Virion Assembly Defect of Polyomavirus hr-t Mutants: Underphosphorylation of Major Capsid Protein VP₁ Before Viral DNA Encapsulation

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Received 29 October 1984/Accepted 11 January 1985

The major capsid protein of polyomavirus, VP₁, was separated into at least four subspecies by isoelectric focusing. One of these subspecies was selectively extracted from purified virions by mild treatment with sodium dodecyl sulfate, leaving a 140S particle enriched in the other three forms. The two most acidic subspecies were labeled in vivo with [³²P]phosphate, and these subspecies are among those identified as being deficient in nontransforming host range (hr-t) mutant virus nonpermissive infection of NIH3T3 cells. Quantitation of VP₁ phosphorylation revealed that hr-t mutant virus VP₁ is phosphorylated to about 40 to 50% of the level of the wild type in NIH3T3 cells, and two-dimensional phosphoamino acid analysis suggested that threonine phosphorylation was affected more than serine phosphorylation. Two results indicate that the VP₁ modifications occur before and independent of virus assembly: (i) modified subspecies were detected during wild-type infection within a 2-min pulse-label with [³²P]methionine, and (ii) VP₁ modifications of temperature-sensitive VP₁ mutants were the same at both restrictive and permissive temperatures for virus assembly. We conclude that most VP₁ modification occurs before viral DNA encapsidation, and that one defect in hr-t mutant virus assembly is in VP₁ phosphorylation, primarily affecting threonine.

Early structural studies on small icosahedral viruses have been seminal in our thinking about virus structure and macromolecular assembly (8). The protein chemistry of virion assembly, however, greatly lags behind our X-ray crystallographic view. The polyomavirus virion was originally thought to be composed of 420 identical VP₁ subunits organized in 60 hexameric and 12 pentameric capsomeres on the surface of a T = 7 icosahedron with quasi-equivalent contacts between subunits (22). However, recent data from X-ray diffraction and electron micrograph image analysis indicate that there are only pentameric surface assemblies, with a total of 320 subunits (27). An all-pentamer icosahedron poses a structural problem that cannot be resolved in a manner consistent with quasi-equivalence and strongly suggests that modification of VP₁ or perhaps the minor capsid proteins, VP₂ and VP₃, play an important role in structure determination (21).

Initial biochemical studies of simian virus 40 (SV40) and polyomavirus virions have utilized disruption of the virion into discrete components and subsequent characterization of these components (7, 9, 12). These approaches have led to a model of a virion structure that is maintained by pH-dependent bonds, calcium ions, and disulfide bridges. Additionally, an outer VP₁ shell can be distinguished from a "core" composed of the viral minichromosome, a subset of VP₁ molecules, and VP₂-VP₃. More recently, less disruptive methods have been used to extract previrion assembly intermediates from infected cells (2, 13, 16, 20). These studies have led to a model where capsid proteins are incrementally added to a 75S viral minichromosome until a final 240S virion structure is assembled. Once initiated, the encapsidation process appears to be very rapid. Recently, assembly intermediates between 75S and 240S have been observed by electron microscopy with temperature-sensitive VP₁ mutants of SV40 (4). For SV40, three VP₁ intracistromic complementation groups have been described, i.e., tsC, tsBC, and tsB. At the restrictive temperature, mutants from each group have distinctive defects in their nucleoprotein assembly intermediates, and Bina et al. (3) have suggested that the VP₁ protein has three domains that function differently during assembly.

The major capsid protein VP₁ has several subspecies identified by their isoelectric point, and for SV40 these subspecies were identified as post-translationally modified forms of a primary translation product (25). The modified subspecies of both SV40 and polyomavirus VP₁ include phosphorylated forms, and Ponder et al. (26) estimated that 12 to 15% of VP₁ monomers in the polyomavirus virion were phosphorylated. Bolen et al. (5) have correlated the phosphorylated VP₁ subunits with the function of cell receptor binding. Recently Anders and Consigli (1) have characterized the phosphopeptides of VP₁ and identified serine and threonine as the phosphate acceptors.

The polyomavirus early proteins, middle and small T antigens, also affect virion assembly. The nontransforming host range (hr-t) mutants, defective in both middle and small T antigens, are blocked in virus assembly when grown on nonpermissive cells (29). This block in assembly is accompanied by a failure to induce the normal stoichiometry of VP₁ subspecies, suggesting that VP₁ is a target for hr-t gene-controlled modification (17). Because the hr-t mutation genetically links virus growth with the ability to transform cultured cells and induce tumors in vivo, the elucidation of the defect in virion assembly may aid in identifying the role of middle and small T antigens in cell transformation. We have therefore further pursued the characterization of polyomavirus VP₁ modification, and we have compared in more detail the differences between hr-t mutant and wild-type (WT) VP₁ modifications. Our results indicate that VP₁ is rapidly modified before its association with the viral mini-

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chromosome. In hr-t mutant nonpermissive infection of NIH3T3 cells, VP1 was underphosphorylated compared with WT VP1, and the phosphorylated subspecies identified by isoelectric point correspond to those previously found deficient by two-dimensional (2-D) gel analysis of [35S]methionine-labeled total cell lysates from hr-t mutant-infected cells (17).

MATERIALS AND METHODS

Cells and viruses. Cell culture was performed as previously described (19). Primary cultures of baby mouse kidney (BMK) cells were prepared as described by Winocour (30). WT viruses were derived from hr-t mutant viruses by marker rescue (14). ts10 and ts59 have been previously described (11). NIH3T3 cells were free of pleuropneumonia-like organisms and were tested routinely for nonpermissivity (17, 19).

Chemicals and isotopes. Electrophoresis reagents were obtained from Bio-Rad Laboratories. [35S]methionine (400 to 600 Ci/mmol), [2-14C]sodium acetate, [3-3H]mannose, [6-3H]glucosamine, [3-3H]galactosamine, [35S]sulfate (10 to 1,000 mCi/mmol), and [32P]phosphate (carrier free) were obtained from New England Nuclear Corp. Ampholines were from LKB Instruments Inc. Rabbit anti-VP1 was the generous gift of Bill Murakami. Anti-poliovirus (strain LID-1) antiserum (goat) was prepared by BBL Microbiology Systems.

Preparation of [35S]methionine-labeled cell extracts for electrophoresis. Cells were plated into 1.5-cm Linbro microwells at 4 × 10^6 cells per well. For labeling periods longer than 2 h, Dulbecco modified Eagle medium without methionine was used; for a labeling period less than 2 h, Hank's balanced salt solution was used. Cells were harvested at given times by washing twice with phosphate-buffered saline (18) and then solubilizing the entire monolayer with 100 μl of lysis buffer (24). [35S]methionine was used at 0.1 to 1.0 mCi/ml.

Sulfate, acetate, and sugar labeling. [35S]sulfate labeling was carried out in Hank's balanced salt solution without sulfate at 2 mCi/ml. [3H]mannose labeling was carried out in Dulbecco modified Eagle medium with low glucose (0.25 mM) 1 at mCi/ml. Acetate (1 mCi/ml) and galactose (250 μCi/ml) labeling were carried out in Dulbecco modified Eagle medium. Except for acetate labeling, cells were preincubated for 3 h before labeling for an additional 5 h. Acetate labeling was for 1 h. Labeling was begun between 24 and 30 h postinfection. At the end of the labeling periods, VP1 was immunoprecipitated as described below, and the immunoprecipitate was electrophoresed on 1-D sodium dodecyl sulfate (SDS) gels.

Electrophoresis. Electrophoresis of proteins was carried out as described by O'Farrell (24). For 2-D analysis, the first dimension (isoelectric focusing) utilized ampholines in the ratio pH 3.5 to 10/pH 5 to 7/pH 7 to 9, 1:2:2 (vol/vol). For 1-D analysis, SDS-polyacrylamide gels were treated with En3Hance (New England Nuclear) for fluorography at −70°C. VP1 immunoprecipitations. Cell monolayers were washed twice with phosphate-buffered saline and then solubilized with RIPA buffer (0.1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris [pH 7.2]) for further analysis. Anti-VP1 antibody (2 μl) was incubated with this extract for 1 to 3 h at 4°C, and immune complexes were then removed with Staphylococcus aureus protein A-Sepharose beads (Pharmacia Fine Chemicals) by a further incubation for 1 h. The beads were washed twice with RIPA buffer, twice with phosphate-buffered saline, and once with water. Immunoprecipitated VP1 was removed from the beads with either O'Farrell lysis buffer (for 2-D analysis) for 5 min at 22°C, or 1-D SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 0.625 M Tris [pH 6.8], 10% glycerol) for 2 min at 100°C.

Virion isolation. Virions were initially isolated from infected cells by the method of Schaffhausen and Benjamin (28), and the virion band from the cesium chloride phase was then banded to equilibrium in a second cesium chloride gradient.

In vivo [32P]phosphate labeling and phosphoamino acid analysis. Cells were grown in 1.5-cm Linbro wells and labeled 35 h after infection with 1 mCi of carrier-free [32P]phosphate in 1 ml of phosphate-free Dulbecco modified Eagle medium with 4% dialyzed calf serum. Labeling was carried out for 4 to 12 h, at which time the VP1 was isolated by immunoprecipitation as described above. After 1-D SDS-polyacrylamide gel electrophoresis the VP1 bands were cut from the dried gel by using the autoradiogram as template. The gel pieces were dialyzed against 10% acetic acid–10% methanol for 2 days to reduce the salt content. The gel slices were then hydrolyzed in 6 N HCl under nitrogen for 2 h at 110°C in a sealed glass ampoule. The samples were subsequently diluted with an equal volume of the first dimension running buffer (7.2% acetic acid, 2.5% formic acid, pH 1.9) and spotted onto a cellulose plate (no. 13255; Eastman Kodak Co.) together with 5 μl of a phosphoamino acid mixture containing 1 mg each of phosphoserine, phosphothreonine, and phosphoserine per ml and electrophoresed on a cooled flat bed apparatus (LKB). After the bromphenol blue tracking dye had migrated 10 cm from the origin, the electrophoresis was stopped, and the plate was dried. The plate was then sprayed with the second-dimension buffer (5% acetic acid, 0.5% pyridine, pH 3.5) and electrophoresed at 90° to the first dimension until the dye had migrated 4 cm toward the anode. The plate was then dried and sprayed with ninhydrin reagent, the marker phosphoamino acids were outlined, and the plate was exposed to Kodak XAR-Omat film with an intensifying screen at −70°C.

VP1 quantitation. Autoradiograms of [35S]methionine- and [32P]phosphate-labeled VP1 were scanned on a Quick-Scanner densitometer (Helena Laboratories). The VP1 band intensities were quantitated by peak weight. Exposures were made in the linear range of the film. Several exposures were quantitated to verify the relative intensities between samples.

RESULTS

Isoelectric subspecies of VP1. Because of difficulties in comparing our 2-D gel VP1 patterns with those of other reports, we defined our VP1 subspecies with respect to their stoichiometry in virions and subviral components. Figure 1 shows the region of 2-D gel autoradiograms of [35S]methionine-labeled VP1. We consistently recognized four distinct subspecies, although several other forms were occasionally seen, representing less than 5% of the total VP1 that can be resolved. The most distinct additional subspecies was located between 3 and 4 and usually merged with subspecies 4. The subspecies in purified virions (Fig. 1A) were present in approximately the same relative stoichiometric ratios as in total infected cell lysates (Fig. 1B) or in immunoprecipitates of total infected cell lysates using anti-VP1 antiserum (Fig. 1C). Thus the relative ratios of subspecies did not vary greatly between the total available pool of VP1 molecules and those assembled into virions. Also, analysis by immunoprecipitation with anti-VP1 antibody does not bias for a particular subspecies.
The subspcies were separated into two groups by using techniques to disrupt intact virions. Figure 2A, B, and C show the VP1 subspcies present in three preparations of disrupted virions that are shown in Fig. 3. The first method (12) uses low-percentage SDS solutions to separate a "shell" of VP1 molecules (140S) from the viral DNA (Fig. 3A). Subspecies 3 was deficient in the shell structures that sediment at 140S (Fig. 2). Figure 2B displays the VP1 subspcies present in the minichromosome preparations of Moyne et al. (23). Using these relatively mild conditions [ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA)]-dithiothreitol (DTT), pH 7.5], many of the viral capsid proteins sedimented with the viral DNA at 140S (Fig. 3B), and all VP1 subspcies present in the intact virion remained associated with this structure. Figure 3C shows the 12S to 18S capsomeres generated by EGTA-DTT disruption at pH 8.5, as described by Brady et al. (7). The VP1 present in these smaller subunits (Fig. 2C) were also depleted in subspcies 3, resembling the pattern found in the SDS-140S shell structures.

Two of the VP1 subspcies are phosphorylated. We attempted to identify the translational modifications that generate the VP1 subspcies by metabolic labeling of infected cells with various substrates. We used [35S]sulfate, [3H]mannose, and [3H]galactose without detectable incorporation (see above). After [3H]mannose labeling, radioactivity was detected in the region of VP1 by 1-D SDS-polyacrylamide gel electrophoresis. However, a similar band was also

FIG. 1. 2-D gel profiles of [35S]methionine-labeled virion and intracellular VP1 subspcies: (A) Purified virions; (B) total lysate from WT-infected NIH3T3 cells; (C) immunoprecipitate of (B) with anti-VP1 antibody. VP1 subspcies are numerically identified, with decreasing pH to the right.

FIG. 2. 2-D gel profiles of VP1 subspcies present in disrupted virion preparations: (A) 140S particles from SDS-dissociated virions (Fig. 3A, fractions 23 through 26); (B) 140S particles from virions dissociated with EGTA-DTT at pH 7.5 (Fig. 3B, fractions 22 through 25); (C) 12S to 18S particles from virions dissociated with EGTA-DTT at pH 8.5 (Fig. 3C, fractions 32 through 33).

FIG. 3. Preparations of disrupted virions. Separate samples of [35S]methionine- and [3H]thymidine-labeled virions were dissociated in (A) 0.5% SDS-20 mM N-2-hydroxyethylpiperaizone-N'-2-ethanesulfonic acid (pH 7.0), (B) 5 mM DTT-10 mM EGTA-150 mM NaCl-50 mM Tris (pH 7.5) or (C) 5 mM DTT-10 mM EGTA-150 mM NaCl-50 mM Tris (pH 8.5) for 30 min at 37°C before sedimentation in 5 to 20% sucrose gradients of the same buffers at 39,000 × g for 50 min in an SW40 rotor. Fractions were collected from below and precipitated with trichloroacetic acid, and radioactivity of the subspcies was analyzed (Fig. 2). Profiles from parallel [3H]thymidine (○) and [35S]methionine (●) gradients are displayed in the same panels.
Phosphorylated VP1 subspecies are decreased in hr-t mutant infection of nonpermissive cells. During hr-t mutant virus infection of nonpermissive NIH3T3 cells, there is a defect in encapsidating the viral minichromosome, accounting for the majority of decreased virus output. Associated with this encapsidation defect, VP1 subspecies 2, 3, and 4 are decreased in abundance compared with WT virus infection of the same cells (17). Two of the deficient forms of VP1 represent phosphorylated subspecies (Fig. 4). In an attempt to quantify this deficiency in phosphorylation, we measured phosphoamino acid analysis by densitometry of the autoradiograms. A systematic error present in this analysis is the inclusion of spot 2, which often was not adequately separated from spots 3 and 4 in the 2-D gel. This error would lead to an overestimate of the relative amount of phosphorylated VP1. In the second method, WT or hr-t mutant-infected cells were labeled with either [32P]phosphate or [35S]methionine, and the amount of VP1 labeled was determined by titrating dilutions of the cell extracts with anti-VP1 antibody to assure antibody excess and then scanning VP1 bands from autoradiograms of 1-D SDS-polyacrylamide gel electrophoresis of the immunoprecipitates in a densitometer. The principal error in this analysis derives from the need to determine a ratio (32P/35S) of ratios (hr-t mutant/WT). The relative autoradiographic densities of phosphorylated VP1 to total VP1 for hr-t mutants (NG59, B2) normalized to the WT (59 RA, R4) were as follows: for method 1, 0.48 ± 0.08 for nonpermissive (NIH3T3) cells and 0.75 ± 0.12 for permissive (BMK) cells (averages of three experiments ± mean error); for method 2, 0.38 and 0.39 for NIH3T3 cells and 0.49 and 0.63 for BMK cells (results of two experiments). Thus, phosphorylated VP1 subspecies were decreased in hr-t mutant infection of NIH3T3 cells by 40 to 50% compared with the WT with either method of analysis. This decrease in hr-t mutant VP1 phosphorylation is less apparent when mutant and WT viruses are compared in permissive BMK cells. To investigate these differences further, VP1 phosphorylation was compared by phosphoaminoacid analysis.

VP1 is phosphorylated on serine and threonine. Previous analysis by 1-D phosphoaminoacid analysis of polyomavirus VP1 from virions determined that serine and threonine were the phosphate acceptors (1). We confirmed that result by using 2-D analysis. Figure 5 shows the phosphoaminoacids present in VP1 isolated by immunoprecipitation of in vivo [32P]phosphate-labeled, infected BMK cell extracts. Figure 5A shows a typical WT pattern with phosphothreonine in slight excess over phosphoserine, consistent with the previously reported ratio determined in purified virions (1). Figure 5B shows a representative hr-t mutant pattern with a decreased threonine-serine ratio. This decreased ratio was also seen in hr-t mutant-infected nonpermissive NIH3T3 cells. The differences in VP1 phosphorylation between WT and hr-t mutant nonpermissive infection (see above) may therefore reflect a relative decrease in threonine phosphate compared with serine as well as a decrease in the total amount of serine and threonine residues phosphorylated.

VP1 modification is rapid and independent of virus assembly. We studied VP1 modification in relation to virion assembly by using two independent approaches. First, temperature-sensitive VP1 mutants defective in virus assembly were studied for the presence of VP1 modifications. Two such mutants demonstrated the presence of modified VP1.
subspecies in NIH3T3 cells at 39.5°C (Fig. 6). At this restrictive temperature, 75S minichromosome complexes accumulate, but are not encapsidated to form 240S virions (18). The patterns of VP1 subspecies were identical at both permissive and restrictive temperatures, as expected based on the presence of normal hr-t function in these mutants. In related studies (R. L. Garcea and M. Bina, manuscript in preparation), SV40 ts VP1 mutants of all three complementation groups also show modified forms at the restrictive temperature for virion assembly.

The second approach utilized pulse-labeling of infected cells with [35S]methionine, isolation of VP1 by immunoprecipitation, and determination of the synthesis kinetics of the different VP1 subspecies by 2-D gel electrophoresis. All four major subspecies were detected after a 2-min pulse-label (Fig. 6D). The pulse-label pattern did not differ from that in a continuous label, and subspecies could not be chased into a stoichiometry different from the pulse profile. These kinetics are consistent with the ts VP1 data, and support rapid cotranslational or post-translational modification of VP1 that is uncoupled from ongoing virus assembly.

DISCUSSION

Previous studies (17) have defined two defects in virus assembly during hr-t mutant virus infection of nonpermissive cells: (i) a 3-fold decrease in viral DNA synthesis, and (ii) 6- to 10-fold decrease in the encapsidation of the viral minichromosome to a complete virion. Associated with these defects is a decrease in modified subspecies of the major capsid protein, VP1, although the total amount of VP1 accumulated is normal. Based upon these results we have hypothesized that VP1 is a target for hr-t gene-controlled modification and that modified forms of VP1 are essential for encapsidation. Results presented here define further the modifications of VP1 in the context of hr-t mutant virus infection.

A possible explanation for coincident defects in virus assembly and VP1 modification is that the hr-t gene products control a step in the addition of the capsid proteins to the viral minichromosome, and that VP1 is modified as this step proceeds. We therefore attempted to determine whether VP1 modification is dependent upon ongoing virion encapsidation. Two results indicated that VP1 modifications occur independently of assembly. First, VP1 modifications occur within a 2-min pulse-labeling of WT virus-infected cells. Thus, modification of VP1 probably occurs cotranslationally and is spatially and temporally independent of viral DNA encapsidation ongoing in the nucleus. Second, temperature-sensitive VP1 mutants modified their VP1 in a similar manner at permissive and nonpermissive temperatures for virus assembly. Consistent with earlier observations demonstrating complementation between hr-t and ts VP1 mutants (11, 15), we interpret these VP1 modifications as reflecting the action of the hr-t gene, which is normal in ts VP1 mutants. Since modified VP1 subspecies are deficient in hr-t mutant nonpermissive infection, the hr-t gene must act at the point of VP1 modification rather than at a step-in assembly per se. Thus, the defects of ts VP1 and hr-t mutants, although grossly similar in that 75S minichromosomes accumulate, clearly occur at different stages of virion assembly.

Because the modification of VP1 is related to hr-t gene action we attempted to define these reactions further. Using in vivo [32P]phosphate labeling we have identified subspecies 3 and 4 as being phosphorylated. Subspecies 3 is preferentially lost from purified virions by mild SDS treatment in the absence of a reducing agent (12) and is also present in decreased amounts in capsomere preparations (7). This subspecies may therefore have a distinct structural role, possibly serving as a bridge between the minor capsid proteins (VP2 and VP3) bound to the minichromosome and the outer VP1 shell comprised of subspecies 1, 2, and 4. Acetylation, previously described for VP1 (5), is a likely modification accounting for subspecies 2. The phosphorylated subspecies 3 and 4 are among those previously identified as being deficient in hr-t mutant infection of NIH-3T3 cells with [35S]methionine labeling (17).

We confirmed the hr-t mutant defect in VP1 phosphorylation by measuring in vivo [32P]phosphate incorporation in comparison with that of the WT. We found that total VP1 phosphorylation is decreased 40 to 50% in hr-t mutant infection of NIH3T3 cells. In both nonpermissive NIH3T3 cells and permissive BMK cells, threonine phosphorylation was more affected than serine phosphorylation. Although the differences between the hr-t mutant and the WT are only two- to threefold with respect to phosphorylation in NIH3T3 cells (see above), we would not be able to detect a large change in a minor subspecies, i.e., spot 4, with the present

FIG. 6. VP1 subspecies of ts VP1 mutants and pulse-labeled, WT-infected NIH3T3 cells. NIH3T3 cells infected at 39.5°C with (A) WT, (B) ts59, and (C) ts10 were labeled with [35S]methionine for 6 h beginning at 40 h postinfection. VP1 immunoprecipitates were then subjected to 2-D gel electrophoresis. The VP1 subspecies at 39°C were identical to those obtained at the permissive temperature, 33°C (data not shown). In panel D, NIH3T3 cells infected with the WT at 37°C were pulse-labeled for 2 min with [35S]methionine, and the immunoprecipitate was electrophoresed. The profile remained unchanged for longer pulse periods.
methods of analysis. Such a change in a minor, yet essential, subspecies could have a great effect on virus yields. Because the enzymes carrying out VP1 modification appear to be cellular, a higher constitutive level of expression of these enzymes in permissive BMK cells may compensate for a lack of hr-t gene function by increasing the total level of VP1 phosphorylation. This compensation is not equivalent to having an intact hr-t gene, since threonine phosphorylation of VP1 remains low in mutant infections of BMK cells in comparison to WT infection. Furthermore, in BMK cells hr-t mutant viral DNA synthesis is normal, whereas in NIH3T3 cells the mutants accumulate only 30 to 40% as much viral DNA as does the WT (17). Therefore, the permissivity of BMK cells for hr-t mutant growth results from a general increase in the amount of VP1 phosphorylated in combination with normal levels of viral DNA synthesis.

The mobilization of cell factors to facilitate virus replication and assembly appears to be the primary function of the hr-t gene, and phosphorylation of VP1, is one essential aspect of this gene’s pleiotropic effects. The association of polyomavirus middle T antigen with pp60 src (10) provides a starting point for intervention by the virus in protein kinase pathways of the cell. The possibility that the interaction of middle T antigen with a cellular tyrosine kinase eventually leads to VP1 phosphorylation requires further investigation.

ACKNOWLEDGMENTS

This investigation was supported by grant 5-R01-CA19567 to T.L.B., grant 1-P01-CA18662-08 to R.L.G., and a Swiss National Foundation Fellowship to K.B.-H. We thank Ingrid Lane and Lynne Kilham for their excellent technical assistance and Ireta Ashby for help in preparing the manuscript.

LITERATURE CITED


