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T-Antigen Kinase Inhibits Simian Virus 40 DNA Replication by Phosphorylation of Intact T Antigen on Serines 120 and 123

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Simian virus 40 (SV40) DNA replication begins after two large T-antigen hexamers assemble on the viral minimal origin of replication and locally unwind the template DNA. The activity of T antigen in this reaction is regulated by its phosphorylation state. A form of casein kinase I purified from HeLa nuclear extracts (T-antigen kinase) phosphorylates T antigen on physiologic sites and inhibits its activity in the unwinding reaction (A. Cegielska and D. M. Virshup, Mol. Cell. Biol. 13:1202–1211, 1993). Using a series of mutant T antigens expressed by recombinant baculoviruses in Sf9 cells, we find that the origin unwinding activities of both $T^{S677\rightarrow A}$ and $T^{S677,679\rightarrow A}$ are inhibited by the T-antigen kinase, as is wild-type T antigen. In contrast, mutants $T^{S120\rightarrow A}$ and $T^{S123,679\rightarrow A}$ are resistant to inhibition by the kinase. Thus, phosphorylation of serines 120 and 123 is necessary for inhibition of T-antigen activity. Previous studies of casein kinase I substrate specificity have suggested that acidic residues or a phosphorylated amino acid amino terminal to the target residue are required to create a casein kinase I recognition site. However, we find that the T-antigen kinase can add more than 3 mol of P_i per mol to full-length bacterially produced T antigen and that it inhibits the unwinding activity of $p34^{cdc2}$-activated bacterially produced T antigen. Since no prior phosphorylation is present in this bacterially produced T antigen, and no acidic residues are present immediately amino terminal to serines 120 and 123, other structural elements of T antigen must contribute to the recognition signals for T-antigen kinase. In support of this conclusion, we find that while T-antigen kinase phosphorylates amino-terminal residues in bacterially produced full-length T antigen, it cannot phosphorylate bacterially produced truncated T antigen containing amino acids 1 to 259, a 17-kDa amino-terminal tryptic fragment of T antigen, nor can it phosphorylate denatured T antigen. These findings strongly suggest that the carboxy-terminal domain of T antigen is an important modifier of the recognition signals for phosphorylation of the critical amino-terminal sites by the T-antigen kinase. This conclusion is consistent with previous studies suggesting close apposition of amino- and carboxy-terminal domains of T antigen in the native protein. The three-dimensional conformation of the substrate appears to make a significant contribution to T-antigen kinase substrate specificity.

Biochemical dissection of simian virus 40 (SV40) DNA replication has provided significant insights into the enzymes and mechanisms involved in eukaryotic DNA replication. Viral DNA replication requires a single viral protein, the SV40 large T antigen, that binds to and unwinds the viral origin of replication. The remainder of replication enzymes are supplied by the host cell. Host factors regulate the initiation of viral replication as well (35). It is presumed that a detailed understanding of the biochemical events occurring during initiation and elongation of viral DNA replication will further our understanding of cellular DNA replication as well.

The initiation of SV40 DNA replication in vitro provides an experimentally approachable model for how protein phosphorylation can control the initiation of eukaryotic DNA replication (10, 23). In in vitro assays, unphosphorylated T antigen (expressed in *Escherichia coli*) does not support SV40 DNA replication until phosphorylated on threonine 124 by the $p34^{cdc2}$ kinase (18) (Fig. 1). T antigen purified from mammalian cells is additionally phosphorylated on multiple serine residues as well as threonine 701. This mammalian T antigen is inactive in the initiation of replication until specific inhibitory serine phosphoryl groups are removed by the catalytic subunit of protein phosphatase 2A ($PP2A_c$) (28, 35, 36). The cellular kinase that phosphorylates these regulatory sites and inhibits T-antigen function has recently been purified from HeLa nuclear extracts (6). This T-antigen kinase is an isoform of casein kinase I and phosphorylates T antigen primarily on serine residues on sites that are phosphorylated in vivo and inhibits T antigen's ability to initiate SV40 DNA replication. This inhibition is reversible by $PP2A_c$, suggesting that these cellular enzymes form a regulatory circuit that controls the onset of SV40 DNA replication (34).

Casein kinase I is a widely distributed serine/threonine protein kinase whose role in cellular regulation is not yet well understood (32). Casein kinase I has been identified in nuclei, cytoskeleton, and cytosol and on mitotic spindles. Nuclear substrates of casein kinase I identified in vitro include p53 (20) and RNA polymerases I and II (8); no functional effect of phosphorylation on the activity of these proteins has been demonstrated. A number of highly related isoforms of casein kinase I have been identified by cDNA cloning (15, 24); there appear to be differences in the regulation, tissue distribution, and subcellular localization of these isoforms (3, 4, 15, 32). A *Saccharomyces cerevisiae* homolog of casein kinase I, HRR25, was cloned in a screen for mutants sensitive to double-strand DNA breaks. HRR25 is 56 to 64% identical and 73 to 81% similar over the kinase domains to the various isoforms of

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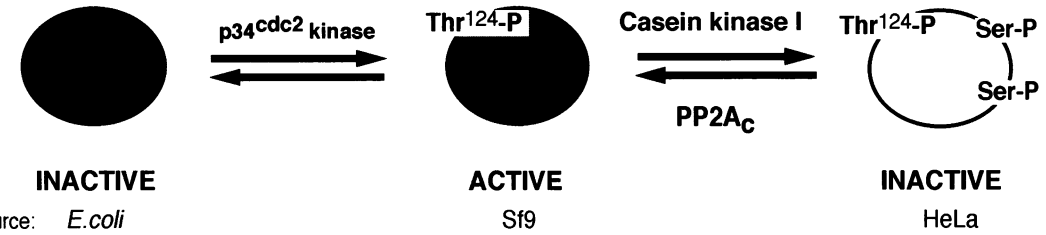

FIG. 1. Control of T-antigen activity by protein phosphorylation. Newly synthesized T antigen is inactive in replication (*E. coli*-produced T antigen) until phosphorylated on threonine 124 (Sf9-produced T antigen). In vitro, phosphorylation of threonine 124 can be catalyzed by $p34^{cdc2}$ kinase. In the nucleus, T antigen is phosphorylated on inhibitory serine residues (HeLa-produced T antigen) by T-antigen kinase. $PP2A_c$ is able to remove the inhibitory phosphoryl groups and activate nuclear T antigen.

casein kinase I. Yeast cells with mutations in HRR25 are sensitive to DNA damage resulting from endonuclease cleavage, DNA-alkylating agents, and X irradiation and have defects in nuclear segregation and meiotic cell division, suggesting an essential role for this kinase in DNA repair (16).

T antigen is phosphorylated on multiple sites by casein kinase I, although the sites that regulate T-antigen activity in the initiation of SV40 DNA replication have not previously been defined (6, 14). Phosphopeptide mapping of T antigen phosphorylated in vitro by casein kinase I purified from rabbit reticulocytes demonstrated that Ser-123 as well as Ser-676, Ser-677, or Ser-679 were targets (14). Additionally, $PP2A_c$, which reverses the inhibition of T-antigen activity by T-antigen kinase, dephosphorylates phosphoserines at positions 120 and 123 followed by 677 and perhaps 679 (28). In in vivo studies, replacement of serines 120 and 123 in T antigen with alanines produced a nonviable SV40 variant with altered replication kinetics (21, 29), suggesting that these sites are important in regulation of viral DNA replication. To identify which of these phosphorylation sites are important in inhibition of T-antigen activity in vitro, we examined the effect of the T-antigen kinase (a human isoform of casein kinase I) on the activity of T-antigen mutants produced with recombinant baculoviruses. We here provide direct evidence that serines 120 and 123 in T antigen are required for T-antigen kinase to inhibit T-antigen activity. An unexpected finding was that the previously identified structural elements that direct casein kinase I phosphorylation of other substrates were insufficient to direct phosphorylation of residues 120 and 123; rather, the three-dimensional conformation of the protein appears to play a major role in determining T-antigen kinase substrate specificity.

MATERIALS AND METHODS

Wild-type and phosphorylation site mutants of T antigen were overexpressed and purified as previously described from recombinant baculovirus-infected Sf9 cells (6, 9, 17, 21), and *E. coli* BL21(DE3)Lys^S carrying pABT7T (18, 22). T-antigen kinase was purified from HeLa nuclei as described previously (6). Glutathione *S*-transferase (GST)-cyclin B/$p34^{cdc2}$ was produced by coinfection of Sf9 cells with recombinant baculoviruses expressing a GST-cyclin B fusion protein and human $p34^{cdc2}$, respectively. The kinase complex was isolated by incubating lysates from infected cells with glutathione-agarose beads as described previously (2).

T-antigen-dependent unwinding of the SV40 minimal origin of replication was assayed as described previously (6). The unwinding substrate contains two ^{32}P-labeled duplex DNA fragments: a 280-bp fragment with 65 bp of SV40 sequence encompassing the minimal origin of DNA replication (SV40 bases 5211 to 32), and a 220-bp fragment derived from the vector that serves as an internal control for nonspecific helicase activity. The DNA fragments are generated by a *Pvu*II-*Asp* 718 digest of pDV.XH and labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP. pDV.XH contains the SV40 minimal origin of replication (nucleotides 5211 to 32), with no additional SV40 sequence, cloned between the *Xho*I and *Hin*dIII sites of pBluescript KS(−) (Stratagene) (37). To assess the effect of T-antigen kinase and $p34^{cdc2}$ on T-antigen unwinding activity, bacterially or Sf9-produced T antigen was incubated for 30 min at 37°C in 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–7 mM $MgCl_2$–4 mM ATP, in the presence or absence of T-antigen kinase, and where indicated with 5 μl (packed volume) of glutathione-agarose beads (Sigma) with or without bound GST-cyclinB/$p34^{cdc2}$. Following the initial incubation, the remainder of the unwinding reaction components were added to give a 20-μl reaction mixture containing 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 4 mM ATP, 2 mM dithiothreitol, 40 mM creatine phosphate, 100 ng of sheared calf thymus DNA, 25 ng of *E. coli* SSB, and 10 ng of ^{32}P-labeled unwinding substrate. After an additional 30-min incubation at 37°C, the reactions were stopped by the addition of sodium dodecyl sulfate (SDS), EDTA, and proteinase K and analyzed by 8% polyacrylamide gel electrophoresis (PAGE) and autoradiography as described previously (6).

Kinase reactions were performed with the indicated substrates for 30 min at 37°C with 20 μM ATP (except where indicated), 5 μCi of [γ-^{32}P]ATP, 30 mM HEPES (pH 7.5), 7 mM $MgCl_2$, and 0.5 mM dithiothreitol in a final volume of 20 μl. The reactions were stopped by the addition of SDS-PAGE sample buffer and then heated to 65°C for 10 min and separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue, and phosphorylated substrates were visualized by autoradiography; incorporated radioactivity was determined by excision of the bands and Cerenkov counting.

Limited tryptic digestion of T antigen was performed for 15 min at 30°C with 5 μg of trypsin (Sigma) per ml in the 20-μl standard kinase reaction mix either before or after phosphorylation of the substrates as indicated. When digestion with trypsin was performed before phosphorylation, the activity of trypsin was inhibited by soybean trypsin inhibitor added to a final concentration of 20 μg/ml.

RESULTS

Identification of the phosphorylation sites that regulate T-antigen activity in the initiation of in vitro SV40 DNA replication. Serines 123, 677, and 679 have been implicated as targets of casein kinase I in vitro, while $PP2A_c$ dephosphorylates serines 120 and 123, followed by serines 677 and 679. The specific phosphoserine residue(s) that inhibits T-antigen func-

TABLE 1. Phosphorylation site mutants of SV40 large T antigen

Residue	Sequence	^{32}P transfer (pmol/pmol)[a]
	120 680	
Wild type	DDEATADSQHSTPP FQAPQSSQSV	3.3
120	DDEATADAQHSTPP FQAPQSSQSV	2.4
123/679	DDEATADSQHATPP FQAPQSSQAV	1.9
677	DDEATADSQHSTPP FQAPQSAQSV	1.9
677/679	DDEATADSQHSTPP FQAPQSAQAV	1.8

[a] Picomoles of ^{32}P transferred to 1 pmol of T antigen from [γ-^{32}P]ATP (final concentration of ATP, 200 μM) in a 40-min kinase reaction at 37°C.

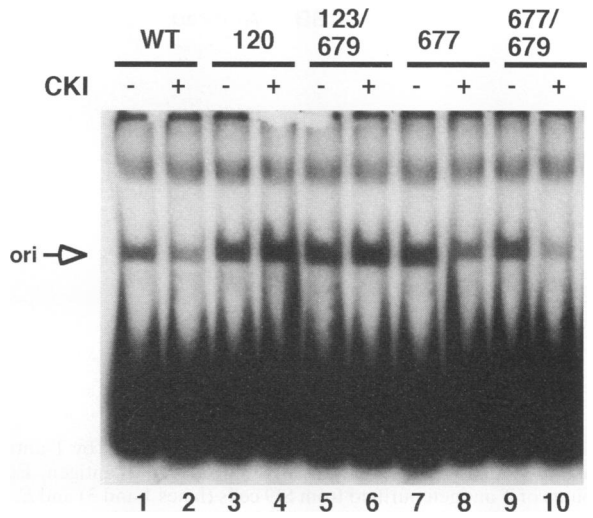

FIG. 2. Identification of serines 120 and 123 as the critical sites that must be phosphorylated by T-antigen kinase in order to inhibit T-antigen activity. Unwinding inhibition assays were performed as described in Materials and Methods with purified Sf9-produced wild-type (WT) T antigen (lanes 1 and 2) and mutant T antigens 120 (lanes 3 and 4), 123/679 (lanes 5 and 6), 677 (lanes 7 and 8), and 677/679 (lanes 9 and 10) (see Table 1). Purified T-antigen kinase was preincubated with the T antigens where indicated. CKI, T-antigen kinase; ori, the unwound 280-bp fragment containing the SV40 minimal origin of replication.

tion has not yet been identified. Wild-type T antigen synthesized in recombinant baculovirus-infected Sf9 cells has been previously shown to be fully phosphorylated on threonine 124 (a p34^{cdc2} site) but relatively underphosphorylated on a number of serine residues (7, 17). Thus, this baculovirus-expressed T antigen is active in SV40 origin unwinding and DNA replication without prior dephosphorylation (Fig. 1) (6, 34). It is readily phosphorylated, and its origin unwinding activity is inhibited by the T-antigen kinase (6). To determine which residues must be phosphorylated by T-antigen kinase to inhibit T-antigen activity, recombinant baculoviruses containing several serine-to-alanine substitutions at known and predicted casein kinase I phosphorylation sites were produced as described previously (17, 21) (Table 1). Mutant and wild-type T antigens were expressed in infected Sf9 cells and purified by affinity chromatography. The wild-type and all of the mutant T antigens were substrates for T-antigen kinase (Table 1) and were capable of supporting in vitro SV40 DNA replication (21).

To test whether mutation of the potential casein kinase I phosphorylation sites prevented inhibition by the T-antigen kinase, purified wild-type and mutant T antigens were preincubated with kinase and ATP and then assayed for the ability to unwind the SV40 minimal origin of replication. Preincubation of wild-type T antigen with T-antigen kinase inhibited origin unwinding activity (Fig. 2, lanes 1 and 2), as previously shown (6). Similar inhibition of origin unwinding activity was seen with T antigen containing the substitution of alanine for serine at position 677 (lanes 7 and 8) and the double mutant with alanine-for-serine substitutions at positions 677 and 679 (lanes 9 and 10). Thus, phosphorylation of T antigen at positions 677 and 679 is not required for inhibition of its replication initiation activity. In contrast, T-antigen kinase did not inhibit the origin unwinding activity of mutant T antigens with alanine for serine at position 120 (lanes 3 and 4) and the double mutant with substitutions at both 123 and 679 (lanes 5 and 6). Similar results were obtained on multiple occasions with different levels of T antigen and T-antigen kinase. At the highest levels of T-antigen kinase, the unwinding activity of wild-type T antigen and mutants 677 and 677/679 was completely inhibited, while the activity of mutants 120 and 123/679 was inhibited by only 25 to 50% (data not shown). The simplest interpretation of these results is that T-antigen kinase inhibits the origin unwinding activity of T antigen by phosphorylation of serine residues 120 and 123.

Unphosphorylated T antigen is a substrate for the T-antigen kinase. Members of the casein kinase I gene family have been shown to phosphorylate serine and threonine residues with acidic domains or phosphorylated residues located two amino acids N terminal, e.g., in the sequence DDDDXXS or S(P)XXS, where X is any amino acid and S(P) is phosphoserine (1, 11–13). The sequences around the known casein kinase I phosphorylation sites in T antigen are shown in Table 1. Neither the N-terminal nor the C-terminal sites appear to contain casein kinase I consensus sequences. How then do T-antigen kinase and other casein kinase I isoforms recognize and phosphorylate these sites? One possibility is that T-antigen kinase has a different substrate specificity than other members of the casein kinase I family. This seems unlikely since bovine casein kinase Iα and the T-antigen kinase phosphorylate T antigen on identical sites, as assessed by two-dimensional peptide mapping (data not shown). Alternatively, serine 120 and serine 677 may be phosphorylated in vivo by other kinases. This would create casein kinase I target sequences in the purified protein, directing in vitro phosphorylation of serine 123 and 679 by T-antigen kinase. Mutation of serine 120 would then block phosphorylation of serine 123, since target sites could not be formed. In support of this hypothesis, Umphress et al. (33) found that the peptide RRASQHSGPP was a very poor substrate for rabbit reticulocyte casein kinase I, while its phosphorylated form RRAS(P)QHSGPP was an excellent substrate. To determine whether prior phosphorylation of the T antigen was required to create a T-antigen kinase recognition site in intact protein, we used a full-length bacterially expressed (and unphosphorylated [13a]) T antigen (Fig. 1).

E. coli BL21(DE3)Lys^S carrying the T7 expression plasmid pABT7T (the gift of D. McVey) was used to produce full-length T antigen essentially as described previously (18). Equal amounts of wild-type T antigen purified from recombinant baculovirus-infected Sf9 cells (Fig. 3, lanes 1 and 3) and *E. coli* (Fig. 3, lanes 2 and 4) were incubated with T-antigen kinase and [γ-^{32}P]ATP and then analyzed by SDS-PAGE and autoradiography. In two separate experiments, T-antigen kinase phosphorylated the two substrates equally well, transferring 3.4 and 2.6 mol of ^{32}P per mol to bacterially produced, unphos-

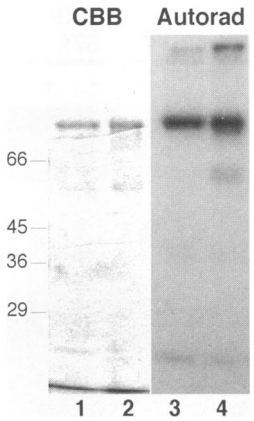

FIG. 3. *E. coli*-produced T antigen is phosphorylated by T-antigen kinase to similar stoichiometry as is Sf9-produced T antigen. Equal amounts of T antigen purified from Sf9 cells (lanes 1 and 3) and *E. coli* (lanes 2 and 4) were phosphorylated by T-antigen kinase and then analyzed by SDS-PAGE, stained with Coomassie brilliant blue (CBB; lanes 1 and 2), and visualized by autoradiography (autorad; lanes 3 and 4). Sizes are indicated in kilodaltons.

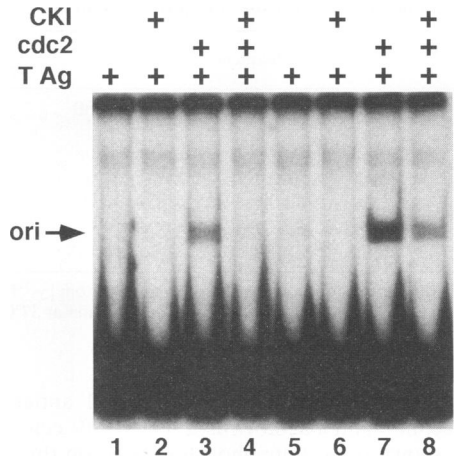

FIG. 4. The origin unwinding activity of bacterially produced T antigen is activated by $p34^{cdc2}$ kinase and subsequently inactivated by T-antigen kinase. Bacterially produced T antigen (0.2 μg [lanes 1 to 4] or 0.5 μg [lanes 5 to 8]) was used in the standard unwinding assay without prior treatment (lanes 1 and 5), after phosphorylation with T-antigen kinase (lanes 2 and 6), after phosphorylation by $p34^{cdc2}$ kinase (lanes 3 and 7), and after phosphorylation by both $p34^{cdc2}$ kinase and T-antigen kinase (lanes 4 and 8). CKI, T-antigen kinase; cdc2, $p34^{cdc2}$; T Ag, T-antigen; ori, the unwound 280-bp fragment containing the SV40 minimal origin of replication.

phorylated T antigen and 3.4 and 3.0 mol of ^{32}P per mol to Sf9-produced T antigen.

This result demonstrates that unphosphorylated T antigen is a reasonable substrate for T-antigen kinase. Two-dimensional phosphopeptide mapping demonstrated that T-antigen kinase phosphorylates the *E. coli*- and Sf9-produced T antigens on the same set of peptides (data not shown), suggesting that prior phosphorylation of T antigen is not required to create the majority of recognition sites for T-antigen kinase.

The origin unwinding activity of unphosphorylated T antigen is activated by $p34^{cdc2}$ and inhibited by the T-antigen kinase. The finding that T-antigen kinase phosphorylates bacterially produced T antigen suggested that T-antigen kinase would phosphorylate the inhibitory sites on T antigen without prior priming phosphorylation. To test this possibility, the origin unwinding activity of *E. coli*-produced T antigen was assessed (Fig. 4). McVey et al. (18) have demonstrated that bacterially produced T antigen is inactive in in vitro SV40 DNA replication until phosphorylated on threonine 124 by $p34^{cdc2}$. Similarly, we find that this unphosphorylated T antigen is inactive in the first steps of replication, unwinding the SV40 origin of replication, until it is activated by phosphorylation by $p34^{cdc2}$ (Fig. 4; compare lane 1 with lane 3 and lane 5 with lane 7). The subsequent phosphorylation of $p34^{cdc2}$-activated T antigen by T-antigen kinase (Fig. 4, lanes 4 and 8) inactivates its origin unwinding activity 85 and 54%, respectively. Higher levels of T-antigen kinase produced greater than 90% inhibition of T-antigen unwinding activity (data not shown). Therefore, T-antigen kinase can directly phosphorylate T antigen on critical regulatory sites without the need for prior upstream phosphorylation.

Amino-terminal sites on T antigen can be phosphorylated only in the presence of carboxy-terminal determinants. To directly examine the effect of sequences surrounding the amino-terminal phosphorylation sites, a truncated version of T antigen containing amino acids 1 to 259 (T259; 31 kDa) was overexpressed in *E. coli* and immunoaffinity purified. The truncated protein has been previously shown to retain SV40 replication origin binding activity (18, 19, 22, 31), indicating at a minimum that the DNA-binding domain (amino acids 131 to 246) folds properly. As shown in Fig. 5, lanes 6 and 7, T259 but not T259 with alanine substituted for threonine at position 124 ($T259^{T124\rightarrow A}$) is readily phosphorylated by $p34^{cdc2}$, as previously demonstrated (18). Additionally, full-length T antigen synthesized in Sf9 cells (lane 5) is poorly phosphorylated by $p34^{cdc2}$ because it is maximally phosphorylated on $p34^{cdc2}$ sites

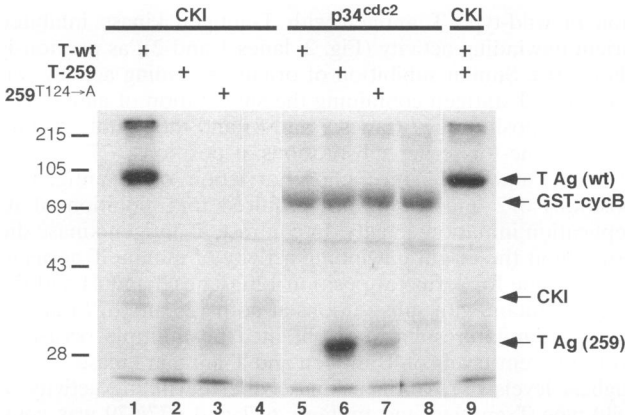

FIG. 5. T-antigen kinase phosphorylates full-length T antigen but not a truncated version (amino acids 1 to 259) containing amino-terminal phosphorylation sites. Full-length T antigen (T-wt; lanes 1 and 5), truncated T antigen containing amino acids 1 to 259 (T-259; lanes 2 and 6), and truncated T antigen with Thr-124→Ala ($259^{T124\rightarrow A}$; lanes 3 and 7) were incubated with [γ-^{32}P]ATP and either T-antigen kinase (lanes 1 to 4) or $p34^{cdc2}$ bound to GST-cyclin B (lanes 5 to 8). Lane 9 represents a mixing experiment in which both full-length and truncated T antigen were incubated with [γ-^{32}P]ATP and T-antigen kinase. Lanes 4 and 8 demonstrate kinase autophosphorylation in the absence of T antigen. CKI, T-antigen kinase; T Ag, T antigen; wt, wild type. Sizes are indicated in kilodaltons.

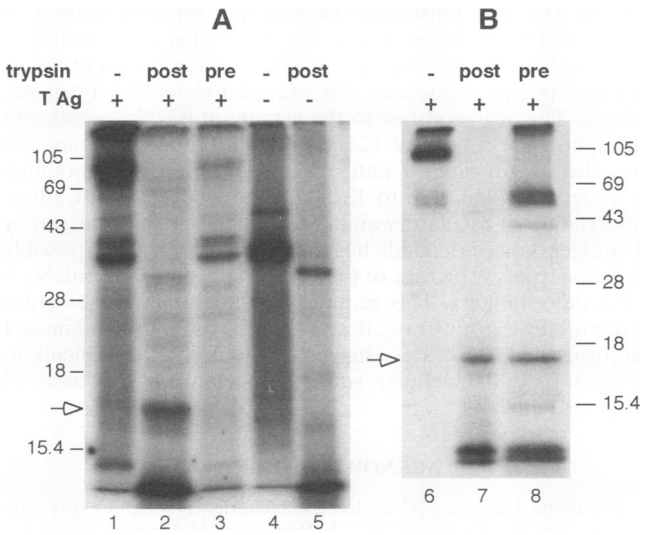

FIG. 6. (A) Amino-terminal sites in T antigen are phosphorylated by T-antigen kinase in intact protein but not in an amino-terminal tryptic fragment. Full-length bacterially produced T antigen (T Ag) was either incubated with [γ-^{32}P]ATP and T-antigen kinase (lane 1) and subsequently partially digested with trypsin (lane 2) or pretrypsinized and subsequently incubated with soybean trypsin inhibitor, [γ-^{32}P]ATP, and T-antigen kinase (lane 3). T-antigen kinase was also autophosphorylated in the absence of T antigen (lane 4) and subsequently treated with trypsin (lane 5). (B) Amino-terminal tryptic fragment of T antigen remains a substrate for an unrelated kinase. Full-length T antigen (produced in Sf9 cells) was phosphorylated by GSK3 and [γ-^{32}P]ATP (lane 6) and subsequently partially digested with trypsin (lane 7) or pretrypsinized and subsequently incubated with soybean trypsin inhibitor, [γ-^{32}P]ATP, and GSK3 (lane 8). Sizes are indicated in kilodaltons.

in vivo (17). Unexpectedly, neither T259 nor T259$^{T124\rightarrow A}$ was phosphorylated by T-antigen kinase (lanes 2 and 3). T259 did not contain an inhibitor of the kinase, since in the mixing experiment (lane 9), autophosphorylation of T-antigen kinase (compare the T-antigen kinase bands; lanes 2 to 4) was not impaired and the kinase remained active on full-length T antigen (compare lane 1). Thus, the T-antigen kinase appeared unable to phosphorylate the truncated T antigen on sites shown to be phosphorylated in full-length protein.

We wished to determine whether (i) full-length T antigen was indeed phosphorylated on the amino terminus and (ii) the presence of the carboxy terminus was crucial for phosphorylation of the amino-terminal sites. We took advantage of the finding that partial proteolysis of native T antigen with trypsin reproducibly generates a 17-kDa amino-terminal fragment, encompassing amino acids N terminal to the nuclear localization signal (amino acids 127 to 131) (30). The 17-kDa peptide is most likely folded properly, since (i) it is cleaved from native protein under nondenaturing conditions and (ii) it forms a stable, trypsin-resistant domain. When full-length T antigen produced in *E. coli* was phosphorylated by T-antigen kinase and [γ-^{32}P]ATP and then partially digested with trypsin, a ^{32}P-labeled 17-kDa peptide was indeed produced (Fig. 6A, lane 2). This result confirms that the T-antigen kinase phosphorylates amino-terminal sites in full-length bacterially produced T antigen. In contrast, when T antigen was first partially digested and then incubated with T-antigen kinase, phosphorylation of the 17-kDa fragment was not observed, although labeling of residual full-length T antigen was still seen (lane 3).

TABLE 2. T-antigen kinase activity on protein substrates is inhibited by low concentrations of SDS[a]

Substrate	Kinase activity (%) in the presence of 0.01% SDS
Autophosphorylation	20.7
D4 peptide	28.4
Casein	1.4
Phosvitin	2.1
T antigen	1.5

[a] T-antigen kinase was incubated in the presence of 0.5 μg of the indicated protein substrate, or 0.5 mM D4 peptide (13), and 20 μM [γ-^{32}P]ATP in the absence or presence of 0.01% SDS for 30 min at 37°C. Phosphorylation of the protein substrates was determined by excision of ^{32}P-labeled bands from SDS-polyacrylamide gels (see Materials and Methods); peptide phosphorylation was determined by isolation of the peptide on P81 paper (5).

This result confirms and extends the finding that the T-antigen kinase does not phosphorylate T259. Inability to phosphorylate the amino-terminal sites in T259 was not simply due to an inhibitory effect of the T antigen DNA binding domain (amino acids 132 to 259), since this region is not present in the 17-kDa peptide. Phosphorylation of the 17-kDa peptide was also seen with full-length but not trypsinized T antigen produced in Sf9 cells, indicating that prior phosphorylation on threonine 124 (Fig. 1) is not sufficient to create a amino-terminal target site for T-antigen kinase (data not shown). Inhibition of trypsin was complete, since no proteolysis of T-antigen kinase was seen (Fig. 6A; compare lanes 3 and 5). The production of the 17-kDa T antigen fragment in lane 3 was confirmed by Coomassie staining of the gel prior to autoradiography (data not shown). The 17-kDa band is derived from the amino terminus of T antigen, since (i) it is ^{32}P labeled in bacterially produced T antigen phosphorylated on threonine 124 by p34^{cdc2} and then trypsinized (data not shown) and (ii) it can be immunoprecipitated by monoclonal antibody PAb419 but not PAb101 (data not shown; PAb419 recognizes an epitope in the amino-terminal region of T antigen, while PAb101 binds to an epitope in the carboxy terminus). In control experiments, we found that the 17-kDa fragment was still a substrate for glycogen synthase kinase 3 (GSK3) (Fig. 6B, lanes 6 to 8). These results confirm that T-antigen kinase phosphorylates sites in the amino-terminal region of T antigen, presumably including serines 120 and 123, but only in the context of full-length T antigen.

Higher-order structure of the substrate protein is the major determinant of T-antigen kinase substrate recognition. The finding that phosphorylation of the inhibitory amino-terminal sites requires determinants in the carboxy terminus of T antigen suggests that the tertiary or quaternary structure of the protein is the major determinant of substrate specificity. This implies that denatured T antigen would not be a substrate for T-antigen kinase. To test this, we initially attempted to heat denature purified T antigen and then phosphorylate it with T-antigen kinase. However, we found that a significant fraction of heat-denatured T antigen was not recovered, as a result of irreversible aggregation and/or adsorption to the test tube walls. We therefore took advantage of the fact that T-antigen kinase retained a significant degree of activity in 0.01% SDS, as assessed by peptide phosphorylation (13) and autophosphorylation activity (Table 2). T-antigen kinase was incubated with [γ-^{32}P]ATP and the indicated substrates in the presence or absence of 0.01% SDS, and then ^{32}P incorporation into each substrate was measured. The presence of SDS disproportionately inhibited T-antigen kinase activity on exogenous protein

substrates, consistent with the hypothesis that higher-order structural elements of the substrate determine T-antigen kinase activity. The inhibition of T-antigen kinase activity by SDS was most likely due to the disruption of the tertiary or quaternary structure of T antigen rather than the masking of phosphorylation sites by detergent binding, since cyclic AMP-dependent protein kinase, GSK3, and casein kinase II all retained 10 to 49% activity on T antigen under these conditions (data not shown).

DISCUSSION

This study demonstrates that the ability of T-antigen kinase, an isoform of casein kinase I, to inhibit the replication initiation activity of SV40 large T antigen requires the presence of serine residues at positions 120 and 123. T-antigen kinase is able to phosphorylate one or both of these sites in unphosphorylated full-length T antigen but not in the context of 17- or 31-kDa amino-terminal fragments of T antigen or short peptides. These results support the hypothesis that the tertiary structure of the intact protein is a critical determinant for substrate recognition by T-antigen kinase. Specifically, these results suggest an interaction between amino- and carboxy-terminal domains in the native protein. This interaction could be supplied in *cis* within each molecule or in *trans*, in the context of ATP-dependent T-antigen hexamer formation.

In the initiation of SV40 DNA replication, two T-antigen hexamers assemble on the minimal origin of replication. Previous studies have shown that removal of inhibitory phosphoryl groups from T antigen by $PP2A_c$ enables functional interactions between the hexamers (28, 35, 37). These interactions are required before the origin region can be unwound to become a template for new DNA synthesis. The effect of phosphorylation of serines 120 and 123 is therefore predicted to be a decrease in double-hexamer formation rather than a decrease in other T-antigen functions. For example, alterations in the phosphorylation state of T antigen do not affect its helicase activity or its ability to form single hexamers (34, 37).

In vivo studies have previously suggested that serines 120 and 123 play an important role in viral DNA replication. Serines 120 and 123 are phosphorylated in the nuclei of infected cells and have a rapid phosphate turnover ($T_{1/2}$, 1.7 h for serine 123) (25, 27). In transfected cells, T-antigen mutants with alanine substituted for serine 120 or 123 could not support lytic viral infection (29), although they did support low levels of viral DNA replication (27). This study provides direct evidence that it is phosphorylation of serines 120 and/or 123 that prevents T antigen from catalyzing the initial steps in SV40 DNA replication.

Studies on the substrate specificity of casein kinase I have shown that a series of acidic residues, or a phosphoryl group two residues amino terminal to the target sites, can direct phosphorylation. Given that T-antigen kinase, which is an isoform of casein kinase I, can (i) phosphorylate amino-terminal sites in and (ii) inhibit the function of bacterially expressed T antigen, it appears that neither of the above targeting mechanisms is operative. It is possible that phosphorylation of serine 120 must precede phosphorylation of serine 123 and that placement of a phosphoryl group at position 123 is the inhibitory step. In support of this view, T antigen with alanine at position 120 is apparently not phosphorylated on serine 123 in vivo (26). In this scenario, T antigen with alanine at position 120 would be uninhibitable because serine 123 could not be phosphorylated. However, serine 120 must still be phosphorylated without an immediately adjacent phosphorylated residue or acidic stretch. The acidic residues at positions 113 to 115 may participate in directing phosphorylation of serine 120 by T-antigen kinase, but this signal is not sufficient since neither T259 nor the 17-kDa tryptic fragment (both containing this sequence) was phosphorylated by T-antigen kinase. This is in contrast to the activity of $p34^{cdc2}$, which can phosphorylate threonine 124 not only in full-length T antigen but also in truncated T antigens and in a peptide containing T-antigen residues 116 to 131 (data not shown). Thus, phosphorylation of regulatory amino-terminal sites in T antigen by T-antigen kinase depends heavily on the tertiary and possibly the quaternary structure of the substrate rather than solely on local determinants. This requirement for intact higher-order structure of the substrate, if also true for other casein kinase I isoforms and substrates, suggests that it will be difficult to predict potential cellular substrates solely on the basis of sequence analysis.

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