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Different Oligomeric Forms of Protein Phosphatase 2A Activate and Inhibit Simian Virus 40 DNA Replication

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The ability of simian virus 40 (SV40) large T antigen to catalyze the initiation of viral DNA replication is regulated by its phosphorylation state. Previous studies have identified the free catalytic subunit of protein phosphatase 2A ($PP2A_c$) as the cellular phosphatase which can remove inhibitory phosphoryl groups from serines 120 and 123. The catalytic C subunit exists in the cell complexed with a 65-kDa A subunit and one of several B subunits. To determine if any of the holoenzymes could activate T antigen, we tested the ability of the heterodimeric AC and two heterotrimeric ABC forms to stimulate T-antigen function in unwinding the origin of SV40 DNA replication. Only free catalytic subunit C and the heterotrimeric form with a 72-kDa B subunit (PP2A-T72) could stimulate T-antigen-dependent origin unwinding. Both the dimeric form (PP2A-D) and the heterotrimer with a 55-kDa B subunit (PP2A-T55) actively inhibited T-antigen function. We found that PP2A-T72 activated T antigen by dephosphorylating serines 120 and 123, while PP2A-D and PP2A-T55 inactivated T antigen by dephosphorylating the $p34^{cdc2}$ target site, threonine 124. Thus, alterations in the subunit composition of PP2A holoenzymes have significant functional consequences for the initiation of in vitro SV40 DNA replication. The regulatory B subunits of PP2A may play a role in regulating SV40 DNA replication in infected cells as well.

Cellular kinases and phosphatases regulate the initiation of simian virus 40 (SV40) DNA replication. Unphosphorylated large T antigen is unable to unwind the viral origin of replication until phosphorylated on threonine 124 by a cyclin-dependent kinase (24). T antigen purified from mammalian cells is heavily phosphorylated on a number of additional serines and threonines (reviewed in reference 11). Phosphorylation on serines 120 and 123 by a nuclear form of casein kinase I (CKI) blocks T antigen's origin-unwinding activity, apparently by preventing functional interactions between T-antigen hexamers bound to the minimal origin of replication (5, 6, 21, 44). This phosphorylation-mediated inhibition of T-antigen activity can be reversed in vitro by treatment of T antigen with alkaline phosphatase (13, 28) or with the isolated catalytic subunit of protein phosphatase 2A ($PP2A_c$) (42, 43). Since the phosphoryl groups on serine 123 (and probably serine 120) of T antigen have a high turnover rate in vivo (31) and T-antigen mutants with alanines instead of serines at these positions are inviable (34), these phosphorylation-dephosphorylation cycles are likely to be physiologically relevant. Two lines of evidence suggest that PP2A is the cellular phosphatase that dephosphorylates serines 120 and 123. First, PP2A was purified from HeLa extracts as a factor required for efficient in vitro SV40 DNA replication (42, 43). Second, addition of okadaic acid but not inhibitor 2 inhibited in vitro SV40 DNA replication (21). Since okadaic acid inhibits both PP2A and protein phosphatase 1, while inhibitor 2 inhibits protein phosphatase 1 but not PP2A, this suggests that PP2A is the sole cellular phosphatase which activates SV40 DNA replication.

The various forms of PP2A are a major component of the intracellular serine/threonine protein phosphatases (9). All members of the family appear to share a ~36-kDa catalytic C subunit. This $PP2A_c$ polypeptide can be dissociated from PP2A regulatory subunits in vitro but does not appear to exist as a monomer in vivo. Rather, the PP2A holoenzymes isolated to date contain both a catalytic subunit and a ~65-kDa regulatory A subunit. These A and C subunits are each encoded by a pair of highly homologous genes (17). In addition, several heterotrimeric PP2A holoenzymes have been purified in a number of laboratories (reviewed in references 29 and 36); these enzymes contain a third B subunit. At least three distinct B subunits of 72, 55, and 54 kDa have been purified to date from skeletal and cardiac muscle and erythrocytes. cDNAs for the 55- and 72-kDa forms (and a 130-kDa splice variant) have been cloned (16, 22). Interestingly, small t antigen of SV40 and small and middle T antigens of polyomavirus can also bind to the A-C complex in place of endogenous B subunits, leading to alterations in phosphatase activity (references 4 and 38 and references therein). Disruption of the gene encoding the 55-kDa B subunit homolog in *Drosophila melanogaster* leads to lethal defects in mitosis and wing development (23, 40), while mutations in the *Saccharomyces cerevisiae* homolog (*CDC55*) lead to defects in cellular morphogenesis (15).

The activity of the PP2A holoenzyme on a variety of substrates is determined by its subunit composition (1, 2, 12, 19, 20, 45). For example, using $p34^{cdc2}$-phosphorylated histone H1 as a substrate, two groups found a relative activity of PP2A-T55 = PP2A-T72 > PP2A-D > $PP2A_c$, where PP2A-D is heterodimeric PP2A containing the 65-kDa A and 36-kDa C subunits; PP2A-T72 is heterotrimeric PP2A containing the A, C, and 72-kDa B subunits; and PP2A-T55 is heterotrimeric PP2A containing the A, C, and 55-kDa B subunits (1, 12, 37). However, using a $p34^{cdc2}$-phosphorylated peptide as a substrate, Agostinis et al. found PP2A-T55 to be much more active than PP2A-T72 (1). With inhibitor 1 as a substrate, PP2A-T72

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was the most active phosphatase (45). These findings suggest that the activity of PP2A on specific substrates is determined by both the nature of the holoenzyme subunit structure and the specific sequence and conformation of the substrate. Specific rules which govern these interactions remain to be determined.

In this study, the ability of the various forms of PP2A to activate the T-antigen-catalyzed unwinding of the SV40 origin of DNA replication was evaluated. $PP2A_c$ activates T-antigen function by dephosphorylation of two of the CKI sites, serines 120 and 123. We found that only one of the holoenzyme forms of PP2A, PP2A-T72, was able to activate T-antigen function. Two alternate PP2A holoenzymes, PP2A-D and PP2A-T55, not only did not activate, but instead inhibited the activity of T antigen. These inhibitory forms of PP2A acted by removing a required phosphoryl group from the $p34^{cdc2}$-phosphorylated threonine 124. These findings demonstrate that changes in the subunit composition of PP2A can shift the phosphatase target site by one amino acid residue with dramatic functional effects and suggest new functions for the 72-kDa B subunit in vivo.

MATERIALS AND METHODS

T-antigen kinase was purified from HeLa nuclei as described elsewhere (6). GST-cyclin B-$p34^{cdc2}$ was produced by coinfection of Sf9 cells with recombinant baculoviruses expressing a glutathione *S*-transferase-cyclin B fusion protein and human $p34^{cdc2}$, respectively (the generous gift of L. Parker and H. Piwnica-Worms). The kinase complex was isolated by incubating lysates from infected cells with glutathione-agarose beads as described previously (5).

Full-length T antigen was immunoaffinity purified from recombinant adenovirus-infected HeLa cells and recombinant baculovirus-infected Sf9 cells, and truncated T antigen containing amino acids 1 to 259 (T259) was immunoaffinity purified from *Escherichia coli* as previously described (5, 6). The phosphorylation state of T antigen purified from these different sources varies and is indicated in Fig. 6.

Multimeric forms of PP2A (see Fig. 1C) were purified from rabbit skeletal muscle, and free catalytic subunit was purified from bovine heart (2, 6, 16). Concentrations of the different forms of PP2A were determined by densitometric quantitation of the 36-kDa catalytic subunit on both immunoblots and silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

T-antigen-dependent unwinding of the SV40 minimal origin of replication was assayed as previously described (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 were 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) (44).

Phosphatase reaction mixtures for reactions performed prior to unwinding assays contained 1 μg of T antigen and the required amounts of various phosphatases in 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.5–7 mM $MgCl_2$–100 μg of bovine serum albumin per ml in a final volume of 10 μl and were incubated for 30 min at 37°C. The T-antigen-dependent unwinding reaction was then initiated by the addition of 10 μl of a cocktail such that the final reaction mixture contained 30 mM HEPES, pH 7.5; 7 mM $MgCl_2$; 4 mM ATP; 15 mM potassium phosphate; 1 mM dithiothreitol; 40 mM creatine phosphate, 50 ng of *E. coli* single-stranded DNA binding protein (SSB); 100 μg of creatine kinase per ml; 200 ng of sheared calf thymus DNA; and 10 ng of unwinding substrate. The unwinding reaction continued for 30 min at 37°C and was then stopped by the addition of a solution containing 2 mg of proteinase K per ml, 2% SDS, and 50 mM EDTA. After an additional 30 min at 37°C, the reaction mixture was heated to 60°C for 5 min and then electrophoresed on an 8% polyacrylamide gel in Tris-borate-EDTA (TBE).

For the phosphatase reactions, the various T-antigen substrates were phosphorylated by T-antigen kinase (6) or 5 μl (packed volume) of GST-cyclin B-$p34^{cdc2}$ bound to glutathione-agarose beads. Each reaction mixture contained 30 mM HEPES, pH 7.5; 7 mM $MgCl_2$; 0.5 mM dithiothreitol; and 20 μM ATP, with 5 μCi of [γ-^{32}P]ATP, and was incubated for 30 min at 37°C. To measure phosphatase activity, the supernatant was transferred to a fresh tube (for GST-cyclin B-$p34^{cdc2}$), unlabeled ATP was added to a final concentration of 4 mM (a 200-fold molar excess over labeled ATP), the various phosphatases were added, and incubations continued for an additional 30 min at 37°C. The phosphatase reactions were stopped by the addition of SDS-PAGE sample buffer, and mixtures were heated to 65°C for 10 min and then separated by SDS-PAGE. The gels were stained with Coomassie brilliant blue, and phosphorylated substrates were visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

Autoradiographs and SDS-PAGE gels were scanned with a Scanmaker IIXE scanner (MicroTek) at 300 dpi and assembled by using Photoshop 2.5.1 software (Adobe). Labels were applied by using Canvas 3.5 (Deneba Software), and images were printed with a Phaser II SDX greyscale laser printer.

RESULTS

Free catalytic subunit and PP2A-T72 stimulate the T-antigen-dependent unwinding of the SV40 origin of DNA replication. Previous studies have shown that heavily phosphorylated T antigen, as purified from mammalian cells, inefficiently unwinds the SV40 origin of replication until phosphoryl groups are removed from serines 120 and 123 by $PP2A_c$ (42, 43). To determine which, if any, of the PP2A holoenzymes could also regulate the initiation of SV40 replication in vitro, T antigen (purified from recombinant adenovirus-infected HeLa cells) was incubated with increasing quantities of the various forms of PP2A (Fig. 1). Following the dephosphorylation reaction, the components of a standard origin-unwinding assay mixture were added (6).

As previously shown (42, 43), $PP2A_c$ activates the T-antigen-catalyzed melting of the origin region, with half-maximal stimulation achieved at approximately 0.8 pmol per reaction mixture (80 nM in the preincubation mixture) (Fig. 1A). The PP2A-T72 holoenzyme was fourfold more active than $PP2A_c$ in activating T-antigen function, with half-maximal stimulation occurring at about 0.2 pmol per reaction mixture (20 nM). PP2A-T55 and PP2A-D failed to activate T-antigen-dependent unwinding significantly, although PP2A-T55 reproducibly stimulated low levels of T-antigen activity at the lowest concentrations tested (0.2 pmol per reaction mixture).

We considered the possibility that the nonstimulatory forms of PP2A either were inactive on T antigen or contained an inhibitor of T-antigen activity. All the phosphatases tested were indeed active under the conditions of the assay, as

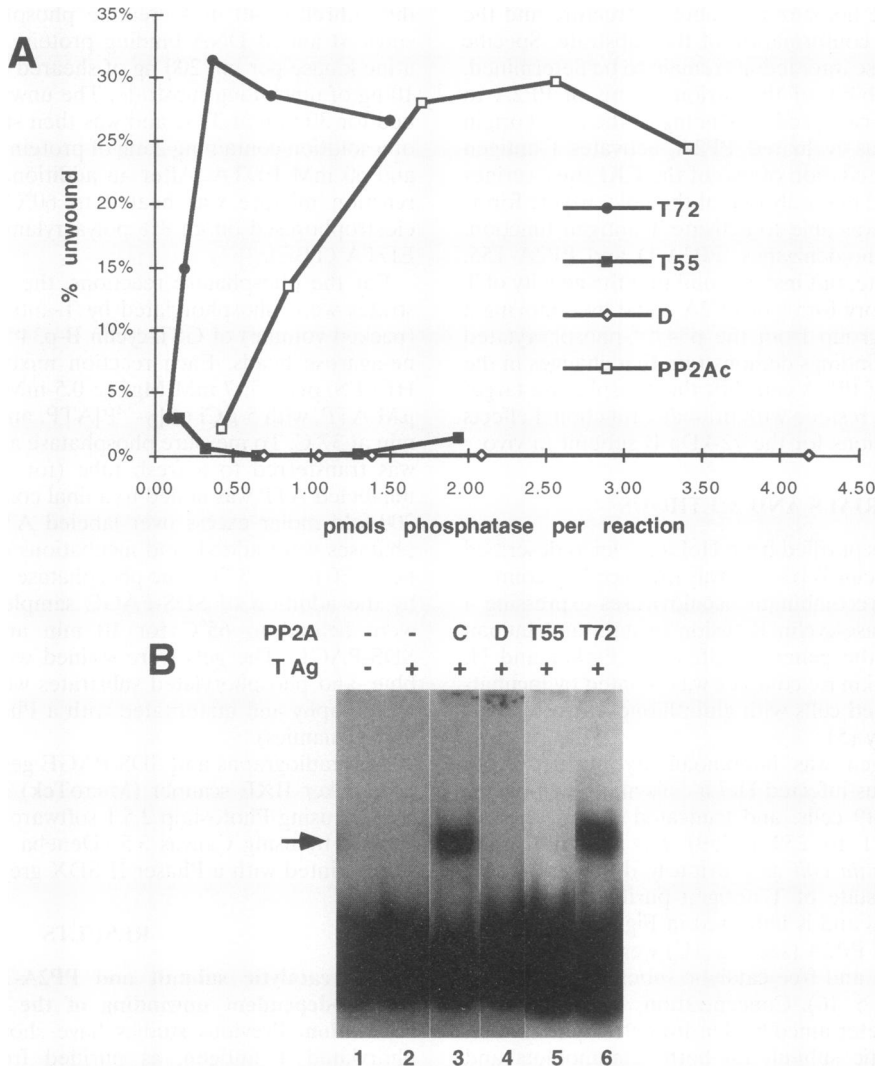

FIG. 1. $PP2A_c$ and PP2A-T72 stimulate the T-antigen-dependent unwinding of the SV40 origin of DNA replication. (A) SV40 large T antigen immunoaffinity purified from recombinant adenovirus-infected HeLa cells was incubated for 30 min at 37°C with the various forms of PP2A as indicated. Following the dephosphorylation reaction, the remainder of the unwinding reaction mixture components were added (see Materials and Methods) and the reaction mixture was incubated for an additional 30 min at 37°C before the products were analyzed by electrophoresis through an 8% polyacrylamide gel. The extent of unwinding of the origin fragment was quantitated with a PhosphorImager (Molecular Dynamics) and plotted as a function of the amount of added phosphatase. (B) Autoradiograph of reaction quantitated in panel A, with 1.4 pmol of each phosphatase form (1.7 pmol of $PP2A_c$). The arrow indicates the unwound (single-stranded) SV40 origin-containing fragment. (C) Purified PP2A holoenzymes used in this study. Lane 1 contains the following prestained molecular mass markers (Gibco-BRL): phosphorylase, 117 kDa; bovine serum albumin, 72 kDa; ovalbumin, 48 kDa; and carbonic anhydrase, 29 kDa. Lane 2, PP2A-D, 5.6 pmol; lane 3, PP2A-T55, 9.2 pmol; lane 4, PP2A-T72, 3.3 pmol. Abbreviations: T Ag, SV40 large T antigen; C, $PP2A_c$; D, PP2A-D; T55, PP2A-T55; T72, PP2A-T72. +, present; −, absent.

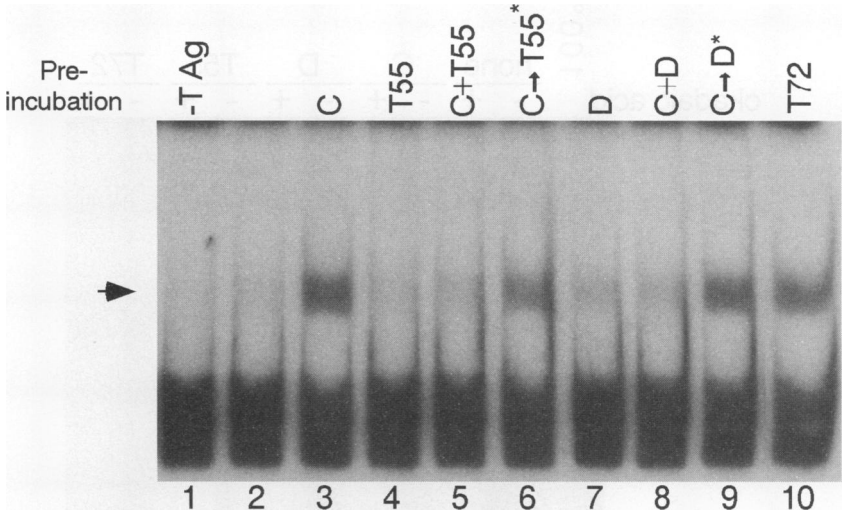

FIG. 2. PP2A-D and PP2A-T55 contain a time-dependent inhibitor of T-antigen activity. T antigen from recombinant adenovirus-infected HeLa cells was incubated either alone (lane 2) or with the indicated phosphatases (preincubation; lanes 3 through 10) for 30 min at 37°C, followed by addition of the unwinding cocktail and a further 30-min incubation at 37°C. Reactions were then analyzed as described in the legend to Fig. 1. For the reactions shown in lanes 5 and 8, the indicated phosphatases were added at the start of the preincubation, while for the lanes marked with an asterisk, PP2A-T55 (lane 6) or PP2A-D (lane 9) was added to the unwinding reaction mixture at the end of the preincubation, at the same time as addition of the unwinding cocktail. Abbreviations are as described in the legend to Fig. 1. The arrow indicates the unwound (single-stranded) SV40 origin-containing fragment.

determined with ^{32}P-phosphorylase *a* as a substrate (data not shown). To evaluate the possibility that the nonstimulatory phosphatases PP2A-D and PP2A-T55 contained an inhibitor of T-antigen activity, mixing experiments were performed (Fig. 2). T antigen (from adenovirus-infected HeLa cells) was preincubated either alone or with the phosphatase(s) for 30 min at 37°C, followed by addition of the unwinding reaction mixture components. As in previous experiments, $PP2A_c$ (Fig. 2, lane 3) and PP2A-T72 (lane 10) activated T-antigen function in the unwinding reaction, while PP2A-T55 (lane 4) and PP2A-D (lane 7) did not. However, when PP2A-T55 or PP2A-D was preincubated together with $PP2A_c$ and T antigen (Fig. 2, lanes 5 and 8), still no stimulation of T-antigen activity was seen. This was not simply due to an inhibitory effect of the buffer, since addition of PP2A-T55 or PP2A-D after T antigen was dephosphorylated by $PP2A_c$ (Fig. 2, lanes 6 and 9) had little effect on the function of T antigen. Thus, PP2A-T55 and PP2A-D appeared to be inhibiting the activity of T antigen in a time-dependent manner.

To determine if PP2A-T55 and PP2A-D were inhibiting T-antigen function by dephosphorylation of a critical site, we utilized an active form of T antigen purified from recombinant baculovirus-infected Sf9 cells. T antigen produced in Sf9 cells is highly phosphorylated on the required $p34^{cdc2}$ site, threonine 124, but is relatively underphosphorylated on the inhibitory CKI sites serines 120 and 123 (5, 8, 18). Thus, unlike adenovirus-produced T antigen, this baculovirus-produced T antigen is able to catalyze unwinding of the SV40 origin of DNA replication without prior phosphatase treatment (41). This T antigen was incubated with the various phosphatases for 30 min at 37°C in the absence or presence of 100 nM phosphatase inhibitor, okadaic acid, and its unwinding activity was assessed (Fig. 3).

Baculovirus-produced T antigen unwound the SV40 origin of replication without prior dephosphorylation (Fig. 3, lanes 2 and 3). Both $PP2A_c$ and PP2A-T72 stimulated this unwinding activity two- to threefold as previously reported (6, 41), and stimulation was blocked by the inclusion of okadaic acid (Fig. 3; compare lane 4 with lane 5 and lane 10 with lane 11). In contrast to $PP2A_c$ and PP2A-T72, both PP2A-D and PP2A-T55 completely inhibited the activity of baculovirus-produced T antigen (Fig. 3, lanes 6 and 8). The inhibitory effect of PP2A-D and PP2A-T55 was blocked by the inclusion of okadaic acid (Fig. 3, lanes 7 and 9). Thus, dephosphorylation of T antigen by PP2A-T55 and PP2A-D inactivates its ability to initiate SV40 DNA replication.

Differential activity of PP2A forms towards the $p34^{cdc2}$-phosphorylated site on T antigen. The activity of T antigen in the initiation of replication is regulated by at least two distinct phosphorylation events. The first event, phosphorylation of threonine 124, is required for T antigen's origin-specific unwinding activity (5, 25, 27). Threonine 124 can be phosphorylated in vitro by the cyclin-dependent kinase $p34^{cdc2}$ (24). The second event, phosphorylation of serines 120 and/or 123 by CKI, down-regulates T-antigen activity (5, 6). These inhibitory phosphoryl groups are removed by $PP2A_c$ (33) and PP2A-T72. PP2A-T55 and PP2A-D may inactivate T antigen by dephosphorylation of threonine 124. Previous studies have shown that although the PP2A forms generally do not dephosphorylate peptides that have phosphothreonine residues with adjacent C-terminal prolines (the consensus $p34^{cdc2}$ site) (2, 3), they can dephosphorylate physiologic $p34^{cdc2}$ sites in intact proteins such as histone H1 (1, 12).

To determine if PP2A-T55 and PP2A-D indeed dephosphorylated threonine 124 of T antigen, we utilized T259. This polypeptide is readily phosphorylated on threonine 124 by $p34^{cdc2}$ (5, 26). Immunoaffinity-purified T259 was phosphorylated by cyclin B-$p34^{cdc2}$ in the presence of [γ-^{32}P]ATP and then incubated with increasing concentrations of the PP2A holoenzymes (Fig. 4).

Both PP2A-T55 and PP2A-D effectively dephosphorylated $p34^{cdc2}$-labeled T259, while PP2A-T72 and $PP2A_c$ had minimal activity. Under the conditions of the assay, approximately 76% of the ^{32}P was removed by PP2A-T55 and PP2A-D, while

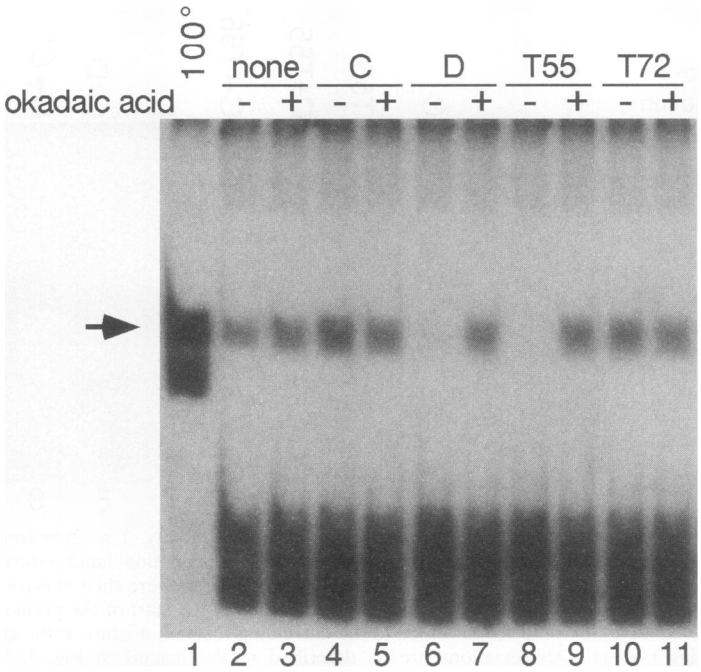

FIG. 3. Inhibition of T-antigen-dependent origin unwinding by PP2A-D and PP2A-T55 requires their phosphatase activity. T antigen from baculovirus-infected Sf9 cells was preincubated with the indicated phosphatases in the absence (−) or presence (+) of 100 nM okadaic acid. Following the 30-min incubation, the unwinding cocktail was added and the incubation continued for an additional 30 min, after which the reaction was analyzed as described in the legend to Fig. 1. The arrow indicates the unwound (single-stranded) SV40 origin-containing fragment. Lane 1 contains boiled (single-stranded) template. Abbreviations are as described in the legend to Fig. 1.

$PP2A_c$ and PP2A-T72 removed 14 and 6% of the label, respectively. The observed dephosphorylation was completely blocked by okadaic acid (data not shown), indicating that the decrease in radioactivity was due to active PP2A-mediated dephosphorylation rather than proteolysis of the substrate.

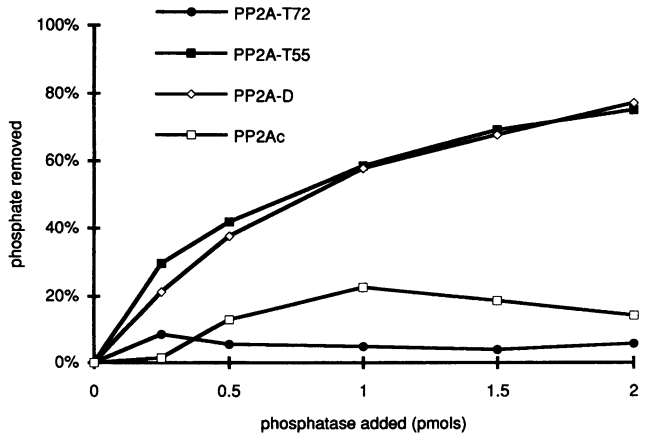

FIG. 4. The inhibitory forms of PP2A dephosphorylate threonine 124. Bacterially produced truncated T antigen (T259) (10 pmol) was incubated for 30 min at 37°C with $p34^{cdc2}$ kinase and [γ-^{32}P]ATP. Following the phosphorylation reaction, $p34^{cdc2}$-labeled T259 was incubated for an additional 30 min at 37°C with the indicated amounts of the various forms of phosphatase in 20-μl reaction volumes containing unwinding buffer (30 mM HEPES, pH 7.5; 7 mM $MgCl_2$; 4 mM ATP). Reactions were stopped by the addition of SDS-PAGE sample buffer. The percent dephosphorylation of ^{32}P-T259 was quantitated with a PhosphorImager (Molecular Dynamics) after SDS-PAGE.

Similar (but less complete) dephosphorylation was obtained when full-length bacterially produced $p34^{cdc2}$-phosphorylated T antigen was used as a substrate (data not shown); we attribute the difference in the degree of dephosphorylation to the presence of additional $p34^{cdc2}$ sites on full-length T antigen. These findings, taken together with the previous results, strongly suggest that PP2A-T55 and PP2A-D inhibit the activity of T antigen by removal of a phosphoryl group from threonine 124.

Differential activity of PP2A forms towards CKI-phosphorylated sites on T antigen. Phosphorylation of serines 120 and/or 123 by a nuclear isoform of CKI inhibits the activity of T antigen in the initiation of SV40 DNA replication (5, 6). CKI phosphorylates additional sites on T antigen, but the effect of these additional phosphorylation events is less clear. The ability of $PP2A_c$ and PP2A-T72 to stimulate T-antigen activity indicates that they are able to dephosphorylate serines 120 and 123. The stimulation of T-antigen activity by low levels of PP2A-T55 suggests that it too can dephosphorylate these sites but that further stimulation of T-antigen activity is blocked by concurrent dephosphorylation of threonine 124. To determine whether PP2A-T55 and PP2A-D could dephosphorylate the CKI sites on T antigen, baculovirus-produced full-length T antigen was phosphorylated by purified T-antigen kinase (CKI) in the presence of [γ-^{32}P]ATP and then incubated with 2 pmol of each of the various phosphatases (Fig. 5). PP2A-T72, which stimulated T-antigen-dependent origin unwinding, removed 58% of the ^{32}P from CKI-labeled T antigen. PP2A-T55, which slightly stimulated T-antigen activity, also removed 25% of the ^{32}P, while PP2A-D removed only 6% of the ^{32}P. These data are consistent with the results shown in Fig. 1 and the conclusion that PP2A-T72, $PP2A_c$, and PP2A-T55 all act on serines 120 and 123. Interestingly, PP2A-D, while very active

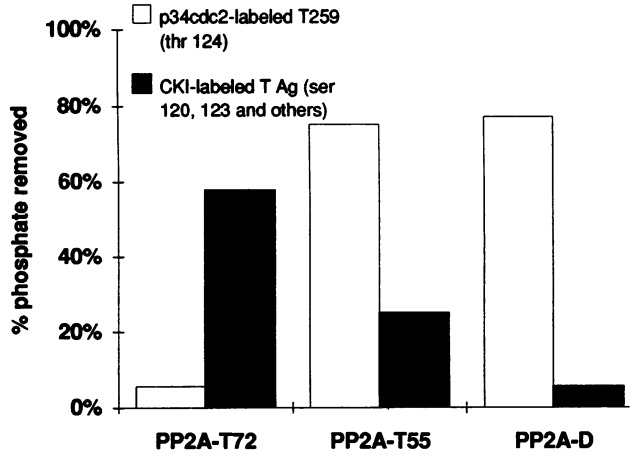

FIG. 5. Comparison of the activities of various PP2A holoenzymes on T antigen labeled with $p34^{cdc2}$ kinase or T-antigen kinase (CKI). T259 ^{32}P labeled with $p34^{cdc2}$ kinase (open bars) and Sf9-produced T antigen ^{32}P labeled with T-antigen kinase (shaded bars) (5) were incubated with 2 pmol of the indicated phosphatases as described in the legend to Fig. 4. Unshaded bars indicate percent ^{32}P removed from $p34^{cdc2}$-labeled T259; shaded bars indicate percent ^{32}P removed from CKI-labeled T antigen.

on threonine 124, appears to have little activity on serines 120 and 123.

DISCUSSION

In this paper, we describe the ability of PP2A to both activate and inhibit SV40 large-T-antigen function in unwinding the origin of replication. This ability of PP2A to switch between activator and inhibitor is due to the nature of the regulatory subunits present in the holoenzyme, which determine the substrate specificity of the phosphatase. PP2A was first identified as a regulator of SV40 replication when its catalytic subunit was purified as a factor which stimulated the ability of large T antigen to unwind the origin of DNA replication (43). The free catalytic subunit is not found in vivo, however, but can be produced in vitro by dissociation of regulatory subunits after exposure to stringent conditions such as ethanol precipitation or chromatography in urea. Two lines of evidence suggested that intact holoenzyme was also capable of activating SV40 DNA replication. First, during the purification of PP2A it was noted that the M_r of the replication-stimulating activity decreased from >100,000 to 34,000 after fractions were exposed to 2 M $MgCl_2$, suggesting dissociation of a larger active complex (43). Second, Lawson et al. found that okadaic acid inhibited SV40 replication in vitro in unfractionated cell extracts, where PP2A holoenzymes might be expected to remain intact (21).

A number of studies have established that the B subunits of the PP2A holoenzyme can regulate its substrate specificity. Further work has demonstrated that the 55-kDa B subunit activated PP2A towards substrates phosphorylated by $p34^{cdc2}$ kinase (1, 12, 37). In the current study, we found that only a single trimeric holoenzyme, that containing the 72-kDa regulatory subunit, was able to dephosphorylate the inhibitory sites on T antigen. An additional finding was that the dimeric AC form, as well as the trimeric form with the 55-kDa subunit, actively dephosphorylated the $p34^{cdc2}$ target site, threonine 124, thereby preventing T antigen from unwinding the origin of replication (Fig. 6). Thus, this study demonstrates that alterations in the subunit composition of the phosphatase holoenzyme have an important functional effect on the initiation of SV40 DNA replication.

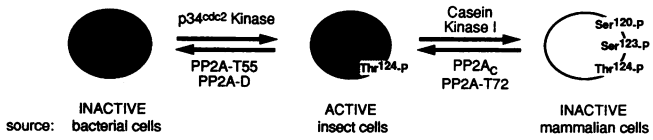

FIG. 6. Model of how cellular kinases and phosphatases control the initiation of SV40 DNA replication. Unphosphorylated T antigen is unable to unwind the SV40 origin of replication until phosphorylated on threonine 124 by a cyclin-dependent kinase. That activating phosphoryl group can be removed by PP2A-D and PP2A-T55. Activated T antigen is also inactivated by further phosphorylation on serines 120 and 123 by a nuclear form of CKI. This fully phosphorylated T antigen can be reactivated by $PP2A_c$ and PP2A-T72. See text for details.

Which cells express which B subunits? The 72-kDa B subunit mRNA is highly expressed only in heart and skeletal muscle, while a prominent splice variant which encodes a 130-kDa polypeptide is expressed in virtually all tissues examined, including brain, placenta, and kidney tissue (16). In tissue culture cells, expression of the mRNA for the 130-kDa form was highly variable while message for the 72-kDa form was not detected. The effect of the 130-kDa B subunit on holoenzyme activity is not known. The message for the α isoform of the 55-kDa subunit (Bα) was ubiquitously expressed in tissue culture cells, while the expression of the Bβ isoform appeared to be restricted to nervous tissue (22). A 74-kDa B subunit from erythrocytes and a 54-kDa subunit purified from bovine cardiac muscle have not yet been as extensively characterized.

How might these changes in the composition of the PP2A holoenzyme regulate the initiation of SV40 DNA replication in the cell? Pulse-chase experiments (31, 32) have shown that threonine 124 is phosphorylated in the cytoplasm soon after T-antigen synthesis and that this threonine phosphate group has a slow turnover rate. T antigen phosphorylated on threonine 124 is then transported to the nucleus, where a nuclear form of CKI phosphorylates it on serines 120 and 123 (6, 31, 32). The phosphate group on serine 123 has a much more rapid turnover rate, suggesting the presence of a specific phosphatase in the nucleus of infected cells. Our finding that PP2A-T72 specifically removes phosphates from serines 120 and 123 indicates that the 72-kDa B subunit may serve to localize holoenzyme to the nucleus and suggests a role for the 72-kDa-subunit-containing holoenzyme in dephosphorylating nuclear substrates. While several studies have in fact suggested a role for PP2A in the regulation of the cell cycle, no cell-cycle-dependent alterations in the quantity of the A, C, and 54-kDa B subunits have been found (30, 35a, 42). The regulation of the 55-, 72-, and 130-kDa B subunits has not yet been extensively examined. However, this study demonstrates that the substrate specificity provided by the B subunit can be a critical factor in the ability of the phosphatase to control the initiation of SV40 DNA replication. One noteworthy precedent is the regulation of the SIT4 protein phosphatase (39). SIT4 encodes an *S. cerevisiae* phosphatase 55% identical to mammalian $PP2A_c$. Growth of *S. cerevisiae* cells with temperature-sensitive mutations in SIT4 is arrested in late G_1 at the nonpermissive temperature. While SIT4 protein does not vary through the cell cycle, it associates with two high-molecular-weight phosphoproteins as the cells enter S phase. Activation (or alteration of the substrate specificity) of SIT4 may be achieved by association with specific regulatory subunits. Thus, it will be important in future studies to determine the quantity

and subcellular localization of the B subunits and the subunit composition of the PP2A holoenzyme through the cell cycle.

Several additional mechanisms that regulate the activity and substrate specificity of PP2A have been described. Receptor tyrosine kinases have been shown to phosphorylate the carboxy-terminal tail of $PP2A_c$ and reversibly inhibit its catalytic function (7). Guo and Damuni have identified a 36-kDa autophosphorylation-activated serine/threonine protein kinase which also inactivates a dimeric form of PP2A (14). Serra and coworkers purified from rat liver a 20-kDa protein which inhibited PP2A activity against one substrate (hydroxymethylglutaryl coenzyme A reductase) but not another (phosphorylase *a*) (35). Brain cytosolic trimeric PP2A containing a 54-kDa regulatory subunit can be activated by micromolar levels of specific stereoisomers of ceramide (10). More recently, Sontag and coworkers found that SV40 small t antigen bound to PP2A-T55 by displacement of the 55-kDa Bα subunit. This modified PP2A holoenzyme no longer was able to inactivate mitogen-activated kinases, thus stimulating cell growth (38). These studies indicate that a number of additional regulatory mechanisms exist which may influence the ability of PP2A to regulate the initiation of SV40 and perhaps cellular DNA replication.

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