. Results from this experiment are presented in Figure 2. This figure illustrates that cellular dUTPase protein is present in an HSV-infected cell for at least 12 h post-infection. The amount of cellular dUTPase protein that remains in an infected cell is equivalent to that seen in a mock infection (Fig. 2, lane 1 vs. lane 2). This datum illustrates that the ^{35}S-methionine label, associated with cellular dUTPase, is stable for at least 12 h in both mock-infected cells and in cells infected with HSV. In order to show that synthesis of cellular dUTPase is not occurring during the infective phase, HeLa cells were infected with HSV and subsequently labeled with ^{35}S-methionine. Evidence from a 6 h labeling of cells during a 3 to 9 h period post-infection indicated that cellular dUTPase is not being made during infection (data not shown). This indicates that the labeled band, seen in Figure 2, lane 2, is not the

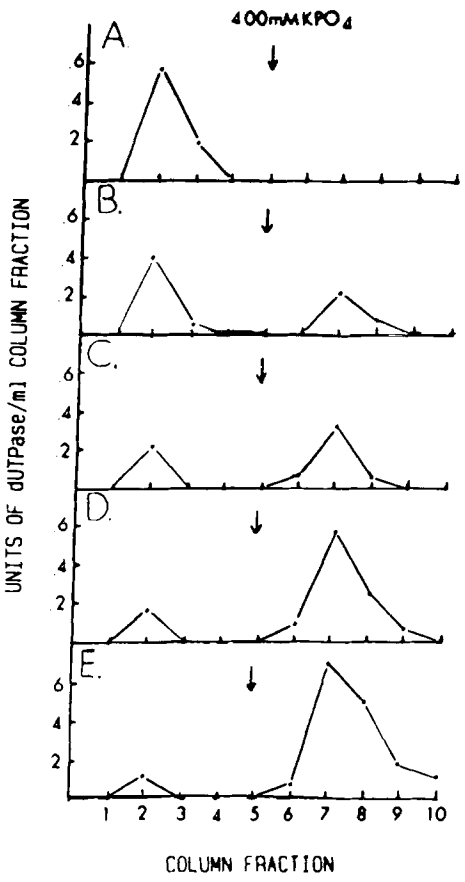

Fig. 1. Relative activities of cellular and HSV dUTPase as a function of time post-infection with HSV-1. Virus and cellular (HeLa) forms of dUTPase were separated by phosphocellulose column chromatography. Equal amounts of crude extract protein (0.5 mg), derived from each time point, were applied to a 0.2 ml phosphocellulose column in 20 mM potassium phosphate buffer, pH 7.5. The columns were then washed successively with 20 mM and 400 mM potassium phosphate buffer at pH 7.5. Cellular dUTPase activity elutes in the unadsorbed fractions whereas HSV dUTPase activity elutes with the 400 mM wash. Letters refer to hours post-infection, **A**, 0 h; **B**, 3 h; **C**, 6 h; **D**, 9 h; **E**, 12 h.

steady-state result of synthesis and degradation of the cellular dUTPase during infection. What is evident from the findings presented in Figures 1 and 2 is that a potential post-translational mechanism for shutoff of cellular dUTPase activity may exist during an infection of cells with HSV.

Phosphorylation of Cellular dUTPase

As a first approach to determine if cellular dUTPase is post-translationally modified, HeLa cells were labeled with ^{32}P-orthophosphate and the dUTPase protein was immunoprecipitated. Results from this experiment indicate that cellular dUTPase is phosphorylated in the uninfected cell. ^{32}P-labeled dUTPase that had been immunoprecipitated was further fractionated by SDS polyacrylamide gel electrophoresis. The radiolabeled band was cut out of the gel. The protein was then extracted, hydrolyzed,

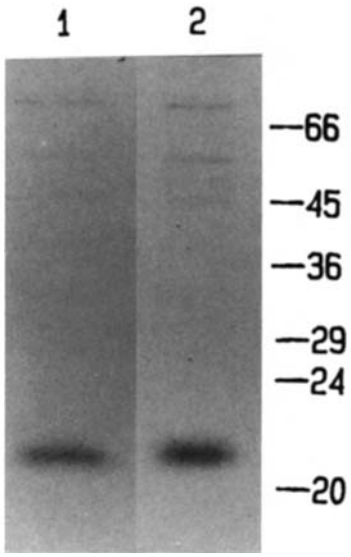

Fig. 2. Analysis of cellular dUTPase protein in infected cells. Fluorograph of an SDS polyacrylamide gel fractionation of immunoprecipitates derived from mock-infected or HSV-1 (KOS)-infected HeLa S3 cells. Cells were prelabeled with ^{35}S-methionine for 6 h. The radiolabel was removed by several washes with medium containing unlabeled methionine and then infected (mock-infected) for 12 h. Extracts were prepared and immunoprecipitation was performed as described in the Methods section. **Lane 1:** Mock-infected cells harvested at 12 h, after label was removed. **Lane 2:** HSV-1 (KOS)-infected cells harvested at 12 h. Equivalent total cpm of ^{35}S-methionine in the extract were immunoprecipitated and applied to each lane. The gel was processed and exposed to Kodak XAR X-ray film for 48 h at −80°C by utilizing intensifying screens. Markers are in kilodaltons.

and analyzed for phosphoamino acid content according to the procedures of Hunter and Sefton [20]. As illustrated in Figure 3, autoradiographic analysis indicates that the phosphorylated amino acid residue of the dUTPase protein is serine.

In an attempt to determine if HSV infection alters the phosphorylation of cellular dUTPase, cells were infected with the virus and then pulse labeled for 4 h at different time intervals, post-infection. Figure 4 illustrates the results from this experiment. Uninfected cells pulse labeled for 4 h reveal significant phosphorylation of cellular dUTPase (Fig. 4, lane 2). However, infected cells show a significant decrease in the phosphorylation rate of cellular dUTPase as time elapses, post-infection. These results are identical when either the type 1 or type 2 strain of HSV is used. The data, so far, indicate a correlation between a decrease in the phosphorylation of cellular dUTPase and a concomitant decrease in dUTPase activity. Both are a function of time post-infection.

There are two possibilities that can explain the observations of Figure 4. The first is that phosphorylation occurs on nascent dUTPase protein during or just after translation. Once phosphorylation occurs, the phosphate moiety remains stable until the dUTPase protein is degraded. In this instance, the observation seen in Figure 4 can be explained by a decrease in dUTPase synthesis during infection and thus a decrease in the phosphorylation rate of that protein. The second possibility is that the phosphate moiety on the cellular dUTPase protein is not stable and the rate at which phosphorylation and dephosphorylation occurs is dependent on the environment of the cell.

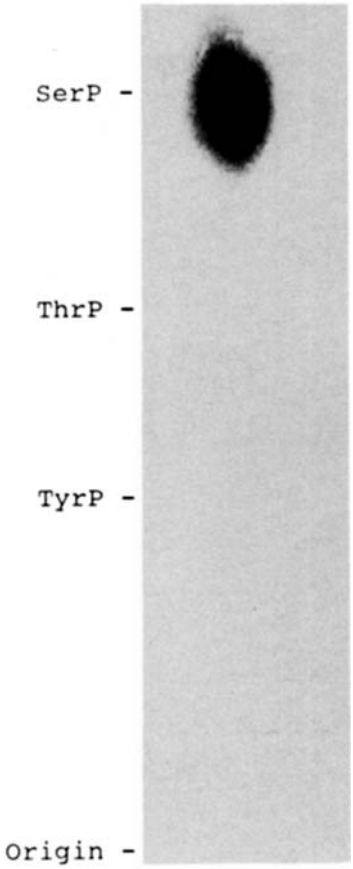

Fig. 3. Phosphoamino acid analysis of immunopurified and SDS-PAGE-purified HeLa dUTPase. Hydrolysates of gel-purified dUTPase were analyzed as described in Methods. Approximately 5,000 cpm of total radioactivity was electrophoresed. Exposure of x-ray film was for 24 h at −80°C. Markers: tyr-P, phosphotyrosine; thr-P, phosphothreonine; ser-P, phosphoserine.

In an effort to test these possibilities, HeLa cells were prelabeled for 12 h with ^{32}P-orthophosphate. This long labeling period should result in steady-state levels of ^{32}P-radiolabeled dUTPase protein [26]. ^{32}P-labeling for either 6 h or 12 h results in roughly equivalent intensities of labeled dUTPase protein (data not shown). Therefore it can be assumed that 12 h labeling has reached steady-state levels for the dUTPase protein. Subsequent to this labeling period, the cells were washed extensively with medium containing unlabeled phosphate and either mock-infected or infected with HSV type 1. Cells were harvested at 0 h or 6 h post-infection and cellular dUTPase was analyzed for phosphate content. Results from this experiment are shown in Figure 5. Cellular dUTPase derived from mock-infected cells, either at 0 h or 6 h post ^{32}P labeling, contain significant levels of ^{32}P label (Fig. 5, lanes 1 and 2). There is a decrease at 6 h post-labeling. However, significant phosphate remains with the dUTPase protein. This indicates that the 6 h chase interval did not result in a major turnover of the phosphate moiety. In contrast, lanes 3 and 4 of Figure 5 reveal that HSV infection results in a significant decrease in the phosphate moiety associated with the dUTPase protein. It can

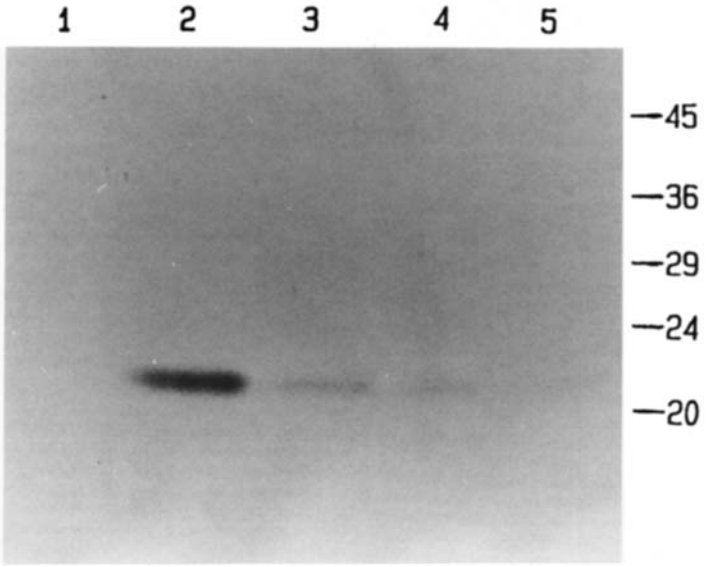

Fig. 4. ^{32}P-orthophosphate pulse labeling of HeLa cells uninfected or infected with HSV-1 (KOS). HeLa cells were infected with HSV and then pulse labeled for 4 h intervals. Subsequent to labeling, cells were harvested and extracted and cellular dUTPase was immunoprecipitated. **Lane 1:** Uninfected cells labeled for 4 h and immunoprecipitated with non-specific mouse IgG. **Lane 2:** Uninfected cells labeled for 4 h and immunoprecipitated with antibody against cellular dUTPase. **Lane 3:** Infected cells labeled at 0 h and harvested at 4 h post-infection. **Lane 4:** Infected cells labeled at 4 h and harvested at 8 h post-infection. **Lane 5:** Infected cells labeled at 8 h and harvested at 12 h post-infection. Immunoprecipitates were fractionated by SDS polyacrylamide gel electrophoresis and the dried gel was exposed to x-ray film for 48 h at −80°C. Markers are in kilodaltons.

be seen from this figure that at 6 h post-infection (and post-labeling) there is very little phosphate label associated with the cellular dUTPase protein. These results point to a specific dephosphorylation of cellular dUTPase protein as a result of infection by wild-type HSV.

Characteristics of Virus Mutants Lacking HSV dUTPase

Studies by Marshall Williams [13] indicate that HSV mutants lacking virus dUTPase retain cellular dUTPase activity. Our next step was to determine if HSV mutants, defective in HSV dUTPase, retain the phosphorylated form of cellular dUTPase along with measurable cellular dUTPase activity. As described in the Methods section, four viable mutants of HSV were generated by insertional inactivation of the HSV dUTPase gene. Figure 6 describes the location of the HSV dUTPase gene on the genomic map. The gene for HSV dUTPase is situated at about 0.7 map units on the prototypic arrangement of the HSV genome [11]. Figure 6 describes, as well, the position in the open reading frame where insertional inactivation occurs. Foreign DNA was inserted at the KpnI site within the coding region for HSV dUTPase (Fig. 6B,C). The insertion was verified by Southern blot analysis in each of the mutants studied (data not shown). Two virus mutants were derived from each of two DNA transfections and each mutant was derived from a different dish of transfected cells. Therefore, four independent isolates were derived from the DNA transfections.

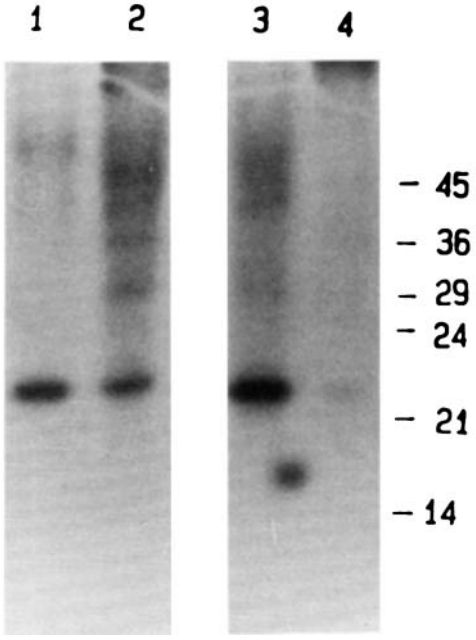

Fig. 5. Dephosphorylation of cellular dUTPase protein as a function of HSV infection. HeLa cells were prelabeled for 12 h with ^{32}P-orthophosphate. The label was removed and the cells were either mock-infected or infected with HSV-1 (strain KOS). At either 0 h or 6 h post-infection, the cells were harvested and dUTPase was extracted and immunoprecipitated. The immunoprecipitation was then fractionated on a 12.6% SDS polyacrylamide gel. **Lane 1,** 0 h post mock-infection. **Lane 2,** 6 h post mock-infection. **Lane 3,** 0 h post HSV1-infection. **Lane 4,** 6 h post HSV1-infection. The gel was dried and exposed to x-ray film for 48 h at −80°C.

In contrast to the wild-type virus (Fig. 7A), these mutants do not express HSV dUTPase protein as visualized by western blot analysis (Fig. 7B) and do not express any HSV dUTPase activity but express significant cellular dUTPase activity throughout the infection period (analyzed to 12 h post-infection). This was determined by phosphocellulose column chromatography (data not shown; however, the profile is identical to the profile seen in Fig. 1A).

In an effort to determine the state of phosphorylation of cellular dUTPase in these mutant viruses, cells were prelabeled with ^{32}P-orthophosphate for 12 h and then infected with the mutant viruses. As seen in Figure 8A, significant dephosphorylation of cellular dUTPase does not occur in any of the mutant virus infections. Surprisingly, when HeLa cells were infected with these mutants for 4 h and then labeled with ^{32}P-orthophosphate from 4 to 12 h post-infection, significant phosphate label became associated with the cellular dUTPase protein (Fig. 8B). Since (as with the wild-type infection) mutant-virus-infected cells are not synthesizing cellular dUTPase protein as judged by a lack of incorporation of ^{35}S-methionine (data not shown), phosphorylation of pre-existing cellular dUTPase must be occurring.

In summary, these results indicate that: 1) The phosphorylation rate of cellular dUTPase in a wild-type HSV infection decreases dramatically with time post-infection

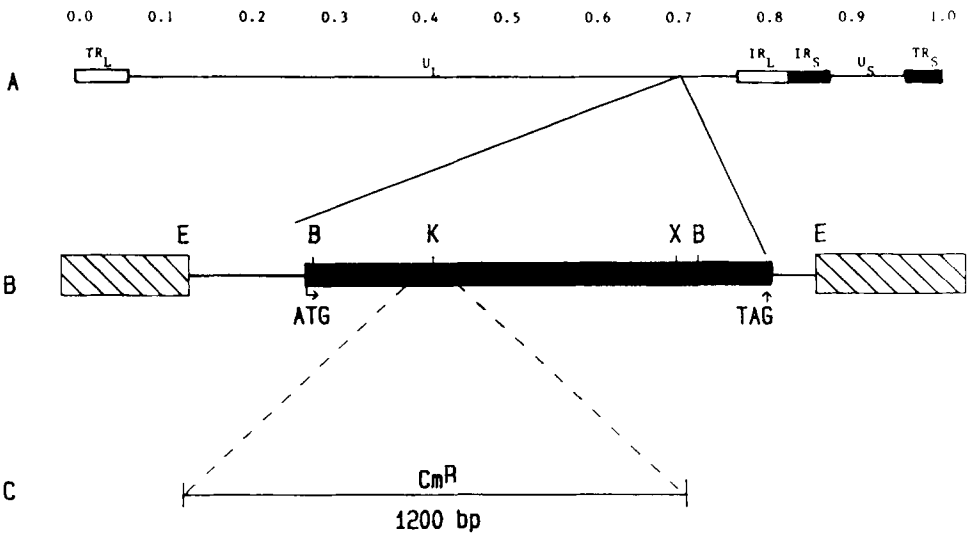

Fig. 6. Insertional inactivation of the HSV dUTPase gene. **A:** Map of the prototypic arrangement of the HSV genome and location of the HSV dUTPase gene. **B:** Map of a plasmid clone of the cDNA for HSV dUTPase (hatched area, plasmid sequence; heavy dark line, open reading frame of HSV dUTPase gene; E, EcoRI; B, BamHI; K, KpnI; X, XbaI; ATG, translation start; TAG, translation stop). **C:** Position of insertion of the 1,200 base pair chloramphenicol resistance gene (CmR) in the open reading frame of the dUTPase gene.

(Fig. 4). 2) Wild-type HSV infection of cells results in a significant dephosphorylation of cellular dUTPase (Fig. 5). 3) In a mutant-infected cell, lacking HSV dUTPase, significant dephosphorylation of cellular dUTPase is not occurring (Fig. 8A). 4) Results presented in Figure 8B indicate that some fraction of the cellular dUTPase is being phosphorylated in the mutant-infected cells without concomitant synthesis of cellular dUTPase protein.

The data infer that phosphorylation of cellular dUTPase in the mutant-virus-infected cell allows that cell to retain cellular dUTPase activity. This allows the cellular dUTPase to substitute for the defective viral enzyme in the production of progeny virus.

DISCUSSION

Sequence analysis of the herpes simplex virus genome has revealed approximately 70 open reading frames [27]. Protein products from seven of these open frames have been shown to be essential for HSV origin-dependent replication of plasmid DNA [28,29]. This group of replication functions, along with regulatory proteins expressed immediately after infection [30] and surface glycoproteins of the virus envelope [18], appears to be essential for production of progeny virus.

In contrast, several of the virus genome's open reading frames have been shown to encode proteins which do not appear to be essential for virus growth, at least in cultured cells. Several investigations, utilizing deletion or insertion mutagenesis of the HSV genome, have shown that certain genes can be inactivated with no apparent effect on virus propagation. These are genes involved in nucleotide metabolism [12,31,32], genes

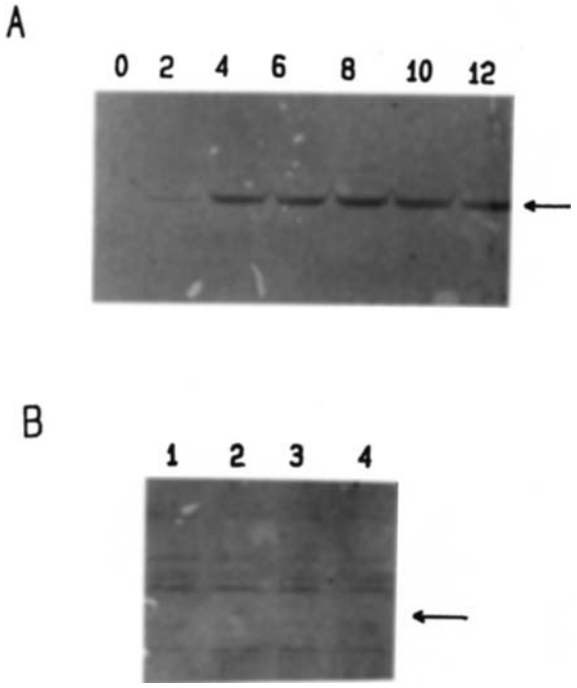

Fig. 7. Western blot analysis of HSV dUTPase. **A:** Time course of infection of HeLa cells with wild-type HSV-1 (strain KOS). Numbers above lanes refer to hours post-infection. Arrow indicates 39,000 dalton HSV dUTPase protein. As can be seen, this band intensity increases as a function of time, post-infection. **B:** Western analysis of four mutant HSV isolates, constructed by insertional inactivation of the HSV dUTPase. No 39,000 dalton band is visualized. The observed background bands, in this over-exposed blot, are also seen with non-specific antisera. **Lanes 1–4,** virus isolates 2.12, 1.4, 2.3, 5.6. Markers are in kilodaltons.

specifying structural components of the virus, as well as genes of unidentified function [33,34].

The premise which instigated these studies attempts to discriminate between two possibilities. The first is that the virus encodes redundant functions which are not necessary, due to the presence of the counterpart cellular function. The second possibility is that the environment of the infected cell precludes, in certain situations, the utilization of cellular enzymes for virus propagation. This requires that the virus encode functions similar to cellular enzymes whose physical properties allow for activity in the infected cell environment. It appears that, at least for the dUTPase function, the latter possibility is correct. We have shown, in this study, that activity of cellular dUTPase in a wild-type HSV infection diminishes to background levels even though cellular dUTPase protein remains stable. In addition, these studies indicate that dephosphorylation of cellular dUTPase protein occurs subsequent to infection. These observations indicate that post-translational phosphorylation of cellular dUTPase appears essential for its activity. In addition, dephosphorylation by wild-type HSV infection is responsible for the decrease in cellular dUTPase activity in the infected cell. In previous studies we have shown that enzymatically active cellular dUTPase is a dimeric protein composed of two equal molecular weight subunits [2]. Association of the subunits into the active enzyme is

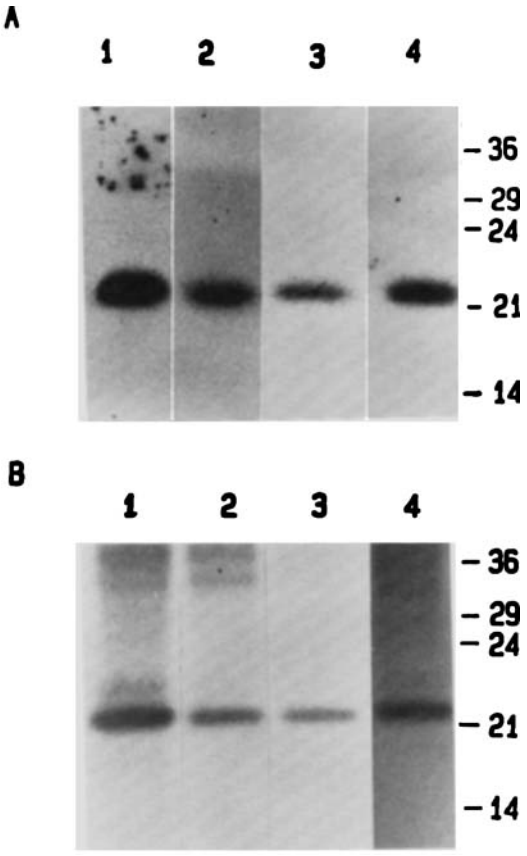

Fig. 8. Phosphorylation state of cellular dUTPase in mutant-virus-infected cells. **A:** HeLa cells were prelabeled with ^{32}P-orthophosphate for 12 h. The label was removed and the cells were infected with each of the mutant virus isolates. Cells were harvested at 6 h post-infection and dUTPase was immunoprecipitated and analyzed by SDS polyacrylamide gel electrophoresis. **Lanes 1–4,** mutant isolates 2.12, 1.4, 2.3, 5.6. Markers are in kilodaltons. **B:** ^{32}P-orthophosphate pulse labeling of mutant-HSV-infected HeLa cells. HeLa cells were infected with each of the mutant virus isolates defective for HSV dUTPase for 4 h. At this time cells were labeled and then harvested at 12 h post-infection. Immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. **Lanes 1–4,** mutant isolates 2.12, 1.4, 2.3, 5.6. Markers are in kilodaltons. Each gel was dried and exposed to x-ray film for 48 h at −80°C.

promoted by Mg^{2+}. It is conceivable that phosphorylation may play some role in subunit association and that dephosphorylation by HSV prevents this association.

The generation of HSV mutants by insertional inactivation of the HSV dUTPase gene appears to have also reversed the dephosphorylation event of cellular dUTPase in the mutant-infected cell. The mutant-infected cell contains significant cellular dUTPase activity along with elevated dUTPase phosphoprotein, compared to wild-type infection. These results support and extend the earlier observations of Williams [13] who showed that other mutants defective in HSV dUTPase contain significant cellular dUTPase activity. The possibility exists that during the generation of the mutant virus, independent mutation occurs at a genetic locus of the virus which inactivates a dephosphorylat-

ing function. This, in turn, allows for the stabilization of the serine phosphate on the cellular dUTPase protein. Since it appears that the dUTPase enzyme is an essential function, only those viruses which do not have dephosphorylating ability allow the cellular form of the enzyme to exist in the infected cell. Thus double mutants may have been selected which allow the substitution of the cellular dUTPase for the inactivated virus function.

Because of the facile nature of generating HSV mutants completely devoid of activities such as dUTPase, thymidine kinase [32], and ribonucleotide reductase [31], it has been postulated that development of chemotherapeutic agents against these viral targets would not prove fruitful. In light of the data presented here a revaluation of methods used to determine the essential nature of virus functions appears appropriate. It may turn out that many more targets for herpesvirus chemotherapy are exploitable.

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