Localization of the Phosphorylations of Polyomavirus Large T Antigen

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Polyomavirus large T antigen is phosphorylated on both serine and threonine residues at a ratio of approximately 6 to 1. This phosphorylation could be resolved into a series of nine *Staphylococcus aureus* V8 phosphopeptides. All of these were found in an N-terminal chymotryptic fragment with a molecular weight of 57,000. A C-terminal formic acid fragment of 50,000-molecular-weight lacked phosphate. Therefore, unlike simian virus 40 large T antigen, polyomavirus large T antigen has no significant C-terminal phosphorylation. Limited V8 and hydroxylamine cleavage showed that the phosphorylations can be localized to two different portions of the molecule. A significant fraction of the phosphate was localized in the N-terminal portion of the molecule before residue 183. Within this region V8 peptides 4, 8, and 9 represented phosphorylations that were more proximal, while peptides 1, 2, and 3 included more distal phosphorylations. None of these phosphorylations appeared analogous to those of simian virus 40 large T antigen. V8 phosphopeptides 5 and 7 were more distal and could be distinguished in biological experiments from the N-terminal phosphorylations. Formic acid mapping suggested that much, if not all, of this phosphorylation is located between residues 257 and 285.

In the previous paper (3) we introduced the patterns of polyomavirus (Py) large T antigen phosphorylation and discussed their possible relationship to large T antigen function. *tsa* mutants were defective in phosphorylation at the nonpermissive temperature; V8 phosphopeptides 5 and 7 were not observed in digests of *ts616* large T antigen labeled at the nonpermissive temperature. Phosphorylation of large T antigen appeared to be dependent to some extent on the growth state of the cell. Early in infection of serum-starved 3T6 cells or resting confluent baby mouse kidney cells, the phosphorylations represented by sites 5 and 7 were at best poorly represented. Later on, along with the induction of cell replication, peptides 5 and 7 became prominent in the digests. This suggests that the virus stimulates kinase activities as part of the process of stimulation of cell growth. Because *hrt* mutants encoding large T antigen, but not small T or middle T antigens, behaved qualitatively like wild-type virus, large T antigen itself appeared to be involved in this induction of kinase(s) that is responsible for its phosphorylation. Differences in the kinetics of infection between *hrt* and wild-type virus indicate that small T antigen, middle T antigen, or both may modulate the cell response.

In the study described here we characterized these phosphorylations and positioned them along the large T antigen molecule. While this report was being prepared, Hassauer and colleagues (10) presented evidence on the phosphorylation of large T antigen. Some of their conclusions are similar to those reached here: the overall pattern of phosphorylation of Py large T antigen and simian virus 40 (SV40) large T antigen is different. Much of the Py large T antigen phosphorylation is in the N-terminal region of the molecule, in the region which has no sequence homology to SV40. There is no C-terminal phosphorylation in Py large T antigen of the kind observed in SV40 large T antigen. However, we also characterized a second region of phosphorylation. This region, which is further down the molecule, appears to be of great interest based on the biological results described above. Some of this phosphorylation may

be more related in a functional way to some of the phosphorylation observed for SV40 large T antigen.

MATERIALS AND METHODS

Cells and viruses. Virus infections were routinely carried out on 3T6 cells grown in Dulbecco modified Eagle medium supplemented with 5% calf serum. In some experiments NIH 3T3 cells or Fischer rat embryo fibroblasts (F111) (8) were used. Baby mouse kidney cells were prepared by the method described by Winocour (31) and grown in medium supplemented with 10% horse serum.

Virus stocks were prepared on 3T6 cells or baby mouse kidney cells. Wild-type virus NG59RA (7) was derived from *hrt* mutant NG59 (2) by marker rescue. *dl45* is a wild-type deletion strain isolated by Bendig and colleagues (1). *dl8* (9) and *dl1015* (15, 16) are deletion mutants for which the large T-antigen function may be affected, as judged by the growth properties of the virus. *dl23* (9) is a deletion mutant that is transformation defective as a result of its middle T antigen defect, but its large T antigen appears unaffected by the mutation.

Antisera. Rabbit antiserum prepared by immunization with small T antigen produced in *Escherichia coli* (18) was generally used. Anti-T antigen fluid was extracted from Brown Norwegian rats bearing a mixed solid ascites tumor as described previously (24).

T-antigen analysis. The procedures for radiolabeling of cells and T-antigen extraction and immunoprecipitation have been described in detail (20-24). In pulse-labeling experiments with [³⁵S]methionine, cells were labeled in Hanks salts (Flow Laboratories, Inc., McLean, Va.) containing 50 μ Ci of [³⁵S]methionine (New England Nuclear Corp., Boston, Mass.) per ml for 90 min. For labeling with ³²PO₄, cells were incubated in phosphate-free medium containing 200 to 400 μ Ci of ³²PO₄ per ml. T antigens were routinely extracted in Nonidet P-40-containing buffer (0.137 M NaCl, 0.01 M Tris hydrochloride [pH 9.0], 0.001 M MgCl₂, 0.001 M CaCl₂, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40). In phosphate labeling experiments the extraction buffer was supplemented with 0.02 M NaF to reduce phosphatase activity in

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the extracts. Incubations with antiserum and protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J. [Div. Pharmacia, Inc.]) were generally carried out for 1 h. Immune complexes were then washed with phosphate-buffered saline, twice with 0.5 M LiCl–0.01 M Tris hydrochloride [pH 7.0], and then with distilled water. Finally immunoprecipitates were treated with a sodium dodecyl sulfate (SDS) dissociation buffer (0.0625 M Tris hydrochloride [pH 6.8], 5% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.015% [wt/vol] bromophenol blue) at 100°C for 2 min. The samples were then analyzed on discontinuous buffer SDS gels (14).

Partial proteolysis of large T antigen on SDS gels was carried out as described previously (21, 22). Proteins to be analyzed were resolved first on cylindrical SDS gels. The samples were then placed horizontally on top of a slab gel and digestion was carried out by overlaying an enzyme solution on the cylindrical gel. The digestion solution contained 0.0125 M Tris hydrochloride (pH 6.8), 0.01 M EDTA, 20% (vol/vol) glycerol, 0.015% (wt/vol) bromophenol blue, and 50 µg of bovine serum albumin per ml, as well as 100 µg of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) per ml. Electrophoresis was carried out at 50 V until the bromophenol blue reached the bottom of the gel.

For enzymatic digestion in the immune complexes, immunoprecipitates were boiled in 1% (vol/vol) SDS–1% (vol/vol) 2-mercaptoethanol for 2 min and cooled to room temperature. Enzyme was then added for 10 min. The samples were then either electrophoresed directly or retreated with boiling SDS dissociation buffer to inactivate the protease.

Hydroxylamine cleavages (4) of proteins in gels were performed by first equilibrating the gel in 0.2 M K₂CO₃ (pH 9.0) and then incubating in 0.2 M K₂CO₃ (pH 9.0) containing 2 M hydroxylamine (Kodak) for 3 h at 45°C. Hydroxylamine was removed by washing for 1 h with two changes of 0.125 M Tris hydrochloride (pH 6.8)–0.1% (wt/vol) SDS–0.1% (vol/vol) 2-mercaptoethanol. The gels were then set on top of a slab gel and sealed in place with 1% agarose in 0.125 M Tris hydrochloride (pH 6.8)–5% (vol/vol) 2-mercaptoethanol. Hydroxylamine cleavage on beads was accomplished as described above, but the immunoprecipitates were then washed with phosphate-buffered saline and distilled water and finally treated with SDS dissociation buffer.

Formic acid cleavage (12) on gels was carried out with 70% (vol/vol) formic acid (J. T. Baker Diagnostics, Bethlehem, Pa. [Div. J. T. Baker Chemical Co.]) for 1 to 2 h at 45°C. The gels were then washed and treated as described above. Because treatment of immunoprecipitates with formic acid released peptides from the beads, the eluate was dried, dissolved in distilled water, and lyophilized before treatment with SDS sample buffer.

Phosphoamino acid analysis was performed on large T antigen extracted from cylindrical gels by a modification of the procedure described by Weiner and co-workers (30). Gel pieces were homogenized in 0.5% sodium bicarbonate–1 mM dithiothreitol–0.1% SDS (wt/vol) (pH 7.0). After acrylamide pieces were removed by centrifugation, the protein was precipitated with nine volumes of acetone. Acetone-washed pellets were dissolved in distilled water and dialyzed against 0.5% (wt/vol) ammonium bicarbonate.

Lyophilized large T antigen was digested with 6 N HCl for 2 h at 110°C under nitrogen. Phosphoamino acids were separated by electrophoresis (formic acid–acetic acid–water; 78:25:897) on thin-layer cellulose plates (MN300; Analtech) for approximately 4,000 V h, followed by chromatography

FIG. 1. (A) Phosphoamino acid analysis of Py large T antigen. 3T6 cells infected with wild-type Py virus were labeled for 2 h with ³²PO₄ starting at 22 h after infection. Large T antigen was isolated and subjected to phosphoamino acid analysis as described in the text. Unlabeled phosphoserine (PSER), phosphothreonine (PTHR), and phosphotyrosine (PTYR) were added to the sample; their positions, indicated by the dotted lines, were established by ninhydrin staining. (B) Phosphopeptides obtained by *S. aureus* V8 protease digestion of large T antigen labeled for different periods of time. Actively growing 3T6 cells were infected with wild-type virus and labeled continuously in phosphate-free medium, beginning 22 h after infection, with ³²PO₄ for 30 min (lane A), 2 h (lane B), or 4 h (lane C). Cells were labeled continuously in complete medium for 19 h (lane D). Isolated large T antigens were digested with 100 µg of V8 protease per ml, as described in Materials and Methods.

(0.5 M ammonium hydroxide–isobutyric acid; 3/5) (11). Amino acid standards were visualized with ninhydrin spray (0.25 g/100 ml of *n*-butanol).

RESULTS

Large T antigen is phosphorylated on serine and threonine residues. Large T antigen phosphorylated in vivo was isolated from SDS-polyacrylamide gels. After limited digestion with 6 N HCl, phosphoamino acids were separated by high-voltage electrophoresis and chromatography (Fig. 1A). Phosphoserine and phosphothreonine were detected in a ratio of approximately 6:1. The ratio varied somewhat with the conditions of labeling and digestion; this was not surprising, because as shown below, multiple sites with different kinetics of labeling were involved. When the immunoprecipitates were labeled in vitro with [γ-³²P]ATP, large T antigen, as well as middle T antigen, was labeled. After such labeling, the amounts of phosphothreonine and phosphoserine in large T antigen were approximately equal (data not shown). This suggests that some of the kinase(s) responsible for phosphorylating large T antigen may remain associated with it during the immunoprecipitation. In addition to the phosphoserine and phosphothreonine, a limited amount of phosphotyrosine was occasionally observed in the in vitro-labeled large T antigen, suggesting that the middle T-pp60^{c-src} complex can use large T antigen as a substrate.

The most useful method for routine analysis of large T antigen phosphorylations was digestion with *S. aureus* V8 protease. Analysis with this enzyme, which cleaves primarily at the C-terminal side of glutamic acid residues, yielded

FIG. 2. Peptides of large T antigen generated by different digestions. The sequence of the large T antigen is shown. Above the large T sequence the positions of deletion mutants *dl8*, *dl45*, *dl23*, and *dl1015* are shown. The principal peptides generated by *S. aureus* V8 protease (V8), hydroxylamine (NH₂OH), and formic acid (HCOOH) are shown.

nine principal fragments with the following molecular weights; 1, 22,500; 2, 20,500; 3, 19,000; 4, 18,000; 5, 16,000; 6, 15,000; 7, 14,000; 8, 13,500; 9, 11,500 (Fig. 1B). This pattern of nine peptides has been used for routine analysis. In addition to these peptides minor fragments with mobilities slightly greater than 5 and 7 could often be detected in wild-type digestions. Fragment 6 (Fig. 1B, lane C) was an intermediate digestion product and was often not observed even in the initial digestions. Because large T antigen is known to behave anomalously on SDS polyacrylamide gels (24), these molecular weights must be regarded as tentative. The apparent mobility of fragment 4, for example, varied with the gel conditions, migrating more rapidly relative to the markers or other peptides on 12.5% than on 15% gels. Potential biases introduced by phosphatases are always a concern. Although the yield of large T antigen phosphate was increased slightly by the addition of sodium fluoride, a phosphatase inhibitor, to the extraction buffer, the V8 phosphopeptide pattern was not altered. Similarly, the same V8 pattern was observed when large T antigen was isolated from cells that were treated directly with boiling SDS for 2 min to inactivate phosphatases.

Analysis of the labeling patterns in exponentially growing cells revealed that the rate of phosphorylation of individual peptides varied. Fragments 5 and 7 were intensely labeled in 15 to 30 min of continuous labeling; fragments 4, 8, and 9 were not heavily labeled until 120 min. By densitometry fragments 4, 8, and 9 represented 22% of the label in large T antigen labeled in 30 min, but 50% of the phosphate labeled in 19 h. Peptides 1, 2, and 3 were labeled within 15 to 30 min, and the intensity of label did not vary significantly. Pulse-chase experiments showed results that were in accord with the labeling patterns (data not shown). Phosphorylated fragments 5 and 7 showed the shortest half-life, while fragments 4 and 9 displayed the longest. Because fragments 2 and 3 can be derived from fragment 1 by additional V8 cleavage (see below), it is interesting that peptide 2 retained label longer

FIG. 3. *S. aureus* V8 protease analysis of large T antigen. (A) On the left is shown large T antigen, labeled as described in Fig. 1, digested with V8 protease (100 μ g of V8 protease per ml). After initial digestion with V8 protease in the immunoprecipitates, the phosphopeptides were resolved in a cylindrical gel and then again digested with V8 protease. This is shown on the right. The positions of the principle V8 peptides in the first-dimension digests (peptide 1, the precursor to 5 and 7, and peptide 4) are indicated by the vertical lines. Although the peptide giving rise to 5 and 7 was completely digested in the second dimension, undigested remnants of 1 and 4 can still be seen along the diagonal. The positions of the V8 peptides in the second dimension are indicated by the arrowheads. (B) Large T antigen of the wild type (lanes 1 and 3) or *dl23* (lanes 2 and 4) was digested in the immunoprecipitates with V8. V8 protease (1 μ g) was used for lanes 1 and 2 and 4 μ g of V8 protease was used for lanes 3 and 4. The fragments were then separated on a 7.5% acrylamide SDS gel. The positions of the intact large T antigen (LT) and the 59K, 41K, and 38K fragments are indicated by the arrowheads. The position of a 44K fragment derived from the 59K fragment is indicated by the bar. (C) V8 protease digestion of the 59K, 44K, and 38K fragments generated by V8 digestion of immunoprecipitates. The vertical lines show the positions of the fragments in the first dimension, while the arrowheads show the position of the V8 fragments in the second dimension. (D) V8 protease digests of large T antigen labeled with ³²P or [³⁵S]methionine were compared. The positions of the V8 peptides are shown with the arrowheads. The absence of a ³⁵S-labeled band corresponding to V8 phosphopeptide 7 is indicated by the bar.

than peptide 3. This suggests that these peptides have some distinct phosphorylations.

Large T antigen phosphorylation sites are localized in two regions of the molecule. Chemical and enzymatic digestions were used to position the large T antigen phosphorylations on the molecule. Partial digestion and double digestion procedures were used to relate the phosphopeptides to each other. Where possible, large T antigen deletion mutants were used in positioning the phosphorylations. The basic arrangement of the peptides to be discussed is shown in Fig. 2.

FIG. 4. Hydroxylamine digestion of large T antigen. (A) Large T antigen from infected 3T6 cells was labeled with $^{32}\text{PO}_4$ or ^{35}S methionine at 22 h after infection. After immunoprecipitation large T antigen was resolved on a 12.5% acrylamide SDS gel. After digestion with hydroxylamine in the gel, the fragments were resolved on a 10% acrylamide SDS gel. The positions of intact large T (LT), the C-terminal fragment (C; residues 211 to 785), and the N-terminal fragment (N; residues 1 to 210) are indicated by the arrowheads. The additional fragment observed in longer hydroxylamine digests is indicated by the bar. (B) Wild-type large T antigen labeled with ^{35}S methionine (lane A) or with $^{32}\text{PO}_4$ (lane B) was digested with hydroxylamine, and the products were compared with those of phosphorylated *dl45* large T antigen (lane C) or *dl1015* large T (lane D) on a 12.5% acrylamide SDS gel. Phosphorylated wild-type hydroxylamine fragments (lane E) were also compared with those of *dl1015* (lane F) on a 10% gel to show more readily the difference in the C-terminal fragments.

***S. aureus* V8 analysis shows two regions of phosphorylation.** Some of the V8 peptides were related to each other (Fig. 3A, V8/V8). Fragment 1 could be further digested to yield fragments 2 and 3, while fragment 4 could be digested to give fragments 8 and 9. Phosphopeptides 5 and 7 could be derived from a precursor with a mobility slightly faster than that of peptide 2.

More limited digestion showed that phosphorylation occurred in two distinct areas of the molecule. Very limited digestion with V8 protease yielded two principal phosphopeptides (Fig. 3B). One had a molecular weight of approximately 38,000 (38K fragment). This fragment, which was smaller for mutants such as *dl23*, came from the N terminus of the molecule. There was an additional minor N-terminal fragment of approximately 41,000 in molecular weight. The second major species had an apparent molecular weight of 59,000. It must have arisen from sequences distal to the *dl23* deletion because it was not shortened in that mutant. This peptide, in turn, could be cleaved to a smaller fragment with a molecular weight of 44,000 (bar in Fig. 3B and 3C). Double V8 digestion oriented these peptides with respect to smaller partial fragments (Fig. 3C). The 38K fragment was digested to give fragments 1 to 4, 8, and 9. These fragments therefore represented phosphorylations that were clustered in the N-terminal end of the molecule. Fragments 5 and 7 were not observed in digests of the 38K fragment. However, the 59K fragment was digested to give these V8 fragments. *hrt* mutant large T antigen can show a major peptide (5') with a mobility that is just faster than that of fragment 5 (3); in double digests 5'-like fragment 5 was also found in the 59K fragment (data not shown).

The distribution of methionine residues within large T antigen is highly asymmetric (6). There are four methionines in the first 71 amino acids, one at residue 240, five between residues 347 and 422, one at residue 439, and four between residue 555 and 689. Some of the phosphopeptides such as peptide 4 matched strongly ^{35}S methionine-labeled bands (Fig. 3D). Because that peptide came from the large N-terminal V8 fragment, this suggests that it was derived from

the N terminus. Other fragments such as 2 had no strongly ^{35}S methionine-labeled counterpart. This peptide must have arisen downstream of the N-terminal methionine-rich region. Fragment 7 appeared to lack methionine completely. The significance of this observation is apparent below.

The clustering of phosphorylation toward the N terminus and the separation of the phosphorylations represented by peptides 5 and 7 from those represented by peptides 1 to 4, 8, and 9 was confirmed by the analysis of a naturally occurring fragment of large T antigen (data not shown). A 42K fragment was an artifact of isolation that was usually present in low concentration. Incubation of the extract at room temperature or for long periods of time increased the yield of this fragment. It came from the N terminus of the molecule, because it was smaller for large T antigens of N-terminal deletion mutants and because it was immunoprecipitated with antibody to a β -galactosidase fusion protein containing only the 70 N-terminal amino acids of the large T antigen. V8 mapping of the 42K fragment gave a pattern very similar to that of the intact molecule. In particular, fragments 1 to 4, 8, and 9 were present in yields similar to that of the intact large T antigen molecule, localizing these phosphorylations to the N-terminal region. In contrast, peptides 5 and 7 were missing from the fragment. This positioned the phosphorylation represented by these fragments elsewhere on the molecule.

Hydroxylamine cleavage of large T antigen confirms the separation of sites. Hydroxylamine preferentially cleaves at Asn-Gly sequences (4). Py large T antigen possesses a potential cutting site at residue 210. The sequences derived by Soeda and colleagues (29) and Deininger and co-workers (6) differ at position 649; a second cleavage would be expected from the former, but not the latter, sequence.

Cleavage with hydroxylamine for a limited period (30 min) released two principal phosphorylated fragments (Fig. 4). The smallest fragment had an apparent molecular weight of 33,000. This fragment represents the N terminus of the molecule arising from cleavage at residue 210. It was shortened for the large T antigen from mutant *dl45*, which has a

deletion proximal to residue 210, but was of wild-type length for *dl1015*, which has a distal deletion. It was absent in digestions of *dl23* large T antigen, which lacks the sequence at Asn-Gly 210–211. This 33K fragment was heavily labeled with $^{32}\text{PO}_4$, confirming the phosphorylation that occurs in the N-terminal end. Digestion of this N-terminal hydroxylamine fragment with V8 yielded fragments 4 and 9, as well as a new peptide representing the cleavage of fragments 1 to 3 with hydroxylamine (Fig. 5).

The shift in electrophoretic mobility of large T antigen has been described in the previous report (3). Although not entirely responsible, the region represented by the N-terminal hydroxylamine fragment is involved in the shift in mobility seen for the intact molecule. In pulse-chase experiments the N-terminal fragment showed a decreased electrophoretic mobility with time (data not shown). Furthermore, treatment of large T antigen with alkaline phosphatase caused the electrophoretic mobility of this fragment to increase substantially (data not shown). This is reminiscent of the shift in mobility observed for an N-terminal tryptic peptide observed by Simmons for SV40 large T antigen (28).

In addition to the N-terminal peptide released by hydroxylamine, a second phosphorylated peptide with a molecular weight of approximately 69,000 was released (Fig. 4). This fragment was released with the same kinetics as the N-terminal fragment, suggesting that it represented the remainder of the molecule (amino acids 211 to 785). The behavior of large T antigen mutants is consistent with this interpretation. The 65K fragment was shortened in *dl1015*, unaffected for *dl45*, and absent for *dl23*. This peptide also had an electrophoretic mobility that varied with time of labeling; furthermore, alkaline phosphatase treatment altered the mobility of the band. The anomalous behavior of large T antigen on gels therefore was not restricted to the N-terminal portion of the molecule. Double digestions with V8 demonstrated that this

fragment contained peptides 5 and 7 (Fig. 5A). This again places them farther down the molecule than the other V8 peptides. No substantial amount of phosphorylation of peptides other than 5 and 7 was observed, suggesting that the phosphorylation of the last 575 amino acids of large T antigen is largely limited to that site(s).

Hydroxylamine cleavage of large T antigen labeled with [^{35}S]methionine yielded peptides that matched electrophoretically the two phosphorylated fragments. A third fragment (60K fragment) was also observed (Fig. 4A, bar). This fragment appeared later in the course of digestion with hydroxylamine. Based on the sequence derived by Soeda et al. (29), it is possible that this could represent utilization of a site at residue 650, generating either a fragment from residue 1 to 650 or one from residues 210 to 650. Either possibility is unlikely. The fragment was too small to extend from the N terminus as far as residue 650. The fragment could not be derived from the Asn-Gly starting at residue 210, because the fragment was present and undeleted in the large T antigen of *dl23*, which lacks the site at residue 210. It is probable that this represents some internal fragment arising from a sensitive sequence other than Asn-Gly, because its appearance coincided with the appearance of minor N-terminal fragments in the 40,000-molecular-weight range. No Asn-Gly sequences are expected in that portion of the molecule (6, 29).

Partial chymotryptic and formic acid digestions help to position the downstream phosphorylations. Because fragments 5 and 7 were lacking in the N-terminal fragments, these must be derived from sequences farther along the molecule. From the V8 digestions it can be concluded that they must arise after the N-terminal 38K fragment. Partial chymotryptic digestion of large T generated two phosphorylated fragments, one with a molecular weight of 65,000 and the other with a molecular weight of 57,000. When these were digested with V8 (Fig. 5B), they yielded both the N-terminal fragments as well as peptides 5 and 7. This means that fragments 5 and 7 are located within the first 57,000 daltons of sequence, proximal to amino acid residue 520. This also shows that there is little phosphorylation in the C-terminal portion of the molecule.

Formic acid analysis supported this conclusion. Digestion with formic acid preferentially cleaves proteins at Asp-Pro sequences (12). Py large T antigen possesses seven such sequences. Although the digestion pattern was somewhat complex, formic acid analysis was quite informative and provided support for the conclusions reached above.

The most intense formic acid fragment (peptide E) had an apparent molecular weight 24,000 and contained both phosphate and methionine (Fig. 6). This phosphopeptide was derived from cleavage at the Asp-Pro starting at residue 183. It could be derived from the 38K N-terminal V8 fragment (Fig. 7) or from the hydroxylamine fragment from residue 1 to 210 by a digestion with formic acid (data not shown). Digestion of the large T antigen of *dl45*, which lacks the sequence at residue 183, resulted in the disappearance of this fragment. As expected, the large T antigen of *dl23*, for which only sequences distal to residue 183 (residues 194 to 228) were deleted, retained this peptide intact (Fig. 7). This again placed a major fraction of large T antigen phosphorylation in the N-terminal region of the molecule.

Several additional peptides were observed that contained the N-terminal portion of the molecule (Fig. 6 and 7). Phosphopeptides C and D were affected for mutants such as *dl23*. Each could be digested further to give the N-terminal fragment E (residues 1 to 183). Peptide B is an N-terminal

FIG. 5. (A) V8 protease digestion of hydroxylamine fragments. Phosphorylated large T antigen was treated with hydroxylamine as described in the text. The resulting products were resolved in a 10% acrylamide gel. The fragments were then digested with V8 protease, and the fragments were separated on a 12.5% acrylamide SDS gel. The positions of the V8 fragments are indicated by the arrowheads. The positions of intact large T antigen (L), the C-terminal piece (C; residues 211 to 785), and the N-terminal fragment (N; residues 1 to 210) are indicated by the vertical lines. (B) V8 protease digestion of chymotryptic (CHT) peptides of phosphorylated large T antigen. Phosphorylated large T antigen was digested with chymotrypsin in the immunoprecipitate. The fragments were resolved on a 10% acrylamide SDS gel. The fragments were then redigested with V8 protease, and the fragments were separated on a 12.5% acrylamide gel. The position of the V8 fragments are shown by the arrowheads. The positions of the undigested 57K and 65K chymotryptic fragments are shown by the vertical lines.

FIG. 6. Formic acid digestion of $^{32}\text{PO}_4$ - or ^{35}S methionine-labeled large T antigen (LT). Large T antigen from wild-type infected 3T6 cells labeled with $^{32}\text{PO}_4$ or ^{35}S methionine was immunoprecipitated and resolved on a 10% acrylamide gel. The large T antigen was digested in formic acid for 2 h at 60°C , and the fragments were electrophoresed on a 12.5% acrylamide SDS gel. The positions of the major phosphopeptides are indicated by the arrowheads. The major ^{35}S methionine peptide lacking any phosphate is indicated by the bar.

peptide with a molecular weight of 59,000. Like the chymotryptic peptides of 57,000 and 65,000 molecular weight, this fragment could be digested with V8 to give phosphopeptides 5 and 7 (data not shown). This again placed a distal limit on the phosphorylation sites represented by V8 fragments 5 and 7.

The positioning of V8 fragments 5 and 7 toward the N-terminal side of the molecule was supported by the demonstration of a ^{35}S -labeled peptide with a molecular weight of approximately 53,000 that lacked phosphate (Fig. 6, bar). Because it appeared even with extremely limited formic acid digestion, it presumably arose from a single cleavage. It must derive from the C-terminal portion of the molecule, because it is not deleted in any of the N-terminal

FIG. 7. Formic acid digestion of intact large T antigen and the initial V8 protease phosphopeptides. Phosphorylated large T antigen of wild type (WT) or *d123* were digested with formic acid either before or after limited V8 protease digestion. The positions of the smaller formic acid fragments are indicated with the arrowheads; C, D, and F are shortened for *d123*. The positions of the N-terminal V8 fragments are indicated by the vertical lines.

FIG. 8. Formic acid double digestion. Phosphorylated large T antigen was digested with formic acid on the beads. The fragments were resolved on a 12.5% acrylamide cylindrical gel. After redigestion with formic acid, the fragments were resolved on a 15% acrylamide SDS gel. The positions of the fragments in each dimension are indicated.

deletion mutants. The C-terminal region therefore contained little, if any, phosphate.

Formic acid double digestion demonstrated the interrelationships of several of the fragments and localized the downstream phosphorylation sites even more precisely (Fig. 8). As indicated above, peptides C (44,000 molecular weight) and D (36,000 molecular weight) are overlapping N-terminal fragments. This was demonstrated by the fact that peptide E, representing residues 1 to 183, can be derived from each of them. Peptide C contained phosphate that was not present in peptide D. Phosphopeptide F was derived from peptide C (44,000 molecular weight) but not from peptide D (36,000 molecular weight) by a second digestion with formic acid. Neither peptide F nor a shortened version of peptide F was derived from peptide D, indicating that fragment C contained phosphorylation sites that were missing from the shorter fragment D. Peptide F itself was deleted in *d123*, was missing from *d145* digests, and was not derived from the N-terminal peptide E by a second digestion with formic acid. This is the expected behavior of a peptide extending toward the C terminus from amino acid 183. From measurement of fragment size, the C-terminal end of fragment F is most likely the C-terminal end of fragment C. Less probably, it could lie within the distinct region between fragments B and C. In either case, this places some phosphorylation in the region of the molecule 36,000 to 44,000 daltons from the N terminus. These phosphorylations should represent the V8 phosphorylations of fragments 5 and 7. A simple demonstration of this fact was not possible because formic acid apparently cleaves within the V8 peptides 5 and 7. Formic acid fragment F was not derived from the 38,000-molecular-weight V8 precursor (data not shown). This suggests that the ends of fragments 5 and 7 overlap the formic acid cleavage site that generates fragment C.

Positioning of the phosphorylations in the N-terminal regions. The combination of chemical and enzymatic digestion with mutant analysis further positions the N-terminal phosphorylations represented in the 38,000-molecular-weight V8 precursor peptide.

As shown by the phosphorylation of formic acid fragment E, most of the phosphorylation represented in the 38,000-molecular-weight V8 fragment came from sites proximal to a

formic acid cleavage site at residue 183. A cleavage even more proximal was also expected at the Asp-Pro sequence at residue 125. The smallest formic acid fragment (peptide G) observed in this digest was likely to represent this cleavage. As expected, it could be derived from fragment E by a second formic acid digestion (Fig. 8). Like the larger fragment E it could be derived from the hydroxylamine peptide from residue 1 to 210, but it was not affected by the deletion in *dl45*. As expected from the amino acid sequence, it was labeled with [³⁵S]methionine. The phosphorylation of this peptide demonstrates that some phosphorylation occurs in the N-terminal 125 amino acids.

Cleavage of the V8 38K peptide with formic acid yielded principally two formic acid peptides (E and G) that arose from sequences proximal to residue 183. This placed most of the phosphorylation in the 38K fragment proximal to residue 183. This conclusion was confirmed in two ways. Hydroxylamine cleavage of the V8 38K fragment showed the labeled N-terminal sequence (residues 1 to 210). Similarly, formic acid peptide C is an N-terminal 37K fragment; redigestion of peptide C with formic acid yielded only the two N-terminal peptides.

Large T-antigen deletion mutants (*dl8*, *dl45*, and *dl23*) that lack the sequences between amino acids 150 and 223 are available. Each mutant large T antigen had wild-type-sized fragments 4, 8, and 9. This was particularly significant for *dl8*, the most proximal of the deletion mutants (Fig. 9). This places fragments 4, 8, and 9 in the proximal portion of the molecule. Given its size (18,000 daltons), it is likely that fragment 4 represents the N terminus. Because four methionines are clustered in the first 70 amino acids, it would be expected that fragment 4 could be readily labeled with [³⁵S]methionine. This was indeed the case, as was discussed earlier (Fig. 3).

Fragments 1, 2, and 3 must be more distal. None of the mutants demonstrated an intact fragment 1, 2, or 3. Fragment 1 was lightly labeled with [³⁵S]methionine, but fragment 2 was not. This is consistent with the idea that these phosphopeptides arise from the methionine-poor region stretching from amino acids 71 to 341. V8 peptides 1 through 3 overlapped N-terminal hydroxylamine and formic acid cleavage sites. Unlike fragments 4 and 9, intact fragments 1 to 3 were not observed when either the N-terminal hydroxylamine (Fig. 5) or formic acid fragments were di-

gested with V8. Instead, a major new peptide was observed in each case, representing the cleavage of fragments 1 to 3 with either hydroxylamine or formic acid.

DISCUSSION

The results described here demonstrate two main regions of phosphorylation of the large T antigen. The first of these was N terminal to the hydroxylamine cleavage site at amino acid residue 210 and the formic acid cleavage site at residue 183. These phosphorylations were represented by V8 fragments 1 to 4, 8, and 9. Although the fragments themselves were partially overlapping, peptides 1 to 3 contained additional phosphorylations distal to those represented in 4, 8, and 9. No common phosphopeptides were obtained by extended digestion of fragments 1 to 3 and 4. Also fragments 1 to 3 label and turned over more rapidly than fragments 4, 8, and 9. V8 fragments 1 to 3 could not extend beyond amino acid 70 toward the N terminus. The amino acids in this stretch of large T antigen had no counterparts in SV40 large T antigen. These phosphorylations occurred early in infections of growth-arrested cells (3). Phosphorylation in this region of the molecule could be affected by mutations farther away on the molecule. The quantitative defect was also observed in deletion mutants in the region from amino acid residues 300 to 500 (P. Rose, B. Bockus, and B. Schaffhausen, unpublished data). As stated in the previous report (3), *tsa* mutants are quantitatively deficient in these N-terminal phosphorylations under nonpermissive conditions. Even at the permissive temperature there was a slight, apparent decrease in specific activity. The significance of this observation therefore is not clear, although it should be noted that *tsa* mutants do not autoregulate early transcription in the same way as the wild type (5).

The second region of phosphorylation was more distal. V8 fragments 5 and 7 could be derived from N-terminal chymotryptic fragments as small as 57,000 daltons. From the other direction, they could be derived from a C-terminal V8 fragment of 59,000 daltons. This limits that phosphorylation to a central region of the molecule. The location of at least some of this phosphorylation can be defined more precisely. Formic acid fragment C, which has a molecular weight of 44,000, must contain phosphate not found in the N-terminal region. Digestion of fragment C but not the overlapping 36,000-dalton peptide D gave rise to peptide F. This shows that there is phosphate in the region that is present in C and not in D, that is the region nominally between 36,000 and 44,000 daltons from the N-terminus. Neither the 58,000- nor the 38,000-dalton V8 fragment could be digested to give formic acid fragment F. This is entirely consistent with the notion that the fragment extends from 183 (in the 38K fragment) to the 44,000-dalton cutting site which would be beyond the V8 cleavage site. Similarly, V8 fragments 5 and 7 could be derived from the 58K fragment but not the N-terminal formic acid 44K fragment. This result, taken with the demonstration that the 44K fragment contains distal phosphorylation, suggests that the sequences in V8 fragments 5 and 7 span the formic acid 44K fragment cutting site.

Where exactly are these cleavages? Formic acid cleaves preferentially at Asp-Pro sequences. There are three relevant sites: residues 434, 285, and 257. There calculated sizes are consistent with the molecular weight of fragments B, C, and D, respectively, when the demonstrated N-terminal anomaly is taken into account. The phosphopeptide containing fragment 7 contains no detectable methionine. There is a

FIG. 9. V8 protease digestion of wild type (WT) and *dl8* large T antigens. After isolation on 10% acrylamide SDS gels, phosphorylated large T antigen from wild type or *dl8* was digested with V8 protease on a 12.5% acrylamide second-dimension SDS gel.

major methionine-rich region from amino acids 350 to 430. The V8 fragment must come from either side of that fragment. Therefore, fragment 7 presumably arises distal to the sequences at residue 257 and proximal to residue 350. In this region there are several candidate sites, and more detailed analysis will be required to identify the residues involved. Judged from formic acid fragments C, D, and F, some or all of this phosphorylation must occur between residues 257 and 285. Digestion of fragment C with V8 yields quantitatively fewer phosphopeptides than expected from the amount of V8 peptides 5 and 7 obtained from intact large T antigen; this suggests that additional phosphorylation occurs beyond residue 285 as well.

This distal phosphorylation is of particular interest. *tsa* mutants do not show these peptides when they are labeled at the nonpermissive temperature, and the activity leading to this phosphorylation must be induced in growth-arrested cells (3). It was not detected by Hassauer and colleagues (10). There are two likely reasons for this. The first is that longer labeling periods used in their studies favor the labeling of the N-terminal region because of differential turnover of the sites (3). Second, judging from the published tryptic digestion patterns, it appears that the distal sites may have remained in larger fragments that do not separate in the thin-layer system.

The detailed arrangement of sites in SV40 large T (25, 26) is different from that for Py large T antigen. SV40 large T antigen has a major region of C-terminal phosphorylation that is lacking in Py. Py N-terminal phosphorylation occurs in a region for which SV40 has no homologous sequence. These differences may be related to different functions for the protein. It appears likely that SV40 large T antigen functions directly in transformation, whereas the role of Py large T antigen in transformation appears to be subordinate to that of the middle T antigen. At the level of DNA binding, there appear to be clear differences between the two proteins (27).

It is less clear that the downstream phosphorylation is entirely different. There are several phosphorylation sites in SV40 that are near the nuclear localization signal (13). Py large T antigen apparently has two nuclear localization signals (19). One of these (at about amino acid Lys-282) is similar to that found in SV40 large T antigen. The SV40 signal and this Py signal have phosphorylation sites (residues 257 to 285) in the vicinity. When the sequences of the SV40 phosphorylation sites are compared with those of Py, it can be seen that only the threonine residue at residue 279 is conserved between the two viruses. Phosphorylation beyond residue 285 would have no counterpart in SV40. With regard to the nuclear localization sites, it is also interesting that the N-terminal phosphorylation sites are near the secondary signal (near amino acid 190). It is quite plausible that the function of the phosphorylation is conserved, even though the exact sequence is not.

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