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Biochemical Characterization of Phosphorylation Site Mutants of Simian Virus 40 Large T Antigen: Evidence for Interaction between Amino- and Carboxy-Terminal Domains

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The simian virus 40 large T antigen is phosphorylated at eight or more sites that are clustered in an amino-terminal region and a carboxy-terminal region of the protein. Mutants carrying exchanges at these phosphorylation sites have been generated in vitro by bisulfite or oligonucleotide-directed mutagenesis and analyzed for their phosphorylation patterns. Two-dimensional phosphopeptide analyses of the mutant large T antigens confirmed most of the previously identified phosphorylation sites, namely, serine residues 106, 112, 123, 639, 677, and 679 and threonine residues 124 and 701. In addition, serine residue 120 was identified as a new site, whereas serines residues 111 and 676 were excluded. Interestingly, several of the mutants exhibited secondary effects in that a mutation in the amino-terminal region affected phosphorylation at distant and even carboxy-terminal sites and vice versa. Thus, the amino- and carboxy-terminal domains appear to be in close proximity in the three-dimensional structure of large T antigen. The possible consequences of the above findings and the role of phosphorylation are discussed.

The simian virus 40 (SV40) large T antigen (LT) is a phosphoprotein that displays various functions during lytic infection and transformation (for reviews, see references 3, 8, and 37). It is involved in initiation and elongation of viral DNA replication (50; reviewed in references 2 and 5) and regulation of transcription of the viral genome. The functions in replication are in part mediated by specific binding of LT to control sequences at the origin of replication (9, 53, 55) and in part through its intrinsic ATPase (56) and helicase (6, 49) activities and the interaction with the cellular DNA polymerase *a* (48). Regulatory functions during transcription also include DNA binding (9) and presumably interaction with or modulation of cellular transcription factors (16, 31). LT affects cellular DNA and RNA synthesis as well and is capable of inducing malignant transformation (3). Transformation seems to require specific interaction with at least two cellular proteins, p53 and the Rb protein (7, 27; for reviews, see references 20 and 38).

Some of these functions seem to reside in distinct domains of the LT molecule (3, 11, 33–35). In addition, LT exists in various forms that differ in their subcellular distribution (3, 51), their degree of oligomerization (12, 21), and their DNA-binding properties (13, 17, 41). Some of these subclasses appear to result from posttranslational modifications, mainly phosphorylation (12, 21, 41, 54, 60). Additional modifications include ADP ribosylation (18), glycosylation (44), and fatty acid acylation (25).

Phosphorylation of LT occurs at eight or more sites that are clustered between residues Ser-106 and Thr-124 in the amino-terminal region and between residues Ser-639 and Thr-701 in the carboxy-terminal region (26, 40, 57). Phosphorylation takes place in a sequential manner in the cytoplasm and in the nucleus (43). Moreover, phosphorylation of LT is reversible (10), and the individual phosphorylation sites exhibit different turnover rates (39, 58). Recent studies suggest that phosphorylation of LT modulates its activities in DNA binding (23, 24, 30, 32, 42, 47) and replication (19, 30, 32) and, perhaps, the formation of oligomers (12, 21, 41) and association with p53 (4). In particular, the phosphorylation state of some of the amino-terminal sites (Ser-123 and Thr-124, which are close to the origin binding domain of LT [1, 34, 46, 52]) strongly correlates with its DNA-binding activity (24, 41). A role for the carboxy-terminal phosphorylation sites is less evident. Viable deletion mutants lacking some of those sites exhibit reduced DNA replication (35), suggesting that phosphorylation of these sites, although not essential, might exert some modulatory effect, too.

To acquire more insight into the functional roles of individual phosphorylation sites, mutants were created in vitro by using bisulfite (22) or oligonucleotide-directed mutagenesis (45). In this investigation we analyzed the phosphorylation patterns of these mutant LTs, first, to confirm previous mapping data and, second, to analyze possible secondary effects. Surprisingly, several mutants exhibited such effects in that a mutation at an amino-terminal site affected phosphorylation at carboxy-terminal sites and vice versa. This phenomenon suggests that the amino- and carboxy-terminal domains are in close proximity in the three-dimensional structure of LT.

MATERIALS AND METHODS

Cell lines and virus. The transformed rat cell lines C8, K5, K6, W7, and D29 (derivatives of rat-1 cells [22]) and R106, R111/112, R123, R124, R676, R677, R679, and R701 (derivatives of rat-2 cells [45]) that had been transformed by the

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various LT mutants via Ca-phosphate transfection were used as the source of the mutant LTs. For infections with wild-type SV40 (strain 776 or VA 45-54), rat F111 or monkey TC7 cells were used. Cells were grown on 9-cm plastic dishes in Dulbecco modified Eagle medium supplemented with 5% or, in some cases, 10% fetal calf serum (Boehringer, Mannheim, Germany).

Labeling of cells. Metabolic labeling of cells was carried out in deficient medium supplemented with dialyzed fetal calf serum. $^{32}P_i$ was applied at 1 mCi per plate, and [^{14}C] glutamine was applied at 125 μCi per plate, in 1.5 ml of medium for 4 h; radiochemicals were purchased from Amersham.

Isolation of LT. Extracts from radiolabeled cells were prepared essentially as described previously (40, 43); briefly, cells were washed with cold phosphate-buffered saline and lysed in isotonic lysis buffer (10 mM $NaPO_4$ [pH 8], 140 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% Nonidet P-40, 50 μM leupeptin [Sigma]); cellular residues were scraped off the plate, nuclei were removed by low-speed centrifugation, and the supernatant extract containing soluble cytoplasmic and nucleoplasmic proteins was collected. Isolation of LT by immunoprecipitation with hamster antitumor serum and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (40, 42).

Two-dimensional phosphopeptide analysis. Phosphopeptide analysis was carried out as described previously (40). Briefly, after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, LT was extracted from the gel, precipitated with 20% trichloroacetic acid, oxidized with 3% performic acid, and digested either with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington) alone or sequentially with trypsin and pronase E (Merck, Darmstadt, Germany). The digestion mixtures were separated on cellulose thin-layer plates by electrophoresis and chromatography. Electrophoresis was carried out at pH 1.9 (6% [vol/vol] formic acid, 1.25% [vol/vol] acetic acid, 0.25% [vol/vol] pyridine) at 1,000 to 1,300 V for 20 to 25 min. Ascending chromatography was performed in isobutyric acid buffer (isobutyric acid-pyridine-acetic acid-butanol-water [65:5:3:2:29]) for 5 h. The phosphopeptides were visualized by autoradiography with intensifying screens.

Phosphoamino acid analysis. Individual phosphopeptides that had been separated as described above were localized by autoradiography, eluted from the cellulose plates, and hydrolyzed in 5.6 N hydrochloric acid at 105°C for 2 h in sealed glass vials. The hydrolysis products were analyzed by electrophoresis on cellulose thin-layer plates and autoradiography as described previously (40).

RESULTS

Phosphorylation patterns of mutants in the amino-terminal domain of SV40 LT. Figure 1 shows an assignment of the phosphorylation sites of wild-type (wt) SV40 LT to individual phosphopeptides as previously determined (40–42) with minor modifications, as stated in the legend to Fig. 1. These phosphorylation sites have been mutated to get insight into their biological and biochemical functions (22, 45). The mutants and their phenotypes are listed in Table 1. Since several of the mutants were replication defective, rat cell lines that had been individually transformed with either one of the mutants were used as a source for LT.

To rule out the possibility that the kinases that phosphorylate LT have different specificities or activities in permissive monkey and nonpermissive rodent cells, we first compared the phosphopeptides of wt LT from infected monkey TC7 and rat F111 cells. Cells were labeled with $^{32}P_i$, and LT was isolated and subjected to two-dimensional peptide mapping as outlined in Materials and Methods. The phosphorylation patterns of LTs from the two sources were almost identical (Fig. 1). Similar results were obtained with other rat cell lines (data not shown). However, it should be noticed that there are always minor variations, presumably due to incomplete or alternate proteolysis.

The patterns obtained with the mutants in the amino-terminal region of LT (Ser-106 to Thr-124) are shown in Fig. 2 and 3 in sequential order. The LT of mutant C8 (Ser-106→Phe) (Fig. 2) lacked phosphopeptide 1, the position of which is indicated by an arrow, which in wt LT contains the phosphorylated Ser-106 (40). All other phosphopeptides identified in wt LT were present. However, there were additional quantitative differences from wt LT. The ratios between peptides 7, 11, and 12 were drastically altered, and peptide 2 was relatively weakly labeled compared with the wt pattern (Fig. 1). As pointed out previously, peptides 7, 11, and 12 reflect different phosphorylation states of the same peptide; peptide 12 is phosphorylated at only one site (Thr-124), and peptides 11 and 7 are phosphorylated at two and three sites (Thr-124 and one and two of the neighboring serine residues), respectively (41; see below). Thus, the lower abundance of peptides 7 and 11 relative to peptide 12 indicated inefficient phosphorylation of the respective serine residues in the C8 mutant. Moreover, the low intensity of peptide 2, which is phosphorylated at Ser-639, suggested that the mutation exerted some effect on phosphorylation in the carboxy-terminal domain. On the other hand, when mutant SV106, in which Ser-106 was replaced by Ala, was analyzed the same way, no such secondary effects were observed (Fig. 2), suggesting that not the lack of phosphorylation at Ser-106 but rather the introduction of the bulky Phe residue was responsible for the effects seen with C8.

In our previous studies, it remained unclear whether Ser-111 or Ser-112 or both were phosphorylated. The mutants K5 and K6 carrying mutations at the respective residues served to clarify this question. Mutant K5 (Ser-111→Phe) (Fig. 2) lacked peptide 6, as expected, since this peptide contains Ser-111 and probably Ser-112 in wt LT (40). Instead, a prominent peptide designated 6a appeared on the left of peptide 7. We assume that this peptide represents an altered form of peptide 6 and that its different mobility during electrophoresis and chromatography results from the mutation of Ser-111 to a hydrophobic residue. Consequently, peptide 6a (and its counterpart in wt LT) must be phosphorylated at Ser-112. It should be pointed out that in this and all other cases in which peptides migrated off their normal position on the thin-layer plates, mixed peptide analyses of wt and mutant LTs were performed to exclude any abnormalities during the experimental procedure (data not shown). Mutant K5 also exhibited a slight overrepresentation of peptide 12, whereas peptide 2 was relatively weakly labeled and peptide 1 was not detectible. The other phosphopeptides appeared with the usual intensities.

When the mutant K6 (Ser-112→Asn) was analyzed (Fig. 2), peptide 6 was also missing, but there was no new peptide instead and the overall pattern was maintained. Similarly, LT carrying a double mutation of Ser-111 and Ser-112 to Ala and Cys, respectively, exhibited a rather normal phosphorylation pattern except for the lack of peptide 6 and a slight overrepresentation of peptide 12 (data not shown). These results strongly suggest that in wt LT, Ser-112 but not Ser-111 is phosphorylated. However, other interpretations

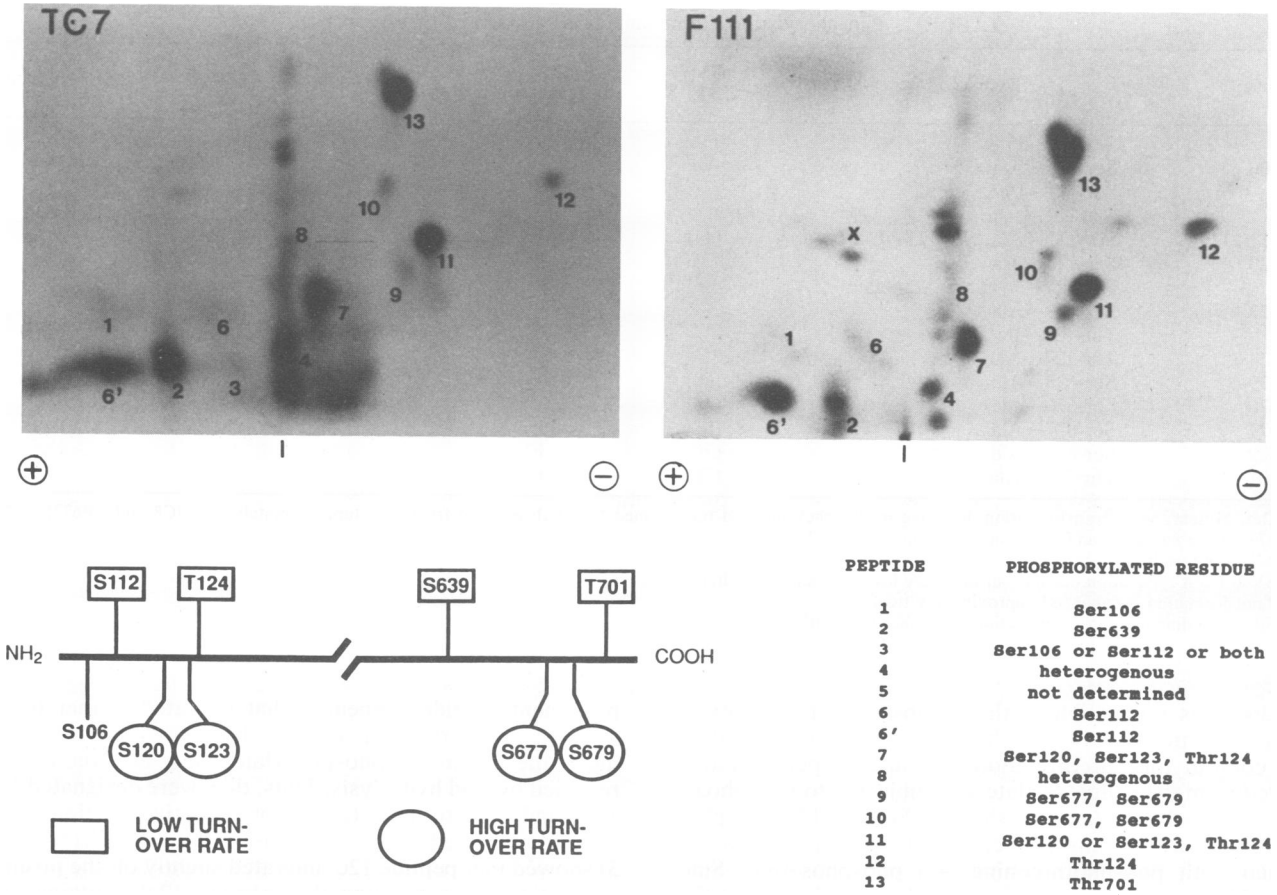

FIG. 1. Comparison of phosphopeptides of SV40 LT from monkey and rat cells and assignment of phosphorylation sites. Monkey TC7 or rat F111 cells were infected with wt SV40. Two days postinfection cells were labeled with $^{32}P_i$ for 3 h, and LT was isolated by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and processed for two-dimensional peptide mapping as described previously (41). Electrophoresis was done in the horizontal direction, and chromatography was in the vertical direction (41); the vertical dash indicates the origin. The phosphopeptides were visualized by autoradiography with intensifying screens. The nomenclature refers to that in reference 41. However, it should be noticed that peptide 1, which is sometimes barely visible and which had been erroneously assigned to peptide 6' in previous publications (39, 41, 43), has now been reassigned. Peptides 6 and 6' obviously represent two forms of the same or overlapping peptides with varying relative abundance. Peptide "x" on the phosphopeptide map from F111 cells appeared to be characteristic of rat cells because it was never observed in monkey cells. Its nature has not been investigated. The scheme at the bottom shows the location of the phosphorylation sites within the linear arrangement of the LT polypeptide chain. The relative metabolic stabilities of individual phosphate residues (39) are also indicated. The list on the right shows which phosphorylation sites are represented by the individual phosphopeptides (40, 41; this study).

are also possible, e.g., it cannot be ruled out that in wt LT, both Ser-111 and Ser-112 are phosphorylated and that the mutation at Ser-112 prevents phosphorylation at both residues, whereas the mutation at Ser-111 still allows phosphorylation of Ser-112 (see Discussion).

Identification of Ser-120 as a new phosphorylation site. Analysis of the following set of mutants carrying mutations at Ser-120, Ser-123, and Thr-124 revealed some unexpected results that were only partly compatible with previous mapping data. Ser-123 and Thr-124 had been identified as phosphorylated residues in peptides 7, 11, and 12, whereas Ser-120 had been excluded (40, 41). This conclusion was derived from the amino acid composition of these peptides, as determined by differential amino acid labeling. The amino acid sequence in this region is Thr-117-Ala-Asp-Ser-120-Gln-His-Ser-123-Thr-124-Pro-Pro-Lys-127 (14, 36). We found proline and lysine in peptide 7 and proline, lysine, and histidine in peptide 11 (40). The relationship of peptide 12 to peptides 7 and 11 was revealed in subsequent studies (41). Since glutamine had not been detected, Ser-120 was thought to be absent from these peptides. Their different charges and polarities had been ascribed in part to differential phosphorylation and in part to the absence of histidine in peptide 7 (the absence of a positively charged histidine would have an effect on the electrophoretic mobility similar to that of the presence of an additional phosphate residue). Based on this interpretation, one can predict the phosphorylation patterns of the mutants in this region.

The double mutant W7 with exchanges of His-122 and Ser-123 to Tyr and Phe, respectively should reveal the absence of peptides 7, 11, and 12 and the appearance of only one new peptide, since there should be only one phosphorylatable residue (Thr-124) and the replacement of His would extinguish possible effects of this residue. Indeed, mutant W7 revealed the absence of peptides 7, 11, and 12 (Fig. 3). However, two new peptides appeared instead. Assuming that these represented altered forms of peptides 11 and 12, they were designated 11a and 12a, respectively. Their mi-

TABLE 1. Phosphorylation site mutants of SV40 LT and their properties

Mutant	Exchange	Viability (%)	DNA replication	ori binding		Transformation[a]
				Site I	Site II	
C8	Ser-106→Phe	−	−	+	+	Def
SV106	Ser-106→Ala	+	+	+	+	+
K5	Ser-111→Phe	+	+	NT[b]	NT	+
K6	Ser-112→Asn	+	+	NT	NT	+
SV111/112	Ser-111→Ala, Ser-112→Cys	+ (44)	+	+	+	+
SV120	Ser-120→Ala	−	+[c]	+	+	+
W7	His-122→Tyr, Ser-123→Phe	−	−	+	+	+
SV123	Ser-123→Ala	−	+[c]	+	+	+
D29	Thr-124→Ile	−	−	+	+	+
SV124	Thr-124→Ala	−	−	+	−	+
SV639	Ser-639→Ala	+ (59)	+	+	+	++[d]
SV676	Ser-676→Ala	+ (48)	+	+	+	++[d]
SV677	Ser-677→Ala	+ (67)	+	−	+	Def
SV679	Ser-679→Ala	+ (70)	+[e]	+	+[e]	Def
SV701	Thr-701→Ala	+ (78)	+	+	+	+

[a] Def, Mutants were transformation defective in that they induced transformed foci with reduced frequency (approximately 10% [C8 and SV677] or 36% [SV679] of the wt value) and after longer latency periods (22, 45).
[b] NT, Not tested.
[c] SV40 DNA replication occurs, but at a very low level and with altered kinetics (58a).
[d] Transformation is enhanced approximately twofold.
[e] Site II binding and DNA replication are higher than with wt LT.

gration was compatible with the absence of a positively charged histidine and the exchange to hydrophobic residues. To confirm this interpretation, the altered peptides were eluted from the cellulose plate and subjected to phosphoamino acid analysis (data not shown). Peptide 12a was phosphorylated at threonine, as expected, but peptide 11a contained both phosphothreonine and phosphoserine. Since Ser-123 was mutated, we assumed that in this mutant Ser-120 might be phosphorylated instead. This unexpected result could be a consequence of the mutation, resulting in both altered phosphorylation and altered proteolytic cleavage. In addition to these effects, the W7 mutant LT exhibited reduced phosphorylation of all sites, including Thr-701 (in peptide 13). This latter effect was probably due to partial degradation of LT before or during extraction, whereby the carboxy terminus is specifically removed (unpublished observation), indicating that W7 LT was more sensitive than wt LT to proteases.

We next analyzed mutant SV123, which bears a more subtle exchange of Ala for Ser-123. The overall phosphorylation pattern was roughly maintained in SV123, except that peptide 7 was missing and two prominent peptides appeared that migrated slightly off the positions of peptides 11 and 12, as determined in mixed peptide analyses (Fig. 3). They were designated 11b and 12b, to distinguish them from the corresponding peptides of W7. Phosphoamino acid analysis of peptide 12b revealed only phosphothreonine, as expected. But again, peptide 11b contained both phosphoserine and threonine, suggesting that in this mutant, too, Ser-120 was phosphorylated. Peptide 11b migrated sometimes as a doublet; the difference between the two variants remains unclear, however. Additional quantitative changes included overrepresentation of peptide 6 and reduced labeling of peptides 9 and 10, indicating effects on distant sites.

To further clarify the above findings, mutant SV120, carrying an exchange of Ala for Ser-120, was investigated. Based on our previous interpretations, this mutation should not have any effects on the overall phosphorylation pattern, unless it caused conformational alterations. However, phosphopeptides 7, 11, and 12 were absent (Fig. 3). Instead, three prominent peptides appeared that migrated farther to the cathode and farther up during chromatography. Surprisingly, these were all phosphorylated only at threonine, as revealed by acid hydrolysis. Thus, they were designated 12c, 12c′, and 12c″, to indicate their presumptive relationship to peptide 12. Mixed peptide analysis of SV120 and wt LT (Fig. 3) showed that peptide 12c′ migrated slightly off the position of peptide 12, suggesting that peptide 12c′ contained the mutated residue 120. Thus, we concluded that peptide 12 in wt LT does also contain residue 120. Peptides 12c and 12c″ were probably generated by alternative proteolytic cleavage, a phenomenon that was reproducibly observed in cases where peptide 12 was overrepresented, in other words, where Ser-123, Ser-120, or both were underphosphorylated (for comparison, see the peptide maps of mutants C8 [Fig. 2] and SV677 [see Fig. 7]).

It was surprising that there was no peptide corresponding to peptide 11, which was phosphorylated on Thr-124 and Ser-123. This finding can be explained in two ways. Either Ser-120 and not Ser-123 is the phosphorylated residue in peptides 7 and 11 or both Ser-120 and Ser-123 can be differentially phosphorylated (one of them in peptide 11 and both in peptide 7). In the latter case, the mutation at Ser-120 would prevent phosphorylation at Ser-123, whereas the mutation at Ser-123 would not affect phosphorylation at Ser-120, as revealed from mutants W7 and SV123. In both cases, however, peptides 7, 11, and 12 should contain glutamine, which we did not detect in previous analyses (40).

The apparent discrepancies with previous results and conclusions led us to reinvestigate the composition and phosphorylation state of peptides 7, 11, and 12. Labeling of wt LT with [^{14}C]glutamine and mixed peptide analyses with ^{32}P-labeled LT (40) revealed a weak Gln signal in peptides 11 and 12 (Fig. 4), whereas in peptide 7 it was hardly detectable, probably due to the lower abundance of the latter peptide.

To determine more precisely the relative amounts of phosphoserine and phosphothreonine in these peptides, they were isolated and subjected to phosphoamino acid analysis (Fig. 5). Peptide 12 revealed only phosphothreonine, and peptides 11 and 7 gave rise to phosphothreonine and phos-

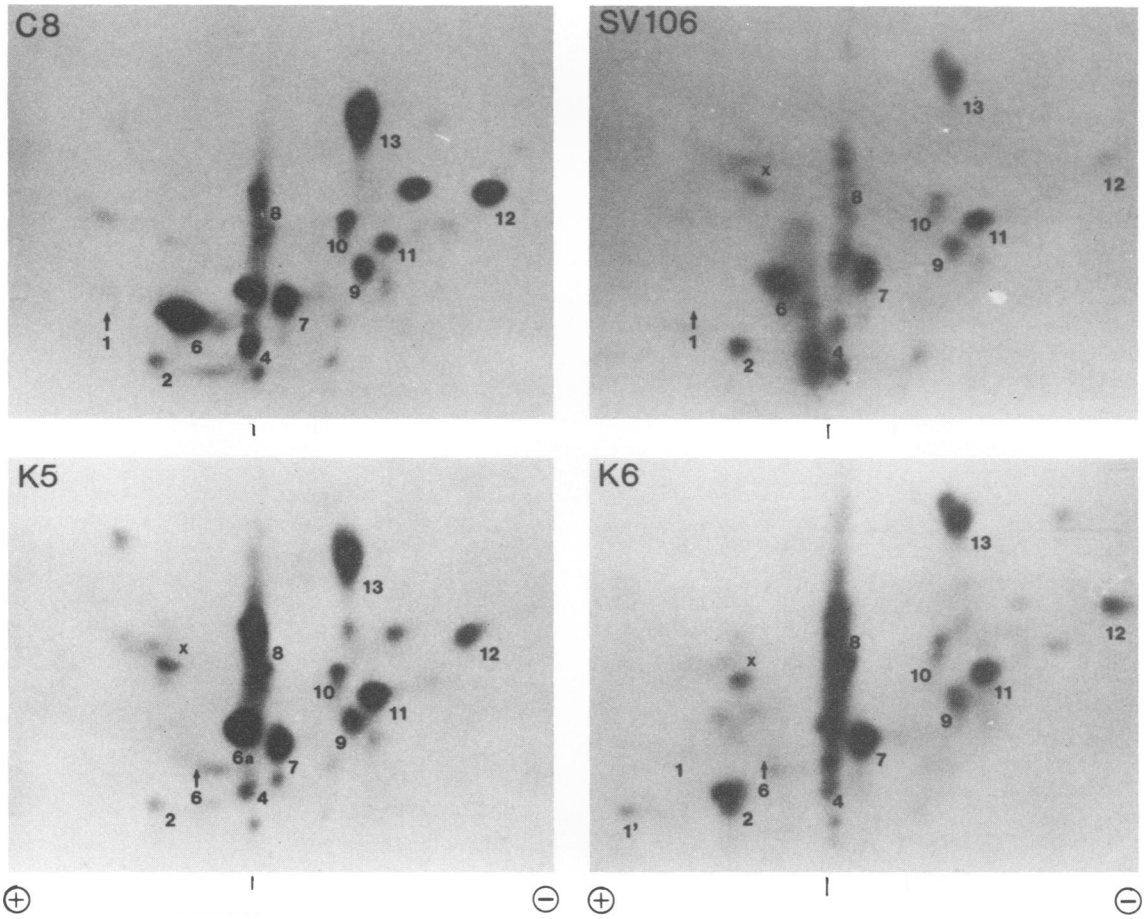

FIG. 2. Phosphopeptide analysis of LT mutants with mutations at Ser-106, Ser-111, and Ser-112. Rat cells transformed with mutants C8, SV106, K5, and K6 (Table 1) were labeled with ^{32}P, and the mutant LTs were isolated and analyzed as described in the legend to Fig. 1 for wt LT. The arrows point to the positions of phosphopeptides that are missing and that represent the mutated phosphorylation sites.

phoserine at ratios of 1:3 and 1:6, respectively. Thus peptide 7 contained twice the amount of phosphoserine label as peptide 11. The discrepancy with the expected ratios of 1:1 and 1:2 resulted probably in part from incomplete hydrolysis of the threonine-proline bonds (see sequence of the peptides above), since there was always some uncleaved material remaining at the origin during electrophoresis. Consequently, phosphothreonine would be underrepresented. In addition, the threonine phosphate has a lower turnover than the serine phosphates (39), resulting in different amounts of ^{32}P incorporation during a 4-h labeling period.

In an additional analysis, the isolated peptides 7, 11, and 12 were subjected to partial acid hydrolysis in 6 N hydrochloric acid at 37°C for 20 h as described previously (40), and the products were analysed by peptide mapping. Each peptide gave rise to an individual pattern (Fig. 6). Peptides 7 and 11 gave rise to six products, designated p through u, four of which were identical (Fig. 6a and b, respectively). Peptide 12 (Fig. 6c) revealed only one phosphorylated product. The precise relationship to the other peptide fragments deriving from peptides 7 and 11 could not be determined, but we assume that the cleavage product of peptide 12 is identical to peptide r or s. Taken together, these results strongly suggested that in wt LT both Ser-123 and Ser-120 are phosphorylated, in a differential manner.

The analyses of mutants with exchanges at Thr-124 revealed the expected results with respect to peptides 7, 11, and 12. Mutant D29 (Thr-124→Ile) revealed the absence of peptides 7, 11, and 12 (Fig. 3). Instead, two new peptides, designated 11d and 12d, appeared. These were phosphorylated exclusively at serine residues, suggesting that they represented the altered forms of peptides 11 and 12, being phosphorylated either at both Ser-120 and Ser-123 or only at Ser-120 or Ser-123, respectively. Additional alterations included relatively low intensities of peptides 2, 9, 10, and 13, deriving from the carboxy-terminal region and overrepresentation of peptide 6. Thus, this mutation seemed to have a rather strong effect on phosphorylation of the carboxy-terminal sites. Mutant SV124 (Thr-124→Ala) showed a rather normal peptide pattern (Fig. 3, panel SV124), except that peptide 1 was overrepresented and peptide 7 was missing. Peptides 11e and 12e, which migrated close to the positions of the authentic peptides 11 and 12, contained only phosphoserine, as expected. These latter results confirmed that in wt LT, Thr-124 is phosphorylated in these peptides.

Mutants in the carboxy-terminal domain. Five mutants in the carboxy-terminal domain carrying exchanges at Ser-639, Ser-676, Ser-677, Ser-679, and Thr-701 into Ala (45) were analyzed for alterations in their phosphorylation patterns as above. Mutant SV639 showed a normal phosphopeptide pattern, except that peptide 2, which in wt LT carries the phosphorylated Ser-639, was missing (data not shown).

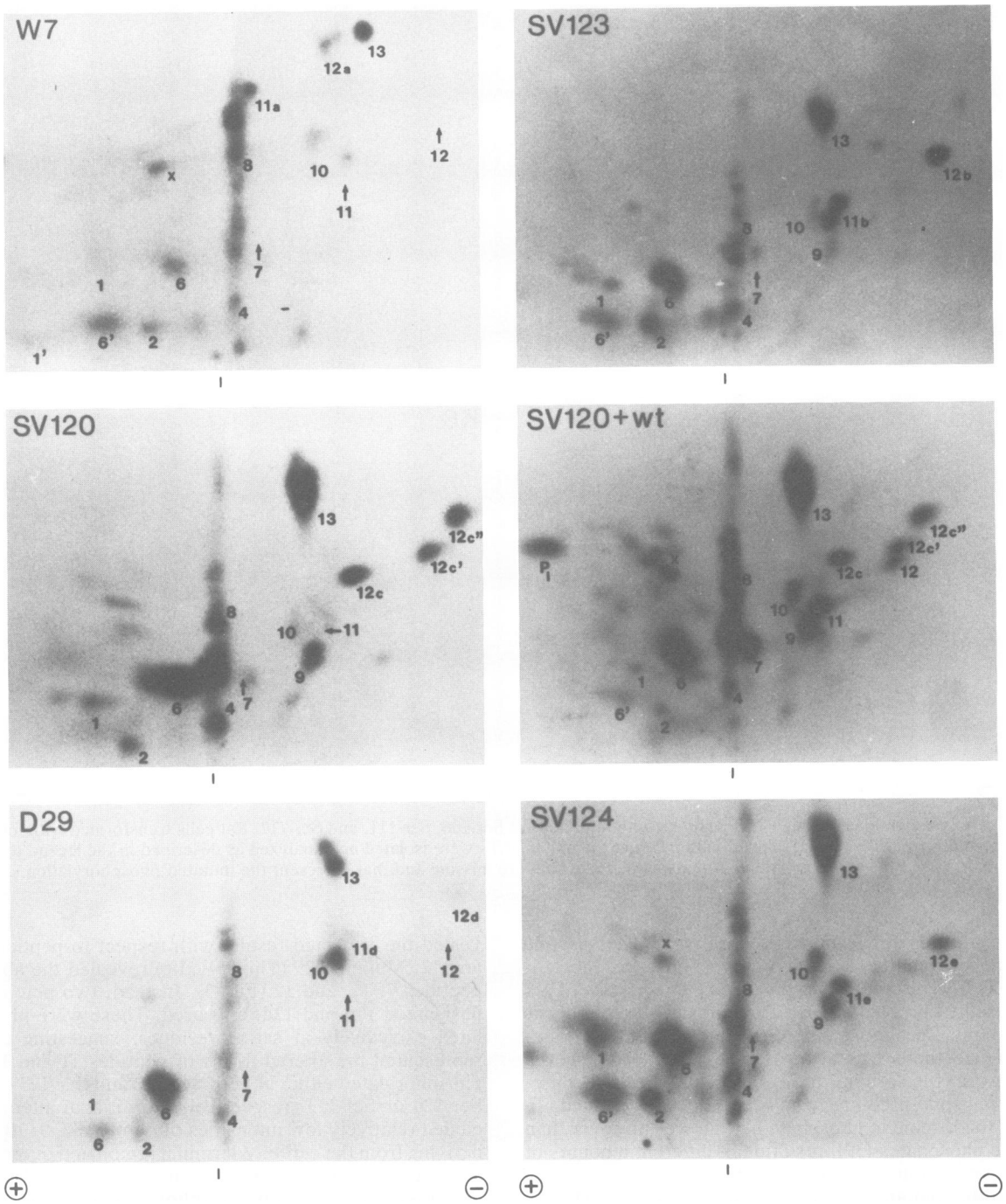

FIG. 3. Phosphorylation patterns of LT mutants carrying mutations at Ser-123, Ser-120, and Thr-124. ^{32}P-labeled LT was isolated from cells transformed with mutants W7, SV123, SV120, D29, and SV124 (Table 1) and analyzed as described in the legend to Fig. 1. SV120+wt represents a mixture of phosphopeptides derived from SV120 and wt LT, to demonstrate the relative locations of peptides 12c.

Mutations at residues Ser-676, Ser-677, and Ser-679 served to clarify which of these serines are phosphorylated. Mutant SV676 exhibited a normal peptide pattern (data not shown), indicating that in wt LT Ser-676 is not phosphorylated. In contrast, mutant SV677 revealed the lack of pronase peptides 8, 9, and 10 and peptides designated "x," which are probably related to the former (Fig. 7). Thus we conclude that Ser-677 is phosphorylated in wt LT. Surprisingly, the mutation at Ser-677 led also to drastic changes in the amino-terminal domain; phosphopeptides 7 and 11 were hardly detectable, but peptides 12 and 6 were overrepresented. Thus, this mutant represented a counterpart of some of the amino-terminal mutants, which affected phosphorylation in the carboxy-terminal domain. Interestingly, the pronase peptides 12c and 12c″ observed on the peptide map of SV120 (Fig. 3) were also present in relatively high amounts,

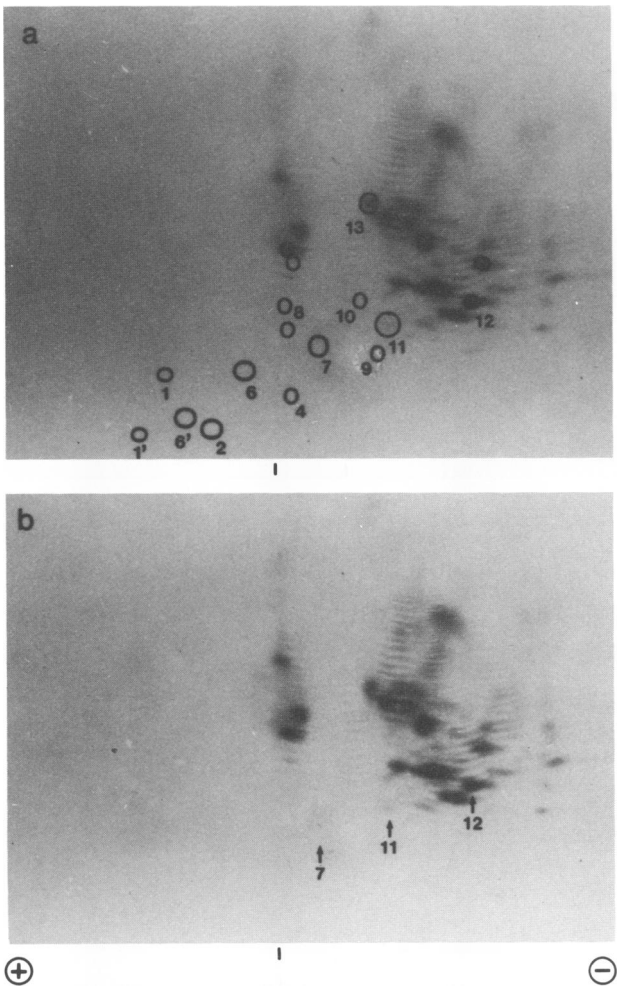

FIG. 4. Analysis of phosphopeptides of wt LT for the presence of glutamine. SV40-infected TC7 cells were labeled with [^{14}C]glutamine, and LT was isolated and processed for peptide mapping as described for the phosphopeptides in the legend to Fig. 1. Samples were run on TL cellulose plates with and without ^{32}P-labeled peptides to localize comigrating peptides. The peptide maps were exposed to X-ray film before and after decay of the ^{32}P label. In panel a, the locations of the phosphopeptides are indicated by circles. In panel b, the positions of phosphopeptides 7, 11, and 12 are indicated by arrows.

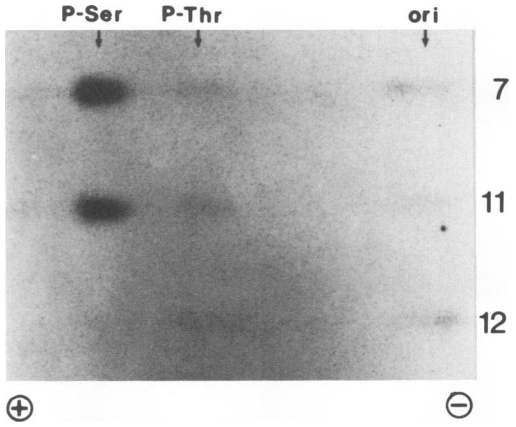

FIG. 5. Phosphoamino acid analysis of peptides 7, 11, and 12. Phosphopeptides were separated on TL cellulose plates, localized by autoradiography, eluted, and hydrolyzed as described in the text. Phosphoamino acids were separated on TL cellulose plates by electrophoresis at 1,000 V for 60 min in the presence of unlabeled phosphoserine and phosphothreonine.

supporting the above-mentioned assumption that lack of phosphorylation at Ser-120 or Ser-123 leads to altered cleavage by pronase.

LT encoded by SV679 revealed a rather normal pattern of pronase peptides, except that peptides in the center of the map seemed to be lacking, as indicated by arrows, and peptide 1 was hardly detectible (Fig. 7). Thus, it cannot be concluded with certainty that Ser-679 is phosphorylated in wt LT. However, there were additional changes in that this mutant, too, exhibited a slight overrepresentation of peptides 11 and 12, although not to the same extent as that in mutant SV677.

In mutant SV701, carrying a Thr-701→Ala exchange, phosphopeptide 13 was missing, as expected (Fig. 7). Again, peptide 12 was overrepresented, indicating an effect on amino-terminal sites too. In addition, peptides 1 and 2 were only weakly labeled.

Taken together, these analyses confirmed most of the phosphorylation sites that had been determined previously: serine residues 106, 112, 123, 639, 677, and perhaps 679, and threonine residues 124 and 701. Ser-120 was identified as a new site. On the other hand, Ser-111 and Ser-676 were excluded. Based on the overall effects of a given mutation, the mutants fell into two classes: those affecting only the mutated site and those showing secondary effects on phosphorylation of distant sites. This latter observation indicates that the two phosphorylation domains, which are more than 500 residues apart in the primary sequence, interact with each other, perhaps due to their close proximity in the three-dimensional structure of the LT molecule.

DISCUSSION

Reevaluation of phosphorylation sites of LT. The phosphorylation sites of SV40 LT, as previously identified (26, 40, 57), had been mutated to investigate their biological role (22, 45). In the present investigation, we analyzed the phosphorylation patterns of these mutant LTs; first, to confirm previous assignments of the phosphorylation sites and, second, to investigate possible secondary effects of the mutations, i.e., structural alterations, that might be reflected by altered phosphorylation patterns. Indeed, the phosphopeptide analyses revealed two categories of mutants. Mutants of the first category lacked only phosphopeptides in which the phosphorylation site was mutated. Mutants of the second category showed additional alterations in that phosphorylation at distant sites was affected.

The phosphopeptide pattern obtained from mutants C8, SV106, SV111/112, D29, SV124, SV639, and SV701 were compatible with previous mapping data in that they lacked the peptides to which we had assigned Ser-106, Ser-111 or Ser-112, Thr-124, Ser-639, and Thr-701, thus confirming these residues as phosphorylation sites in wt LT (40, 42). In the cases of Serine residues 111 and 112 in the amino-terminal region and serine residues 676, 677, and 679 in the carboxy-terminal region, it remained unclear which and how many of these residues were phosphorylated. Mutants K5, K6, SV676, SV677, and SV679 served to clarify this question. Mutants K5 and K6, carrying mutations at Ser-111 and

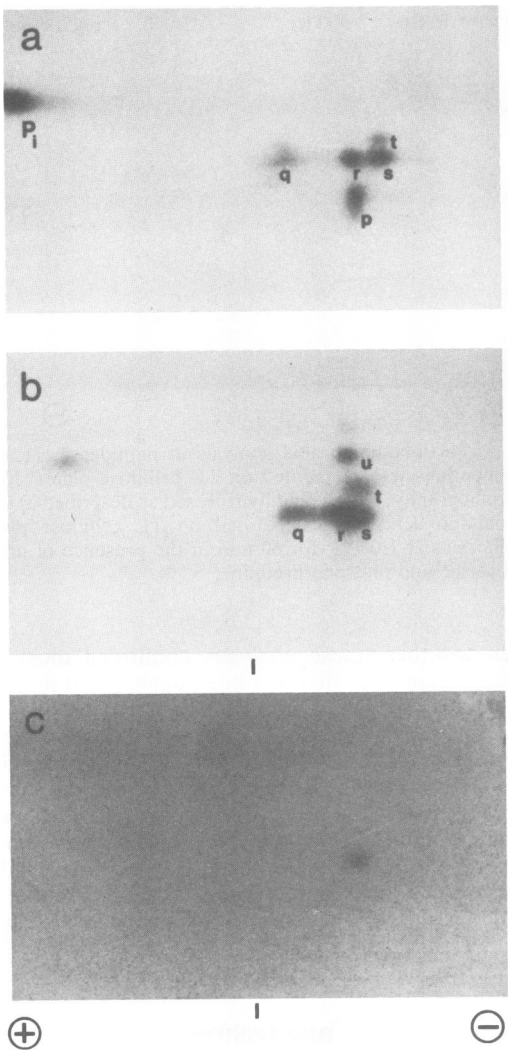

FIG. 6. Analysis of phosphopeptides 7, 11, and 12 by partial acid hydrolysis. Phosphopeptides were isolated as described in the legend to Fig. 5 and subjected to partial acid hydrolysis in 6 N HCl at 37°C for 20 h (41). The fragments obtained were analyzed by two-dimensional peptide mapping as described before for the trypsin-pronase peptides: a, cleavage products of peptide 7; b, products of peptide 11; c, product of peptide 12. The peptide fragment in panel c was not marked because its precise relationship to the fragments in panels a and b could not be determined.

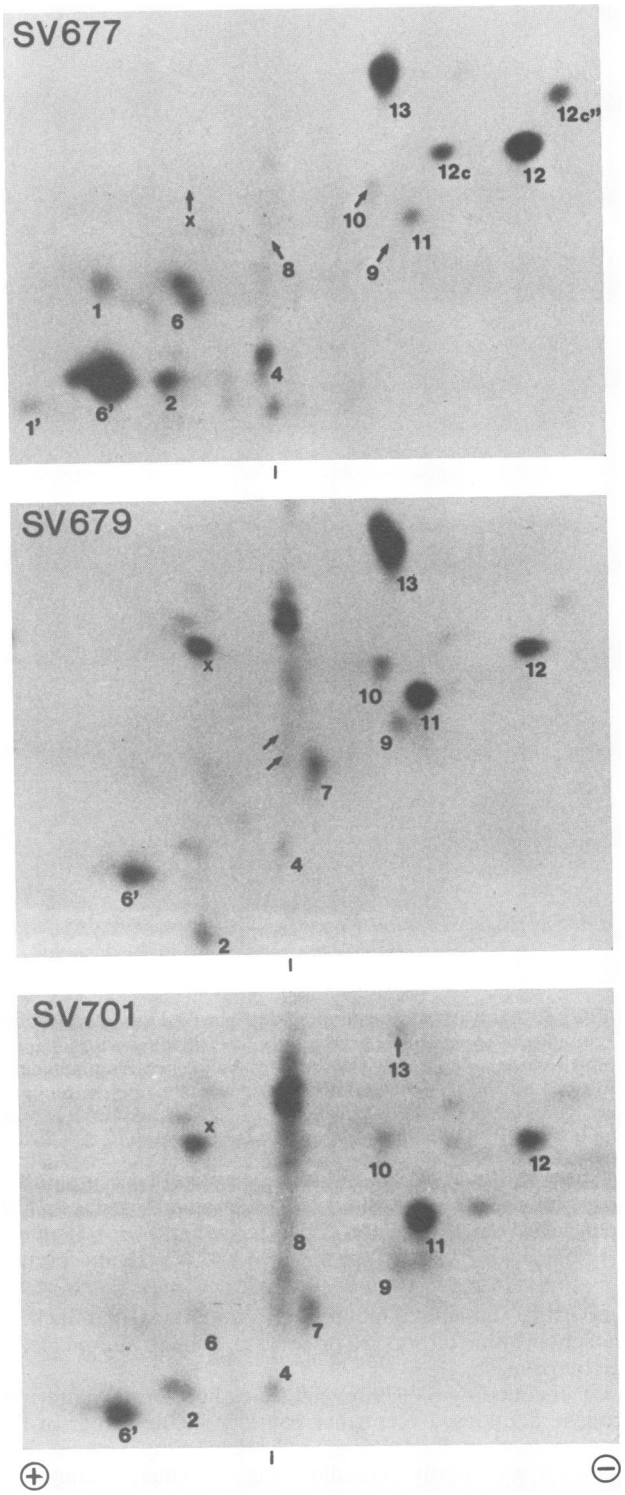

FIG. 7. Phosphopeptide analysis of LT mutants in the carboxy-terminal region. Mutant SV677, SV679, and SV701 LTs were isolated from the respective transformed cell lines and subjected to peptide mapping as described in the legend to Fig. 1.

Ser-112, respectively, both lacked phosphopeptide 6, to which we had assigned these residues (40). However, K5 gave rise to a new phosphopeptide that must be phosphorylated at Ser-112, whereas K6 did not. Thus, the simplest interpretation of the data is that in wt LT only Ser-112 but not Ser-111 is phosphorylated. The physical properties of the altered peptide 6 (6a in Fig. 2) are compatible with the exchange of the polar serine to the hydrophobic phenylalanine residue. However, we cannot completely rule out the more complex interpretation that in wt LT, Ser-111 or even both Ser-111 and Ser-112 are phosphorylated and that the mutation at Ser-111 allows or leads to alternative phosphorylation of Ser-112, whereas the mutation of Ser-112 prevents phosphorylation at residue 111.

The serine residues 676, 677, and 679 have been assigned to two overlapping tryptic peptides (5 and 5′ [40]). Cleavage by pronase of the tryptic peptide 5′ gave rise to pronase peptides 9 and 10, whereas cleavage of the more negatively charged tryptic peptide 5 revealed a more complex pattern including pronase peptides 2, 8, 9, and 10 (40). The hetero-

geneity of these peptides and their interrelationships strongly suggested that at least two of the above residues are phosphorylated. The phosphopeptide pattern of mutant SV676 was indistinguishable from that of wt LT, indicating that Ser-676 is not phosphorylated. Mutant SV677 clearly lacked peptides 8, 9, and 10 (and the tryptic peptides 5 and 5a; data not shown), thus strongly suggesting that in wt LT Ser-677 is, indeed, phosphorylated. The results with mutant SV679 were less clear cut, since only peptides designated "8" were missing or reduced, whereas all other peptides appeared to be present. Our previous analyses suggested that the phosphorylated residue in peptide 8 differs from that in peptides 9 and 10 (40). Since the latter two peptides seem to be phosphorylated only at Ser-677 (see above), Ser-679 appears to be the phosphorylated residue in peptide 8. The lack of peptide 8 in mutant SV677 may be actually a secondary effect in that this mutation prevents phosphorylation at Ser-679. Thus, we assume but have no clearcut evidence that both Ser-677 and Ser-679 are phosphorylated in wt LT. Interestingly, mutants SV677 and SV679 have similar phenotypes with respect to viability and transforming capacity but are quite different in DNA binding and replication. The LT of SV677 does not bind site I but exhibits normal site II binding and replication. On the other hand, mutant SV679 is even more active in site II binding and DNA replication than wt LT (45) (Table 1). These properties suggest that both Ser-677 and Ser-679 play a regulatory role and are subject to reversible phosphorylation.

Identification of Ser-120 as an additional phosphorylation site. In previous studies, we had determined that Ser-123 and Thr-124 are differentially phosphorylated in pronase peptides 7, 11, and 12 (40, 41), whereas Ser-120 had been excluded, as described in Results. However, Ser-123 mutants retained a phosphorylated serine in the respective peptides, whereas the Ser-120 mutant did not. One possible interpretation of these results is that the mutation at Ser-123 leads to alternative phosphorylation of Ser-120, whereas the mutation at Ser-120 would prevent phosphorylation of Ser-123. An alternative possibility is that in wt LT Ser-120 but not Ser-123 is phosphorylated. The detection of glutamine in peptides 7, 11, and 12 means that Ser-120 can no longer be excluded. However, the following findings strongly suggest that both Ser-120 and Ser-123 can be phosphorylated. (i) The three peptides migrate on a diagonal, indicating that they differ from each other by the same degree of charge and polarity, which is compatible with their being phosphorylated at one (peptide 12), two (peptide 11), or three (peptide 7) residues. (ii) Peptides 11 and 7, indeed, showed increasing amounts of phosphoserine relative to phosphothreonine. (iii) Partial acid hydrolysis of the isolated peptides 7, 11, and 12 revealed different phosphorylated cleavage products for each peptide. (iv) When we treated wt LT with increasing amounts of the catalytic subunit of protein phosphatase 2A, we found a sequential conversion of peptide 7 to 11 and then to 12; the resistant phosphate in peptide 12 was linked to threonine, indicating successive dephosphorylation of the two serine residues (43a, 59). (v) Finally, the SV120 mutant exhibits functional defects similar to those of SV123 (45). Thus, peptides 7, 11, and 12 reflect three different phosphorylation states of LT; peptide 12 is phosphorylated only at Thr-124, and peptides 11 and 7 are phosphorylated at Thr-124 and one and two of the adjacent serine residues, respectively. At present, we cannot distinguish whether peptide 11, which represents the intermediate state, is phosphorylated at one specific serine residue or randomly at either Ser-120 or Ser-123. However, the finding that the mutation at Ser-120 obviously prevented phosphorylation of Ser-123 suggests that phosphorylation of Ser-120 is a prerequisite for phosphorylation of Ser-123. Thus, peptide 11 may be phosphorylated at Ser-120 rather than Ser-123.

In summary, our analyses confirmed the previously identified serine residues 106, 112, 123, 639, 677, and presumably 679 and threonine residues 124 and 701 as phosphorylated residues; Ser-676 and, presumably, Ser-111 were excluded, whereas Ser-120 was identified as a new phosphorylation site. It should be pointed out that this and previous studies dealt only with phosphopeptides that are clearly and reproducibly detected. There are always minor peptides that might be either alternative cleavage products generated during proteolytic digestion or might represent additional phosphorylation sites that are modified only in a particular subfraction of LT. For example, peptide 12 is usually barely detectible in bulk LT preparations but becomes a prominent peptide in the monomeric or DNA-binding fraction of LT (41).

Effects of mutations on distant phosphorylation sites. Several mutations showed more or less pronounced effects on distant phosphorylation sites. The mutation of Ser-120→Ala seemed to prevent phosphorylation of Ser-123. The mutations in C8 (Ser-106→Phe) and, to a lesser extent, K5 (Ser-111→Phe) led to reduced phosphorylation of Ser-120 and Ser-123 in the amino-terminal region and even Ser-639 in the carboxy-terminal region. Similarly, the mutations in W7 and D29 showed an effect on carboxy-terminal sites. On the other hand, mutations at Ser-677, Ser-679, and Thr-701 exhibited an influence on sites in the amino-terminal domain; the most pronounced effect was seen with mutant SV677, in which phosphorylation of Ser-120 and Ser-123 was drastically reduced and that of Ser-112 seemed to be enhanced. In one case (SV124), peptide 1, representing Ser-106, was more heavily labeled than usual.

Short-range effects could be due to the mutation per se or to the lack of phosphorylation of a particular site. In the case of C8 and K5, the mutations to the bulky and hydrophobic phenylalanine seem to be responsible for these effects, since the mutants SV106 and SV111/112 (Ser→Ala) exhibited rather normal phosphorylation patterns. The phenylalanine residue may cause structural distortions and thus alter the accessibility of some sites for the respective kinases or phosphatases.

The secondary effects seen with SV120 and SV677 are probably due to the lack of phosphorylation of these sites: first, because the changes of Ser→Ala are rather subtle and, second, because the mutations at adjacent residues (Ser-123 or Ser-676) did not reveal similar effects. The prevention of phosphorylation of Ser-123 in SV120 suggests that the phosphorylation of Ser-120 might serve as a recognition signal for subsequent phosphorylation of Ser-123.

The long-range effects of amino-terminal mutations on carboxy-terminal sites and vice versa suggest that the two phosphorylation domains might be in close proximity in the three-dimensional structure of LT. As a result, changes in one domain could induce structural alterations in the other. The drastic reduction of phosphorylation in the amino-terminal region seen with mutant SV677 is especially interesting. First, it suggests that Ser-677 must be phosphorylated in order to achieve phosphorylation of Ser-120 and Ser-123. However, it is unlikely that phosphorylation of Ser-677 is a direct recognition signal for the kinase(s) that phosphorylates Ser-120 and Ser-123. Rather, we assume that phosphorylation of Ser-677 induces structural changes in the amino-terminal domain, which might lead to exposure of the

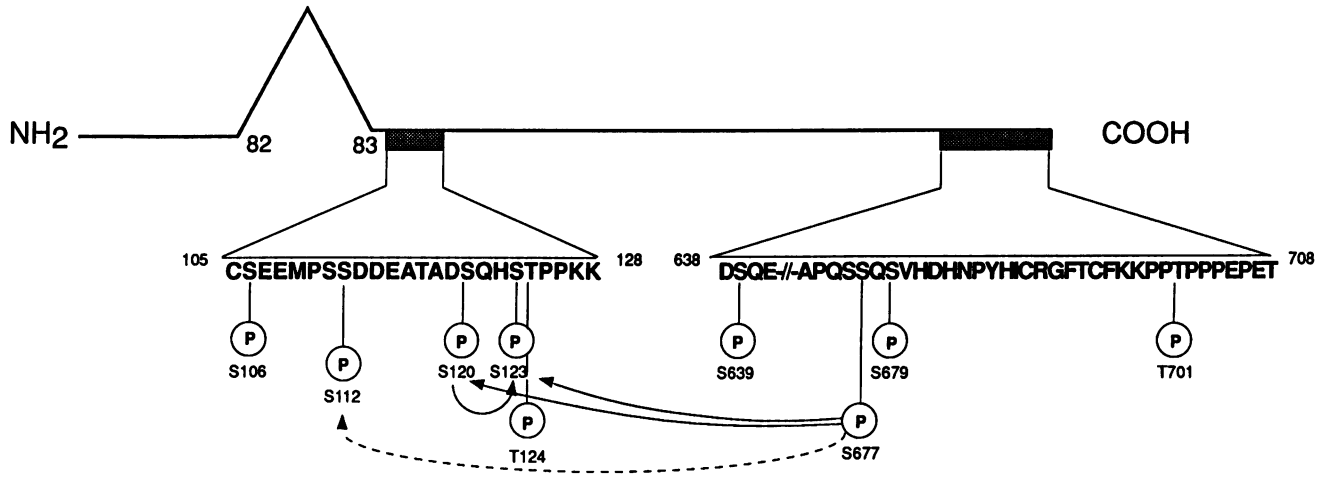

FIG. 8. Model indicating the major interactions between phosphorylation sites in the two phosphorylation domains. The upper line represents LT, divided into the two domains that are encoded by the two exons. The regions containing the phosphorylation sites are shown enlarged with the amino acid sequence in the one-letter code. Phosphorylation sites, as confirmed or determined (for Ser-120) in the present study, are indicated by encircled P's. Arrows between phosphorylation sites indicate an influence of individual phosphorylation events on distant sites, as concluded from the present results: (——) negative effects, (– – –) enhancing effect. Minor or less pronounced effects were not included.

phosphorylation sites. This view is supported by other findings: oligomerization of LT seems to induce (or require) conformational changes, and the cellular proteins p53 and Rb associate only with the oligomeric form of LT (4, 28, 29). Moreover, antibodies against phosphorylation sites induced by corresponding synthetic peptides show a remarkable preference for the tetrameric form of LT (15). Interestingly, SV677-encoded LT is defective for oligomerization (39a), perhaps because it is incapable of inducing the appropriate conformational changes. The major interactions of individual phosphorylation sites and their influence on each other, as deduced from these data, are depicted in Fig. 8.

Correlation with functional defects: role of individual phosphorylation sites in DNA binding. Previous studies suggested that the phosphorylation state of the neighboring residues Ser-123 and Thr-124 might be important for the DNA-binding activity of LT (24, 41). Phosphorylation at Thr-124 seems to be required for site II DNA binding (30), whereas phosphorylation at Ser-123, and presumably the newly identified Ser-120, appears to have a modulatory effect by decreasing the affinity or activity of binding (24, 32, 41), particularly to site II (24, 32). Consistent with this view is the high turnover in vivo of these serine sites (39). Furthermore, all mutants with exchanges at Ser-123, Thr-124, and Ser-120 exhibited functional defects, either in DNA binding or replication (22, 45) (Table 1). Mutant SV124 bound exclusively to binding site I, whereas mutants SV106, SV120, and SV123 bound better to binding site II than did wt LT (45), thus providing additional support for the above concept. The functional defects of other mutants, C8 and SV677, were unexpected. C8 LT bound to origin sequences but was replication defective (22, 34). The LT of mutant SV677 did not bind to site I but bound very efficiently to site II, as did SV679 LT (45). Since the ori-binding domain of LT has been mapped in the amino-terminal third of the molecule (1, 22, 34, 46, 52), it is unlikely that the carboxy-terminal phosphorylation domain excerts a direct effect on DNA binding. Rather, at least some of the above findings can now be explained by the secondary effects on phosphorylation of Ser-120 and Ser-123. The failure of SV677 to bind to site I might also be due to its defect in oligomerization (39a), because this site is preferentially bound by oligomeric forms of LT (13). In conclusion, both phosphorylation domains seem to regulate the DNA-binding and replication functions of LT in a concerted fashion.

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