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Metabolic Turnover of Phosphorylation Sites in Simian Virus 40 Large T Antigen

FRANS VAN ROY, LUCIE FRANSEN, AND WALTER FIERS*
Laboratory of Molecular Biology, State University of Ghent, B-9000 Ghent, Belgium

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Four (groups of) phosphorylation sites exist in the large T antigen of simian virus 40, and they involve at least two serine and two threonine residues (Van Roy et al. J. Virol. **45**:315–331, 1983). All the phosphorylation sites were found to be modified and again dephosphorylated at discrete rates, with phosphoserine residues having the highest turnover rate. The measured half-lives ranged between 3 h (for the carboxy-terminal phosphoserine site) and 5.5 h (for the amino-terminal phosphothreonine site). The influence of four temperature-sensitive A mutations on phosphorylation of large T antigen was also examined. At restrictive temperature, phosphorylation of the carboxy-terminal phosphoserine in mutated large T antigen was found to be particularly impaired. These data emphasize the physiological importance of the latter phosphorylation site.

Simian virus 40 large T antigen is a key protein with multiple functions during both productive infection and oncogenic transformation (recently reviewed in references 5 and 13). It is clear that the biological activity of such a protein must be rigorously and adequately controlled. One way in which this may be achieved is by a posttranslational modification such as phosphorylation. Large T antigen is indeed a phosphoprotein (11), and we have recently been able to localize three domains in the large T molecule, bearing at least four discrete phosphorylation sites (14, 15). At least some of these phosphorylation sites have a high turnover rate (2, 8), which is consistent with a role in metabolic regulation by posttranslational modification. With the hope of revealing subclasses of large T antigen with differential states of phosphorylation, possibly related to discrete biological activities, we studied both the kinetics of modification and the metabolic stability of the four (groups of) phosphorylation sites recognized so far.

We have also analyzed the influence of temperature-sensitive mutations in the viral *A* gene (*tsA*) on the phosphorylation sites. Walter and Flory (16) have shown previously that the specific activity of ^{32}P-labeled *tsA*-encoded large T molecules drops to some extent when the temperature is shifted from the permissive to the restrictive one. However, the phosphorylation of Thr^{701} seemed to be unaffected. This suggests a differential phosphorylation defect in *tsA*-encoded large T antigen at the nonpermissive temperature. Other investigators have also mentioned a negative effect of the *tsA* mutation on phosphorylation of large T antigen (2, 4). We have reinvestigated this phenomenon at the level of four (groups of) phosphorylation sites by using four different *tsA* mutants.

Equal aliquots of permissive monkey cells (CV-1) were infected with wild-type simian virus 40 and labeled with $^{32}P_i$ for increasing lengths of time, as previously described (14). Alternatively, each of several infected cultures was ^{32}P labeled for 7 h and then chased with an excess of unlabeled phosphate for different periods of time. Large T molecules from these cell lysates were purified and digested with large amounts of *Staphylococcus* V8 protease as described in the legend to Fig. 1. Then the phosphoamino acid contents of the resulting 18,000 (18K) phosphopeptide and the 11K + 13K doublet were determined. We have recently reported conclusive evidence for the presence of the amino (N)-terminal phosphoserine (P-Ser) and phosphothreonine (P-Thr) residues in the 18K molecule, whereas the 11K + 13K doublet is diagnostic for carboxy (C)-terminal phosphorylation events, including a P-Ser residue(s) and P-Thr^{701} (15). The relative stability of these fragments makes them fairly easy to isolate with a reproducible high yield. From the data obtained, the radioactivity associated with each of the four phosphorylation sites of large T could be calculated as described in the legend to Fig. 1. These results were plotted either linearly or semilogarithmically, as depicted in Fig. 1.

It is evident that the rate of phosphorylation of both P-Ser sites, especially the N-terminal P-Ser site, is rapid. The N-terminal P-Thr is slowly modified, and the reaction continues for a long

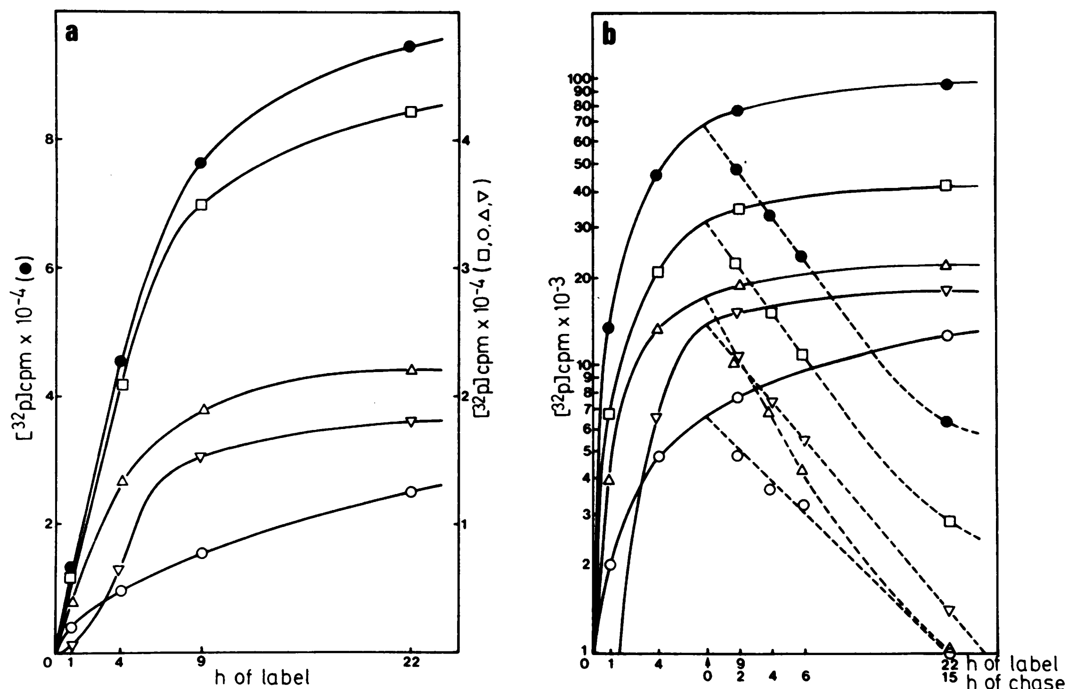

FIG. 1. Metabolism of phosphorylated large T molecules and its diverse phosphorylation sites. Simian virus 40 776-infected cells were ^{32}P labeled at 42 h postinfection for increasing lengths of time. Alternatively, infected cells were ^{32}P labeled at 42 h postinfection for 7 h and then chased by washing twice with complete regular medium containing an excess of unlabeled phosphate. The cells were further incubated in the same medium for increasing lengths of time. To prevent depletion of phosphate, cells were labeled in this experiment in phosphate-free medium containing 2.5% dialyzed calf serum and 0.1% normal Dulbecco modified Eagle medium. Large T molecules from the diverse cell lysates were immunoprecipitated and gel purified as described previously (14, 15). The radioactivity of the excised protein bands was measured by Čerenkov counting. They were then digested with *Staphylococcus* V8 protease during reelectrophoresis (14, 15). This results in a quantitative yield of 18K and 11K + 13K phosphopeptides, which contain N-terminal and C-terminal phosphorylation sites, respectively (14, 15). At that stage, the ratio of radioactivity in 18K peptides to radioactivity in 11K + 13K peptides was determined both by Čerenkov counting of excised gel bands and microdensitometry of suitable autoradiographs. The peptides were then eluted, desalted, and acid hydrolyzed. Their phosphoamino acids were analyzed, and the P-Ser/P-Thr ratio was determined as described previously (14, 15). This ratio was reproducible and was unaffected by any loss during preparation and analysis of the sample. Given the initial amount of radioactivity in the undegraded large T molecules, the ratio of 18K to 11 + 13K peptides, and the ratio of P-Ser to P-Thr in these peptides, the radioactivity associated with each of the four phosphorylation sites of large T was calculated. Thus, for all lysates, the total ^{32}P radioactivity incorporated in 88K large T molecules (●) is shown, as well as the ^{32}P radioactivity associated with the N-terminal P-Ser site (□), the N-terminal P-Thr site (○), the C-terminal P-Ser site (△), and the C-terminal P-Thr site (▽). These data were plotted either linearly (a) or semilogarithmically (b). Data of the chase experiment are connected by dashed lines. Note the scale difference between left and right ordinates in panel a.

time, whereas the C-terminal P-Thr is modified much more rapidly, but apparently only after a lag period. It is difficult to present a detailed interpretation of this picture, as we do not know what fraction of the radioactivity is incorporated by newly made molecules and what fraction by the preexisting molecules. The latter could be still completely unphosphorylated, partly phosphorylated, or already partly dephosphorylated. Scheidtmann et al. (8) measured both the unphosphorylated and phosphorylated forms of a C-terminal peptide. In this way, they found that within a labeling period of 24 h, up to 93% of newly made large T molecules were phosphorylated at the C-terminal site Thr^{701}. This phosphorylation site is fairly stable (see below; 8). It can be seen in Fig. 1a that after labeling for 9 or 22 h, the C-terminal Thr^{701} residue had incorporated about 20% of the total ^{32}P radioactivity in the large T antigen. Given the completeness of the kinase reaction at that particular site, one can deduce that large T molecules contain an average of five phosphate groups (on five or more different P-Ser and P-Thr sites). This num-

ber agrees well with the 4.19 phosphates per molecule of large T antigen estimated by Walter and Flory from their determination of specific radioactivity (16). Moreover, it is clear from the data in Fig. 1 that at least phosphorylation of Ser residues in the N-terminal region occurs on multiple sites. These Ser residues contain approximately 40% of the total ^{32}P radioactivity incorporated, which corresponds to a minimum of two phosphorylated sites.

From the pulse-chase experiment, the following order of half-life times ($t_{1/2}$) can be deduced. The C-terminal P-Ser is most labile, with a $t_{1/2}$ of about 3 h. This is followed by the N-terminal P-Ser and C-terminal P-Thr sites, with $t_{1/2}$ of 4 and 4.5 h, respectively. The most stable site is the N-terminal P-Thr ($t_{1/2}$ = 5.5 h). The biphasic turnover of total phosphate in large T was reported previously (2, 8). Although our data are limited, they suggest that N-terminal and C-terminal P-Ser sites also turn over in a biphasic way, in contrast to both P-Thr sites. The biphasic turnover is probably caused by heterogeneity of the P-Ser sites due to either the multiplicity of these sites or to the presence of an identical P-Ser site on different conformational forms of large T antigen.

To study the influence of *tsA* mutations on phosphorylation, four different mutants were used: *tsA*255, *tsA*239, *tsA*209 (1; gift of R. G. Martin), and *tsA*58 (10; gift of P. Tegtmeyer). Edwards et al. (2) have reported that *tsA* large T molecules tend to turn over as fast as their phosphate. This makes correct quantitative interpretation of phosphorylation in pulse-chase experiments complicated. Instead, we preferred to measure the fraction of radioactivity incorporated in each of the four (groups of) phosphorylation sites during temperature shift-up experiments. If all phosphorylation sites are affected equally by the *tsA* mutation or are equally unaffected, one still must consider that at restrictive temperature a majority of short-lived molecules is examined. This suggests that the ratio of P-Ser to P-Thr should increase considerably. P-Ser residues are indeed found to be labeled at a high rate, whereas their metabolic lability has less effect on a molecule with a short half-life (see above).

Monkey cells were infected with wild-type or *tsA* viruses and then labeled with ^{32}P for 8 h at 32.5°C or for 4 h at different times after temperature shift to 41.0°C. Large T molecules were extracted, gel purified, and digested with high amounts of V8 protease during reelectrophoresis as described previously (14, 15). The 18K and 11K + 13K phosphopeptides, diagnostic for N-terminal and C-terminal phosphorylation sites, respectively, were recovered, and their P-Ser/P-Thr ratio was determined. This ratio was calculated as the percentage of total radioactivity in large T antigen and is depicted in Fig. 2. Phosphorylation of wild-type large T antigen turned out to be largely temperature independent, although clearly the extent of total phosphorylation could vary. In contrast to this and to the situation expected for short-lived molecules (see above), mutant-encoded large T molecules seemed to have temperature-independent N-terminal phosphorylation sites, whereas their C-terminal sites were affected reproducibly. This could mean that the Thr^{701} site in the mutated molecules was labeled more quickly at a higher temperature, or that it turned over even more slowly, and vice versa for the C-terminal P-Ser site. Since our values are relative, it is also possible that Thr^{701} is just as stable here as in the wild-type large T molecule. Then all other sites, especially the C-terminal P-Ser, would be very thermolabile as part of the *tsA* phenotype.

This finding was further elaborated by an analysis of the phosphorylation patterns of proteolytically degraded 83K large T molecules. These can be generated by a cellular protease during extraction, and they lack the C-terminal $P\text{-}Thr^{701}$ site (15). Thus, cells were infected with either wild-type virus or *tsA* mutants and then ^{32}P labeled at either permissive or nonpermissive temperature or after temperature shift-up. The yield of 83K large T molecules was improved by harvesting cells at a lower pH and in the absence of protease inhibitors (15). N-terminal 31K and C-terminal 55K phosphopeptides were obtained therefrom by mild V8 proteolysis as described previously (15). Their P-Ser/P-Thr ratio and the fraction of total radioactivity incorporated in both peptides were determined. The C-terminal 55K phosphopeptide is of interest, as it contains, in principle, no P-Thr residues, and this allows a direct comparison of the C-terminal P-Ser phosphorylation site(s) with the N-terminal sites. From the results in Table 1, it can be concluded that at nonpermissive temperature, phosphorylation of the C-terminal P-Ser in particular is indeed impaired in the large T antigen of *tsA* mutants.

We have recently mapped the N-terminal P-Ser and P-Thr residues in a region extending from position Thr^{81} to Thr^{124} (15). This segment still contains a total of 10 phosphorylatable residues (5 Thr and 5 Ser), which is ample for the three phosphate groups (2 P-Ser and 1 P-Thr) quantified so far in the N-terminal half of large T antigen. The region essential for specific binding of large T antigen to the viral origin resides between approximate positions 82 and 140 (7 and discussion in reference 15). Specific DNA binding of large T antigen probably influences several of its other physiological functions. Thus, the metabolic importance of this region

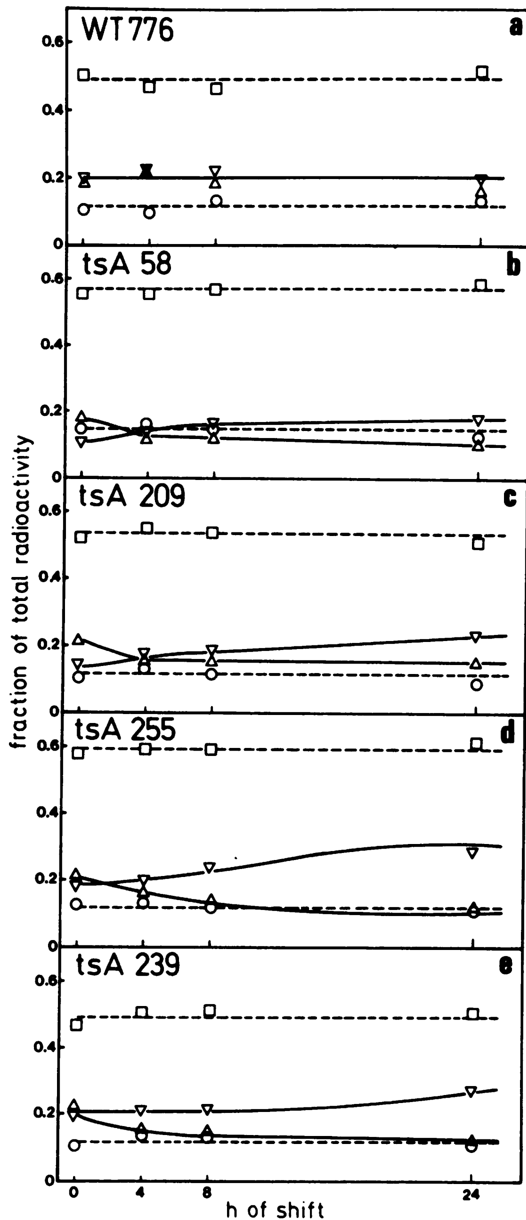

FIG. 2. Fraction of ^{32}P radioactivity in diverse phosphorylation sites of wild type (WT)- and *tsA*-encoded large T molecules during shift-up of the incubation temperature. The *tsA* virus stocks used were checked for conditional lethality and were found to be suitable by three methods (data not shown): (i) inhibition of plaque formation at nonpermissive temperature; (ii) overproduction of ^{35}S-labeled large T antigen during a short pulse at the nonpermissive temperature (12); and (iii) metabolic lability of these pulse-labeled molecules during chase at the nonpermissive temperature (12). Replicate cell cultures were infected with WT776 (a), *tsA*58 (b), *tsA*209 (c), *tsA*255 (d), or *tsA*239 (e) virus and grown for 43 h at 32.5°C. The cells were then ^{32}P labeled for 8 h at 32.5°C (0 h of shift), or the incubation temperature was shifted to 41.0°C. The shifted cultures were incubated at the high temperature for 4, 8, or 24 h, followed each time by a 4-h pulse period at the same temperature. Radioactivity in each of four phosphorylation sites of large T antigen is expressed as the fraction of total ^{32}P radioactivity incorporated in 88K large T molecules. The following sites were quantitated as described in the text: N-terminal P-Ser (□), N-terminal P-Thr (○), C-terminal P-Ser (△), and C-terminal P-Thr (▽). Data on N-terminal sites are connected by dashed lines, and data on C-terminal sites are connected by solid lines.

TABLE 1. Effect of incubation temperature on phosphorylation sites of wild type- and *tsA*-encoded 83K large T molecules

Infecting virus	Incubation temp[a] (°C)	Relative amt of phosphoamino acid[b]			
		N-terminal sites		C-terminal sites	
		P-Ser	P-Thr	P-Ser	P-Thr
776 (wild type)	32.5	52	20	28	0
	Shift	50	20	30	0
	41.0	48	22	30	0
*tsA*58	32.5	49	17	34	0
	Shift	60	26	14	0
	41.0	59	26	15	0
*tsA*239	32.5	47	20	33	0
	Shift	54	26	20	0
	41.0	56	25	19	0

[a] The infecting conditions were as follows: (i) 32.5°C for 64 h, followed by a labeling period of 6 h at the same temperature; (ii) 32.5°C for 56 h, followed by a 9-h shift to 41.0°C and a labeling period of 3 h at 41.0°C; (iii) 41.0°C for 29 h, followed by a labeling period of 3 h at the same temperature.

[b] The amount of each phosphoamino acid is expressed as a percentage of the total ^{32}P radioactivity incorporated in 31K and 55K V8 proteolytic fragments of the 83K large T molecule. Additional data are given in the text.

can explain the high turnover rate of the N-terminal P-Ser sites, which constitute a major phosphorylation site of large T antigen.

Furthermore, we found that the C-terminal P-Ser site(s) also had a high rate of synthesis, and somewhat surprisingly, also the highest rate of degradation. This suggests that this site also has an important regulatory function. In addition, it is striking that modification of this C-terminal P-Ser site(s) was particularly impaired in *tsA*-infected cells at the nonpermissive temperature. Three of the *tsA* mutants used in our study were each reported to have unique but mutually different nucleotide changes in their genomes (9). These changes, and also the *tsA*58 mutation, mapped in the *Hin*dII/III restriction fragment I (6), which extends from position 360 to 447 (3)

and codes for the unphosphorylated center of the large T molecule. In contrast, we mapped the C-terminal P-Ser site(s) in a region extending between approximate positions 500 and 640 (15). One must assume, therefore, that an altered tertiary structure of *tsA*-encoded large T molecules at nonpermissive temperature either prevents efficient phosphorylation or favors dephosphorylation of the C-terminal P-Ser site(s). Viral DNA replication, transformation efficiency, tumor-specific transplantation, and U antigen expression are altered under the influence of the *tsA* mutation at restrictive temperature (reviewed in reference 5). However, with the present data, it is not possible to establish whether the thermolabile structure of large T antigen alone or the lack of phosphorylation (and its influence on an additional structural change) is related to any of the changes mentioned.

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