Protein Phosphatase 2A Dephosphorylates Simian Virus 40 Large T Antigen Specifically at Residues Involved in Regulation of DNA-Binding Activity

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Treatment of purified simian virus 40 large T antigen (LT) with protein phosphatase 2A stimulates LT-dependent DNA unwinding and replication (D. M. Virshup, M. G. Kauffman, and T. J. Kelly, EMBO J. 8: 3891–3898, 1989). The specificity of the catalytic subunit of protein phosphatase 2A toward LT was investigated by two-dimensional peptide mapping. Increasing amounts of phosphatase sequentially removed the phosphates from serine residues 120, 123, 677, and perhaps 679, residues which have been implicated in regulating the DNA-binding activity of LT.

The simian virus 40 (SV40) large T antigen (LT) plays an essential role in the viral life cycle, particularly in DNA replication and regulation of transcription (for reviews, see references 1, 2, 5, and 19). These functions are mediated by interaction with specific sequences in the viral origin of DNA replication (reviewed in reference 5) and by cooperation with cellular replication and transcription factors (15, 28). LT is differentially and reversibly phosphorylated at eight or more sites that are clustered in two separate regions (6, 12, 21, 23, 24, 25, 29, 30).

Initial studies on the functional significance of phosphorylation revealed an inverse correlation between the degree of phosphorylation of LT and its DNA binding activity, suggesting that phosphorylation downmodulates DNA binding (24, 27). Subsequent studies in which unspecific alkaline or acid phosphatases were used to dephosphorylate purified LT in vitro revealed different effects: alkaline phosphatase removing most or all of the serine phosphates (7, 11) led in two cases to an increase in DNA binding, particularly to site II (11, 16), which is involved in initiation of DNA replication, whereas no effect was seen in a third study (7). The reason for these discrepancies is not clear. Furthermore, dephosphorylation of LT by alkaline phosphatase stimulated DNA replication in vitro (7, 16), whereas ATPase or helicase activities were not affected (7, 11). On the other hand, further dephosphorylation by acid phosphatase at Thr-124 reversed the enhancing effect of alkaline phosphatase on origin binding, suggesting that phosphorylation at this residue is required for efficient binding (11). This latter suggestion was further supported by recent data showing that bacterially expressed, and hence unphosphorylated, LT exhibited low activities in site II binding and initiation of DNA replication and that both these activities were greatly stimulated after specific phosphorylation of Thr-124 by cdc2 kinase in vitro (14). Although these data indicate an influence of the overall phosphorylation state of LT on its binding to site II and some function in DNA replication, they do not allow an assignment of these effects to specific residues. Earlier biochemical (24) and genetic (10, 26) studies seem to

indicate a regulatory role of phosphorylation at serine residues 120, 123, 677, and 679, at least in DNA and viral replication in vivo (26).

We (Virshup and Kelly [32]) have recently isolated a replication factor, RP-C, from HeLa cells which was capable of stimulating LT-dependent DNA replication in vitro. In particular, RP-C shortened or eliminated the lag phase prior to the onset of replication, presumably by enhancing the unwinding activity of LT (32). This factor proved to be the catalytic subunit of protein phosphatase 2A (PP2A_C [31]). In a further study, the enhancing effect was correlated with a limited degree of dephosphorylation (31). These results further support the hypothesis that the functional state of LT is modulated by reversible phosphorylation (for a recent review, see reference 18). In the present study, we determined more precisely which phosphorylation sites on LT were most readily dephosphorylated by RP-C/PP2A_C.

To investigate whether phosphatase 2A (PP2A_C) dephosphorylates particular sites of LT, monkey TC7 cells were infected with SV40, cells were labeled with ³²P_i in vivo, and LT was purified from cell extracts by immunoprecipitation and treated with increasing amounts of PP2A_C, as described in the legend to Fig. 1. Extensive treatment of LT (approximately 1 µg) with about 3 ng of PP2A_C resulted in removal of 30% of the LT-bound phosphate label. When we used eight times more enzyme, an additional 10 to 15% of phosphate was removed. This is in agreement with previous results showing that in contrast to alkaline or acid phosphatases, which are capable of removing 80 or 95% of the LT-bound phosphate, respectively (7, 11), dephosphorylation with PP2A_C is much more limited (31).

LT was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, digested with trypsin and pronase E, and analyzed by two-dimensional peptide mapping as described previously (23). Fig. 1a shows the phosphopeptide pattern of untreated LT. Figures 1b and c show the patterns obtained after treatment with increasing amounts of PP2A_C. The major alterations in the peptide pattern, as compared to the untreated control, occurred in peptides 7, 11, and 12, representing the phosphorylation sites Ser-120, Ser-123, and Thr-124 (see below), in peptides 9 and 10 and those above 8, phosphorylated at Ser-677 and presumably Ser-679 (22–24). Peptides 7 and 11 decreased

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whereas peptide 12 increased in relative intensity. Obviously, peptides 7 and 11 were sequentially dephosphorylated and converted to peptide 12 in a dose-dependent manner (see below). Similarly, peptides 9 and 10 and those above 8 were greatly reduced after treatment with 3 ng of PP2A_c and disappeared completely with greater amounts of enzyme. The phosphate residues in the other peptides appeared to be rather resistant to the enzyme.

As shown previously, peptides 7, 11, and 12 represent three different phosphorylation states of the same peptide which encompasses Ser-120, Ser-123, and Thr-124; peptide 12 is phosphorylated only at Thr-124, while peptide 11 is phosphorylated at one and peptide 7 is phosphorylated at two additional serine residues (22, 23). Theoretically, conversion of peptides 7 and 11 to peptide 12 could be achieved by dephosphorylating serine or threonine residues since the overall composition and properties of the end product, peptide 12, would be the same, regardless of whether it were phosphorylated at Thr-124 or at one of the serines. To investigate whether the phosphate in peptide 12 was bound solely to Thr-124 or to serine residues as well, peptides 11 and 12 of PP2A_c-treated LT were eluted from the thin-layer plate, hydrolyzed in 5.6 N hydrochloric acid, and analyzed for the presence of phosphoserine or phosphothreonine by thin-layer electrophoresis. As shown in Fig. 2, peptide 11 revealed phosphoserine and traces of phosphothreonine, as expected. Peptide 12 revealed only phosphothreonine, thus indicating that this residue was resistant to the enzyme and that peptides 7 and 11 were dephosphorylated only at serine residues. As controls, peptides 7, 11, and 12 from untreated LT were analyzed the same way, showing ratios between serine and threonine phosphate as expected from previous analyses (24).

In summary, the protein phosphatase PP2A_c used in this study showed a remarkable specificity towards serine residues 120 and 123 in the amino-terminal region and serine residues 677 and presumably 679 in the carboxy-terminal region of LT. The other phosphorylation sites were rather

FIG. 1. Phosphopeptide analyses of untreated and phosphatase PP2A_c-treated LT. Monkey TC7 cells were infected with SV40. Two days postinfection, cells were labeled with ³²P_i for 4 h and LT was purified by immunoprecipitation with hamster anti-SV40 tumor serum and fixed *Staphylococcus aureus* as described previously (23). The immune complexes were washed three times with detergent buffer (buffer J or RIPA buffer [23]) and two times with phosphatase buffer (30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] pH 7.4, 7 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol [32] in the presence of 50 μM leupeptin [Sigma] and 1 μg of aprotinin [Boehringer] per ml as protease inhibitors; bovine serum albumin was omitted). Subsequent treatment with PP2A_c was in 60 to 200 μl at 37°C for 60 min at enzyme concentrations of 50 (panel b) or 400 (panel c) ng/ml and enzyme to substrate ratios of approximately 1:160 and 1:20, respectively. Reactions were stopped by adding 10 volumes of buffer J containing 10 mM EDTA. The complexes were washed once more, and LT was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After elution from the gel, LT was digested sequentially with trypsin and pronase E and the resulting peptides were separated by two-dimensional peptide mapping as described previously (23). Electrophoresis was carried out in the horizontal direction at pH 1.9 and 1,300 V for 25 min, and ascending chromatography was carried out in isobutyric acid buffer for 5 h; the vertical dash indicates the origin. The phosphopeptides were visualized by autoradiography using intensifying screens. The nomenclature refers largely to that in reference 24 with the exception of peptide 1, which has been relocated (22).

FIG. 2. Phosphoamino acid analysis of phosphopeptides 7, 11, and 12 prior to and after treatment with PP2A_C. Phosphopeptides were separated on thin-layer cellulose plates, localized by autoradiography, eluted, and hydrolyzed in 5.6 N hydrochloric acid at 110°C for 2 h. Phosphoamino acids were separated on thin-layer cellulose plates by electrophoresis at 1,000 V for 60 min, in the presence of unlabeled phosphoserine and phosphothreonine as markers.

resistant to the enzyme. Whether this specificity is determined by primary or secondary structure of the respective phosphorylation sites cannot be assessed from these data alone, although the sites share certain residues: the sequence around Ser-120 runs Ser-120-Gln-His-Ser-123-Thr-124-Pro-Pro-, and that around Ser-677 is Ser-Ser-677-Gln-Ser-679-Val-His. But in general, phosphatases are presumed to have broad specificities (for reviews, see references 3 and 4). Thus, it is more likely that the preference of PP2A_C for particular sites of LT is determined by secondary structure.

It is striking that PP2A_C has been isolated as a replication factor (32). Thus, it may be concluded that those phosphorylation sites in LT that are preferentially dephosphorylated by PP2A play a role in regulating functions related to DNA replication. In fact, the limited extent of dephosphorylation shown in Fig. 1c can be well correlated with previous data that show that a similar degree of dephosphorylation of LT led to stimulation of its functions, presumably unwinding (32), in DNA replication (31). Which of the affected phosphorylation sites are important in this respect cannot be assessed from these data. However, biochemical and genetic studies suggest that amino-terminal sites (Ser-120, Ser-123) as well as carboxy-terminal sites (Ser-677, Ser-679) play a regulatory role (10, 24, 26, 27). Consistent with this view are recent findings suggesting that the two phosphorylation domains of LT interact with each other in the three-dimensional structure (22) and thus might cooperate in modulating LT's interactions with DNA and cellular replication and transcription factors (15, 28).

Whether PP2A is indeed involved in regulation of viral and perhaps cellular DNA replication *in vivo* remains to be investigated. The sites dephosphorylated by PP2A_C *in vitro* show high turnover rates *in vivo* (21). Recent data obtained by using the PP2A-specific inhibitor okadaic acid suggest that PP2A is indeed present and plays a stimulatory role in cell extracts employed in LT-dependent SV40 DNA replication *in vitro* (13). Moreover, the findings of Roberts and D'Urso (20) that S-phase cell extracts stimulate replication *in vitro*, whereas G1 extracts do not, and that G1 extracts gain stimulatory activity after supplementation with PP2A suggest that PP2A indeed participates in the regulation of cellular DNA replication during the cell cycle.

The question which arises from these observations is how the activity of PP2A might be regulated. The level of expression of the catalytic subunit seems to be constant throughout the cell cycle (31). Either the enzyme is regulated by the A or B subunit associated with the catalytic subunit (for a review, see reference 3), or PP2A is itself subject to reversible posttranslational modification. In this context, it is noteworthy that the small T antigen of SV40 and the small and medium T antigens of polyomavirus bind to PP2A, particularly to the two-subunit form (9, 17, 21a, 22, 33, 34). The significance of these associations is not clear yet, but it is tempting to speculate that the small T antigens indirectly regulate the phosphorylation state and thereby the functional state of the large T antigens via this interaction. An alternative possibility is that the action of PP2A is determined by its subcellular distribution or attachment to cellular structures. This would be compatible with our earlier findings that LT from different nuclear compartments has different phosphorylation states (24; unpublished observations). Another possibility is that the kinases rather than the phosphatases are regulated during the cell cycle. Investigating these interesting questions might yield further insights into the complex network of cell cycle regulation and growth control.

From the data collected so far, a model of how LT might be regulated by reversible phosphorylation can be envisioned: LT becomes partially phosphorylated in the cytoplasm (25) at Ser-112, perhaps at Ser-106 by casein kinase II (8), at Thr-701 (by an unidentified kinase), and at Thr-124 by the cdc2 kinase (13), with the last-mentioned phosphorylation event being a prerequisite for DNA binding and replication. LT migrates in the nucleus, and a fraction of it binds rather tightly to the viral origin of DNA replication, binding site II. LT might then be phosphorylated *in situ* at additional sites (e.g., Ser-120, Ser-123, Ser-677, or Ser-679), perhaps by casein kinase I (8), rendering binding more reversible or even leading to dissociation of LT from the DNA. After dephosphorylation at particular sites by PP2A (and perhaps other phosphatases) LT might regain its original tight binding status and reenter a new functional cycle.

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