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Phosphorylation Sites in Polyomavirus Large T Antigen That Regulate Its Function in Viral, but Not Cellular, DNA Synthesis

ALAKANANDA CHATTERJEE, BEVERLY J. BOCKUS,† OLE V. GJØRUP,‡ AND BRIAN S. SCHAFFHAUSEN*

Department of Biochemistry, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, Massachusetts 02111

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Polyomavirus large T antigen (large T) is a highly phosphorylated protein that can be separated by proteolysis into two domains that have independent function. A cluster of phosphorylation sites was found in the protease-sensitive region connecting the N-terminal and C-terminal domains. Edman degradation of ^{32}P-labeled protein identified serines 267, 271, and 274 and threonine 278 as sites of phosphorylation. Analysis of site-directed mutants confirmed directly that residues 271, 274, and 278 were phosphorylated. Threonine 278, shown here to be phosphorylated by cyclin/cyclin-dependent kinase activity, is required for viral DNA replication in either the full-length large T or C-terminal domain context. The serine phosphorylations are unimportant in the C-terminal domain context even though their mutations activates viral DNA replication in full-length large T. This finding suggests that these sites may function in relating the two domains to each other. Although the phosphorylation sites were involved in viral DNA replication, none was important for the ability of large T to drive cellular DNA replication as measured by bromodeoxyuridine incorporation, and they did not affect large T interactions with the Rb tumor suppressor family.

Large T antigen (large T) of murine polyomavirus (Py) is multifunctional. Its most obvious role is to initiate viral DNA replication (7). Large T also stimulates host cell DNA synthesis (9, 35, 42) and transactivates a variety of cellular promoters (17, 19, 20, 24, 34, 35). Such effects on the host cell are reflected in large T's ability to immortalize primary cells (37) and to inhibit differentiation of myoblasts (28). Both of these effects depend on interactions with members of the Rb tumor suppressor family (8, 23, 27).

The functions of Py large T are assorted to different structural domains within its 785-amino-acid sequence (Fig. 1). Proteolysis experiments point to a division between an N-terminal domain (NT) and a C-terminal domain (CT) around residue 260 (14). NT is sufficient for immortalization by Py large T (14) and for the stimulation of cell cycle progression in serum-starved cells (9). It contains a binding motif for Rb family proteins and a DnaJ domain that provides a binding site for hsc70 (43a). CT is sufficient for viral DNA replication in growing cells (9) and contains the DNA binding domain, zinc finger, and ATP binding region. Py large T is localized to the nucleus and contains two independent nuclear localization signals (16, 38).

It has long been known that Py large T is highly phosphorylated (40). Most of the phosphorylation occurs after large T is translocated to the nucleus (16). There is ample reason to suspect that the phosphorylation may be functionally significant. Both correlation and direct evidence suggest a role for large T phosphorylation in DNA replication. A slower-migrating form resulting from phosphorylation (2) predominates after the initiation of viral DNA replication, while a faster-migrating form is seen early in infection. A number of mutants defective in DNA replication also show altered patterns of phosphorylation (2, 39). Wang and colleagues have shown that phosphatase treatment of large T affects its activity in DNA replication assays in vitro (49).

Although phosphorylation of large T has attracted considerably attention, the sites have not been identified. Previous work showed substantial phosphorylation in two regions, one in NT and one more C terminal, near the junction of the two domains (1). Work by Hassauer et al. (12) implicated residues 81 and 187 as sites of phosphorylation in NT. The work presented here identifies the more C-terminal phosphorylations and tests their function. These phosphorylations appear important in large T function in initiating viral, but not cellular, DNA replication.

MATERIALS AND METHODS

Cells, viruses, and plasmids. NIH 3T3 and 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% calf serum (Hyclone) (3T3 cells) or with 10% fetal calf serum (293 cells). Transfections were performed by the calcium phosphate precipitation method of Chen and Okayama (6). Cells were harvested after 48 h for transient assays. Stable 3T3 cell lines expressing wild-type and mutant large T's were selected with geneticin as described previously (14).

Spodoptera frugiperda Sf9 cells were maintained at 27°C in Grace's insect cell culture medium (GIBCO) supplemented with 10% fetal calf serum, Yeastolate, and lactalbumin hydrolysate. The Sf9 cells were infected with the recombinant baculoviruses and used 48 h after infection.

Baculovirus-infected cells expressing a glutathione *S*-transferase (GST)–CT fusion was used for Edman degradation. Fusion to GST was done with pGEX 3X (Pharmacia) as a parent vector. CT of Py large T (residues 264 to 785) was cloned into the *Bam*HI site of pGEX 3X as a *Bam*HI/*Bcl*I fragment to generate pGEX 3X-CT. The baculovirus vector bGST-CT was constructed by PCR amplification using pGEX 3X-CT as the template, digesting the product with *Nhe*I/*Bam*HI, and cloning it into the baculovirus transfer vector pVL1392 which has been digested with *Xba*I and *Bam*HI. The resulting transfer vector was cotransfected with BaculoGold genomic DNA (Pharmingen) to yield the recombinant baculovirus expressing GST-CT. The recombinant baculoviruses expressing p34^{cdc2} kinase and GST-cyclin B were a gift from H. Piwnica-Worms.

Wild-type pCMV-LT and pCMV-CT have been described previously (9). Site-directed mutagenesis to obtain mutants in the phosphorylation sites was carried out on pCMV-LT by PCR, using the site overlap extension technique. 5′-GCGCGCGCTAGCTGATCATGGATAGAGTTCTGAGCAGAG-3′ and

* Corresponding author. Mailing address: Department of Biochemistry, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6876. Fax: (617) 636-6409. E-mail: bschaffh_pol@opal.tufts.edu.

† Present address: Genzyme Corporation, Framingham, MA 01701.

‡ Present address: University of Copenhagen, 1353 Copenhagen K, Denmark.

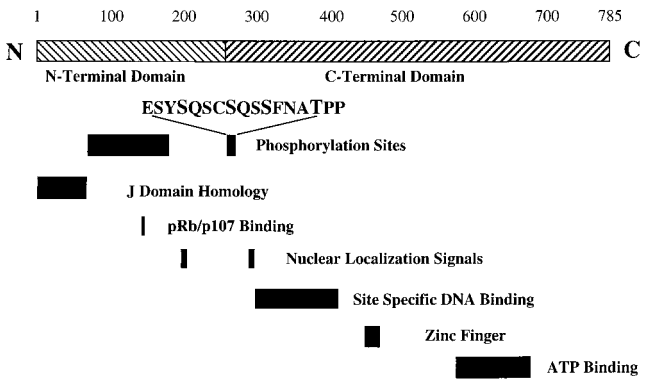

FIG. 1. Schematic diagram of Py large T indicating the structural domains and functional motifs. The amino acids in boldface indicate the sites of phosphorylation identified in this study.

5′-GGAAGCGCTAGCATCCGGGATCCGGGGGACCCTGATATGACGC GC-3′ (LT6) were used as outside primers. Conversion of T278 to alanine, glutamate, and aspartate was done by using oligonucleotides 5′-TCAATGCA GCGCCACCTAA-3′, 5′-TCAATGCAGAGCCACCTAA-3′, and 5′-TCAAT GCAGACCCACCTAA-3′, respectively, and a common antisense primer, 5′-AG GCATTTGAATTCGGGCCTGAAC-3′. A triple mutant converting serines 267, 271, and 274 to alanine was made by PCR using a double mutant at serines 271 and 274 as the template, plus oligonucleotide 5′-GAGAGCTACGCACAGA GCTGC-3′ and its complement. A quadruple mutant converting serines 267, 271, 274, and threonine 278 to alanine was made by PCR using the triple mutant as the template and oligonucleotide 5′-TCAATGCAGCGCCACCTAA-3′. The PCR products were digested with *Esp*I and *Nsi*I, and the fragment containing the mutations was recovered and cloned into pCMV-LT digested with *Esp*I and *Nsi*I. The N-terminal mutagenic oligonucleotide 5′-GGGCTAGCGGATCCATCAT GGAGAGCTACGCACAGAGCTGCGCTCAGAGGCATTCAATGCAACG-3′ was used to convert serines 267, 271, and 274 to alanines in CT, while the single mutant at threonine 278 was constructed by using pCMV-LT T278A as the template and the N-terminal oligonucleotide 5′-CGATCGGCTAGCTGATCAT GGAGAGCTACTCACAGAGCTGCTCTCAG-3′. LT6 was used as the C-terminal oligonucleotide.

pUC-ori contains the Py origin of replication from the *Bcl*I site at nucleotide 5024 to the *Sph*I site at nucleotide 163, cloned into the *Eco*RI site of pUC12. Plasmid pA10-E2F-CAT (gift of A. Yee) consisted of the −85 to −30 adenovirus E2 promoter sequence and a minimal simian virus 40 (SV40) promoter fused to a chloramphenicolacetyltransferase (CAT) gene (26).

Cells, transfections, metabolic labeling, and protein analysis. Labeling, extraction, and immunoprecipitation of Py large T have been previously described (1). Briefly, mammalian or insect cell proteins were labeled metabolically with carrier free [^{32}P]orthophosphate (Dupont-NEN) in phosphate-free DMEM or RPMI. For labeling insect cells, the pH of the phosphate-free medium was reduced to 6.1. Cells were labeled with 500 to 1,000 μCi per ml in a 100-mm-diameter dish for 3 h. Large T was extracted in T extraction buffer (137 mM NaCl, 10 mM Tris-Cl [pH 9.0], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40) supplemented with 20 mM NaF for 20 min on ice. Py large T was immunoprecipitated with a polyclonal antiserum and protein-A Sepharose (Pharmacia). After immunoprecipitation, the samples were subjected to chemical and enzymatic digestion and analyzed on discontinuous buffer sodium dodecyl sulfate (SDS)-gels (22).

Procedures for the mapping of Py large T with hydroxylamine or *Staphylococcus aureus* V8 protease have been described previously (1). Hydroxylamine cleavage of Py large T was done by treating immunoprecipitates with 2 M hydroxylamine in 0.2 M K_2CO_3 (pH 9.0) for 3 h at 45°C. The hydroxylamine fragments of Py large T remain attached to the beads. After digestion, the beads were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide cylindrical gels. The cylindrical gel was then placed horizontally onto a 15% polyacrylamide gel, overlaid with 2 ml of enzyme solution (125 mM Tris-Cl [pH 6.8], 10 mM EDTA, 20% [vol/vol] glycerol, 0.015% [wt/vol] bromophenol blue, 100 μg of *S. aureus* V8 protease per ml), and electrophoresed at 50 V. The gels were dried, and the maps were visualized on Kodak XAR5 film or a PhosphorImager with ImageQuant software (Molecular Dynamics).

Western analysis was performed by SDS-PAGE followed by blotting onto nitrocellulose (46). The blot was then blocked in Tris-buffered saline–Tween (50 mM Tris-Cl [pH 7.5], 0.15 M NaCl, 0.05% [vol/vol] Tween 20) containing 5% (wt/vol) dried milk (Carnation) for 1 h at room temperature. The blot was then incubated with 1:50 anti-large T monoclonal antibody PN-116 followed by 1:10,000 horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham). Protein was detected by enhanced chemiluminescence.

Edman degradation. Edman degradation was performed to localize phosphoamino acids in CT by the method of Sullivan and Wong (45). Briefly, labeled CT (released with factor Xa) was subjected to SDS-PAGE. The band corresponding to CT was excised, and CT was eluted in 0.1% SDS–100 mM sodium acetate (pH 8.5) by incubation for 16 h at 37°C. The eluate was dried, resuspended in 30 μl of 50% acetonitrile, and spotted onto a 1,4-phenylene diisothiocyanate-Sequelon disc (Millipore). Covalent linkage was accomplished by adding *N*-methylmorpholine for 5 min at 50°C. After 30 min at room temperature, the disc was washed extensively with water and then extracted five times with trifluoroacetic acid (TFA) to remove unbound peptide. The disc was then extracted with methanol and subjected to Edman degradation. Edman degradation of the immobilized peptide was carried out by the following cycles of degradation: incubation with 0.5 ml of coupling reagent (methanol-water-triethylamine-phenylisothiocyanate [7:1:1:1, vol/vol]) for 10 min at 50°C (step 1), five washes with 1 ml of methanol (step 2), drying under vacuum for 5 min (step 3), incubation with 0.5 ml of TFA for 6 min at 50°C (step 4), extraction with 1 ml of TFA–42.5% phosphoric acid (9:1, vol/vol) (step 5), and finally Cerenkov counting of the disc and of washes from steps 4 and 5. At this stage, the disc was either stored in methanol at −20°C or washed six times with 1 ml of methanol before the next cycle was started.

In vitro kinase reactions. p34^{cdc2} and GST-cyclin B were produced in Sf9 cells. The active kinase complex was precipitated on glutathione (GSH) beads and washed with cdc2 kinase buffer (20 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$). The kinase complex was then incubated with factor Xa-cleaved GST-CT along with 50 μCi of [γ-^{32}P]ATP in 200 μl of kinase buffer for 15 min at room temperature. The reaction product was then subjected to SDS-PAGE and Edman degradation.

Replication assays. Replication assays were carried out as modifications of published procedures (9, 36, 50). NIH 3T3 cells were plated at 4×10^5 to 5×10^5 cells per 60-mm-diameter dish; 12 to 24 h later, the cells were cotransfected with a large T or CT expression plasmid and pUCori with a total of 10 μg of DNA per 60-mm-diameter dish. The total amount of transfected expression plasmid was normalized by the addition of pCMV vector without coding sequence. At 48 to 72 h after transfection, low-molecular-weight DNA was isolated by a modified Hirt procedure (13). After digestion with *Dpn*I and *Hin*cII, the DNA obtained from 1/6 to 1/20 of a 60-mm-diameter dish was examined by Southern blotting. The DNA fragments were separated on a 1.2% agarose gel in 0.5× Tris-borate-EDTA buffer, transferred to a nylon membrane by capillary blotting overnight, and probed with a ^{32}P-labeled random hexamer-primed 454-bp *Eco*RI restriction fragment from pUCori containing the entire Py origin. The washed blots were used to expose Molecular Dynamics PhosphorImager screens.

Measurement of cellular DNA synthesis. Cellular DNA synthesis was determined as described previously (9). NIH 3T3 cells were plated on coverslips and transfected at 15 to 25% confluence; then 200 μl of calcium phosphate precipitate (10 μg of cytomegalovirus [CMV]-based pCMV-LT/ml) was added to 2 ml of medium in a 30-mm-diameter dish containing coverslips. The precipitate was left on the cells for 5 h, after which the cells were washed twice with phosphate-buffered saline and fed with DMEM containing 0.2% calf serum. Approximately 40 h after transfection, the cells were labeled with 100 mM bromodeoxyuridine (BrdU) for an additional 8 h. The cells were then fixed in 3.7% formaldehyde and permeabilized with methanol-acetone (3:7) for 10 min at −20°C. Cells were simultaneously stained for Py large T with a polyclonal anti-T serum and for BrdU with monoclonal antibody BU-1 (Amersham). The cells were then stained with a mixture of anti-rabbit fluorescein isothiocyanate-conjugated and anti-mouse tetramethylrhodamine isothiocyanate-conjugated secondary antibodies. Nuclear fluorescence was visualized with a Zeiss microscope.

CAT assays. NIH 3T3 cells in 100-mm-diameter dishes were transfected at 15 to 25% confluence with 5 μg of expression plasmid pCMV-LT and 5 μg of pA10-E2F-CAT. Cells were harvested 48 h posttransfection. CAT activity was measured by standard chromatographic techniques (10). Thin-layer plates were quantitated with ImageQuant software (Molecular Dynamics) to determine the percentage of acetylated [^{14}C]chloramphenicol.

RESULTS

Identification of phosphorylation sites in CT. Previous work had indicated that there was considerable phosphorylation between residues 257 and 285 of Py large T. This phosphorylation was reflected in two V8 phosphopeptides, 5 and 7 (1). Characterization of the large T variant LT 97 (24), which is deleted of residues 271 to 280, narrowed the region of interest further. Figure 2 shows partial V8 maps of wild-type and LT 97 large T labeled in vivo with $^{32}PO_4$. It is quite clear that V8 peptides 5 and 7, the peptides from the 257-to-285 region, are absent.

Most of this region is contained in CT, starting at residue 264. CT is fully functional in DNA replication in growing cells (9), so studies concentrated on these sequences. Baculovirus vectors were used to obtain enough material for N-terminal amino acid sequencing. The patterns of phosphorylation in

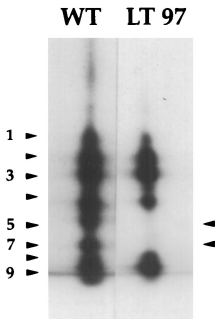

FIG. 2. *S. aureus* V8 protease maps of Py large T. NIH 3T3 cell lines were labeled with $^{32}PO_4$, and the mutant and wild-type Py large T's were immunoprecipitated and electrophoresed on 7.5% polyacrylamide cylinders. The cylinders were then placed horizontally on top of a 10% acrylamide slab gel and electrophoresed in the presence of 200 μg of V8 protease. WT refers to wild-type Py large T; LT 97 refers to mutant that is deleted of residues 271 to 280 of Py large T. The numbers and positions of the V8 phosphopeptides are shown on the left.

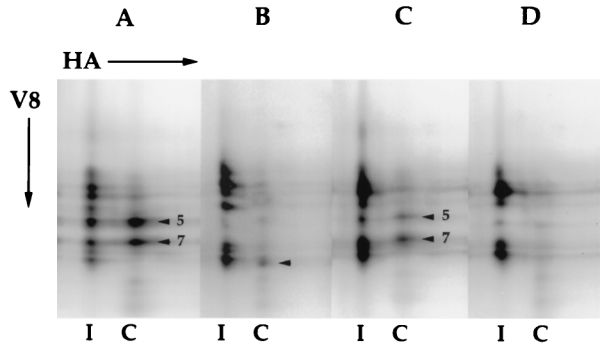

FIG. 4. Two-dimensional phosphopeptide mapping of large T phosphorylation mutants. 293 cells were transfected with Py large T expression vectors and labeled with $^{32}PO_4$. The mutant and wild-type Py large Ts were immunoprecipitated, treated with hydroxylamine (HA), and electrophoresed on 7.5% polyacrylamide cylinders in the direction indicated by the arrow labeled HA. The cylinders were then placed horizontally on top of 15% acrylamide slab gels and electrophoresed in the presence of 200 μg of V8 protease. (A) Wild-type Py large T; (B) triple mutant S267A,S271A,S274A; (C) single mutant T278A; (D) quadruple mutant S267A,S271A,S274A,T278A I, intact Py large T that was not cleaved by hydroxylamine; C, the C-terminal hydroxylamine fragment. The positions of V8 phosphopeptides 5 and 7 are indicated.

insect cell systems for SV40 large T are qualitatively similar to those in mammalian systems (15), and the same is true for Py (not shown). Further, baculovirus-expressed Py large T is functional in in vitro viral DNA replication (49). For ease of purification, a baculovirus-expressed GST-CT fusion including amino acids 264 to 785 of Py large T was used. After in vivo labeling of infected Sf9 cells with $^{32}PO_4$, the fusion protein was collected on GSH beads. CT was then released from the GST by cleavage with factor Xa. After separation by SDS-PAGE, CT was eluted from the gel and subjected to manual Edman degradation (45). Application of the manual degradation procedure resulted in four peaks of phosphate release, at turns 6, 10, 13, and 17 (Fig. 3). These peaks correspond to the serine residues 267, 271, and 274 and threonine 278 in Py large T. The threonine phosphorylation peak was the highest even though its yield might have been expected to be lower since it came at a later sequencing turn. While this finding suggests greater phosphorylation at threonine 278 relative to the serines, it is not possible to draw firm conclusions about relative stoichiometry. The extent of labeling could depend on the rate of turnover at individual sites. In any case, quantitative differences exist in SV40 large T phosphorylation in Sf9 cells compared to monkey cells (15). This is likely to be true for Py as well.

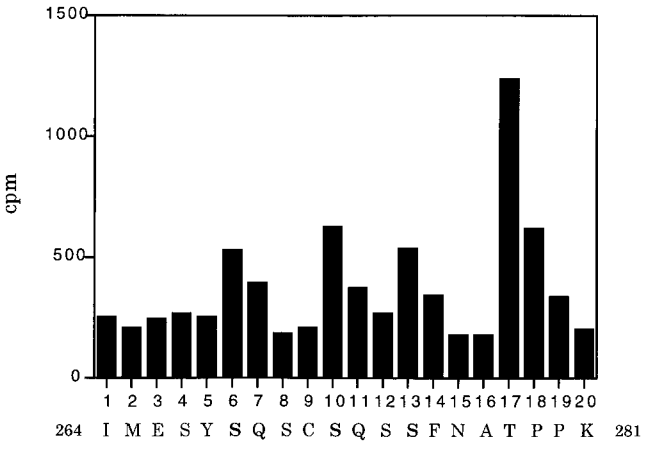

FIG. 3. Edman degradation of phosphorylated CT produced in Sf9 cells. The ^{32}P released by each turn of Edman degradation was measured by Cerenkov counting. Each cycle of Edman degradation is shown, with the corresponding Py large T sequence indicated underneath. The first two amino acids indicated do not belong to the Py sequence but occur as a result of the in-frame GST fusion. The four sites of phosphorylation are indicated in boldface.

Mutation of the phosphorylation sites. To assess the phosphorylation sites identified in the baculovirus system, a series of mutations were created both at individual sites and at sets of sites (triple serine mutant S267A/S271A/S274A and quadruple mutant S267A/S271A/S274A/T278A). The threonine at 278 was changed to alanine, asparagine, glutamate, and aspartate. After transient transfection, both single- and multiple-site mutants were generally expressed at levels similar to the wild-type level as measured by Western blotting (not shown). The one exception, single mutant S267A, was expressed at a level fivefold lower than the wild-type level.

To test the effects of the mutations on phosphorylation, large T labeled in vivo with $^{32}PO_4$ was analyzed first by partial proteolysis with *S. aureus* V8 protease. V8 gives a series of phosphopeptides (1), including peptides 5 and 7, that comes from the more C-terminal cluster. No single mutation at 267, 271, 274, or 278 abolished labeling of these peptides. However, a double mutant lacking S271 and S274 did not shown peptides 5 and 7 (not shown). This was unexpected in view of the baculovirus results showing phosphorylation at 267 and 278 and suggested that the assay lacked sufficient resolution. Therefore, double digestion of Py large T with hydroxylamine and *S. aureus* V8 protease was carried out. Hydroxylamine cleavage separates large T into two fragments (residues 1 to 210 and 211 to 785) (1). This makes it possible to examine the C-terminal phosphorylations without the N-terminal phosphopeptide background (Fig. 4). The wild-type V8-NH_2OH phosphopeptide pattern of large T (Fig. 4A) shows that peptides 5 and 7 arose from the C-terminal hydroxylamine fragment. Mutation of threonine 278 left a pattern of V8 cleavage that resembled the wild-type pattern qualitatively (Fig. 4C). Mutation of the three serines (Fig. 4B) resulted in loss of peptides 5 and 7, consistent with the result seen in the one-dimensional V8 map. However, a new phosphopeptide with a mobility similar to that of N-terminal V8 peptide 9 (Fig. 4B, arrowhead) was now resolved. This new phosphopeptide corresponded to the phosphorylation at threonine 278, since it disappeared when T278 was next mutated to alanine to give

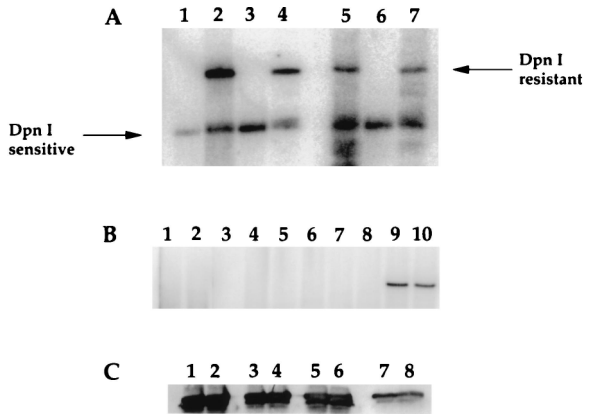

FIG. 5. *In vivo* replication assays of Py large T phosphorylation mutants. Southern blots show low-molecular-weight DNA from 3T3 cells transfected with Py origin plasmid (pUCori) and Py large T expression vectors. Extracted DNA was cut with *Hin*cII and *Dpn*I. The blot was probed with ^{32}P-labeled Py origin DNA (bp 5024 to 163). (A) Lane 1 is a control with pUCori and the CMV expression vector without Py large T coding sequences. All the other lanes are from cells transfected with pUCori plus S267A,S271A,S274A large T (lane 2), T278A large T (lane 3), wild-type large T (lane 4), S267A,S271A,S274A CT (lane 5), T278A CT (lane 6), and wild-type CT (lane 6). The positions of the *Dpn*I-sensitive and -resistant bands are indicated. (B) In vivo replication assay of mutants at T278. Cells were transfected with pUCori plus the CMV expression vector without large T coding sequences (lanes 1 and 2), T278A large T (lanes 3 and 4), T278E large T (lanes 5 and 6), T278D large T (lanes 7 and 8), and wild-type T (lanes 9 and 10). (C) Western blot showing the amount of protein expressed in the replication assay in panel B. Cells were transfected with pUCori plus T278A large T (lanes 1 and 2), T278E large T (lanes 3 and 4), T278D large T (lanes 5 and 6), and wild-type large T (lanes 7 and 8).

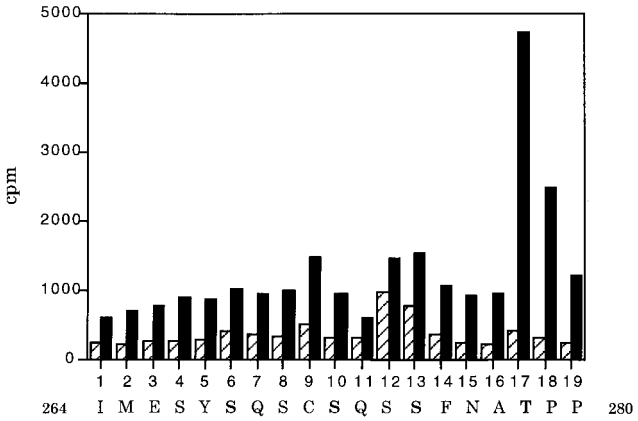

FIG. 6. Edman degradation of CT produced in Sf9 cells and phosphorylated in vitro by GST-cyclin B and p34^{cdc2}; hatched bars represent CT phosphorylated by GST-cyclin B alone. Each cycle of Edman degradation is shown, with the corresponding Py large T sequence indicated underneath. The first two amino acids indicated do not belong to the Py sequence but occur as a result of the in-frame GST fusion. The four sites of phosphorylation identified in Fig. 2 are indicated in boldface.

the quadruple mutant (Fig. 4D). There are at least two explanations for the difference in mobility of the 278-containing peptide in the triple serine mutant and in the wild type. The most likely possibility is that the mutations altered the site of partial proteolysis, leading to a different fragment size. Another possibility is that the mobility of the phosphopeptide depends on serine phosphorylation. Since the wild type does not show peptides 5 and 7 as well as the new phosphopeptide, phosphorylation would have to be functionally processive, with phosphorylation of 278 leading to rapid phosphorylation on serine.

Taken together, these results showed phosphorylation of wild-type large T on threonine 278 and serines 271 and 274. The phosphorylation of serine 267 could be neither directly confirmed nor ruled out in this mapping.

Mutant analysis demonstrates that T278 is critical for viral DNA replication. The next issue is the importance of the phosphorylation to large T function. Since there is already evidence connecting large T phosphorylation to viral DNA replication (2, 9, 39, 49), in vivo assays of Py DNA replication were performed. Large T expression vectors with full-length or C-terminal large T constructs were cotransfected with a plasmid containing the Py origin of DNA replication. After 48 h, cells were extracted and replication was measured by conversion of input *Dpn*I-sensitive DNA to replicated *Dpn*I-resistant DNA. Figure 5A shows that mutation of T278 to alanine (or asparagine [not shown]) led to large T that was inactive in DNA replication (compare lane 3 with lane 4). The same result was obtained in a C-terminal domain construct (lane 6). By contrast, large T mutants lacking S267, S271, and S274 were replication competent as either full-length or CT constructs (Fig. 5A, lanes 2 and 5).

Since phosphorylation can sometimes be mimicked by replacement with a negative charge (43), T278D and T278E were constructed. These two mutants were no more active than the alanine mutant in DNA replication (Fig. 5B). All three mutants were essentially inactive, although Western blotting (Fig. 5C) shows protein levels even higher than the wild-type level.

Replication assays were also performed after cotransfection of wild-type and T278A large T (not shown). There was no indication of a substantial dominant negative effect that might have been expected if each subunit of the large T hexamer needed to be wild type.

Threonine 278 is an in vitro site for cdc2 kinase. Threonine 278 lies in the sequence (S/T)PX(R/K), which is a consensus sequence for the cyclin/cyclin-dependent kinase (cdk) family of kinases (18). Also, this sequence of Py large T (TPPKK) is identical to the SV40 large T sequence starting at T124; T124 is an in vitro site for p34^{cdc2} (29). To test the Py 278 sequence, CT was tested as a substrate for cyclin B and p34^{cdc2}. CT generated from the baculovirus GST-CT construct was used in an in vitro kinase experiment using baculovirus-expressed GST-cyclin B and p34^{cdc2}. After labeling, CT was subjected to manual Edman degradation. As a control for endogenous phosphorylation, CT was phosphorylated with GSH beads incubated with cyclin B alone (Fig. 6). While none of the serines identified previously were phosphorylated by cyclin B and p34^{cdc2}, T278 was highly phosphorylated. Thus, threonine 278 of Py large T is a site of phosphorylation by the cyclin/cdk family of kinases.

Mutations of S267, S271, and S274 activate DNA replication. As shown above, even the Py large T mutant at the three serine phosphorylation sites is able to mediate viral DNA replication. In fact, from Fig. 5A, mutation of the serines appeared to stimulate viral DNA replication. Therefore, we compared the dose responses of the triple serine mutant and wild-type Py large T (Fig. 7A). The results indicated that the triple serine mutant could stimulate as much as fivefold with respect to viral DNA replication. Mixtures of the triple serine mutant and wild type gave the higher level of replication. These results are in one sense different from the result for SV40. Mutation of serines 120 and 123 results in loss of DNA replication (43). However, in another sense they are similar. As for Py, removal of phosphate from wild-type SV40 large T is activating (41, 47, 48).

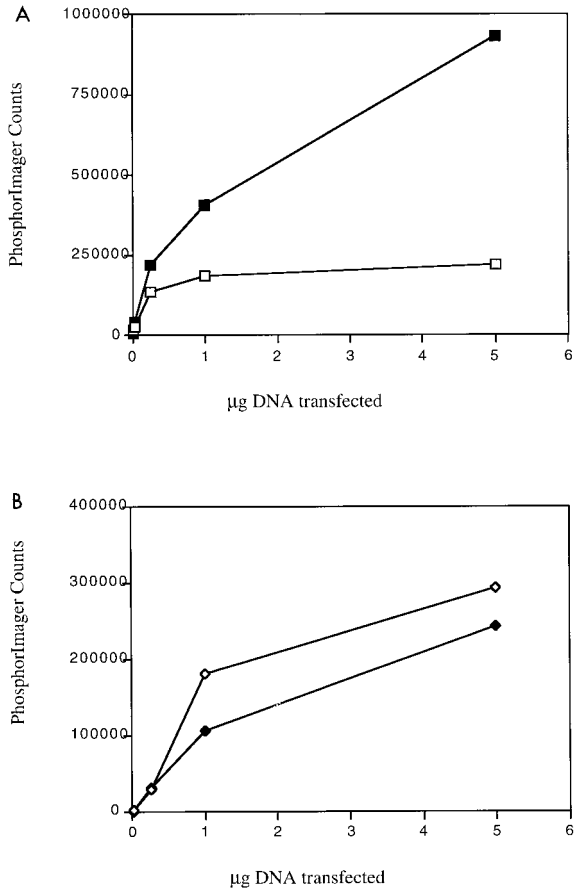

FIG. 7. Dose responses of viral DNA replication by the S267A,S271A,S274A mutant and the wild type. (A) In vivo replication assay done in the large T context. Solid squares represent the S267A,S271A,S274A mutant large T; open squares represent wild-type large T. (B) In vivo replication assay done in the CT context. Solid diamonds represent the S267A,S271A,S274A CT mutant; open diamonds represent wild-type CT. The *y* axis shows replication measured in arbitrary counts assigned by PhosphorImager scans of *Dpn*I-resistant replicated DNA.

Interestingly, the same kind of dose-response experiment done in the context of the serine phosphorylation mutants in the C-terminal background showed no significant difference between mutant and wild type (Fig. 7B). This result implies that the role of these phosphorylations is connected to the relationship between NT and CT.

Mutation of C-terminal serine or threonine phosphorylation sites does not seem to affect large T functions related to cellular DNA replication. Large T not only is essential for viral DNA replication but also stimulates cellular DNA replication (9, 35, 42). Given the importance of T278 to viral DNA replication, it was of interest to determine what, if any, contribution it makes to large T's effect on cell cycle progression. BrdU incorporation was used to measure cellular DNA replication. 3T3 cells were serum starved for 40 h after transfection and then labeled with BrdU for 8 h. Double immunostaining was used to score both expression of large T and DNA replication in individual cells. Table 1 shows that the wild type, T278A, the triple serine mutant, and a quadruple mutant lacking all four sites were capable of efficiently promoting cellular DNA replication.

One important function of large T is to interfere with tumor suppressors of the Rb family. As shown by the Rb^- large T, the growth assay was done in cells where inactivation of Rb family function is necessary for cell cycle progression. This suggests that mutation of these phosphorylation sites did not compromise large T's ability to affect Rb family members. This possibility was confirmed directly by using a CAT reporter assay measuring E2F-mediated transcription. Wild-type large T transactivates the A10 CAT construct in a manner dependent on binding to Rb family members (reference 16 and Table 1). As for cell cycle progression, mutation of the phosphorylation sites had no effect on the ability of large T to transactivate.

TABLE 1. Activities of phosphorylation site mutants

Construct	A10 E2F CAT assay (% conversion)[a]	S-phase induction assay (% BrdU)[b]
pCMV	2	7[c]
pCMV-T278A-LT	45	60
pCMV-S267A,S271,S274A-LT	44	45
pCMV-S267A,S271,S274A,T278A-LT	46	51
pCMV-LT-Rb^-	0	5
pCMV-LT	44	52

[a] Conversion of nonacetylated [^{14}C]chloramphenicol to acetylated forms.
[b] Number of large T-positive cells that were also positive for BrdU.
[c] The BrdU background was measured by the number of BrdU-positive cells per 100 cells.

DISCUSSION

This work characterizes the cluster of phosphorylation sites which are found in the protease-sensitive region connecting NT and CT of Py large T. Edman degradation of labeled protein identified serines 267, 271, and 274 and threonine 278 as sites of phosphorylation. Labeling of mutant large T's confirmed 271, 274, and 278 as sites of phosphorylation. The threonine at 278 is required for viral DNA replication in either the full-length or CT context, suggesting it is formally part of CT. The serine phosphorylations are unimportant for viral DNA replication in the CT context even though their mutation activates full-length large T. This finding suggests that these sites may regulate some aspect of domain-domain communication. None of these sites affect the ability of large T to drive the cell cycle as measured by BrdU incorporation or the ability to affect interactions with the Rb tumor suppressor family. This finding is consistent with the view that these are largely properties of NT.

For both Py and SV40, the regions N terminal to their homologous nuclear localization signals, residues 280 to 286 in Py and 126 to 133 in SV40, are regions of high phosphorylation. The threonine at 278 that we have characterized aligns with the threonine at 124 of SV40 precisely. The alignment of the serines is less clear. In SV40, major sites are found at 120 and 123 (SQHS) compared to the Py sites 271 and 274 (SQSS), which are further from the critical threonine. The phosphorylation of Py residue 267 occurs in a second SQS motif that is not apparent in SV40. Interestingly, the E1 replication protein of bovine papillomavirus is quite similar to those of both Py and SV40, having a sequence (TPVK) analogous to threonine 278 and a proximal serine sequence (SQNS) that could be aligned with either Py or SV40 large T.

Edman degradation, peptide mapping of large Ts phosphorylated in vivo, and analysis of mutants show that threonine 278 is a site of phosphorylation. Mutation at threonine 278 completely abolished the ability of Py large T to mediate viral DNA replication in both full-length and CT constructs. Substitutions

of a negative charge at this site was ineffective in replacing this function. These results support the view that phosphorylation of threonine 278 is absolutely required for Py large T to perform its replication function. As with Py large T, mutation of threonine 124 in SV40 large T abolishes viral DNA replication. The DNA replication defect in the threonine 124 mutant has been shown to lie in the unwinding of the viral origin (30, 32). Recently it was shown that threonine 124 phosphorylation enhances the interaction of the minimal DNA binding domain with the core origin of replication, possibly stimulating double hexamer assembly and thus DNA unwinding (31). It is likely that the DNA replication defect in T278 is similar to that of T124 in SV40 large T. Since this phosphorylation is required in the CT context, it is puzzling that the comparable phosphorylation site, in E1, which has been shown to be phosphorylated, is not required for bovine papillomavirus replication (25).

Threonine 278 is a form of biosensor connecting large T function in DNA replication to the state of the cell cycle. CT is capable of efficient replication in growing cells but not in resting cells (9). It can be complemented by a variety of genes, such as the NT, E1A, E7, or E2F1, that stimulate the cell cycle. This complementation is accompanied by a shift in CT mobility that suggests phosphorylation. Here we have shown that CT containing threonine 278, but not serines 267, 271, and 274, is fully capable of replication. Further, threonine 278 was identified as a substrate for the cell cycle-regulated kinase $p34^{cdc2}$. Threonine 124 in SV40 large T antigen (29) is also a substrate for the cell cycle-regulated kinase $p34^{cdc2}$.

Mutation of serine phosphorylation sites at positions 267, 271, and 274 has a less dramatic effect on DNA replication than the mutation at threonine 278. Further, these mutations result in stimulation, not inhibition, of DNA replication. This observation is likely related to the earlier result that treatment of Py large T with calf intestinal alkaline phosphatase (CIAP) at low levels resulted in a stimulation of viral DNA replication in vitro (49). CIAP preferentially removes phosphates from serines and leaves threonine phosphorylations intact. In that work, Wang and coworkers implicated phosphorylations lying between residues 89 and 110 and 133 and 167 in the stimulation of viral DNA replication. It could well be that phosphorylation in the N terminus as well as at 267, 271, and 274, can also affect DNA replication. A stimulatory effect of phosphatase treatment was also seen in the case of SV40 large T, where treatment with either CIAP or the catalytic subunit of protein phosphatase 2A enhanced its ability to mediate SV40 origin-dependent replication in vitro (11, 21, 33, 41, 44). Subsequent work has shown that phosphorylation on serines 120 and 123 by a casein kinase I isoform causes inhibition of unwinding and DNA replication which can be reversed by protein phosphatase 2A (3–5). No molecular explanation for this inhibition is currently available. Since the triple serine mutation had little effect on DNA replication when placed in the context of CT, it may be that phosphorylation regulates the connections between NT and CT.

The data presented here connect the phosphorylation of large T at the N terminus of CT to viral DNA replication. Experiments to examine the ability of large T to promote cell cycle progression or to activate E2F sites suggest that these sites are not important for such processes. This is reasonable in view of the known ability of NT to act on the cell. NT is also highly phosphorylated. It will be of considerable interest to determine what contributions those phosphorylations have on large T function.

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