Effects of In Vitro Dephosphorylation on DNA-Binding and DNA Helicase Activities of Simian Virus 40 Large Tumor Antigen

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Simian virus 40 large T antigen is a phosphoprotein with two clusters of phosphorylation sites. Each cluster includes four serine residues and one threonine residue. In vitro treatment with intestinal alkaline phosphatase removes the phosphate groups from the serine but not from the threonine residues. Potato acid phosphatase additionally dephosphorylates the phosphothreonine (Thr-124) in the N-terminal cluster but does not attack the phosphothreonine in the C-terminal cluster (Thr-701). Two biochemical functions of untreated and partially dephosphorylated T antigen were assayed, namely, its specific DNA-binding property and its DNA helicase activity. After treatment with alkaline phosphatase, T antigen had a severalfold higher affinity for the specific binding sites in the viral genomic control region, in particular, for binding site II in the origin of replication. However, T antigen, when dephosphorylated by acid phosphatase, had DNA-binding properties similar to those of the untreated control. Neither alkaline nor acid dephosphorylation affected the DNA helicase activity of T antigen.

Large T antigen, a phosphoprotein encoded by an early gene of simian virus 40 (SV40), performs several functions in the infected host cell. It regulates viral gene expression and performs essential functions in the viral DNA replication process. T antigen also induces the expression of some host cell genes and stimulates host cell DNA replication (39).

T antigen is a DNA-binding protein with a marked preference for two independent high-affinity binding sites in the genomic control region of SV40 DNA. Both sites contain several copies of the recognition pentamer GAGGC (6, 37, 38). However, the sites have different molecular architectures and functions.

Site I, which is located in the early section of the genomic control region, consists of two tandemly oriented GAGGC elements, separated by a 7-base-pair AT-rich spacer (27). T antigen, which is bound to site I, autoregulates its synthesis by attenuating early SV40 gene expression (7, 20, 26).

Site II is part of the viral origin of replication and contains four GAGGC pentamers which are separated from each other by only 1 base pair. The pentamers in site II are arranged as 2 base pairs which are inverted relative to each other, forming a perfect palindrome. T-antigen binding to site II is a necessary first step in the initiation of viral DNA replication (20, 41), leading to a separation of the DNA duplex and to the establishment of the replication forks (5). The mechanism of binding of T antigen to site II is probably quite different from its binding to site I (37), as might be expected from the different structures and functions of the two sites.

A second biochemical property of T antigen is its ability to unwind DNA duplexes in a reaction that requires magnesium ions and ATP hydrolysis (34). The DNA helicase activity of T antigen is responsible for the parental strand unwinding reaction at the origin of replication (5, 42) and at replication forks (35, 40). In addition, T antigen possesses an ATPase activity (3, 11) which is most likely connected to its DNA unwinding function.

Thus, T antigen performs several functions. One might expect that these functions are independently regulated. One possible mechanism for regulation would be the differential posttranslational modification of amino acid side chains. Indeed, T antigen is a phosphoprotein with two clustered regions of phosphorylation sites. The N-terminal cluster includes amino acid residues Ser-106, Ser-111, Ser-112, Ser-123, and Thr-124. Amino acids Ser-639, Ser-676, Ser-677, Ser-679, and Thr-701 are in the C-terminal cluster of phosphorylation sites (28). Phosphorylation of T antigen occurs in a sequential manner, with Thr-124 and Thr-701 (and, possibly, Ser-111) being phosphorylated in the cytoplasm, whereas the other phosphate groups are probably added in the nucleus (10, 30). Newly synthesized, underphosphorylated "young" T antigen binds more tightly than "old," fully phosphorylated T antigen to its specific binding sites in the SV40 genomic control region (23, 29), suggesting that phosphorylation may modulate the specific DNA-binding activity of T antigen. Moreover, higher-phosphorylated T antigen is more likely found in an aggregated, mostly tetrameric form compared with underphosphorylated T antigen, which is mostly mono- or dimeric (9). These observations suggest that the state of phosphorylation has profound effects on the structure and function of T antigen.

Several research groups have tried to support this conclusion with biochemical experiments. Shaw and Tegtmeyer (31) first reported experiments in which they used alkaline phosphatase to dephosphorylate ³²P-labeled T antigen in vitro. They noted that T antigen could not be completely dephosphorylated by alkaline phosphatase. They could not detect significant effects on the specific binding of T antigen to SV40 DNA. Likewise, Baumann (1), working with the T-antigen-related D2 protein, did not detect changes in the DNA-binding properties after treatment with alkaline phosphatase. However, by using acid phosphatase, Baumann obtained a more complete dephosphorylation of the D2 protein and found a strong reduction in specific binding of the D2 protein to SV40 DNA, although the general, unspecific DNA-binding property of the D2 protein was not significantly affected by dephosphorylation. An effect of acid

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phosphatase treatment on the ATPase activity of D2 protein was not detected (1). More recently, Mohr et al. (22) showed that dephosphorylation by alkaline phosphatase increases the affinity of T antigen to the SV40 binding site II and, furthermore, that the activity of dephosphorylated T antigen in an in vitro replication system was severalfold higher than the activity of untreated T antigen. Mohr et al. (22) also found that dephosphorylation had no effect on the ATPase activity of T antigen.

The relationship of the results of these biochemical experiments to the in vivo observations remains uncertain. For example, it is not known which phosphate groups are removed by alkaline or acid phosphatase and, consequently, whether the in vitro-dephosphorylated T antigen forms are comparable to the newly synthesized underphosphorylated young T antigen mentioned above.

We report here that in vitro dephosphorylation with alkaline phosphatase removes most phosphate groups from serine but not threonine residues of ^{32}P -labeled T antigen. Treatment with alkaline phosphatase increases the affinity of T antigen for the specific binding sites on SV40 DNA, in particular, for binding site II. These data confirm the results of Mohr et al. (22). Acid phosphatase causes a more complete dephosphorylation, leaving Thr-701 as the only remaining phosphoamino acid. Interestingly, T antigen, when treated with acid phosphatase, binds to SV40 DNA much like the untreated control. Dephosphorylation does not affect the DNA helicase activity of T antigen.

MATERIALS AND METHODS

T antigen. The African green monkey cell line TC7 was grown as monolayers on plastic dishes in Dulbecco modified Eagle medium with 5% fetal bovine serum and infected under standard conditions with SV40 (multiplicity of infection, 10). The cells were harvested 48 h after infection and used for T-antigen preparation. T antigen was prepared in two different ways, either by differential ammonium sulfate precipitation and column chromatography, essentially by the method of Baumann (1), or by a modification of the immunoaffinity procedure of Simanis and Lane (32). The former procedure gave a T-antigen preparation of at least 50% purity that was free from other contaminating DNA-binding proteins. T antigen prepared by the immunoaffinity procedure was essentially pure, as demonstrated previously (34).

For [^{32}P]phosphate labeling, SV40-infected cells were transferred at 44 h after infection to phosphate-free medium and labeled for 4 h with 0.5 mCi of $^{32}\text{P}_i$ per plate. ^{32}P -labeled T antigen was then extracted from infected cells and isolated as described above.

Phosphatase treatment. Potato acid phosphatase (grade I; Boehringer GmbH, Mannheim, Federal Republic of Germany) was dialyzed for 1 h against 50 mM sodium acetate buffer (pH 5.6). For dephosphorylation, 1 μg of T antigen was incubated with 0.5 units of acid phosphatase at room temperature in a 50- μl volume of 50 mM sodium acetate (pH 5.6)–10% glycerol–80 μg of bovine serum albumin.

Bovine intestinal alkaline phosphatase (type VII S; Sigma Chemical Co., St. Louis, Mo.) was dialyzed before use against alkaline phosphatase buffer (20 mM Tris hydrochloride [pH 8.0], 1 mM MgCl_2 , 0.1 mM ZnCl_2). T antigen (1 μg) was treated with 5 units of alkaline phosphatase for 1 h at room temperature in 50 ml of alkaline dephosphorylation buffer containing 10% glycerol and 80 μg of bovine serum albumin.

Phosphopeptides. ^{32}P -labeled T antigen was purified as described above and, in addition, by sodium dodecyl sulfate

(SDS)-polyacrylamide gel electrophoresis. T antigen was eluted from the gel, precipitated with trichloroacetic acid, oxidized with performic acid, and digested with trypsin and pronase E as described previously (28).

The resulting phosphopeptides were separated by two-dimensional electrophoresis-chromatography and visualized by autoradiography by using intensifying screens (28). T-antigen preparations purified by either one of the procedures described above gave identical results. The results were indistinguishable from those obtained before immunoprecipitated T antigen was used (28), indicating that dephosphorylation did not occur during our purification procedures.

DNA. Plasmid pSV contains wild-type SV40 DNA cloned via its *Bam*HI restriction site into pAT153 (13). Plasmid pSV(cs1097) carries the SV40 mutant cs1097 DNA in pAT153. The cs1097 mutant has a deletion of SV40 DNA nucleotides 5178 to 5208, including the high-affinity T-antigen-binding site I (7). Plasmid pSV(cs1097) was kindly supplied by E. Fanning. Restriction of DNA was performed by standard procedures (18). The resulting DNA fragments were 3' end-labeled by using the Klenow fragment of DNA polymerase, [^{32}P]dATP or [^{32}P]dCTP, and unlabeled nucleotides as described previously (18).

Assays. The DNA-binding property of untreated or dephosphorylated T antigen was assayed by using restricted and ^{32}P -end-labeled SV40 DNA, pSV DNA, or pSV(cs1097) DNA. Stringent conditions (8) for the binding of T antigen consisted of a solution containing 80 mM KCl, 10 mM Tris hydrochloride (pH 7.8), and a 100-fold excess of competing sonicated salmon sperm DNA. Nonstringent conditions (8) consisted of a solution containing 10 mM Tris hydrochloride (pH 6.5), no KCl, and no competing DNA. T antigen-DNA complexes were immunoprecipitated by the procedure of McKay (21) or a modification thereof (15). In earlier experiments we used polyclonal antibodies from mouse tumor serum, as described previously (8). In more recent experiments monoclonal antibody PAb101 was used for immunoprecipitation (15). This antibody reacts with an epitope at the C-terminal end of the T antigen (14) and has no effect on its DNA-binding activity (15). The immunoprecipitated DNA was recovered and displayed by polyacrylamide gel electrophoresis and autoradiography (13).

The DNA helicase activity of T antigen was determined exactly as described by Stahl et al. (34) by using M13 mp8 DNA carrying a hydrogen-bonded, ^{32}P -labeled complementary strand of 300 nucleotides as the substrate (M. Wiekowski, M. Schwarz, and H. Stahl, *J. Biol. Chem.*, in press).

RESULTS

Dephosphorylation. To investigate the efficiency of the in vitro dephosphorylation reactions, we incubated [^{32}P]phosphate-labeled T antigen with acid or alkaline phosphatase for various times to determine the remaining radioactivity in the trichloroacetic acid-precipitable protein. Maximal dephosphorylation was achieved after 60 to 90 min, when alkaline phosphatase had removed about 90% and acid phosphatase about 95% of the ^{32}P label originally present in the T-antigen preparation (Fig. 1). These values, which were obtained by trichloroacetic acid precipitation, were confirmed by SDS-polyacrylamide gel electrophoresis of ^{32}P -labeled T antigen before and after 60 min of incubation with phosphatases (Fig. 1, insert). The loss of radioactivity was not due to a degradation of T antigen since electrophoretic analysis showed that Coomassie blue- or silver-stained T antigen was present in similar quantities before and after dephosphory-

FIG. 1. Dephosphorylation of ^{32}P -labeled T antigen. Samples of 1 μg of chromatographically purified T antigen were incubated with alkaline (●) or acid (○) phosphatase as described in the text. At the indicated times, samples were diluted into 20 mM phosphate buffer containing 0.1 μg of bovine serum albumine per ml and precipitated with 10% trichloroacetic acid. The precipitates were collected on nitrocellulose filters and counted in a scintillation spectrometer. The 100% value corresponds to ca. 2×10^4 cpm in 1 μg of T antigen. (Insert) Untreated, ^{32}P -labeled T antigen (T-Ag) (lane C), ^{32}P -labeled T antigen incubated in alkaline buffer with (+) and without (−) alkaline phosphatase (Alk. P'ase), and ^{32}P -labeled T antigen incubated in sodium acetate buffer (pH 5.6) with (+) and without (−) acid phosphatase (Acid P'ase). Two different batches of acid phosphatase were used. The results shown here were after SDS-polyacrylamide gel electrophoresis (17) and autoradiography.

lation (data not shown). The results (Fig. 1) were obtained in several independent experiments with chromatographically or immunologically purified T antigen (1) and with a 10-fold higher phosphatase concentration.

We tried to determine whether the phosphatase attacked each of the 10 phosphoamino acids with a similar probability, or whether some sites were preferentially dephosphorylated. For this purpose, T antigen was enzymatically cleaved to obtain phosphopeptides for two-dimensional peptide mapping (28) (Fig. 2). Untreated, ^{32}P -labeled T antigen or T antigen incubated in alkaline or acid buffer without phosphatase yielded all the phosphopeptides described and identified previously (28), representing the entire spectrum of phosphorylation sites in the N-terminal and C-terminal clusters. Alkaline phosphatase completely released the phosphate groups from most serine residues represented by peptides 1 through 7 and 9 and 10 (28) and partially desphosphorylated Ser-639 (Fig. 2, peptide 4). However, the radioactivity in the peptides containing Thr-701 (peptide 13) and Thr-124 (Fig. 2, peptides 8, 11', 12 and 12') did not change or increased (as in peptides 12 and 12'). This latter effect can be attributed to the conversion of the doubly phosphorylated peptides 7 and 11, which were phosphorylated at Ser-123 and Thr-124 to peptides in which only the phosphate at Thr-124 remained, as confirmed by acid hydrolysis (29). Thus, both threonine residues seemed to be resistant to alkaline phosphatase treatment.

Peptide analysis of T antigen treated with acid phosphatase showed only two remaining phosphopeptides, including partially desphosphorylated Ser-639 (peptide 4) and the phosphoamino acid Thr-701 (Fig. 2, peptide 13).

Thus, peptide analysis showed once again that acid dephosphorylation is more effective than alkaline dephos-

phorylation (Fig. 1). More importantly, the data in Fig. 2 demonstrate that in vitro dephosphorylation is not a random reaction but leads to T antigens with defined phosphoamino acid contents.

DNA-binding properties of dephosphorylated T antigen. We investigated the specific DNA-binding properties of T antigen both before and after dephosphorylation. Specific DNA-binding was determined by using *Hind*III-restricted, ^{32}P -, 3'-end-labeled pSV DNA and T antigen which was incubated in alkaline dephosphorylation buffer with or without enzyme. The binding conditions chosen for these experiments were considered to be stringent for a specific T antigen-DNA association (8): high pH (10 mM Tris hydrochloride, pH 7.8), high salt (80 mM KCl), and a 100-fold excess of competing unspecific salmon sperm DNA. The T antigen-DNA complexes that were formed under these conditions were collected by immunoprecipitation (15) by using the T-antigen-specific monoclonal antibody PAb101 (14). The DNA fragments in the immunoprecipitates were identified by polyacrylamide gel electrophoresis and autoradiography. As expected from similar experiments done previously (1, 13), the SV40 *Hind*III C fragment, which contains both high-affinity binding sites, was recovered in the immunoprecipitates (Fig. 3), demonstrating that T antigen binds specifically to this part of the SV40 genome. Results of the experiment with restricted pSV DNA showed that alkaline phosphatase-treated T antigen binds significantly more of the *Hind*III C fragment than does an equal amount of the untreated control T antigen (Fig. 3b). We performed more than 20 experiments of this type and consistently found a 2- to 5-fold (average, 2.6-fold) higher amount of *Hind*III C fragment bound by alkaline phosphatase-treated T antigen than by untreated control T antigen.

To determine whether alkaline dephosphorylation affects the binding of T antigen to both sites, we used *Hind*III-restricted pSV(cs1097) DNA, which does not possess T-antigen-binding site I. We found that untreated T antigen bound poorly to the mutant *Hind*III C fragment (Fig. 3a), in agreement with earlier observations showing that T antigen has a severalfold higher affinity to binding site I than to binding site II (37). However, T antigen treated with alkaline phosphatase bound quite efficiently to the mutant *Hind*III C fragment (Fig. 3a), suggesting a marked preference of dephosphorylated T antigen for the binding site in the origin of viral replication (22). To investigate whether alkaline dephosphorylation had any effect on the association of T antigen to binding site I, we isolated and 3' end-labeled the fragment between the *Hind*III restriction site at nucleotide 5171 and the *Dde*I site at nucleotide 5228. This fragment included the entire binding site I but did not contain the SV40 origin. DNA-binding studies revealed that alkaline phosphatase-treated T antigen also bound better (1.4- to 2.0-fold) than untreated T antigen to this fragment (Fig. 3c).

It appears that alkaline dephosphorylation has a more pronounced effect on the binding of T antigen to site II than it does to site I. To confirm this conclusion, we used *Dde*I-restricted SV40 DNA for binding experiments. Restriction endonuclease *Dde*I cuts SV40 DNA at 20 sites, including a site (nucleotide number 5228) which separates binding site I and binding site II. A 310-base-pair *Dde*I fragment (nucleotide coordinates 4918 to 5228) contained the entire binding site I, whereas a 302-base-pair fragment (nucleotide coordinates 5228 to 287) carried the origin of replication, including binding site II. Untreated T antigen bound efficiently to the larger but only poorly to the smaller one of the two fragments (Fig. 4), as expected from results of

FIG. 2. Phosphopeptide analysis of ^{32}P -labeled T antigen before and after dephosphorylation. Purified T antigen was split into three equal parts. One part was used directly and the second and third parts were used after treatment with alkaline and acid phosphatase, respectively, as outlined in the text (28). After digestion with trypsin and pronase E, the phosphopeptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 (cathode on the right) and ascending chromatography in isobutyric acid buffer (28). The plates were autoradiographed with intensifying screens. The phosphopeptides were identical to those described and characterized previously by Scheidtmann et al. (28, 29). We also used the numbering system of those investigators (28, 29). Peptides 7 and 11 of the control T antigen contained phosphate groups at Ser-123 and Thr-124. Peptides 8, 11', 12, and 12' of dephosphorylated T antigen contained a phosphothreonine at residue 124. Peptide 13 contained a phosphothreonine at residue 701. Note that digestion with pronase E generated overlapping peptides, several of which included Ser-123 and Thr-124 (28, 29).

earlier studies in which it was shown that T antigen has a higher affinity for site I than for site II (37, 38, 41). After alkaline dephosphorylation, however, T antigen bound significantly more of the origin-containing 302-base-pair fragment.

Dephosphorylation of T antigen by acid phosphatase was more complete than dephosphorylation by alkaline phosphatase, resulting in the removal of all phosphate groups except for that on Thr-701 (Fig. 2). T antigen that was dephosphorylated by acid phosphatase was used for DNA-binding studies similar to those described above. Surprisingly, T antigen bound only slightly (average, 1.5-fold) better to the *Hind*III C fragment after acid dephosphorylation than did the untreated control T antigen (Fig. 5). With some T-antigen preparations we observed a reduction of specific DNA binding after acid phosphatase treatment. This effect was not

due to the low pH conditions which were necessary for acid dephosphorylation, since the control sample was incubated under identical conditions (except for the absence of the enzyme). Thus, dephosphorylation of Thr-124 by acid phosphatase (Fig. 2) caused at least a partial reversion of the stimulatory effect on DNA binding that was observed on the dephosphorylation of phosphoserines by alkaline phosphatase.

General (unspecific) DNA binding. So far we have shown the specific binding of T antigen to sites in the viral genomic control region. Since T antigen is known to be a general DNA-binding protein, we thought it would be interesting to determine whether the state of phosphorylation affects this property of T antigen. To investigate the unspecific DNA binding of T antigen, we used *Hind*III-digested SV40 DNA fragments as described above (Fig. 3), but we employed

FIG. 3. DNA-binding properties of T antigen treated in alkaline buffer with (+) and without (-) alkaline phosphatase (P'tase). Plasmids pSV and pSV(cs1097) were restricted with *Hind*III and 3' end-labeled with [32 P]dATP and the Klenow fragment of DNA polymerase for fill-in synthesis. The DNA fragments were mixed with T antigen under stringent binding conditions. The resulting protein-DNA complexes were immunoprecipitated as described previously (21). The precipitated DNA was extracted with 0.5% SDS and used for gel electrophoresis (7% polyacrylamide, 90 mM Tris borate, 10 mM EDTA [pH 8.3]) and autoradiography. Lane M shows *Hind*III fragments before immunoprecipitation. The fragments have the alphabetical designations described previously (13). The 32 P-labeled DNA fragment carrying SV40 binding site I (BSI) was prepared as described in the text and used for DNA binding. The results of an immunoprecipitation experiment are shown (21).

nonstringent binding conditions (10 mM Tris hydrochloride [pH 6.5], no KCl, no competing DNA) (8). Results of an experiment performed with *Hind*III-restricted SV40 DNA are shown in Fig. 6, in which it is demonstrated that essentially all fragments could be recovered in the immunoprecipitate and, furthermore, that untreated and acid phosphatase-treated T antigen are equally efficient in this reaction. Results of this and several similar experiments with alkaline phosphatase-treated T antigen (data not shown)

FIG. 4. DNA-binding properties of T antigen treated in alkaline buffer with (+) or without (-) alkaline phosphatase (P'tase). SV40 DNA was restricted with endonuclease *Dde*I and 3' end-labeled with [32 P]dCTP as the radioactive nucleotide. Since the *Dde*I recognition site is C/TNAG, only a subset of the 21 *Dde*I fragments was radioactively labeled. The indicated fragments contained the high-affinity binding sites I (BSI) and II (BSII), as described in the text. Lane M shows the labeled DNA fragments before T antigen was added. The lanes marked + and - show the immunoprecipitated DNA fragments. Note that the distance between the BSI- and BSII-carrying fragments in these lanes is somewhat larger than their distances in the marker lane. This is due to the presence of proteins which migrate in SDS-polyacrylamide gels like DNA fragments of ca. 300 base pairs.

FIG. 5. DNA-binding properties of T antigen treated in acid buffer with (+) and without (-) acid phosphatase (P'tase). *Hind*III-restricted and 32 P end-labeled pSV(cs1097) was used in this experiment, which was performed exactly as described in the legends to Fig. 3 and 4.

demonstrated that dephosphorylation has no effect on the general unspecific DNA-binding activity of T antigen (1).

DNA helicase activity of dephosphorylated T antigen. The DNA helicase activity of alkaline dephosphorylated T antigen was assayed by using as substrate circular M13 mp8 DNA that carried a hydrogen-bound, radioactively labeled complementary strand (34; Wiekowski et al., in press). In the presence of ATP, T antigen unwound the double-stranded region of the substrate, releasing the radioactive complementary strand, as identified by gel electrophoresis and autoradiography (34). We found that alkaline dephosphorylation had no effect on the extent or the kinetics of the unwinding reaction (Fig. 7). The data obtained with acid phosphatase-treated T antigen were identical to those shown in Fig. 7 for T antigen dephosphorylated with alkaline phosphatase (data not shown).

DISCUSSION

Alkaline phosphatase preferentially dephosphorylated the phosphoserine residues of large T antigen. Peptide mapping of proteolytically cleaved, 32 P-labeled T antigen showed that all peptides carrying phosphoserines are dephosphorylated after extensive treatment with alkaline phosphatase. An

FIG. 6. The general (unspecific) DNA-binding properties of dephosphorylated T antigen. Shown here is an experiment with T antigen treated with acid phosphatase (P'tase) (1, 2, and 5 units, as indicated). *Hind*III-restricted, 32 P-labeled SV40 DNA was used for DNA binding under nonstringent conditions. Immunoprecipitation was performed as described previously (21).

FIG. 7. DNA helicase activity of T antigen. The substrate (native) used was M13 mp8 DNA carrying a 300-nucleotide-long, ^{32}P -labeled complementary strand (34; Wiekowski et al., in press) which could be identified after heat denaturation of the substrate (denat.). (a) In the presence of 0.05 μg of T antigen, untreated (–) or dephosphorylated (+), the labeled complementary strand was displaced in a reaction depending on magnesium ions and ATP hydrolysis, as described previously (34). After 20 min of incubation, the DNA was extracted with 1% SDS–50 mM EDTA and investigated by polyacrylamide gel electrophoresis. (b) Kinetics of unwinding. T antigen treated in alkaline buffer with (+) and without (–) phosphatase (P'tase) was incubated with the M13 mp8 substrate at 37°C for the indicated times. DNA was extracted and analyzed as described above.

exception was the peptide containing Ser-639, which was not fully dephosphorylated. In contrast, the radioactivity recovered in peptides with Thr-124 and Thr-701 did not change during alkaline dephosphorylation, indicating that the two phosphothreonines are completely resistant to alkaline phosphatase. Acid dephosphorylation was more complete since the phosphate on Thr-124 was also removed, but the phosphothreonine at residue 701 survived an incubation with acid phosphatase. We cannot say whether the differential effects of phosphatases on T antigen reflects intrinsic specificities of the enzymes or conformational changes induced in T antigen due to the different pH values of the reaction buffers. In any case, it became clear that treatment with different phosphatases reduces the phosphate content of T antigen in a well-defined way. It is therefore possible to compare T antigen that is dephosphorylated *in vitro* with the forms of T antigen found in infected cells.

Fischer-Fantuzzi et al. (10) noted that the T antigen produced by a nuclear transport mutant of SV40 is mainly phosphorylated on residues Thr-124 and Thr-701, and concluded that phosphorylation of these amino acids is catalyzed by a cytoplasmic protein kinase. The additional phosphorylation of serines occurs later in the nucleus and is done by one of the various nuclear protein kinases (36). Furthermore, the phosphorylation state of T antigen correlates with its ability to specifically interact with SV40 DNA because newly synthesized ("young") T antigen, phosphorylated at the two threonine residues, has a higher affinity for SV40 DNA than fully phosphorylated ("old") T antigen (23, 29).

It appears now that T antigen treated with alkaline phosphatase resembles young T antigen. In both cases, amino acid residues Thr-124 and Thr-701 were fully phosphorylated, whereas most of the serine phosphorylation sites were

not, and furthermore, T antigen that was dephosphorylated by alkaline phosphatase bound with a higher affinity to the SV40 control region, just like young T antigen.

The earlier *in vivo* (10, 23, 29) and the present *in vitro* data suggest an order of events with regard to the function of T antigen in replication. According to this scheme, newly synthesized T antigen is phosphorylated in the cytoplasm at threonine residues 124 and 701 and is transported into the nucleus, where it is directed specifically to the binding site at the SV40 origin. T antigen unwinds the origin (5, 42) as a first step to establishing replication forks. T antigen remains on replicating DNA molecules (35), serving as a DNA helicase to separate parental DNA strands (40).

Since the state of amino acid phosphorylation does not affect the helicase activity, the fact that the phosphorylation of serines occurs on DNA-bound T antigen cannot be excluded. It may be more likely, however, that the serine residues are phosphorylated at the end of the replication cycle. In any case, fully phosphorylated T antigen is unlikely to initiate new replication rounds because its affinity to the origin-binding site is greatly reduced. Instead, T antigen accumulates in the cell in a highly phosphorylated aggregated (probably tetrameric) form (9). The function of the aggregated T antigen is unknown. It may well be a reservoir of T antigen which can be activated and reused for DNA replication after dephosphorylation. The aggregated form of T antigen may also be important for the regulation of viral and cellular gene expression.

How could phosphorylation influence the DNA-binding activity of T antigen? It has been noted previously (19, 22, 24, 28, 29) that neither the N-terminal nor the C-terminal cluster of phosphorylation sites overlaps with the DNA-binding region of T antigen, which begins at amino acid residue 139 (24) and may reach as far as amino acid 371 (33). Thus, direct effects of phosphate groups on protein-DNA interactions appear to be unlikely. It is more likely that phosphorylation induces conformational changes, the nature of which must still be examined.

Work with SV40 mutants suggests that the N-terminal section of T antigen plays an important role in the stabilization of the tertiary structure of the proteins (4). Moreover, several monoclonal antibodies reacting with the extreme N terminus have been shown to partially inhibit the DNA helicase activity of T antigen (40), even though the DNA helicase-ATPase domain is probably much closer to the C-terminal than to the N-terminal side of the protein (25).

The importance of the N-terminal section for the conformation of T antigen may be the reason that dephosphorylation studies with the T antigen-related D2 protein gave results (1) that differed from those reported here. The D2 protein, a product of an SV40-adenovirus hybrid, shares its C-terminal 594 amino acids, including the domains for specific DNA binding and for DNA helicase-ATPase activity, with authentic T antigen (2). Most of the 122 N-terminal amino acids of D2 protein are derived from an adenovirus protein and are not related to those in the N-terminal section of T antigen. Alkaline dephosphorylation of the D2 protein had no effect on specific DNA binding, but dephosphorylation with acid phosphatase caused a drastic reduction of specific DNA binding (although the general DNA-binding activity of the D2 protein was not affected). The only remaining phosphoamino acid in the D2 protein treated with acid phosphatase was Thr-701 (unpublished data), as was the case with authentic T antigen (Fig. 2). The loss of specific DNA-binding activity of the D2 protein after treatment with acid phosphatase may be due to the effects that the foreign

N-terminal region exerts on the overall conformation of the protein.

The results described here for authentic T antigen suggest that Thr-124 plays an important role in the regulation of the function of T antigen in DNA replication. When Thr-124 was not phosphorylated, such as when T antigen was treated with acid phosphatase, we detected no particularly high affinity to binding site II in the SV40 origin. However, T antigen efficiently bound to the origin site when Thr-124 was phosphorylated. The increased affinity for site II was reversed when the adjacent serine residues were also phosphorylated.

The proposed regulatory role of Thr-124 phosphorylation is consistent with the behavior of the nucleotide exchange mutants studied by Kalderon and Smith (16) and Paucha et al. (24). These investigators found that a mutation in the T-antigen gene leading to a replacement of Thr-124 by Ile destroyed the replication function of the mutant protein (D29 mutant) (16), although its specific binding to SV40 DNA remained intact (24). In the study of Paucha et al. (24), no distinction was made between an association of the D29 mutant T antigen to binding site I or to the origin-binding site II. We expect that the Thr-to-Ile mutant lost its ability to bind to site II and is therefore replication negative. Incidentally, an exchange of adjacent Ser-123 to Phe reduces but it did not completely abolish SV40 DNA replication (24). In contrast, replacement of Ser-106 and Ser-112 had no effect on either specific DNA binding or on DNA replication (16, 24), suggesting that the function of phosphorylation at Ser-106 and Ser-112 appears to be the downregulation of the replicative function of T antigen.

In summary, we conclude that the phosphorylation of amino acids in the N-terminal cluster of the phosphorylation sites plays an important role in the regulation of the function of T antigen in DNA replication or, more specifically, in directing T antigen to the origin of replication.

While this report was under revision, we saw the article by Grässer et al. (12), who showed, in agreement with the data presented here, that alkaline phosphatase dephosphorylates the phosphoserines but not the phosphothreonines of T antigen. They also showed that T antigen treated with alkaline phosphatase stimulates SV40 DNA replication in vitro. However, Grässer et al. (12) did not observe an effect of dephosphorylation on the specific DNA binding of T antigen. The discrepancy between their data and the results reported here cannot be explained at present.

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