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Phosphorylation of Threonine in the Proline-Rich Carboxy-Terminal Region of Simian Virus 40 Large T Antigen

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The position of phosphothreonine in the predicted primary structure of simian virus 40 large T antigen was determined by different methods. After digestion of large T antigen with trypsin and subsequent two-dimensional peptide mapping, a single peptide containing phosphothreonine could be separated from the bulk of phosphoserine-containing peptides. Its amino acid composition was determined by differential labeling with various amino acids in vivo. The high yield of proline (4.5 mol) within the phosphothreonine peptide indicated that it was derived from the carboxy terminus of large T antigen and had in its unphosphorylated form the sequence Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr-COOH. A phosphopeptide generated by chymotrypsin could be converted into the tryptic phosphothreonine peptide, indicating that the latter was part of the chymotryptic peptide. The origin of the phosphothreonine-containing peptides was independently confirmed by using an antiserum directed against the carboxy terminus of large T antigen. This serum reacted specifically with the proline-rich, phosphothreonine-containing peptides. Further analysis by partial acid hydrolysis indicated that the internal threonine was phosphorylated. The unusual amino acid composition on both sides of the phosphothreonine and the possible function of this phosphorylation site are discussed.

Simian virus 40 (SV40) large T antigen is involved in various functions during the growth cycle of the virus. It is required for the initiation of viral DNA replication (8, 58), it regulates transcription of its own gene (1, 30, 51, 61), and may also play a role in transcription of the late genes (45), functions that are probably mediated by interaction of large T antigen with the origin of replication on the SV40 genome (27, 46, 50, 63). Large T antigen also seems to interact with host DNA, since it stimulates both cellular DNA and RNA synthesis (10, 22, 25, 64). It promotes human adenovirus replication in monkey cells by enhancing the expression of several late adenovirus genes (20, 31, 32, 48). Moreover, large T antigen is required for the initiation and maintenance of malignant transformation (2, 4, 6, 7, 41, 44, 59). So far, no clue exists as to how these different functions are exerted. Either large T antigen may consist of several structural or functional domains, or modification could lead to different forms of this protein, each involved in a specific function.

Several observations support the latter possibility (but do not rule out the former): large T antigen is a phosphoprotein (60) containing, on the average, four phosphate groups that are bound to serine and threonine residues (65); these phosphate groups turn over more rapidly than large T antigen itself (17), indicating that phosphorylation is reversible, which is a prerequisite for a regulatory role of any modification; moreover, subclasses of large T antigen have been described which differ in their biochemical and biophysical properties (i.e., their phosphorylation state [23] or their DNA-binding capacity [43, 47]), and a direct correlation between the degree of phosphorylation and the affinity for DNA has been demonstrated recently (42). Thus, it is conceivably the phosphorylation state of large T antigen that determines which of its potential functions a certain molecule will fulfill.

To correlate a specific phosphorylation event with one of the various functions of large T antigen, we have focused on the precise localization of the individual phosphate residues within the polypeptide chain. We report here on the mapping of phosphothreonine. Evidence is presented that the phosphothreonine is located within the proline-rich carboxy-terminal region of large T antigen.

MATERIALS AND METHODS

Cell line and virus. The TC7 subclone of the CV1 African green monkey kidney cell line was used in all experiments. The cells were grown in Dulbecco's modification of Eagle minimal essential medium (DMEM) supplemented with 10% fetal calf serum. The large-

plaque strain of SV40, originally obtained from P. Tegtmeyer, was propagated in TC7 cells. Virus stocks had a titer of approximately 5×10^7 PFU/ml.

Infection and labeling of cells. Confluent TC7 cells growing on 9-cm plastic petri dishes were infected with SV40 at a multiplicity of 5 to 10 PFU/cell. Cells were routinely labeled between 45 and 55 h postinfection in 1.5 ml of MEM with 10% fetal calf serum (MEMFS) for 3 h. Labeling with carrier-free $^{32}P_i$ was carried out at 1 mCi/plate in phosphate-free MEMFS. ^{3}H-labeled essential amino acids were used at 1 mCi/plate (specific activity, 16 to 60 Ci/mmol) in MEMFS lacking the respective amino acid. Labeling with the nonessential ^{3}H-labeled amino acids was at 1 mCi/plate in 3 ml of complete DMEMFS from 40 to 60 h postinfection, [^{35}S]methionine and [^{35}S]cysteine were used at 250 μCi/plate (specific activity, ~950 and ~560 Ci/mmol, respectively), and [^{14}C]proline label was used at 125 μCi/plate (specific activity, 280 mCi/mmol). $^{32}P_i$ and ^{3}H-labeled amino acids were obtained from Amersham Buchler, Braunschweig, West Germany; [^{35}S]methionine, [^{35}S]cysteine, and [^{14}C]proline were obtained from New England Nuclear Corp.

Isolation of large T antigen. After labeling, the cells were carefully washed and lysed on the plate in 1 ml of pH 8 low-salt extraction buffer (10 mM Tris-hydrochloride, pH 8, 140 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 1 U of aprotinin per ml). The lysate was scraped off the plates, and nuclei were removed by low-speed centrifugation. Large T antigen was obtained from the cytoplasm by immunoprecipitation with antiserum from hamsters bearing SV40-induced tumors and *Staphylococcus aureus* and by subsequent electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels as described previously (36, 38, 40). After autoradiography of the unfixed dried gel, large T antigen was eluted in buffer containing 50 mM NH_4HCO_3, 0.1% sodium dodecyl sulfate, and 5% 2-mercaptoethanol; it was precipitated with 20% trichloroacetic acid and oxidized with 3% performic acid.

Peptide mapping. Digestion with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin or chymotrypsin was carried out in 0.5 ml of 50 mM NH_4HCO_3, pH 7.9, at an enzyme-to-substrate ratio of 1:2 (based on the concentration of carrier protein) at 37°C for at least 10 h. In sequential digests, the enzyme used first was inactivated by boiling. The digestion products were separated by two-dimensional fingerprinting on thin-layer cellulose plates as described by Gibson (21). Electrophoresis was performed in 1% (wt/vol) NH_4HCO_3, pH 8.9 (buffer A), or in 6% formic acid–1.25% acetic acid–0.25% pyridine (vol/vol), pH 1.9 (buffer B), at 1,000 V for the times indicated in the figure legends. Chromatography was done in 37.5% butanol–25% pyridine–7.5% acetic acid (vol/vol) (buffer C) or in 39.3% butanol–30.4% pyridine–6% acetic acid (vol/vol) (buffer D). The position of ^{35}S- or ^{14}C-labeled peptides was visualized by fluorography (3); ^{32}P-labeled peptides were subjected to autoradiography directly. ^{3}H-labeled samples were mixed with ^{32}P-labeled digests of large T antigen and fingerprinted as described above; ^{32}P-labeled spots were eluted from the cellulose and analyzed for their ^{3}H content by liquid scintillation counting.

Determination of phosphoamino acids. Acid hydrolysis of isolated phosphopeptides was performed in 5.6 N HCl at 110°C for 2 h in sealed glass tubes under a nitrogen atmosphere. The hydrolysates were mixed with unlabeled phosphoserine and phosphothreonine and electrophoresed in 7.8% acetic acid–2.5% formic acid (vol/vol), pH 1.9 (buffer E), at 1,000 V for 70 min. The markers were visualized with ninhydrin.

Immune reaction with specific antisera. Antisera directed against the amino and carboxyl ends of large T antigen were obtained by immunizing rabbits with synthetic peptides corresponding to the seven amino-terminal and the 11 carboxy-terminal amino acid residues of large T antigen. The synthesis of these peptides, their coupling to bovine serum albumin for immunization, and the specificities of the antisera obtained have been published (66). In our study, the antisera were used for immune reactions with tryptic and chymotryptic digests of large T antigen to identify the carboxy-terminal peptides. The antisera were first incubated with fixed *S. aureus* to immobilize the immunoglobulins. The complex was washed twice with 50 mM NH_4HCO_3, pH 7.9, resuspended in the same buffer, and incubated with tryptic or chymotryptic digests of ^{32}P- or [^{14}C]proline-labeled large T antigen in the presence of 1 U of aprotinin per μg of trypsin or chymotrypsin. The *S. aureus*-immune complex was pelleted, and the supernatant was lyophilized and subjected to fingerprinting.

RESULTS

Separation of the phosphothreonine peptide. Growing TC7 cells were infected with large-plaque SV40 at a multiplicity of 5 to 10 PFU/cell and labeled with $^{32}P_i$ from 50 to 53 h after infection. Large T antigen was isolated from the cytoplasmic extract by immunoprecipitation with hamster antitumor serum and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Materials and Methods. After digestion with trypsin and subsequent two-dimensional fingerprinting on thin-layer cellulose plates, a single phosphopeptide could be separated from the bulk of phosphopeptides (Fig. 1). This phosphopeptide appeared to be rather acidic since it migrated to the anode during electrophoresis at pH 8.9 (Fig. 1a), whereas most tryptic peptides of large T antigen were neutral or migrated to the cathode at this pH (compare Fig. 3a, b). The phosphate in the single peptide was bound to threonine, as has been demonstrated by Walter and Flory (65). The unresolved material represented a heterogeneous mixture of phosphoserine-containing peptides. They seemed to be very polar and did not migrate in a variety of chromatography systems (see Discussion).

With a pH 1.9 buffer system for electrophoresis in which only amino and phosphate groups were charged, again a single phosphopeptide could be separated from the residual unresolved

phosphate-labeled peptides (Fig. 1b). It seemed to have a net positive charge at pH 1.9 as judged from its migration to the cathode. This was to be expected, however, since any tryptic peptide except the carboxy-terminal one should have an extra positive charge due to its carboxy-terminal basic residue unless it is compensated by a second phosphate or a cysteic acid residue. The isolated peptide contained mainly phosphothreonine, as shown by acid hydrolysis and subsequent electrophoresis (Materials and Methods). The phosphothreonine-containing peptides will be referred to hereafter as phosphothreonine peptides.

Analysis by sequential proteolytic cleavage. When ^{32}P-labeled T antigen was digested with chymotrypsin, a distinct phosphopeptide was obtained which migrated at pH 1.9 further to the cathode than the tryptic phosphothreonine peptide, indicating either an additional positive charge(s) or lower molecular weight (Fig. 2a, b). The phosphate in this chymotryptic peptide was bound to threonine, as shown by acid hydrolysis. Sequential cleavage by chymotrypsin and trypsin generated two additional phosphopeptides (Fig. 2c), whereas no change in the peptide pattern was observed when tryptic digestion was followed by chymotryptic cleavage (data not shown).

One of the additional phosphopeptides obtained in the sequential digest comigrated with the tryptic phosphothreonine peptide when analyzed by mixing the double digest with the tryptic digest (Fig. 2d). Acid hydrolysis revealed that it contained phosphothreonine, indicating that it was generated by cleavage within the chymotryptic phosphothreonine peptide. Thus, the difference in migration distance between the chymotryptic and the tryptic phosphothreonine peptides was due to a difference in charge rather than in mass. The other additional peptide contained phosphoserine and must have been derived from one of the phosphoserine peptides that were not resolvable in single digests (Fig. 2a, b). This phosphoserine peptide was not further investigated.

Assay for phosphotyrosine. Since several transforming proteins have been shown to be phosphorylated at tyrosine residues (14, 16, 24, 67) and phosphotyrosine would not have been distinguished from phosphothreonine under the assay conditions used here, the possibility existed that either the presumed phosphothreonine itself was phosphotyrosine or a phosphotyrosine existed that was not identified in former analyses. However, no phosphotyrosine was detectable when a hydrolysate of large T antigen was analyzed under the conditions described by Eckhart et al. (16) (Fig. 3).

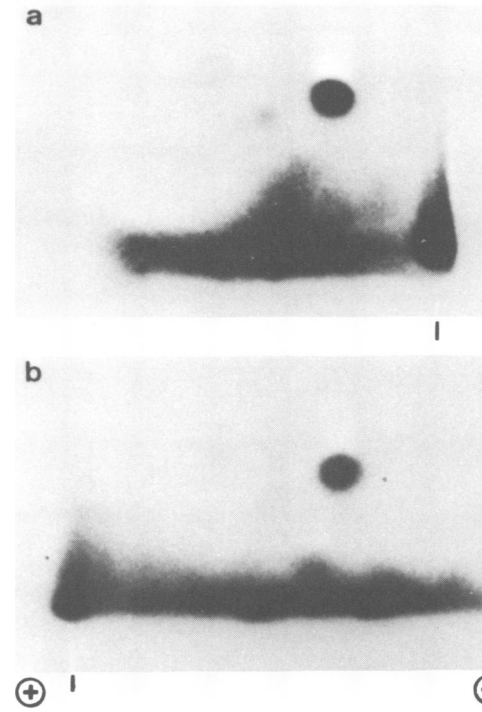

FIG. 1. *Peptide mapping of a tryptic digest of ^{32}P-labeled large T antigen. Growing TC7 cells were infected with SV40 and labeled with $^{32}P_i$ from 48 to 51 h postinfection. Large T antigen was isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, digested with trypsin, and subjected to two-dimensional fingerprinting on thin-layer cellulose plates as described in Materials and Methods. Electrophoresis was carried out in the horizontal direction in buffer A (a), or in buffer B (b) for 25 min. The origin is indicated by a vertical dash. Ascending chromatography was performed in buffer C for 6 h in all cases, unless indicated otherwise.*

Amino acid composition of the phosphothreonine peptide. Since the complete amino acid sequence of large T antigen has been deduced from the DNA sequence (19, 49), one can locate exactly a certain peptide within the polypeptide chain by elucidating its amino acid composition. To determine the composition of the tryptic phosphothreonine peptide, SV40-infected cells were labeled with various radioactive amino acids in separate experiments, and large T antigen was purified by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After digestion with trypsin, the appearance of radioactivity in the phosphothreonine peptide was assayed by two-dimensional fingerprinting. Localization of the phosphothreonine peptide was achieved by mixing tryptic

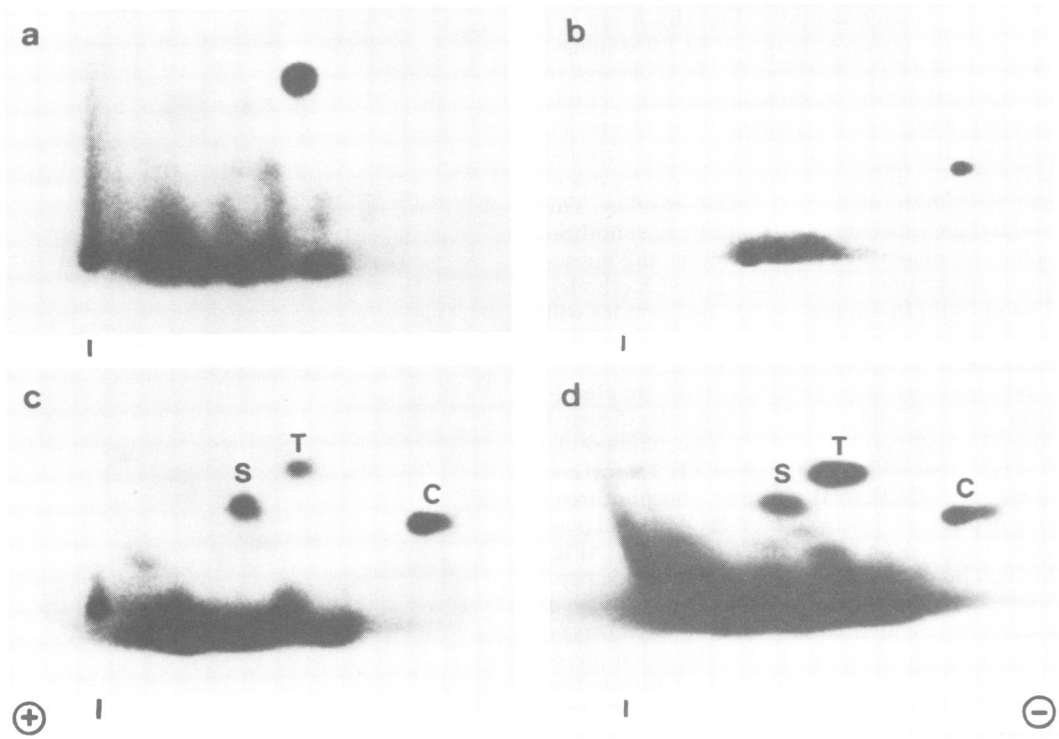

FIG. 2. *Comparison of tryptic and chymotryptic phosphopeptides of large T antigen. ^{32}P-labeled large T antigen was digested with trypsin (a), chymotrypsin (b), or sequentially with chymotrypsin and trypsin (c); in (d), a mixture of a tryptic digest and a chymotryptic-tryptic double digest was analyzed. Electrophoresis in buffer B (25 min) was followed by chromatography. Peptides C and T represent the chymotryptic and the tryptic phosphothreonine peptides, respectively; peptide S contains phosphoserine.*

digests of amino acid-labeled and ^{32}P-labeled large T antigen.

When large T antigen was labeled with [^{35}S]-methionine or [^{35}S]cysteine, no radioactivity appeared in the phosphothreonine peptide (Fig. 4). Therefore, this peptide did not contain methionine and cysteine. From the amino acid sequence of large T antigen, one can predict only seven tryptic peptides that contain threonine, but no methionine or cysteine (Fig. 5). Considering the finding that the threonine peptide was strongly acidic at pH 8.9 and positively charged at pH 1.9, only peptides 3, 4, 5, and 7 remained as possible candidates. Peptide 7, which represented the carboxy-terminal peptide of large T antigen, fell into this category only because Lys-Pro bonds are not cleaved by trypsin. To discriminate between these four possibilities, the following ^{3}H-labeled amino acids were selected for labeling experiments: alanine, histidine, leucine, phenylalanine, proline, and tyrosine. Peptide 3 contained alanine, histidine, phenylalanine, and proline, but not leucine or tyrosine; peptide 4 contained only leucine, and peptide 5 contained none of the six. In peptide 7, five of the amino acids were absent, but proline was represented in high amount. Tyrosine served as a negative control in all cases.

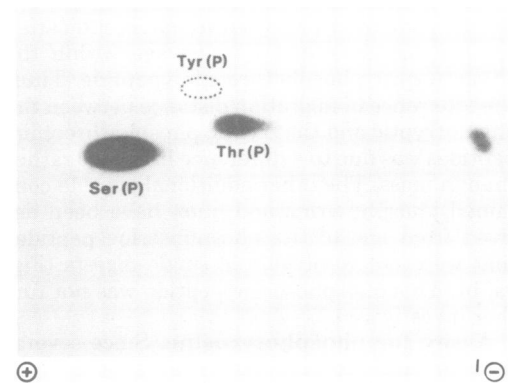

FIG. 3. *Two-dimensional separation of phosphoamino acids of large T antigen. ^{32}P-labeled large T antigen was isolated and hydrolyzed in 5.6 N HCl as described in Materials and Methods. Separation of the hydrolysate by electrophoresis and chromatography was performed according to Eckart et al. (16).*

Tryptic digests of large T antigen labeled with

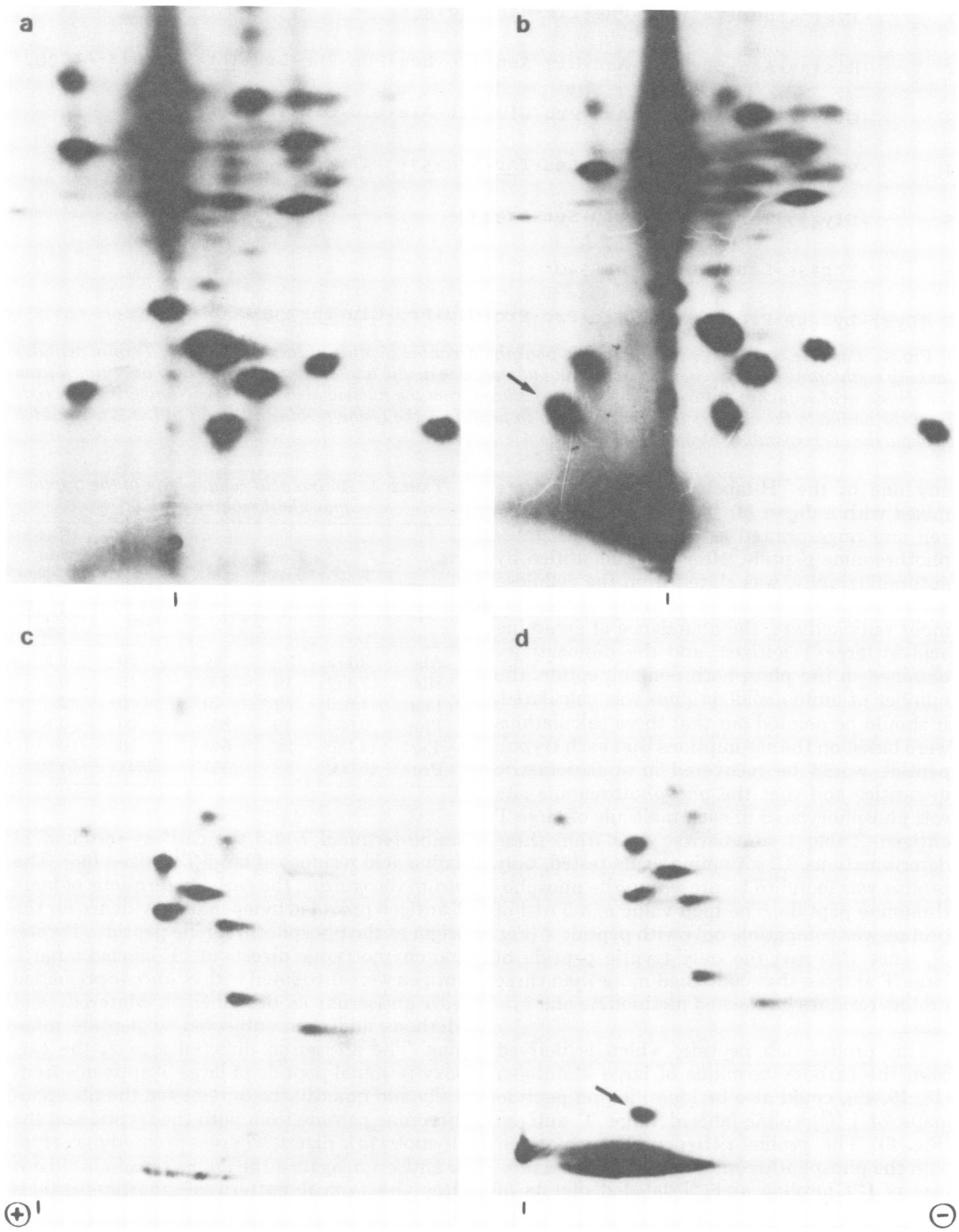

FIG. 4. *Comparison of tryptic peptides of [^{35}S]methionine- and [^{35}S]cysteine-labeled large T antigen with tryptic peptides of ^{32}P-labeled large T antigen. SV40-infected TC7 cells were labeled with [^{35}S]methionine or [^{35}S]cysteine from 52 to 55 h postinfection, and large T antigen was isolated and processed as described in the legend to Fig. 1. The tryptic digests were analyzed by peptide mapping: (a and b) [^{35}S]methionine peptides, electrophoresis in buffer A for 25 min; (c and d) [^{35}S]cysteine peptides, electrophoresis in buffer B for 35 min. In (b) and (d), mixtures of [^{35}S]methionine- or [^{35}S]cysteine-labeled and ^{32}P-labeled digests were analyzed; the phosphothreonine peptide is indicated by arrows.*

1 ·Tyr179·Ser·Val·Thr·Phe·Ile·Ser·Arg186·

2 ·His187·Asn·Ser·Tyr·Asn·His·Asn·Ile·Leu·Phe·Phe·Leu·Thr·Pro·His·Arg202·

3 ·Glu254·His·Asp·Phe·Asn·Pro·Glu·Glu·Ala·Glu·Glu·Thr·Lys266·

4 ·Val350·Asp·Ser·Leu·Glu·Leu·Thr·Arg357·

5 ·Gly477·Thr·Gly·Gly·Glu·Ser·Arg483·

6 ·Thr536·Leu·Glu·Ala·Arg540·

7 (Lys)·Lys698·Pro·Pro·Thr·Pro·Pro·Pro·Glu·Pro·Glu·Thr708·COOH

FIG. 5. *Possible candidates for the tryptic phosphothreonine peptide of large T antigen. Tryptic peptides lacking methionine and cysteine but containing at least one acidic and one basic amino acid were taken from the amino acid sequence of large T antigen as deduced from the nucleic acid sequence. Numbering proceeds from the amino to the carboxy terminus (19, 49). In peptide 7, the lysine residue adjacent to* Lys_{698} *was added to show the corresponding chymotryptic peptide.*

any one of the ^{3}H-labeled amino acids were mixed with a digest of ^{32}P-labeled large T antigen and fingerprinted as described. The phosphothreonine peptide, after being identified by autoradiography, was eluted from the cellulose plate and analyzed for ^{3}H content. From the input radioactivity, the abundance of an amino acid in large T antigen, and the radioactivity obtained in the phosphothreonine peptide, the number of amino acid residues was calculated. It should be pointed out that these calculations were based on the assumptions that each tryptic peptide would be recovered in stoichiometric quantities and that the phosphothreonine site was phosphorylated in each molecule of large T antigen. Table 1 summarizes data from these determinations. Of all amino acids tested, only proline was found to be present in the phosphothreonine peptide. The high value of 4.5 mol of proline was compatible only with peptide 7 (Fig. 5), since this was the only tryptic peptide of large T antigen that contained more than three proline residues but lacked methionine and cysteine.

This proline-rich peptide, which is derived from the carboxy terminus of large T antigen (18, 19, 49), could also be identified on peptide maps of [^{14}C]proline-labeled large T antigen (Fig. 6). The proline-rich peptide comigrated with the phosphothreonine peptide when a mixture of [^{14}C]proline and ^{32}P-labeled digests of large T antigen was fingerprinted (data not shown).

Analysis by antisera specific for the ends of large T antigen. We have recently prepared antisera specific for the amino and the carboxy termini of large T antigen by immunizing rabbits with synthetic peptides corresponding to the amino-terminal 7 and the carboxy-terminal 11 amino acid residues of large T antigen (66). The serum recognizing the carboxy terminus of large T antigen provided independent evidence for the origin of the phosphothreonine peptide. Tryptic and chymotryptic digests of ^{32}P-labeled large T antigen were incubated with *S. aureus* preloaded with antiserum as described in Materials and Methods and then subjected to peptide mapping. The antiserum directed against the carboxy-terminal peptide of large T antigen specifically and quantitatively removed the phosphothreonine peptide from both the tryptic and the chymotryptic digest, whereas preimmune serum or antiserum against the amino terminus did not alter the typical pattern of phosphopeptides (Fig. 7). The same results were obtained with a tryptic digest of [^{14}C]proline-labeled large T antigen (Fig. 6). In this case, the proline-rich peptide and one minor peptide were missing on the fingerprint. The minor peptide may have represented the proline-rich peptide in its unphosphorylated form, because the absence of one

TABLE 1. *Amino acid composition of the tryptic phosphothreonine peptide*

^{3}H-amino acid	Total cpm	Residues/large T	cpm/residue	cpm in phosphothreonine peptide	Residues/phosphothreonine peptide
Ala	3,112	37	84	20	0.2
His	7,472	19	393	19	0.1
Leu	4,816	69	70	0	0
Phe	9,656	36	268	30	0.1
Tyr	14,110	26	543	10	0
Pro	18,000	32	563	2547	4.52

phosphate residue would reduce the negative charge by one at pH 1.9, leading to an increased migration to the cathode; it would also render the peptide less polar, resulting in faster migration during chromatography. In comparing the tryptic and chymotryptic phosphothreonine peptides (Fig. 2), a similar change in migration during electrophoresis due to a one-charge difference was observed. The ratio between the phosphorylated and the presumed unphosphorylated forms of the carboxy-terminal peptide was determined to be 12:1, indicating that more than 90% of the large T antigen molecules were phosphorylated at the threonine site.

Analysis by partial acid hydrolysis. Because the carboxy-terminal tryptic peptide of large T antigen contains two threonine residues (see Fig. 5), it is necessary to determine whether phosphorylation occurs at the terminal or the internal threonine. Treatment with carboxypeptidase A or Y had no effect on the phosphothreonine peptide. Therefore, the isolated peptide was subjected to partial acid hydrolysis in 0.03 N HCl at 105°C for 5 h (54), and the products were analyzed by peptide mapping. About 95% of the phosphate label was released as P_i under these conditions (57). However, two new phosphopeptides were generated which migrated at pH 1.9 slightly farther to the cathode than the "original" phosphothreonine peptide (data not shown), indicating that these peptides had retained both the phosphate label and the positive charge due to the amino-terminal lysine residue. Therefore, cleavage had occurred in the carboxy-terminal portion without removal of the phosphorylated threonine.

DISCUSSION

To elucidate a possible relationship between phosphorylation and the functions of large T antigen, we first would like to determine the sites of phosphorylation and then investigate how phosphorylation at the various sites affects the different functions that can be tested in vivo and in vitro. In this study, we focused on the localization of phosphothreonine in large T antigen. A tryptic peptide containing phosphothreonine could be separated from the bulk of phosphoserine-containing peptides by two-dimensional fingerprinting. We have identified this peptide as the carboxy-terminal 11 amino acid residues of large T antigen by differential labeling with amino acids and on the basis of the predicted amino acid sequence of large T antigen (19, 49).

A phosphothreonine peptide generated by chymotrypsin differed from the tryptic phosphothreonine peptide only by one additional positive charge which was, according to the amino acid sequence, caused by an additional lysine residue at the amino end of the tryptic peptide. This lysine residue could be cleaved off by trypsin, thereby generating the tryptic phosphothreonine peptide. It should be pointed out that amino-terminal lysine residues are cleaved with extremely low efficiency, which explains the low yield of the tryptic peptide in sequential digests.

Independent evidence for the origin of the phosphothreonine peptides was obtained from immunological studies using antiserum against

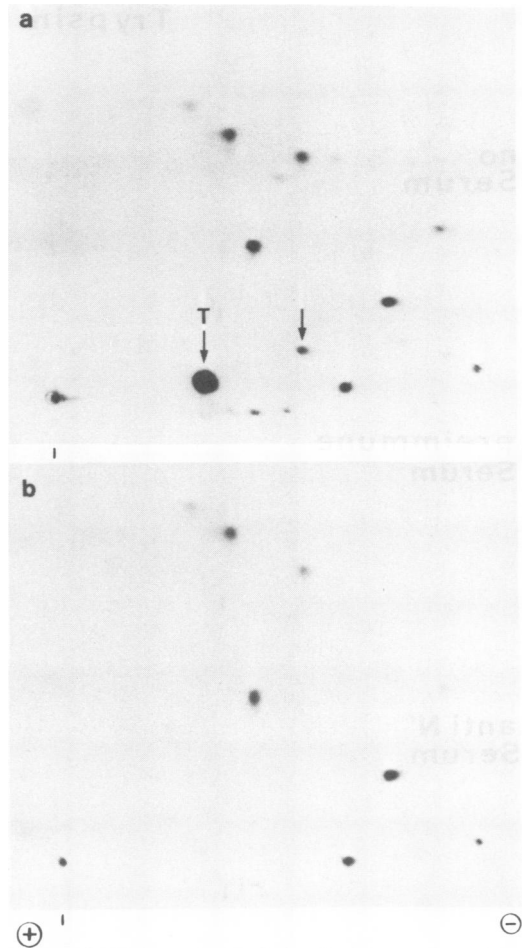

FIG. 6. *Immune reaction of tryptic peptides of [^{14}C]proline-labeled large T antigen. Immune reactions were performed as described in the legend to Fig. 7. Electrophoresis was carried out in buffer E for 25 min; chromatography was done in buffer D for 5 h. Fingerprints of tryptic peptides after incubation with antiserum to the amino terminus (a) or the carboxy terminus (b) are shown. Arrows indicate peptides missing in (b); T indicates the phosphothreonine peptide.*

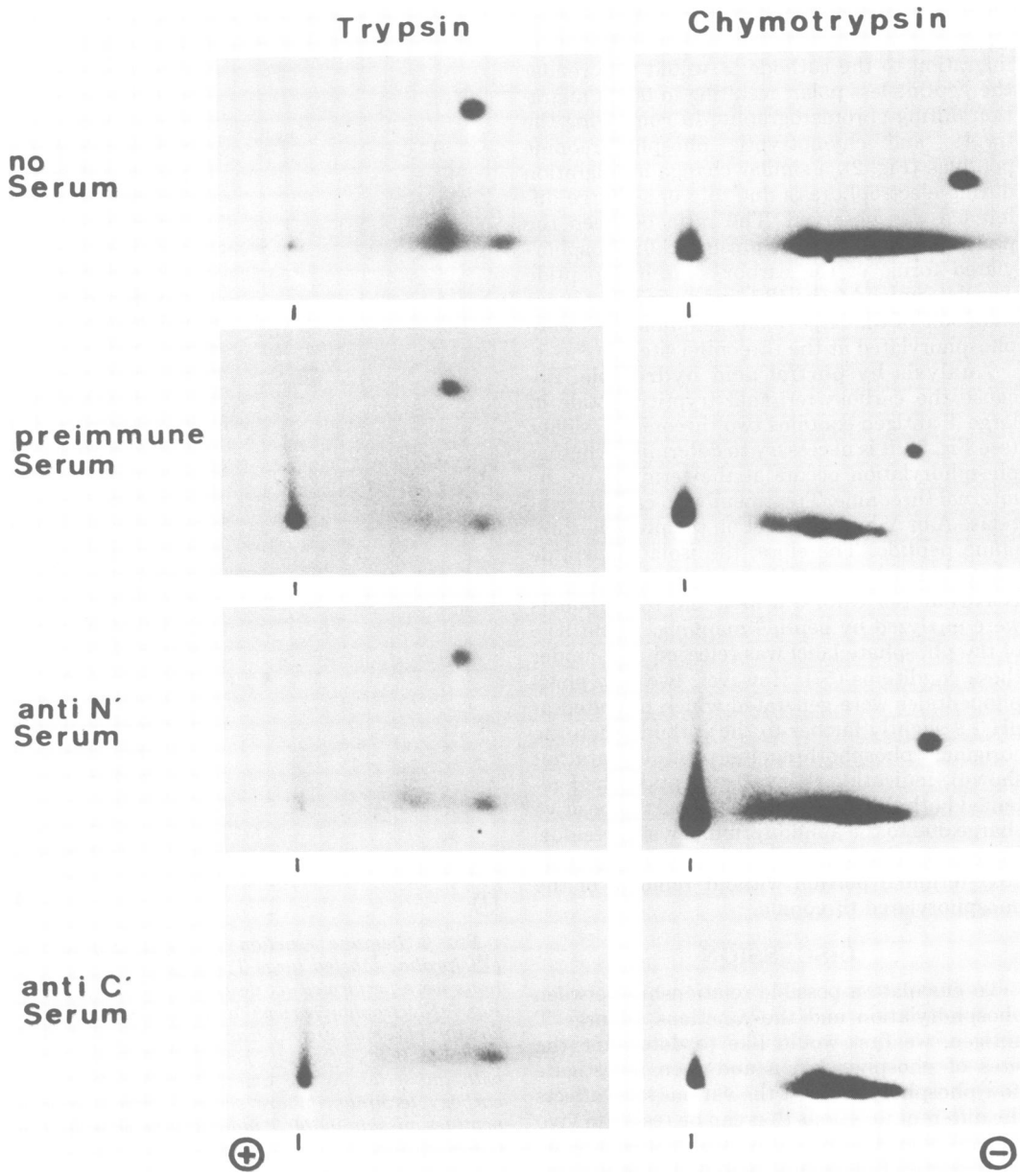

FIG. 7. *Immune reaction of tryptic and chymotryptic phosphopeptides of large T antigen. Rabbit antisera against the carboxy terminus of large T antigen (anti C' serum) was immobilized on fixed S. aureus and incubated with tryptic or chymotryptic digests of ^{32}P-labeled large T antigen as described in Materials and Methods. The unbound peptides were subjected to fingerprinting. Electrophoresis was performed in buffer B for 35 min. As controls, peptide maps without prior incubation with antiserum and after incubation with preimmune serum or with rabbit serum against the amino terminus of large T antigen (anti N' serum) are shown.*

the carboxy terminus of large T antigen. This antiserum reacted specifically with the tryptic and chymotryptic phosphothreonine peptides as well as with the proline-rich tryptic peptide of large T antigen.

Since the carboxy-terminal peptide contains two threonine residues, the question arose as to which of them is phosphorylated. In principle, five different modes of phosphorylation can be envisioned for this peptide: (i) all large T antigen

molecules are phosphorylated at both threonines; (ii) there are two subpopulations of large T antigen, one phosphorylated at both threonines and the other at only one; (iii) only one threonine is phosphorylated, but it could be either one (in this case, phosphorylation of the first site would prevent phosphorylation of the second); (iv) only the carboxy-terminal threonine is phosphorylated; and (v) only the internal threonine is phosphorylated.

The first two possibilities can be ruled out from the electrophoretic mobility and the homogeneity of the phosphothreonine peptide; we observed only one phosphothreonine peptide that migrated to the cathode at pH 1.9 due to a net positive charge. Two phosphate residues would compensate the two positive charges of the amino-terminal lysine residue. As a result, the peptide would be neutral at pH 1.9 and stay close to the origin during electrophoresis. Conclusions about the remaining possibilities cannot yet be made definitely. The properties of the phosphopeptides generated by partial acid hydrolysis indicate that the internal rather than the terminal threonine is phosphorylated. However, this conclusion can be drawn only for a minor fraction of the phosphothreonine peptide since during acid treatment most of the phosphate label had been released as P_i. Direct sequencing experiments are in progress to resolve this question.

The recovery of only 75% (4.5 mol) of the expected proline label in the phosphothreonine peptide (Table 1) may simply have been due to losses during the experimental procedure such as incomplete proteolytic cleavage or solubility, or it may reflect incomplete phosphorylation of the threonine site or partial dephosphorylation either in the cell or during extraction. The ratio between the phosphorylated and the presumed unphosphorylated forms of the carboxy-terminal peptide was found to be 12:1. Therefore, only about 8% of the peptide is in the unphosphorylated form: the incomplete recovery of 75% may be ascribed to losses during purification or to incomplete digestion.

We have not yet been able to analyze the phosphoserine peptides, which contain the majority of phosphate residues in large T antigen (65). They appear on peptide maps as a heterogeneous mixture, barely migrating and poorly resolved. Part of the heterogeneity may reflect different degrees of phosphorylation, as has been reported for intact large T antigen (23, 42). So far, only indirect information exists about the possible location of phosphoserines. Rundell et al. (52) demonstrated phosphorylation of a 33,000-molecular-weight fragment encoded from 0.65 to 0.59 and from 0.54 to 0.43 map units on the SV40 genome. In addition, Walter and Flory (65) have demonstrated that the SV40-specific proteins of the adenovirus type 2 (Ad2)-SV40 hybrid viruses $Ad2^+ND2$ and $Ad2^+ND1$, mapping between 0.44 and 0.17 units on the SV40 genome, are weakly phosphorylated, whereas a protein of $Ad2^+ND4$ encoded from 0.625 and 0.59 and from 0.54 to 0.17 units was strongly phosphorylated. These data suggest that the major phosphorylation sites are located between 0.625 and 0.59 or between 0.54 and 0.44 units (65).

Phosphorylation of threonine in large T antigen appears to be very specific, for only 1 of 31 threonine residues is phosphorylated. Thus, it is likely that some of the amino acids surrounding the phosphothreonine act as a recognition signal for a kinase. From studies on the specificity of protein kinases, it appears that primary and secondary structures serve as recognition determinants. The importance of two basic residues, usually including one arginine, on the amino side of the phosphorylated residue has been demonstrated for cyclic nucleotide-dependent and calcium-dependent kinases (28, 29, 62). These two basic residues are separated from the phosphorylated amino acid by one or two neutral residues. Often, the residues immediately adjacent to both sides of the phosphorylated amino acid are hydrophobic and appear to be in β-turn conformation as far as predictable from the primary structure (35, 39). The few examples of phosphothreonine sites that have been determined as well as the majority of phosphoserine sites follow these rules (11, 15, 35, 55). For the calcium-dependent protein kinases, an additional basic residue (arginine) on the carboxy-terminal side of the phosphorylated amino acid is required for recognition (62). Another type of kinase prefers acidic regions as phosphorylation sites, as reported recently for casein and for two acidic nucleolar proteins (5, 39).

The region surrounding the presumptive phosphothreonine of large T antigen contains two basic amino acid residues (lysine, lysine) on the amino side of phosphothreonine; it is rather hydrophobic and has a high probability of assuming β-turn conformation due to proline residues (9). Thus, it resembles the phosphorylation sites recognized by cyclic nucleotide-dependent kinases and may serve as a substrate for these enzymes. The extraordinarily high proline content, however, is unique for this phosphorylation site.

So far, we can only speculate on the possible function of this phosphorylation site. The finding that phosphorylation of threonine is not affected in *tsA* mutants at the restrictive temperature (65) suggests that the phosphothreo-

nine site is probably not involved in one of the known *tsA* functions, initiation of viral DNA replication, autoregulation of T antigen synthesis, and transformation. Moreover, deletion mutants lacking carboxy-terminal segments of large T antigen do not display any defects, neither during the normal growth cycle of the virus nor in transforming capacity (4, 13). However, the latter mutants are defective in the helper function (12).

The mechanism by which the helper function operates is not known, but recent observations shed some light onto this problem. The block to adenovirus late gene expression in monkey cells is probably due to incomplete maturation of several late mRNA's, especially the fiber message (32). This block can be overcome with the aid of SV40 large T antigen or carboxy-terminal fragments of large T antigen (18, 64), but does not exist for a class of adenovirus host range mutants which code for an altered DNA-binding protein (34). These findings suggested that the adenovirus DNA-binding protein plays some role in RNA processing (splicing), presumably by interacting with other cellular proteins. This interaction could take place properly in human cells, wheras in monkey cells it might be dependent on, or substituted by, SV40 large T antigen (34).

The nature of the alteration in the DNA-binding protein of the host range mutants has not yet been elucidated. In considering that this protein is also a phosphoprotein (26, 37), and assuming that the helper function of large T antigen is dependent on the phosphothreonine-containing region, it is tempting to speculate that the DNA-binding protein of the host range mutants may have acquired a new phosphorylation site similar to the phosphothreonine site in large T antigen, thereby allowing the appropriate interactions to take place.

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