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Polyomavirus Major Capsid Protein VP1 Is Modified by Tyrosine Sulfuration†

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Polyomavirus was propagated in primary mouse kidney cell monolayers and ^{35}S-sulfate labeled by maintaining the infected cells in serum-free Eagle medium supplemented with ^{35}S-labeled sodium sulfate. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of CsCl gradient-purified ^{35}S-sulfate-labeled virions followed by fluorography indicated that the polyomavirus-coded major capsid protein VP1 incorporated this radiolabel. Two-dimensional gel electrophoresis followed by fluorography revealed ^{35}S-sulfate incorporation into only two of the six VP1 isoelectric species (E and F). Amino acid analysis of ^{35}S-sulfate labeled VP1 by enzymatic hydrolysis followed by two-dimensional thin-layer electrophoresis revealed the presence of ^{35}S-sulfate-labeled tyrosine-*O*-sulfate.

The virions of polyomavirus are composed of three virus-coded proteins, VP1, VP2, and VP3, which surround the genomic DNA and four host cell-contributed histones (8, 9, 12, 13, 16). The major capsid protein VP1 can be separated into six species, designated A to F, by isoelectric focusing (IEF) (2, 5, 15, 26, 28). Only a portion of this charge heterogeneity can be attributed to phosphorylation, since only three of the identified species, D, E, and F, are phosphorylated (2, 5, 35). This phosphorylation occurs on serine and threonine residues, and these three species are more acidic in IEF than are the nonphosphorylated species. Previous reports from our laboratory suggested that these three phosphorylated species are involved in both specific and nonspecific virus attachment to cells (1, 5–7).

The purpose of the studies undertaken in this report was to further investigate the charge heterogeneity of the polyomavirus major capsid protein VP1. Since the polyomavirus genome does not code for enzymes capable of modifying its own protein structure, posttranslational modifications, such as the previously reported phosphorylation, must therefore be dictated by the host cell. Although our laboratory as well as others could not demonstrate the presence of tyrosine phosphorylation in polyomavirus-coded structural proteins (2, 25), this study explored the possible presence of another modification of tyrosine residues, tyrosine sulfuration. This modification is of particular interest owing to the molecular resemblance between sulfuration and phosphorylation which, therefore, may contribute to the charge heterogeneity of VP1 as well as influence the tertiary structure of this protein. Evidence is presented that the polyomavirus-coded major capsid protein VP1 is modified by tyrosine sulfuration and that this modification occurs on the VP1 IEF species E and F, which have been implicated in virus attachment to host cells.

Mouse kidney cells used in these experiments were prepared as described previously (22, 33). Wild-type polyomavirus was used to infect cells at a multiplicity of 10. Infected cell cultures were maintained in serum-free Eagle medium. Preparation of radioisotopically labeled virus was accomplished in the following manner. Infected cells were maintained in serum-free Eagle medium supplemented with additional cystine (40 ng/ml), methionine (20 ng/ml), and ^{35}S-sulfate (carrier free; ICN Pharmaceuticals Inc., Irvine, Calif.) at a concentration of 0.16 mCi/ml. Excess cystine and methionine were used to decrease the possibility of ^{35}S labeling of the sulfur-containing amino acids cysteine and methionine. At 18 h postinfection, additional ^{35}S-sulfate-containing medium was added to each infected cell monolayer, raising the concentration to 0.36 mCi/ml. Infected cells and media were harvested 3 to 5 days postinfection when the cells were observed to be liberated from the plastic dish. Virus was purified as previously described (21). CsCl gradients used to purify virus were prepared as described by Brunck and Leick (10) and were described in greater detail previously (8, 9, 34).

Several reports have demonstrated that mammalian cell proteins are modified by tyrosine sulfuration (for reviews, see references 19 and 23), and recently it was demonstrated that virus-transformed cell proteins can also be modified in this manner (14). To determine if our polyomavirus purification procedure was adequate to remove mouse cell proteins that were ^{35}S labeled, we performed mixing experiments in which mouse cells that were prelabeled with ^{35}S-sulfate were mixed with unlabeled purified polyomavirus virions. ^{35}S-sulfate labeling of uninfected cell monolayers began 24 h after seeding of the plastic culture dishes. The culture medium was removed and replaced with Eagle medium containing 5% dialyzed fetal calf serum and 5% fetal calf serum supplemented with the same amounts of cystine, methionine, and ^{35}S-sulfate as were used for maintaining infected cell monolayers. After harvesting of the labeled cells 3 to 5 days after the addition of ^{35}S-sulfate-containing medium and mixing with unlabeled purified virions, this mixture was then purified through a CsCl gradient. Fractionation of the CsCl gradient followed by scintillation counting, hemagglutination of guinea pig erythrocytes, and density determinations to locate the virion-containing fractions revealed that the recovered virus was free of mouse cell ^{35}S-labeled proteins that were prelabeled with ^{35}S-sulfate. Quantitation of ^{35}S-sulfate incorporated into complete polyomavirus virions and empty capsids revealed that complete virions possessed a threefold greater ^{35}S-sulfate counts per minute/hemagglutination unit value than did empty

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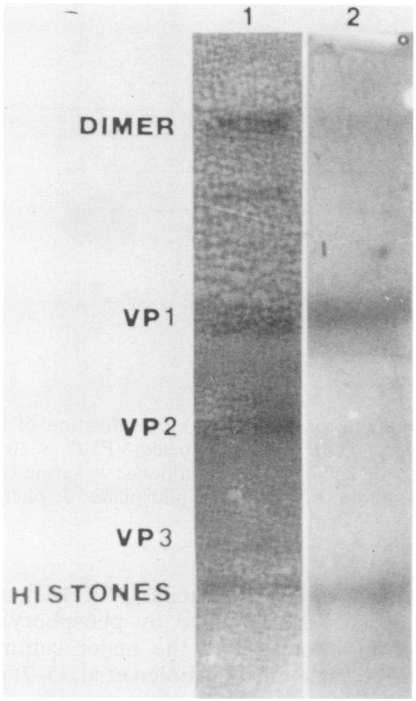

FIG. 1. SDS-PAGE of purified ^{35}S-sulfate-labeled polyomavirus virions. Lanes: 1, visualization of structural proteins by silver staining; 2, autoradiogram of ^{35}S-sulfate-labeled structural proteins (1.2×10^4 cpm).

capsids (13 and 4 ^{35}S-sulfate cpm/hemagglutination unit, respectively) (data not shown).

The proteins of purified ^{35}S-sulfate-labeled polyomavirus virions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 15% acrylamide gel and a 0.2% bisacrylamide cross-linker (9). Silver staining (24) and fluorography (11) were performed to visualize these structural proteins as well as to localize the ^{35}S-sulfate radioactivity. Comparison of the silver-stained gel (Fig. 1, lane 1) with the autoradiogram (lane 2) revealed that the majority of the ^{35}S-sulfate incorporation was in the virus-coded major capsid protein VP1. Some radiolabel also appeared to be incorporated into the dimer. This was not surprising, since the dimer has been shown to contain only VP1 sequences by peptide mapping (18) and immunological studies (7). Another band of radioactivity appeared in the histone region of the gel. It should be mentioned that upon polyomavirus infection, host cell histones have been shown to undergo extensive posttranslational modification (3, 4, 32). This report, however, deals specifically with the sulfuration of the polyomavirus-coded major capsid protein VP1. The appearance of ^{35}S-sulfate label in the host cell-contributed histone region of the gel will be the subject of another investigation. Further examination of ^{35}S-sulfate-labeled polyomavirus capsid protein VP1 by IEF and SDS-PAGE in the second dimension is shown in Fig. 2. Two-dimensional gel electrophoresis was performed as described previously (2, 5, 25, 26) with Polybuffer (Pharmacia, Inc., Piscataway, N.J.) in place of Ampholine (LKB Instruments, Inc., Rockville, Md.) for IEF. Comparison of the silver-stained gel (lane 1) with the autoradiogram (lane 2) revealed that only the acidic species E and F contained radioactivity, with species E containing the majority of this label. No radioactivity could be detected in the remaining four IEF species.

^{35}S-sulfate-labeled VP1 electroeluted from polyacrylamide gels after identification by autoradiography was then subjected to enzymatic hydrolysis with proteinase K by resuspending the lyophilized sample in 50 μl of a 2-mg/ml solution of this enzyme and incubating the mixture at 37°C. After 24 h, another 50 μl of proteinase K was added to the mixture, which was then incubated for an additional 24 h. At the end of these 48 h, 5 volumes of acetone was added to precipitate the excess proteinase K from the mixture. This material was then centrifuged, the supernatant was collected, the acetone was evaporated, and the remainder was lyophilized for examination by two-dimensional thin-layer electrophoresis performed at pH 1.9 (7.8% acetic acid, 2.2% formic acid) in the first dimension and pH 3.5 (5% acetic acid, 0.5% pyridine) in the second dimension (20). In addition to hydrolyzed VP1, each sample contained 5 μg each of phosphorylated serine, threonine, and tyrosine (Sigma Chemical Co., St. Louis, Mo.) as well as sulfurated serine, threonine, and tyrosine synthesized by the method of Reitz et al. (31). At the pHs used, these modified amino acids migrated towards the anode, while all the other amino acids migrated towards the cathode. The results of this analysis are shown in Fig. 3. The autoradiogram of the ^{35}S-sulfate-labeled VP1 hydrolysate (panel B) shows the ^{35}S radioactivity migrating identically to that of the tyrosine-*O*-sulfate standard identified by spraying the cellulose sheet with a solution of 0.25% ninhydrin in 95% ethanol and allowing the color to develop at 80°C for several minutes (panel A). The additional spot on the autoradiogram may be due to incomplete hydrolysis of VP1. Similar two-dimensional thin-layer electrophoresis results were obtained when ^{35}S-sulfate-labeled VP1 was first alkaline hydrolyzed with fresh 0.2 M barium hydroxide at 110°C for 20 h (data not shown). Upon acid hydrolysis (6 N HCl at 105°C for 2 h) of the radiolabeled material recovered from the cellulose sheet as shown in Fig. 3B, followed by two-dimensional thin-layer electrophoresis, no radioactivity could be detected in the region of tyrosine-*O*-sulfate (data not shown). Figure 3C shows two-dimensional thin-layer electrophoresis of proteinase K-hydrolyzed, ^{32}P-labeled VP1, which has previously been shown to be phosphorylated on serine and threonine residues (2, 15). ^{32}P-labeled serine and threonine were distinct from ^{35}S-labeled tyrosine. The additional spot on the autoradiogram is free $^{32}P_i$.

The presence of tyrosine-*O*-sulfate in the glycoproteins of several enveloped viruses has been demonstrated (27, 29, 30). In this report, evidence is presented that the nonenveloped DNA tumor virus polyomavirus is also modified by tyrosine sulfuration. This virus was found by SDS-PAGE and fluorography to incorporate ^{35}S-sulfate in the virus-coded major capsid protein VP1 (Fig. 1). Further examination of VP1 by IEF followed by SDS-PAGE in the

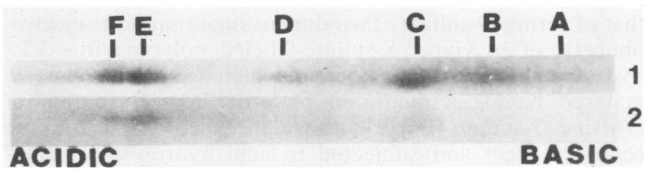

FIG. 2. Two-dimensional gel electrophoresis of purified ^{35}S-sulfate-labeled polyomavirus virions. Lanes: 1, visualization of VP1 IEF species (A to E) by silver staining; 2, autoradiogram of ^{35}S-sulfate-labeled VP1 IEF species (1.4×10^4 cpm).

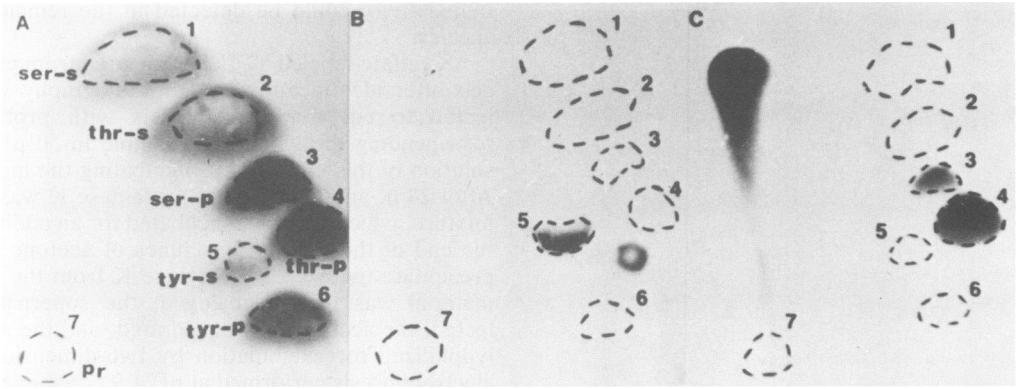

FIG. 3. Two-dimensional thin-layer chromatogram of proteinase K-hydrolyzed polyomavirus VP1. (A) Visualization of amino acid standards (5 μg each) by ninhydrin staining. (B) Autoradiogram of enzymatically hydrolyzed ^{35}S-sulfate-labeled VP1 (7×10^3 cpm). (C) Autoradiogram of enzymatically hydrolyzed ^{32}P-labeled VP1 (6×10^4 cpm). Amino acid standards were as follows: 1, serine-*O*-sulfate; 2, threonine-*O*-sulfate; 3, serine-*O*-phosphate; 4, threonine-*O*-phosphate; 5, tyrosine-*O*-sulfate; 6, tyrosine-*O*-phosphate; 7, phenol red dye marker.

second dimension revealed that only two of the six VP1 IEF species were sulfurated (Fig. 2). In a previous report from our laboratory, peptide mapping revealed that all six of these species have identical amino acid sequences (2). The charge heterogeneity as evidenced by IEF must therefore be due to modifications of this protein. Three of the identified species have been shown to be phosphorylated (2, 5, 35). Anders and Consigli (2) found that these phosphorylated species (D, E, and F) apparently are in identical phosphorylation states. They postulated that the charge heterogeneity results from superimposing phosphorylation on the heterogeneity of the nonphosphorylated VP1 species. While the biochemical basis of this heterogeneity is not clear, it may result from the presence of other posttranslational modifications. Our finding of VP1 modification by sulfuration supports this postulate.

One of the properties of the sulfate-modified amino acid tyrosine is the lability of the ester bond in acid and its stability in alkali (20). Amino acid sequencing of proteins by chemical methods and analysis by high-performance liquid chromatography and thin-layer chromatography generally use acid conditions for hydrolysis. The reason that tyrosine sulfate is not observed when proteins known to contain modified residues are analyzed by these methods may be the acid lability of the ester bond. Initially, digestion of polyomavirus VP1 with proteinase K was chosen to avoid the harsh pH conditions of alkaline and acid hydrolyses. At the time, the possibility of sulfurated serine and threonine could not be ruled out, although no such modified residues have been definitively identified. In preliminary experiments reported by Huttner (20), however, pronase digests of ^{35}S-sulfate-labeled rat brain proteins analyzed by two-dimensional thin-layer electrophoresis and autoradiography revealed a radioactive substance having a mobility similar to that of serine-*O*-sulfate. Two-dimensional thin-layer electrophoresis of in vivo ^{35}S-sulfate-labeled polyomavirus VP1 hydrolyzed by proteinase K revealed ^{35}S-sulfate labeled material having a migration pattern identical to that of tyrosine-*O*-sulfate (Fig. 3) and which, when eluted from the cellulose sheet and subjected to acid hydrolysis, demonstrated lability to this pH condition.

Sulfate and phosphate groups exhibit similar molecular properties; both modifications impart negative charges to proteins. It is therefore not surprising to find the sulfate modification occurring on the acidic VP1 IEF species, as these same species are modified by phosphorylation. An interesting observation is that the major sulfurated VP1 species, E, is the same one that Bolen et al. (5–7) implicated in specific cellular recognition, while the minor sulfurated VP1 species, F, is involved in nonspecific cellular interactions. It is believed that the negative charges imparted to proteins by phosphorylation and sulfuration affect the tertiary structure and thus the activity of the proteins. Relating this possibility to polyomavirus, the negative charge given to the tyrosine of VP1 species E and F by sulfuration, as well as the previously described phosphorylation of serine and threonine residues of these same species (2, 25), may be involved in the folding of the protein in such a way as to facilitate binding of the virus to the host cell. Alteration of the surface charges of cells as a result of sulfuration has already been implied to affect cell-cell interactions (17). While this report provides evidence that the polyomavirus-coded major capsid protein VP1 is modified by sulfuration primarily on the IEF species previously implicated in virus attachment to host cells, the exact function of this modification remains to be determined.

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