Compartmentalization of Spermine and Spermidine in the Herpes Simplex Virion

(nucleocapsid/viral envelope/viral core/DNA phosphate-group neutralization/human carcinoma cells)

WADE GIBSON AND BERNARD ROIZMAN

Department of Microbiology, University of Chicago, Chicago, Illinois 60637

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ABSTRACT Enveloped particles of herpes simplex virus produced in human cells in culture contained spermidine and spermine, in a molar ratio of 1.6 ± 0.2. The spermine present within the nucleocapsid is sufficient to neutralize at least 40% of the viral DNA. Disruption of the envelope with nonionic detergent and urea resulted in the selective loss of spermidine. Exogenous ornithine can function as a precursor to host and viral polyamines only before infection.

Polyamines are known to bind to nucleic acids and phospholipids (1), and may be functional (2) as well as structural (3) components of eukaryotic cell organelles. Polyamines have previously been shown to occur in appreciable amounts in the DNA-containing bacteriophages T2 and T4 (4, 5). They have not been found in poliovirus, tomato bushy stunt, cucumber, and tobacco mosaic virus, all of which contain RNA and replicate in eukaryotic cells. Interestingly, another unenveloped RNA virus, turnip yellow mosaic virus, does contain polyamines, almost exclusively in the form of spermidine (6).

We report here the presence of spermine and spermidine in herpes simplex virus. The virion of herpes simplex consists of a core that contains DNA (10^8 daltons) made in the nucleus, a multilayered capsid assembled in the nucleus from proteins made in the cytoplasm, and an envelope derived from the nuclear membrane modified by virus-specific glycoproteins (7). Of the two polyamines in the virion, spermine appears restricted to the nucleocapsid and spermidine to the viral envelope. This result is in contrast to the finding that putrescine and spermidine are present together in the nucleocapsids of T2 and T4 phages.

MATERIALS AND METHODS

Cell Culture. Human epidermoid carcinoma no. 2 cells were grown as monolayers in minimal essential medium supplemented with 10% calf serum.

Virus and Infection of Cells. The F prototype of the subtype 1 (8) of herpes simplex virus was used in all experiments. The procedure for infection of cells was as follows: Briefly, 4 X 10^6 cells in a uniform monolayer were incubated with the virus suspended in phosphate-buffered saline (pH 7.2) containing 10% glucose and 1% calf serum, at a concentration of 10-20 plaque forming units per cell, for 60 min at 37°C. The inoculum was then removed and the cells were incubated at 37°C in medium 199 containing 1% inactivated calf serum.

Labeling of Cells. Cells were labeled with 4 μCi of [3-3H]L-ornithine per ml of medium at the times indicated in the text. [3-3H]L-ornithine (2.38 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

Purification of Virus. Purified enveloped virus was prepared by a modification of a procedure developed by Spear and Roizman (manuscript in preparation). Briefly, the infected cells were swollen in hypotonic buffer and disrupted by four strokes of a Dounce homogenizer with a tight pestle. The cytoplasm was then separated from the nuclei and large debris by centrifugation, and layered onto a 3-30% (w/w) Dextran 10 (Pharmacia Fine Chemicals, Piscataway, N.J.) gradient prepared in phosphate buffer (0.01 M, pH 7.1) in place of the Tris buffer recommended in the original procedure. The gradients were then centrifuged at 20,000 rpm for 50 min at 5°C in a Beckman SW25.3 rotor. A single band present at the midpoint of the tube contained almost exclusively enveloped nucleocapsids free of host DNA (9) with relatively little contamination by membrane fragments.

Preparation of Naked Nucleocapsids. The envelope of the herpesvirus was disrupted and largely removed by two procedures described by Spear and Roizman (manuscript in preparation). Procedure 1 involved mixing purified enveloped particles with NP-40 (0.5% v/v final concentration; Shell Oil Co., New York, N.Y.), incubating the suspension at 0°C for 15 min, and then pelleting the treated particles for polyamine analysis. This procedure releases some, but not all, viral-envelope glycoproteins. Moreover, electron microscopic examination shows that some unidentified structural components of the envelope remain.

Procedure 2 was designed to increase the purity of the nucleocapsids obtained after NP-40 treatment and involved pelleting the treated viral particles through a barrier of 10% (w/v) sucrose-0.01 M sodium phosphate (pH 7.1)-2 M urea. This procedure considerably enhances the purity of the nucleocapsids, although measurable amounts of envelope glycoproteins still remain.

Sample Preparation. The virus band was aspirated through the wall of the nitrocellulose tube with a syringe. After treatment appropriate to the experiment, the virus suspension was diluted 1:1 with 0.01 M sodium phosphate (pH 7.1) and centrifuged for 2 hr at 25,000 rpm and 5°C in a Beckman 25.3 rotor. The supernatant was discarded and the pellet, containing enveloped nucleocapsids or naked nucleocapsids, was assayed for polyamine content.
**Polyamine Analyses.** Polyamine analyses were done by the method of Seiler and Weichmann (10), as modified and described by Dion and Herbst (11). Briefly, the polyamines were extracted with perchloric acid and dansylated (1-dimethylamino-naphthalene-5-sulfonyl chloride was obtained from Pierce Chemical Co., Rockford, Ill.). The dansylated derivatives were extracted from the reaction mixture with benzene.

Dansylated polyamines were separated by thin-layer chromatography on 250 \( \mu \)m silica gel G plates (20 \( \times \) 20 cm) obtained from Analtech, Inc., Wilmington, Del. The plates were developed three times in ethylacetate-cyclohexane 2:3, with several minutes drying between each development. After they were sprayed with triethanolamine-isopropanol 1:4, and dried overnight in the dark at reduced pressure, the plates were scanned with a Turner fluorometer model 111. The area under each peak was then measured.

When radioisotopically labeled material was analyzed, each spot observed by fluorescence was scraped from the thin-layer plate, dried at 70°C for 1 hr, and suspended in a scintillation fluid. The radioactivity was measured in a Packard Tri-Carb scintillation spectrometer. In a typical experiment, 28% of the tritium counts in the starting acid extract were recovered from the thin-layer plate as the dansylated derivatives of spermine and spermidine.

**Identification of Polyamines.** The primary criterion used to identify the polyamines present in virus extracts was their position on the thin-layer plate relative to that of putrescine, spermidine, or spermine (all obtained as the hydrochloride salts from Sigma Chemical Co., St. Louis, Mo.), spotted at several dilutions on each plate. These three polyamines were well separated from ethanolamine—a possible contaminant—which moved more slowly than spermine.

**DNA Analyses.** DNA determinations were done on the cold-HClO\(_4\)-insoluble pellets by the diphenylamine technique described by Burton (12).

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**RESULTS**

**Polyamine content of enveloped nucleocapsids**

The results of analyses of four different preparations of enveloped viral nucleocapsids are shown in Table 1. The data may be summarized as follows: (i) Spermine and spermidine were present in substantial amounts in all preparations. Trace amounts of a dansylated compound migrating to a position similar to that of putrescine on the thin-layer plates were also noted, but the amounts were insufficient to quantitate. (ii) The molar ratio of spermidine to spermine was nearly constant, with an average value of 1.6 \pm 0.2. (iii) The average molar ratios (calculated with the constants shown in the footnotes to Table 1) of spermine and spermidine nitrogen to DNA phosphate were 0.58 \pm 0.17 and 0.65 \pm 0.12, respectively; these ratios did not vary appreciably from experiment to experiment.

**Characterization of polyamine binding to the virion and sub-viral particle**

In an attempt to determine whether the polyamines are associated with the envelope or with the nucleocapsid of the virus, a large pool of enveloped virions was prepared and divided into three equal aliquots. The first was pelleted and extracted with HClO\(_4\), the second was treated with NP-40 according to Procedure 1 (above), and the third was treated with both NP-40 and urea according to Procedure 2 (above). A portion of the cytoplasmic lysate from which the virus was purified on Dextran gradients was simultaneously extracted and will be referred to as “cell extract”.

Electron microscopic studies of the three preparations showed the presence of enveloped nucleocapsids in the pellet obtained after centrifugation of the untreated virus, and of partially unenveloped nucleocapsids in the material treated with NP-40. The principal features of the experiment, summarized in Table 2, are as follows: The recovery of spermine per unit of DNA phosphate was constant and unaffected by the treatment with NP-40 or by centrifugation through 2 M urea (column 3). By contrast, spermidine concentrations were reduced by NP-40 treatment, and even more strikingly reduced (nearly 30-fold) after centrifugation through 2 M urea (column 4).

**Time of synthesis of viral polyamines**

To establish the time interval during which viral polyamines are synthesized, four sets of cell cultures were handled as follows: set \( A \) (8 \( \times \) 10\(^7\) cells) was labeled with \(^{3}H\)ornithine for 18 hr and then washed, infected, and incubated without label; set \( B \) (8 \( \times \) 10\(^7\) cells) was infected and then labeled for the next 18-hr period; set \( C \) (8 \( \times \) 10\(^7\) cells) was labeled during the same 18-hr interval as set \( A \), but left uninfected; and set \( D \) (10\(^8\) cells) was infected but not labeled; the cytoplasmic extract obtained from these cells served as a carrier. Cell extracts were prepared from all four sets of cultures. Extracts from sets \( A - C \) were each combined with a third of the material from set \( D \). Enveloped virus was then isolated from each extract–carrier mixture by centrifuga-
The main features of the data, presented in Table 3, are as follows: (i) Polyamines present in enveloped virions are largely specific structural components of the virion. This conclusion is based on a comparison of the ratios of the specific activities of polyamines in the crude cell extracts and in the purified virions from each part of the experiment. Specifically, (Table 3, columns 6 and 8), the data show that very little of the spermidine present in the labeled uninfected-cell extract becomes bound to the unlabeled virus mixed with it, and that the ratio of the specific activities for the purified virus from the artificial mixture of unlabeled infected-cell extract and labeled uninfected-cell extract is five times less than that for virus from cells labeled before infection. The data are even more striking for spermine (Table 3, columns 5 and 7). (ii) The metabolic conversion of ornithine into polyamines is markedly reduced in infected cells. This conclusion is based on the observation that the specific activity of polyamines in cells labeled after infection is drastically diminished compared to that of polyamines in cells labeled before infection (Table 3, columns 5 and 6). Polyamine metabolism subsequent to this first step, however, may continue after infection. This possibility is suggested by the near identity of the specific activities of spermine and spermidine in extracts of infected cells labeled before infection and uninfected cells labeled during the same time interval (Table 3, columns 5 and 6). However, specific tests have not been done to confirm whether such continued synthesis does in fact occur. (iii) The spermine preferentially incorporated into the virion is that most recently synthesized by the host cell. This conclusion is supported by the observation that the specific activity of viral spermine was more than three times that of the prelabeled cell extract from which the virus was isolated (Table 3, rows 1a and b, column 5). In contrast, there was little difference between the specific activity of viral spermidine and that of the cell extract (Table 3, rows 1a and b, column 6).

**DISCUSSION**

These data show that (i) herpes simplex virus of subtype 1, a DNA virus infecting animal cells, contains the polyamines spermine and spermidine, (ii) these two polyamines appear to be bound to different substructures of the virion, and (iii) metabolic conversion of ornithine into host and viral polyamines is drastically reduced after infection. These conclusions are supported by the following: (i) Enveloped virions reproducibly contain 1.6-times more spermidine than spermine, and display a nearly constant molar proportionality between DNA phosphate and polyamine nitrogen (Table 1). In
addition, the very low extent of binding of extraneously added polyamines (Table 3) makes it highly unlikely that viral spermine and spermidine occur merely as contaminants. (ii) Treatment of the virions with NP-40 and urea preferentially removed 97% of the spermidine, yet decreased the amount of spermine by just 10% (Table 2, columns 3 and 4). (iii) Enveloped nucleocapsids from cells labeled after infection contained polyamines with specific activities less than 1% of those of virus particles purified from cells labeled before infection. This decrease in specific activity reflects a similar decrease in the extracts of cells labeled after infection (Table 3, columns 5 and 6). It is unlikely that this decrease is the result of a diminished uptake of ornithine by the infected cells, since preliminary experiments (unpublished) indicate that the radioisotope can still be incorporated into acid-precipitable polypeptides.

The conclusions presented in this paper raise two questions. The first relates to the apparent reduction of the metabolic conversion of ornithine into polyamines in infected cells. Dilution of the radioisotope does not appear to explain our findings, since putrescine pools in both infected and uninfected cells appear to be very small in comparison with those of spermine and spermidine. One explanation for this finding concerns the fact that herpesviruses inhibit protein synthesis in the host (7), and that the first enzyme involved in the conversion of ornithine to polyamines—ornithine decarboxylase (EC 4.1.1.17)—has been reported to have a very high turnover rate (19). Hence, it is possible that the enzyme would disappear from the cells very rapidly after infection, blocking subsequent metabolism of labeled ornithine into polyamines.

The possibility of continued polyamine metabolism subsequent to the ornithine decarboxylation step is suggested by the data presented in Table 3 (rows 1a and 2a, columns 5 and 6). While these data are not conclusive, they are consistent with the observation that S-adenosyl-L-methionine decarboxylase, which is involved in the synthesis of spermidine, has a much longer half-life than does ornithine decarboxylase (2).

The second question concerns the apparent segregation of polyamines into different substructures of the virion. We do not know why spermidine is preferentially bound to the envelope, whereas spermine is preferentially found in the nucleocapsid, nor do we know the functional role of either polyamine. However, one point should be made. In view of the constant molar ratios of spermine nitrogen to DNA phosphate, it is tempting to speculate that the function of spermine in this virus is the same as the putative functions of both spermidine and putrescine in T4 phage, i.e., to neutralize the negative charges on the DNA.

Since spermine and spermidine are present in the infected-cell lysate in nearly equal amounts, we anticipated that both would be found closely associated with the nucleocapsid, as is the case with putrescine and spermidine in T4 phage. However, the segregation of the two polyamines found in herpes simplex virus is in striking contrast to the distribution of bacteriophage polyamines.

One hypothesis to explain our finding is that the affinity of spermine for herpesvirus DNA is much greater than that of spermidine. Thus, while both polyamines may be available within the nucleus for incorporation into the nucleocapsid, only spermine is included. This hypothesis is weakened, however, by the following lines of evidence: (i) T4 phage, isolated from bacteria containing nearly 40 times the amount of spermine as putrescine and spermidine combined, contained all three polyamines (5). Thus, even in great excess, spermine did not completely displace the other polyamines. It seems unlikely, therefore, that the stronger binding of spermine to DNA can alone account for the exclusion of spermidine from the nucleocapsid of herpesvirus isolated from cells containing nearly equimolar amounts of spermine and spermidine. (ii) Regarding the possibility that specific polyamines are preferentially bound to certain species of DNA, equilibrium dialysis results showed no significant difference in the affinity of spermine for DNAs of different base compositions (14). Thus, the high (67%) guanine plus cytosine content of herpesvirus DNA (9) may be inconsequential in determining which polyamines are bound to it.

A second and more interesting hypothesis to explain the segregation of spermine and spermidine in the herpesvirus particle is that available polyamines are compartmentalized within the host cell—spermine inside the nucleus and spermidine outside. Thus, for a virus replicating in the nucleus, spermine alone would be available for binding to the viral nucleic acid, either during its synthesis or during morphogenesis of the nucleocapsid, while spermidine alone would be available for binding to the virus during its envelopment at the nuclear membrane and/or its egress from the infected cell.

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