Comparison of Nonphosphorylated and Phosphorylated Species of Polyomavirus Major Capsid Protein VP1 and Identification of the Major Phosphorylation Region†

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The major virion protein of polyomavirus, VP1, consists of about six isoelectric species designated A through F. The minor species D, E, and F are phosphorylated and are thought to serve as viral receptors. We first wanted to distinguish whether all VP1 species are derived by post-translational modification from a common amino acid sequence or whether one or more of the species contain a region(s) of altered amino acid sequence resulting from alternate mRNA processing. We compared the VP1 species by detailed peptide mapping with several combinations of specific protease and radioisotopic labels. This approach enabled us to examine more than 80% of the predicted VP1 sequence, including the amino- and carboxy-termini. We found no evidence of sequence differences among any of the VP1 species. The specific incorporation of $^{32}\text{P}_i$ was found to be the same for all of the phosphorylated species. Comparison of the phosphorylation sites of *in vivo* $^{32}\text{P}_i$ -labeled D, E, and F by peptide mapping showed them to be identical. Each phosphorylated species contained a single major phosphopeptide and several minor phosphopeptides. The major phosphoamino acid, identified by acid hydrolysis, was phosphothreonine, with phosphoserine also present. By using chemical cleavage methods, we localized the major phosphorylation region to a central portion of the VP1 sequence. We discuss some features of this region and relate this information to functional implications of phosphorylation.

Polyomavirus virions are composed of three virus-coded proteins, VP1, VP2, and VP3, surrounding a compacted complex of the genomic DNA and four species of host-contributed histones (6, 7, 16, 19). Current evidence indicates that the outer capsid structure is composed of VP1 and that VP1 forms both the hexavalent and pentavalent capsomers (14, 44). It has been shown that polyomavirus is composed of 12 five-coordinated and 60 six-coordinated capsomers arranged in an icosahedral structure (15, 27). X-ray diffraction studies have provided evidence that both hexavalent and pentavalent capsomers are in fact pentamers (1, 38). These results imply that the principle of quasi equivalence as originally conceived is violated in the case of polyomavirus, since it is not obvious how five subunits in the hexavalent capsomers could interact equivalently with six neighbors (9).

In addition to its complex structural roles, VP1 serves in cell binding, functioning both as hemagglutinin and in recognizing or being recognized by specific host cellular receptors (3-5, 14,

44). Approximately 12 to 15% of the VP1 monomers found in mature virions are phosphorylated (3, 37). Isoelectric focusing resolves six or more VP1 species (3, 22, 35). Phosphorylation appears to account for part of the heterogeneity, since only three of the identified species are phosphorylated, and the phosphorylated species are more acidic in isoelectric focusing than are the nonphosphorylated species (3, 37). Similar results were obtained in the simian virus 40 (SV40) system (35). The phosphorylated species are of particular interest because our laboratory has presented evidence that they correlate with viral cell-binding activity (3). Furthermore, since specific phosphorylation frequently performs its function by inducing conformational alterations in the modified protein, phosphorylation may be a prime candidate in serving as a switching mechanism, changing the bonding specificity of individual capsomere subunits to conform to requirements of the all-pentamer capsid model described by Rayment et al. (38).

We undertook the studies described in this report to further define the phosphorylation of VP1 and the relationship of the phosphorylated species of VP1 to the nonphosphorylated species. As indicated by substantial indirect evi-

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dence, we show that the phosphorylated VP1 species appear to have the same sequence organization as the nonphosphorylated species. The three phosphorylated species are phosphorylated to the same extent and at the same sites. The phosphorylation is site specific and is confined to one major site and at least four minor sites. About 90% of the phosphate incorporated is found in the major site, which lies in a central region of the VP1 sequence.

MATERIALS AND METHODS

Cells and virus propagation. These studies were performed with our laboratory strain of wild-type polyomavirus. Some studies noted in the text were duplicated by using Pasadena strain wild-type polyomavirus. Virus used for these studies was maintained by passage at low multiplicity in primary mouse kidney cells (MKC). The preparation of MKC has been described previously (40). Infected cultures were maintained in serum-free Dulbecco modified Eagle medium.

Virus purification. Virus was purified as previously described (32). CsCl gradients used to purify virus were prepared as described by Brunck and Leick (8).

Preparation of radioactively labeled virus. Virus was labeled *in vivo* with $^{32}\text{P}_i$ by removing the serum-free Dulbecco modified Eagle medium from MKC monolayers infected at a multiplicity of 10 PFU per cell at 18 to 24 h postinfection, by washing the infected monolayers with phosphate-free Earle salts, and by replacing the medium with serum-free, phosphate-free Eagle medium containing 0.5 mCi of $^{32}\text{P}_i$ (carrier free; Amersham Corp.) Before harvesting, the infection was allowed to proceed until all cells were released from the dish, usually 48 to 72 h after the addition of the label.

Virus was labeled with ^{35}S methionine by replacing the serum-free Dulbecco modified Eagle medium with Eagle medium containing 10% of the standard amino acid concentration and 20 μCi of ^{35}S methionine per ml (1,200 Ci/mmol; New England Nuclear Corp.) at 18 h post infection.

Polyomavirus virions were labeled with ^{125}I *in vitro* by the chloramine-T method, as described by Frost and Bourgaux (18).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously in 15% acrylamide gels with a 0.2% bisacrylamide cross-linker (7). Two-dimensional electrophoresis was performed as described by O'Farrell (34) and samples were prepared for two-dimensional electrophoresis as previously described (3).

Two-dimensional peptide mapping. Two-dimensional peptide mapping was performed on ^{125}I -labeled proteins essentially as described by Elder et al. (13), except that proteins were always iodinated before SDS-PAGE. Chymotrypsin, L-(tosylamido, 2-phenyl) ethyl chloromethyl ketone-trypsin, and *Staphylococcus aureus* V8 protease were purchased from Worthington Diagnostics and used at concentrations of 50 $\mu\text{g}/\text{ml}$ in 50 mM ammonium carbonate buffer, pH 8.0, containing 1.5 mM dithiothreitol. After lyophilization, the peptides were dissolved in 20 μl of acetic acid-formic acid-water, 15:5:80 (buffer I), spotted on high-

efficiency thin-layer chromatography silica gel thin-layer plates (10 by 10 cm; Analtech Inc.), and electrophoresed at 500 V on a cooling plate until the lead component of the tracking dye (2% orange G [wt/vol]–1% acid fuchsin [wt/vol]) traversed the plate. Chromatography in the second dimension was performed in butanol-pyridine-acetic acid-water, 32.5:25:5:20 (buffer II). The plates were dried, and labeled peptides were located by autoradiography with Cronex 4 or Kodak XAR-5 X-ray film and Cronex Lighting-Plus screens.

Phosphoamino acid analysis. Lyophilized tryptic peptides eluted from gel slices or spots on thin-layer chromatography plates were dissolved in 50 μl of 6 N HCl, sealed in capillary tubes, and incubated at 105°C for 2 h. The HCl was removed *in vacuo*, the hydrolysate was dissolved in 7.8% (vol/vol) acetic acid–2.5% (vol/vol) formic acid, pH 1.9, containing 1 mg each of phosphoserine and phosphothreonine markers per ml; and the mixture was electrophoresed on cellulose thin-layer plates in 7.8% (vol/vol) acetic acid–2.5% (vol/vol) formic acid for 120 min at 1,000 V. Markers were visualized with ninhydrin and ^{32}P -labeled phosphoamino acids by autoradiography on Kodak XAR-5 film and Cronex Lighting-Plus intensifying screens.

Chemical cleavage. For chemical cleavage studies, VP1 was isolated from purified virions by SDS-PAGE before cleavage. The positions of VP1 in the gel were determined by staining or autoradiography, and the band was excised. For formic acid treatment, gel slices containing VP1 were dried, rehydrated by the addition of 0.1 ml of 75% (vol/vol) formic acid, which was completely absorbed, and incubated in tightly closed polypropylene tubes (12 by 75 mm) at 37°C for 18 to 24 h. Digestion was stopped by removing the formic acid *in vacuo*. The gel slices were then equilibrated with several changes of SDS-gel sample buffer (containing bromophenol blue as a pH indicator) until the indicator remained blue. The cleavage products were analyzed by placing the equilibrated gel slices in the sample wells of a second SDS-PAGE gel, followed by electrophoresis.

Iodosobenzoic acid (IBA) cleavage was performed on SDS-PAGE-purified VP1 as for formic acid cleavage, except that SDS was removed from the gel slices before treatment by washing in 10% (vol/vol) acetic acid–25% (vol/vol) isopropanol or by Coomassie blue staining. This was necessary because solubilization of IBA requires 4 M guanidine, which forms an insoluble gel when mixed with SDS. After the removal of SDS, gel slices containing VP1 were dried, and 0.1 ml of 10 mg of IBA per ml dissolved in 80% (vol/vol) acetic acid–4 M guanidine was added. The gel slices were then incubated in tightly sealed tubes at room temperature for 24 h. Guanidine was removed by washing the gel slices briefly in 80% (vol/vol) acetic acid; the gel slices were dried *in vacuo* and equilibrated, and the products were analyzed as described above for formic acid cleavage.

Quantitative assays. Protein concentrations were determined with Bio-Rad protein assay (Bio-Rad Laboratories) with bovine serum albumin as the standard. Samples labeled with β -emitters were quantitated in toluene-Triton X-100 (3:1) scintillation fluid with a Beckman LS-233 liquid scintillation counter. Samples in gel slices were prepared for counting by incubating slices swollen in deionized water with Protosol (New

FIG. 1. VP1 region of a Coomassie blue-stained O'Farrell (34) two-dimensional gel. Species are identified. Gels were loaded with 2.5 μ g of 32 P-labeled polyomavirus virions disrupted by prior treatment with SDS and β -mercaptoethanol. Loading 2.5 μ g of total virion protein gave sufficient resolution to separate D and D', yet it allowed visualization of all species by Coomassie blue staining. Phosphorylated species were identified by autoradiography to permit positive identification of all species.

England Nuclear Corp.) scintillation fluid (1 liter of toluene–4 g of Omnifluor–60 ml of Protosol) for 24 h at 45°C. In some cases, 32 P was counted directly, without scintillation fluid, in the scintillation counter. 125 I-labeled samples were counted in an LKB 1275 Mini-gamma scintillation counter.

RESULTS

Relationships between the phosphorylated and nonphosphorylated VP1 species. Polyomavirus virion proteins have been studied by using isoelectric focusing and two-dimensional electrophoresis in several laboratories (3, 22, 37). In previous studies in our laboratory, VP1 was resolved into six species designated A through F, three of which were shown to be phosphorylated (3). Figure 1 shows the VP1 region of an O'Farrell (34) two-dimensional gel with a representative pattern. Under the loading conditions used in this study and described in the legend to Fig. 1, the species we previously designated D was resolved into two species, D and D'. D is phosphorylated, but D' is not. When more than 3 μ g of virion protein was applied per gel, D and D' were not resolved. Table 1 presents the approximate apparent isoelectric points, relative abundance in virions, and phosphorylation information for each species. Substantial indirect evidence indicates that all species are VP1 sequences modified post-translation. All species have approximately the same mobility in SDS-PAGE, and all species are immunoprecipitated by anti-VP1 antisera; no similar proteins are precipitated from uninfected cells (3, 37). VP1 mutants with altered charge characteristics show similar charge changes for all species (20, 35). However, no studies have directly compared the different species, and the studies mentioned do not rule out the possibility that some of the species contain a region(s) of altered amino acid sequence due to rare alternative mRNA splice sites.

To directly compare the sequence organization of the different species, we performed detailed peptide analyses of an in vitro iodinated

VP1 species purified by two-dimensional electrophoresis with several different proteases. Under the loading conditions used to prepare the VP1 species for peptide mapping, D and D' were not resolved. When *S. aureus* V8 protease (which cleaves primarily at glutamoyl bonds under the conditions used) (24) was used to cleave the VP1 species, approximately eight major peptides and several minor peptides were produced (Fig. 2). The amino acid sequence from Deininger et al. (12) predicts eight labeled peptides, in agreement with our results, although our assignment of major peptides was somewhat arbitrary due to variation in the relative intensities of several spots. The additional spots were probably produced by cleavage at aspartic acid residues.

We also found no differences between tryptic and chymotryptic maps of [35 S]methionine-labeled VP1 species (data not shown). The most significant result of these experiments is that the peptide maps produced for the respective spe-

TABLE 1. Characteristics of polyomavirus virion VP1 isoelectric species

Species ^a	Apparent pI ^b	% Total VP1	32 P incorporated ^c
A	6.8	12	—
B	6.6	40	—
C	6.4	30	—
D	6.2	7	+
D'	6.1	5	—
E	5.9	4	+
F	5.8	2	+

^a Nomenclature modified from Bolen et al. (3).

^b Apparent pIs of species determined by incubating a gel slice containing respective species in 1 ml of distilled water and measuring the pH at room temperature.

^c Determined by autoradiography of in vivo 32 P-labeled polyomavirus virions electrophoresed on an O'Farrell (34) two-dimensional gel. —, Nonphosphorylated species. +, Phosphorylated species.

FIG. 2. Autoradiography of two-dimensional *S. aureus* V8 proteolytic peptide maps comparing the sequence organization of the species. Purified polyomavirus virions were labeled with ^{125}I by the chloramine-T method in the presence of 0.2% SDS, and VP1 was isolated by SDS-PAGE and applied to O'Farrell (34) two-dimensional gels. VP1 species were identified by autoradiography, excised, and treated with *S. aureus* V8 protease as described in the text. Lyophilized peptides were redissolved in buffer I and spotted on silica gel thin-layer plates. Origin is in the lower right-hand corner. Electrophoresis was performed in buffer I from right to left, and ascending chromatography was performed in buffer II from bottom to top. Panels are identified by the VP1 species name in the upper left-hand corner. The eight major peptides are identified in panel A.

cies were indistinguishable, indicating that the amino acid sequences of the peptides containing tyrosine (the primary labeling site for *in vitro* iodination) or methionine residues were held in common by all of the VP1 species. By extension, this indicates that the sequences of the species are probably identical, since tyrosine and methionine residues are distributed throughout the VP1 sequence (12).

These results were also obtained when chymotrypsin was used to digest the VP1 species

(Fig. 3). Chymotrypsin cleaved iodinated VP1 to produce significantly more peptides than were predicted from the sequence data. This was probably largely due to the lower specificity of chymotrypsin but may also have resulted from cleavage inhibition at iodinated tyrosine residues. However, although the peptide maps for all the species were generally identical, two differences were observed in the chymotryptic peptide maps between the nonphosphorylated and the phosphorylated species. First, for the phosphorylated species D, E, and F, a pair of spots which were not present in maps of nonphosphorylated species appeared. These spots migrated very near the origin, to the same position as the major phosphopeptide spot. In addition, a pair of spots whose original position was above the new spots were significantly reduced in D, E, and F. The relevant peptides are indicated with black arrows in Fig. 3. Second, an additional spot which was not present in the major species B and C, was observed in the phosphorylated species D, E, and F, as well as in A. This spot is indicated in Fig. 3 by white arrows. Theoretically, it should be possible to detect differences between the phosphorylated and the nonphosphorylated species because labeled phosphorylated peptides should behave differently from their nonphosphorylated counterparts; because phosphorylation could be expected to be site specific, only one peptide or a few peptides should behave differently. Since the spots which comigrated with the major phosphopeptide were found only in the phosphorylated species, we believe that they are phosphorylated versions of normal peptides, and we hypothesize that the normal peptides which we found to be considerably reduced in D, E, and F contain the major phosphorylation site. The additional spot seen in the phosphorylated species as well as in the nonphosphorylated species A (Fig. 3, white arrows) may be attributed to an unknown modification other than phosphorylation, since this spot did not appear to comigrate with any of the observed chymotryptic phosphopeptides or chymotryptic peptides. Mixing experiments were performed with the major nonphosphorylated (B and C) and phosphorylated species (D) to control minor differences between individual peptide masses (Fig. 4). These experiments show no differences other than those shown in Fig. 3. These results demonstrate the potential sensitivity of this method for detecting such post-translational modifications as phosphorylation. No shifted peptides were observed when *S. aureus* V8 protease was used (as described above), and no ^{125}I -labeled V8 peptides comigrated with the major phosphopeptide. Presumably, this is due to the absence of a tyrosine residue in the major V8 phosphopeptide.

FIG. 3. Autoradiography of two-dimensional chymotryptic peptide maps comparing the sequence organization of the species. VP1 species were prepared for analysis as described in the legend to Fig. 2, digested with chymotrypsin as described in the text, dissolved in buffer I, and applied to silica gel thin-layer plates. Origin is in the lower right-hand corner. Electrophoresis was performed in buffer I from right to left, and ascending chromatography was performed to buffer II from bottom to top. Panels are identified by the VP1 species name in the upper left-hand corner. Black arrows in panels C and E show peptides missing and appearing in phosphorylated species, respectively. White arrows in panels A and E indicate the position of the additional peptide present in species A and in the phosphorylated species D, E, and F.

Comparison of the phosphorylated species. Earlier studies in our laboratory suggested functional differences among the phosphorylated species D, E, and F, and we hypothesized that changes in the phosphorylation state among the species could account for these functional differ-

ences as well as for their heterogeneous isoelectric behavior (3). To address this question, we first performed peptide mapping studies on purified VP1 species, labeled *in vivo* with ^{32}P , by using several different proteases to compare phosphorylation sites (Fig. 5). The respective

FIG. 4. Autoradiography of two-dimensional chymotryptic peptide maps of VP1 species and mixtures of VP1 species. VP1 species were prepared for analysis as described in the legend to Fig. 2 and digested with chymotrypsin as described in the text. Peptide mapping was performed as described in the legend to Fig. 3. The major nonphosphorylated species B and C and the phosphorylated species D were mapped individually and with the appropriate mixtures, as indicated in the upper left-hand corner of each panel. Equal ^{125}I counts for each species were applied in the mixing experiments.

phosphopeptide maps generated by each of the species appeared to be the same for a given enzyme, indicating that the sites of phosphorylation were the same for each species. With every protease, a major phosphopeptide spot was produced, demonstrating that a single major site, or closely adjacent sites present within a single peptide, is phosphorylated *in vivo*. Several minor phosphopeptides were also observed and were present in all phosphorylated species in roughly the same proportions. These studies indicate that there are not site-specific differences in the phosphorylation of D, E, and F.

To compare the amount of phosphorylation in D, E, and F, we performed double-label experiments with either [^{35}S]methionine *in vivo* or

chloramine-T ^{125}I -labeled VP1 as internal measures of the fraction of total VP1 present in each species. By loading a known quantity of VP1 onto the gels, we were able to determine the protein present and calculate the specific ^{32}P radioactivity for each species. Table 2 summarizes the results of a typical experiment. We have performed this experiment several times and have not observed significant specific activity differences among the species. Although these studies compare the specific activities of the respective species, we cannot estimate the actual number of phosphorylations per molecule, since we do not know the specific activity of total incorporated phosphate. We therefore interpret these results only to mean that there are probably no quantitative differences among the three phosphorylated species which we have resolved.

Identification of the phosphorylated residues present in VP1. Tan and Sokol (42, 43) found phosphate exclusively in serine residues in SV40 VP1. To study the phosphate acceptor sites in polyomavirus VP1, we performed acid hydrolysis on *in vivo* ^{32}P -labeled VP1. We quantitated the phosphoamino acid produced by excising the spots and counting by liquid scintillation. We found the major polyomavirus VP1 phosphoamino acid to be phosphothreonine, as determined by comigration with authentic standards, in contrast to the results obtained for SV40 (Fig. 6, lane 1). Phosphoserine was also present, but it represented only about 30% of the total phosphoamino acids. These results were also ob-

TABLE 2. Comparison of the specific incorporation of ^{32}P into phosphorylated VP1 species

Species	^{125}I cpm (% Total VP1) ^a	μg of VP1 ^b	^{32}P cpm ^c	^{32}P cpm/ μg
D	7.0	0.152	355	2,336
E	3.8	0.082	228	2,780
F	2.0	0.043	77	1,790

^a *In vivo* ^{32}P -labeled polyomavirus virions were iodinated by the chloramine T method in the presence of SDS before two-dimensional electrophoresis. After electrophoresis, species were identified by Coomassie blue staining, excised, and quantitated by gamma counting. Counts for all species were totaled, and the relative percentage was calculated for each species by the following formula: (cpm of species/cpm of total) $\times$ 100.

^b Exactly 2 μg of total VP1 protein was applied to each gel. Protein present in each species was estimated by multiplying the total protein by the percentage of ^{125}I cpm present in the respective species.

^c Determined by scintillation counting of gel slices containing respective species. Variable discriminator was set to exclude ^{125}I counts.

FIG. 5. Autoradiography of two-dimensional proteolytic peptide maps of in vivo phosphorylated VP1 species D, E, and F. In vivo ^{32}P -labeled VP1 species were prepared for analysis as described in the legend to Fig. 2 and digested with the respective enzyme or combination of enzymes, and the resulting peptides were spotted on silica gel thin-layer plates after lyophilizing and redissolving in buffer I. Origin is in the lower right-hand corner. Electrophoresis was performed in buffer I from right to left, and ascending chromatography was performed in buffer II from bottom to top. Columns represent the same VP1 species digested with a different enzyme or enzyme combination, and rows present comparisons of different phosphorylated species digested with the same enzyme. VP1 species identified in the upper left-hand corners, and the enzymes used for digestion indicated at the left of each row. TRP, Trypsin; CHY, chymotrypsin.

tained with Pasadena strain wild-type polyomavirus virions as the source of VP1 (Fig. 6, lane 2). Hydrolysis was allowed to proceed for 2 h at 105°C for most experiments, which was sufficient to yield almost complete cleavage. Under these conditions, approximately 50% of the radiolabel was released as free phosphate. No phosphotyrosine was found, even under the mildest hydrolysis conditions, when we used a two-dimensional system which clearly separated authentic standards, including tyrosine (data not shown).

Localization of the major phosphorylation site(s) within the VP1 sequence. Our laboratory has utilized recently developed methods for the

specific chemical cleavage of polypeptides (29, 31) and the available sequence data (12) to produce a cleavage map of polyomavirus VP1 which is summarized in Fig. 7 of the accompanying paper (2). We produced the map by using 75% formic acid, specific for aspartyl-prolyl bonds, which cleaves at two sites to generate three peptides with molecular weights of 29,000 (29K), 16K, and 2K, and IBA, which cleaves at tryptophan residues and generates five fragments identifiable by SDS-PAGE.

Our standard conditions of formic acid treatment were designed to produce incomplete cleavage, and in addition to the complete peptides, partial products resulting from cleavage at

FIG. 6. Autoradiography of ^{32}P -labeled phospho-amino acids resulting from 6 N HCl digestion of in vivo-labeled VP1. In vivo ^{32}P -labeled VP1 was isolated by SDS-PAGE and trypsinized, and the resulting fragments were lyophilized, redissolved in buffer 1, and electrophoresed at 600 V for 2 h. (Tracks 1) 2-h hydrolysis of our laboratory strain of wild-type VP1; (track 2) 2-h hydrolysis of the Pasadena strain of wild-type VP1. Arrows indicate the positions of phosphoserine (P-SER) and phosphothreonine (P-THR) standards identified by nonhydrazin.

a single site were generated, with molecular weights of 40K and 18K. Cleavage of in vivo ^{32}P -labeled polyomavirus VP1 with formic acid and analysis by SDS-PAGE revealed that ^{32}P comigrates with the 29K peptide as well as with uncleaved VP1 and the 40K peptide, but not with the 18K and 16K peptides (Fig. 7, lane 2). This peptide (29K) extends from the amino terminus through amino acid 231 of the VP1 sequence (2, 12). Cleavage of in vivo ^{32}P -labeled VP1 with IBA resulted in the production of a single-labeled peptide of approximately 11K (Fig. 7, lane 1). Once again, the results obtained by with our laboratory strain were confirmed by cleavage of Pasadena strain VP1 (data not shown). We have shown this peptide (11K) to originate in a central region of the VP1 sequence at the carboxy-terminal end of the 29K formic acid peptide, extending from residues 122 through 228. We conclude from these results that the major phosphorylation site(s) must reside within this region of the VP1 sequence.

DISCUSSION

Relationships among the VP1 isoelectric species. The major virion protein of polyomavirus, VP1, is resolved into about six isoelectric spe-

cies, which were designated A through F in a previous report (3). In the studies presented here, we examined the sequence relationship of the phosphorylated and nonphosphorylated species of VP1 and further defined the nature of the phosphorylation.

We used two-dimensional proteolytic peptide analysis to compare the sequence organization of the species. We estimate that by using the available sequence information (12) to select a combination of radioisotopic labels and proteases predicted to produce overlapping sets of labeled peptides, we were able to compare more than 80% of the total VP1 sequence, including the amino- and carboxy-termini. No major differences in the peptide maps were found. These experiments provide direct evidence that all of the VP1 species observed by isoelectric focusing have the same sequence organization and do not contain any regions of altered amino acid sequence resulting from rare mRNA splicing sites. This supports the fact that no such rare mRNA species have been detected in the elegant studies

FIG. 7. SDS-PAGE analysis of in vivo ^{32}P -labeled VP1 peptides generated by cleavage with formic acid or IBA. VP1 was isolated from in vivo ^{32}P -labeled polyomavirus virions by SDS-PAGE and cleaved with formic acid or IBA, as described in the text, and the cleavage products were analyzed by SDS-PAGE. Resulting peptides were located by Coomassie blue staining, and ^{32}P -containing peptides were identified by autoradiography. (Track 1) Autoradiogram of IBA-cleaved VP1; (track 2) autoradiogram of formic acid-cleaved VP1. Positions of Coomassie blue-stained VP1 proteins are indicated on the left, and positions of Coomassie blue-stained VP1 formic acid peptides are shown on the right. HIS, Histone.

of polyomavirus late mRNAs (21, 23, 25, 26, 30). Only chymotryptic peptide maps of iodinated VP1 species revealed altered peptides in the phosphorylated species, and these differences apparently reflect phosphorylation itself rather than sequence differences, since the new peptides observed comigrated with the phosphopeptide spot and correlated with a significant reduction but not disappearance of peptides found in all species.

We also compared the phosphorylation states of the individual phosphorylated species D, E, and F. In these studies we detected no differences in the phosphorylation among the three phosphorylated species, either in the specific incorporation of $^{32}\text{P}_i$ or in the sites phosphorylated. Although we have not been able to determine the actual number of phosphorylations per molecule, our results are most compatible with a model in which each phosphorylated VP1 polypeptide contains a single phosphate. Evidence indicates that each VP1 species behaves as if it differs from its neighbors by a single charge unit. When the 20 amino acids of the carboxy-terminal are cleaved from VP1 by formic acid treatment and the resulting 40K polypeptide is electrophoresed in O'Farrell (34) two-dimensional gels along with intact VP1, the major 40K isospecies is shifted two positions in the acidic direction with respect to the major VP1 species (data not shown). According to sequence data, the 20 amino acids of the carboxy-terminal should have a net charge of about two positive units in the pH range of VP1 pI (12). Similarly, the addition of a phosphate carrying two negative charges in the pH range of the VP1 pI to the major nonphosphorylated species of VP1, B, should shift its position two units in the acidic direction, to the observed position of the major phosphorylated species, D.

Since all the phosphorylated species apparently are in identical phosphorylation states, we postulate that their charge heterogeneity results from superimposing phosphorylation on the heterogeneity of the nonphosphorylated VP1 species. The biochemical basis of this heterogeneity is not clear. It may result from additional, as yet uncharacterized, posttranslational modifications such as acetylation, carboxylation or decarboxylation, sulfation, methylation, etc. Alternatively, it has been suggested that some of the heterogeneity may result from conformational differences, protein-protein interactions, or ampholyte binding (20). We are continuing to investigate this important question.

Location of the major phosphorylation site(s) within the VP1 sequence. The phosphopeptide mapping studies indicate that a small region of the VP1 sequence contains the majority of the incorporated phosphate. We used three different

proteases and combinations of proteases in these studies. In all three cases, either a single major peptide or a closely related set of peptides which migrated near the origin in our two-dimensional system was produced. We consider it highly unlikely that widely separated sites would generate comigrating peptides with all three proteases, since there are no significant repeated sequences in VP1. We interpret this to mean that a single residue or small set of residues clustered together in the VP1 sequence represents the major phosphorylation site(s) of polyomavirus VP1. These studies also indicate that the phosphorylation of VP1 is highly site specific, since many available sites are not used. This apparent site specificity argues a priori that phosphorylation of VP1 is not fortuitous. Presumably, any such specific phosphorylation serves to modify the conformation of VP1 to perform a specific function(s).

We do not know the origin of the minor phosphopeptides observed in our mapping studies. It is not clear whether they represent phosphorylation sites outside of the major phosphopeptide or if they are produced by aberrant cleavage of the major phosphopeptide; we favor the former explanation since their production is not slower than cleavage of the major phosphopeptide. It is also possible that they are underrepresented in our peptide maps because of low solubility. We are currently extending our studies to characterize these phosphopeptides.

Most of the incorporated phosphate is present within an 11K peptide produced by IBA cleavage. This peptide originates in a central region of the VP1 sequence and is predicted to extend from Glu₁₂₂ through Trp₂₂₈ (Fig. 8) (12). The finding of a single phosphorylated region supports our interpretation of the phosphopeptide mapping results. Chemical modification studies indicate that this region of the sequence is relatively inaccessible in intact virions, implying that it is either buried in the core of the VP1 tertiary structure or involved in intermolecular interactions (e.g., between VP1 monomers in the capsomere, between capsomeres, or both) (2). Interestingly, we have found VP1 phosphorylation to be substantially alkaline phosphatase-resistant when the native virion structure is preserved, as Tan and Sokol found for SV40 (42, 43). Only by denaturing the virion proteins can the phosphorylation be made phosphatase sensitive (data not shown).

Figure 8 shows the amino acid sequence of the 11K IBA peptide predicted by the nucleic acid sequence of Deininger et al. (12). This sequence presents several interesting features which may provide clues to the exact sites of phosphorylation. First, the peptide mapping studies show that the major chymotryptic phosphopeptide co-

FIG. 8. Predicted amino acid sequence of the 11K IBA peptide containing the major phosphorylation sites of polyomavirus VP1. Sequence data are from Deininger et al. (12). Possible sites of phosphorylation, discussed in the text, are underlined. Relevant chymotryptic sites are indicated with solid triangles, V8 sites are indicated with open triangles, and the relevant tyrosine residue is shown with an asterisk.

migrates with iodinated peptides found only in the phosphorylated species, whereas no unique iodinated peptides are produced by V8 cleavage of phosphorylated VP1 species. These results suggest that most of the phosphorylated residues reside within the sequence extending from Asn₁₄₃ through Glu₁₅₉, since this would result in a tyrosine-containing chymotryptic major phosphopeptide and a tyrosine-free V8 major phosphopeptide. This sequence contains four threonine residues and one serine residue, in agreement with our observation that the major phosphate acceptor is threonine. Second, Small et al. (39) observed that phosphorylation sites are generally found in or near sequences predicted to have a high probability of assuming a β -turn conformation. Two of the four threonine residues, Thr₁₄₆ and Thr₁₄₈, lie within a tetrapeptide strongly predicted to assume β -turn conformation, and a third, Thr₁₅₁, lies just outside the same predicted β -turn (10). Third, the sequence between Asn₁₄₃ and Glu₁₅₉ also contains two structures which may serve as specific substrates for protein kinase. The cyclic nucleotide-dependent protein kinases generally recognize the sequence B₁-B₂-X-X-Ser/Thr, in which B₁ and B₂ are basic residues, at least one of which is usually arginine (28). The basic residues may in some cases be separated by additional residues (45). The sequence Asn₁₄₃-Lys-Pro-Thr-Asp-Thr₁₄₈ may function as such a site. These threonine residues fall within the predicted β -turn. Another class of protein kinases, the casein kinases, recognize the general sequence Ser or Thr-X-A, where A represents an acidic residue (33), and the sequence Ser₁₅₅-Thr-Pro-Val-Glu₁₅₉ may be able to function as any acceptor for this class of kinases. Taken together, these points form a substantial argument that these sites may serve as major phosphorylation sites in polyomavirus VP1. We have used the Deininger et al. (12) nucleic acid sequence to make the points listed above. In general, the polyomavirus VP1 sequence of Soeda et al. (41) is substantially the same, differing primarily by

conservative substitutions at individual sites. However, one of the major exceptions falls within the sequence between Asn₁₄₃ and Glu₁₅₉, in which 9 out of 10 residues differ. Nevertheless, despite the extensive changes, the basic structures described above are preserved, including potential kinase acceptor sites and the predicted β -turn. Furthermore, the SV40 VP1 sequence conserves many of these features as well (36).

All of these studies have been performed on VP1 from purified polyomavirus virions. There is some indication in the literature that additional sites may be phosphorylated before assembly. Ponder et al. (37) found a higher proportion of phosphorylated VP1 intracellularly. O'Farrell and Goodman (35) studied the heterogeneity of SV40 VP1, and using pulse and pulse-chase experiments, they demonstrated that the most acidic phosphorylated species appeared first, followed by the appearance of less acidic forms. The position of the most acidic spot is compatible with predictions made for a diphosphorylated species. Interestingly, the polyomavirus VP1 amino-terminus contains a sequence resembling a cyclic AMP dependent kinase site. We are now looking at the intracellular polyomavirus VP1 species to determine if additional phosphorylation sites are present or if some of the undefined minor sites are major sites before assembly.

Functional implications of VP1 phosphorylation. It is our goal to relate information obtained in biochemical and structural studies to biological functions. The obvious question, therefore, is, what do these results tell us about the function or functions of polyomavirus VP1 phosphorylation? Considerable genetic evidence indicates that papovavirus major capsid proteins are made up of two domains. Both polyomavirus and SV40 VP1 structural genes define two complementation groups (11, 17). Furthermore, two major functions of polyomavirus VP1 have been demonstrated; VP1 forms the highly cross-linked protective outer capsid and serves as the hemagglutinin and viral receptor. Therefore,

two possibilities immediately present themselves. First, VP1 phosphorylation may serve in a purely structural role, functioning to switch the bonding specificity of capsomeres to fit into symmetrically distinct environments, as required by the all-pentamer capsid model of Rayment et al. (38). Second, phosphorylation may be involved in cell-binding activity. Our laboratory has presented two lines of evidence suggesting that this may indeed be the case. First, highly purified capsids, which were found to lack a major portion of the phosphorylated VP1 species, failed to bind specifically to MKC and did not compete with virions for specific binding sites on MKC (3, 4). Second, when empty capsids were used to affinity fractionate hemagglutination-inhibiting and neutralizing antibodies, antibodies eluting from the column without binding to the capsids were found to bind to virions and neutralize infectivity but not hemagglutination inhibition and to preferentially immunoprecipitate phosphorylated species of VP1 (5). Antibodies which were bound to the capsids were found to inhibit hemagglutination but not to neutralize infectivity. Both populations of antibodies were specific for VP1. These data suggested that the different populations of antibodies were capable of distinguishing alternate conformational states of VP1 in the virion which were generated by phosphorylation, and furthermore, that the alternate conformational states related to different biological functions.

In this report, we have demonstrated that the major phosphorylation sites lie within a central portion of the VP1 molecule. In the accompanying paper (2), we show evidence that at least some of the neutralizing and hemagglutination-inhibiting determinants reside in the 18K formic acid fragment, which is the carboxy-terminal one-third of the VP1 sequence (Fig. 8), substantially separated from the phosphorylation region. Therefore, if phosphorylation functions strictly to effect receptor activity, it probably acts at a distance, inducing a general conformational change. Biochemical studies show that the amino-terminal 29K formic acid fragment of VP1 is relatively inaccessible to aqueous solvent in intact virions. We hypothesize that the relatively hydrophilic carboxy-terminal portion of VP1 lies on the surface of the virion structure and forms the viral receptor and that slightly over two-thirds of the amino-terminal portion of VP1 forms a core structure responsible for association of VP1 monomers to form the capsomeres and for the contacts between the capsomeres. The major phosphorylation region bridges these two functional domains and is ideally situated to affect their conformational relationship.

We are continuing our biochemical and immunological studies of the organization of VP1 in

virions. We hope that these studies will provide further insight into the functions of VP1 phosphorylation. We eagerly await the eventual solution of the polyomavirus three-dimensional structure which will complement these studies and which will ultimately help provide a complete understanding of the role of polyomavirus VP1 phosphorylation.

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