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Activation of SV40 DNA replication *in vitro* by cellular protein phosphatase 2A

David M.Virshup[1,2], Michael G.Kauffman[1] and Thomas J.Kelly[1]

Departments of [1]Molecular Biology and Genetics and [2]Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

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We have made use of the cell-free SV40 DNA replication system to identify and characterize cellular proteins required for efficient DNA synthesis. One such protein, replication protein C (RP-C), was shown to be involved with SV40 large T antigen in the early stages of viral DNA replication *in vitro*. We demonstrate here that RP-C is identical to the catalytic subunit of cellular protein phosphatase 2A ($PP2A_c$). The purified protein dephosphorylates specific phosphoamino acid residues in T antigen, consistent with the hypothesis that SV40 DNA replication is regulated by modulating the phosphorylation state of the viral initiator protein. We also show that purified RP-C/$PP2A_c$ preferentially stimulates SV40 DNA replication in extracts from early G_1 phase cells. This finding suggests that the activity of a cellular factor that influences the net phosphorylation state of T antigen is cell cycle dependent.

Key words: cell cycle regulation/phosphatase/T antigen

Introduction

The replication of mammalian chromosomal DNA in growing cells is a complex and tightly regulated process. The papovavirus SV40 appears to be an excellent model system for studying this phenomenon. The SV40 genome is a small, circular, double-stranded DNA molecule that encodes only a single replication protein, the SV40 large T antigen (T antigen). All other proteins required for replication of the viral genome are supplied by the host cell and are presumably involved in cellular DNA synthesis as well. The development of a cell-free system for SV40 DNA replication (Li and Kelly, 1984) has permitted a biochemical approach to the identification of cellular replication proteins (for recent reviews see Kelly, 1988; Challberg and Kelly, 1989). Recently, several such proteins have been purified to homogeneity from extracts of human tissue culture cells based upon their requirement for the reconstitution of authentic SV40 DNA replication *in vitro* (Murakami *et al.*, 1986; Prelich *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988; Tsurimoto and Stillman, 1989; Virshup and Kelly, 1989; Wold *et al.*, 1989).

The SV40 T antigen plays a central role in the initial stages of viral DNA replication. T antigen is a multifunctional 90 kd viral-encoded phosphoprotein that recognizes and binds to four pentanucleotide repeats in the SV40 origin of DNA replication (*ori*) (Tooze, 1981; Delucia *et al.*, 1983). The binding of T antigen to *ori* is facilitated by ATP and induces alterations in the local DNA structure (Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988a). In the presence of the cellular single-stranded DNA binding protein replication protein A (RP-A), T antigen catalyzes the ATP-dependent unwinding of the two strands of the duplex in the origin region (Dean *et al.*, 1987; Dodson *et al.*, 1987; Wold *et al.*, 1987; Wold and Kelly, 1988). This unwinding reaction generates a template suitable for the initiation of DNA synthesis by the DNA polymerase α-primase complex (D.H.Weinberg and T.J.Kelly, unpublished results).

A number of studies have suggested that the activity of T antigen in DNA replication can be regulated by changes in its phosphorylation state. The T antigen molecule contains two clusters of phosphoserine and phosphothreonine residues, one near the amino terminus (Ser 106, 111, 112, 123, and Thr 124) and one near the carboxyl terminus (Ser 639, 676, 677, 679, and Thr 701) (Scheidtmann *et al.*, 1982; Van Roy *et al.*, 1983a). Treatment of T antigen with calf intestinal alkaline phosphatase, which removes phosphoryl groups from phosphoserine residues, appears to increase the ability of the molecule to bind to *ori* and initiate DNA replication *in vitro* (Simmons *et al.*, 1986; Grässer *et al.*, 1987; Mohr *et al.*, 1987). On the other hand, treatment of T antigen with potato acid phosphatase, which dephosphorylates Thr 124 as well as the phosphoserines, has little effect on origin binding activity (Baumann, 1985; Klausing *et al.*, 1988). These results suggest that the phosphorylation of T antigen can have both positive and negative effects on its biochemical activities. This possibility is consistent with results of recent studies in which the phosphorylatable residues in T antigen were systematically altered by *in vitro* mutagenesis (Schneider and Fanning, 1988). Mutations in Ser 123 or Thr 124 eliminated viral DNA replication *in vivo*. In contrast, mutations in Ser 677 or 679 enhanced DNA replication *in vivo*. While the Thr 124 mutant displayed reduced affinity for site II (which is absolutely required for origin unwinding and replication), it apparently bound normally to site I. The Ser 679 mutant appeared to bind to the viral origin better than wild-type T antigen. Thus, the regulation of the activities of T antigen by phosphorylation is complex because the net activity of a given molecule may depend on the state of phosphorylation of several independent sites. It follows that the population of T antigen molecules isolated from infected cells is likely to be functionally heterogeneous (Oren *et al.*, 1980; Scheidtmann *et al.*, 1984).

We have recently purified a 34 kd protein from HeLa cells based on its ability to stimulate SV40 DNA replication 5- to 10-fold in reconstitution assays (Virshup and Kelly, 1989). This protein was tentatively named replication protein C (RP-C). Two lines of evidence suggested that RP-C is involved in the early steps of SV40 DNA replication *in vitro*. First, RP-C stimulated the T antigen-catalyzed unwinding of *ori*-containing DNA in a reaction requiring ATP and RP-A (or *Escherichia coli* SSB). Second, pre-incubation of *ori*-

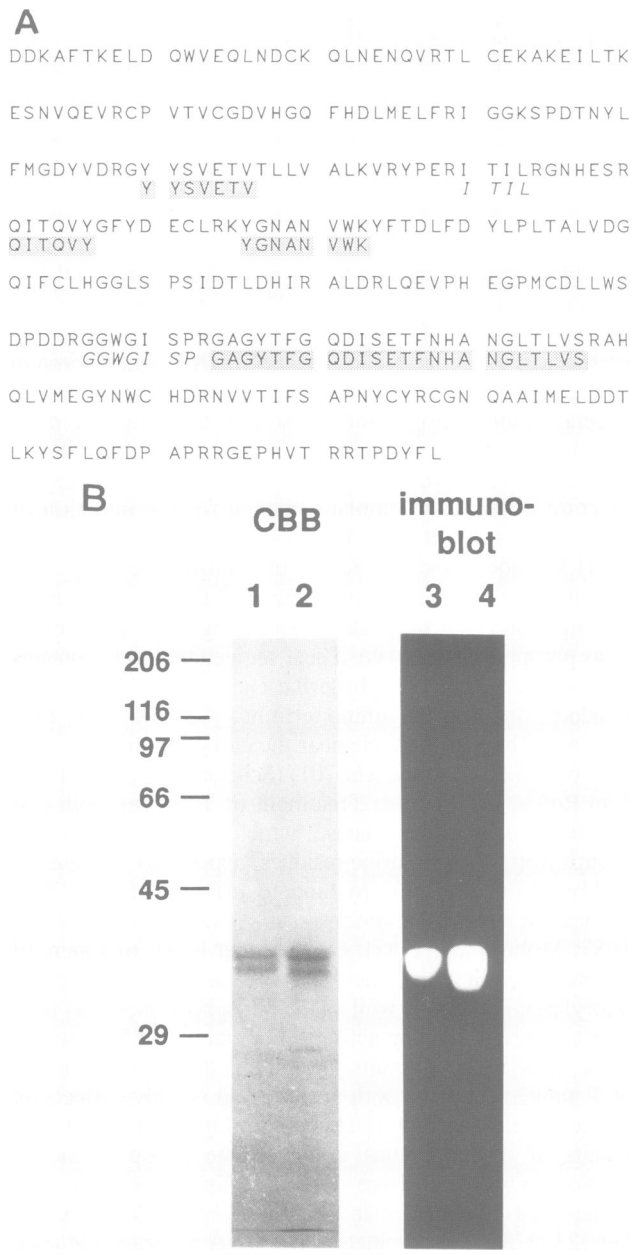

Fig. 1. RP-C is identical to the catalytic subunit of PP2A. (**A**) Peptide sequence of RP-C was obtained as described (see Materials and methods) and found to match the deduced amino acid sequence of the catalytic subunit of human intracellular protein phosphatase 2A ($PP2A_c$) (GenBank accession numbers J03804 and J03805) (Arino *et al.*, 1988). The deduced sequence of the human liver clone HL-1 of $PP2A_c$ is shown in plain letters. Peptide sequence obtained from RP-C is shown below. Unambiguous peptide sequence is shown in shaded letters. In one instance, two amino acid residues were identified per sequencing cycle, indicating the presence of two peptides in the sample. These residues could be completely assigned to deduced peptides in $PP2A_c$ and are indicated in italics. (**B**) RP-C and bovine heart $PP2A_c$ were subjected to SDS–PAGE on a 10% gel (Laemmli, 1970) and either stained with Coomassie Brilliant Blue R-250 (CBB) or immunoblotted with monoclonal antibody PTC-1 directed against bovine heart $PP2A_c$ (Mumby *et al.*, 1985). HeLa RP-C, **lanes 1** and **3**; bovine $PP2A_c$, **lanes 2** and **4**,.

containing DNA with T antigen, RP-C and ATP greatly reduced the time lag usually seen prior to the onset of DNA synthesis in an SV40 replication reaction. Since RP-C does not appear to bind to DNA (our unpublished results), these findings suggested that RP-C stimulates initiation of SV40 DNA replication by interacting with or modifying T antigen (Virshup and Kelly, 1989).

In this report we demonstrate that RP-C is identical to the catalytic subunit of cellular protein phosphatase 2A ($PP2A_c$). The sequences of four tryptic peptides recovered from homogeneous RP-C were identical to sequences predicted from cDNA clones of $PP2A_c$. In addition, RP-C co-migrated with authentic bovine heart $PP2A_c$ on SDS–PAGE and reacted with an anti-$PP2A_c$ monoclonal antibody. Two-dimensional phosphopeptide analysis indicated that RP-C/$PP2A_c$ dephosphorylates SV40 large T antigen in a site-specific manner. These results, together with our previous data, support the hypothesis that RP-C/$PP2A_c$ activates SV40 DNA replication by modulating the phosphorylation state of the viral initiation protein T antigen. Interestingly, purified RP-C/$PP2A_c$ stimulated DNA replication by crude extracts derived from early G_1 phase cells (Roberts and D'Urso, 1988) to a much greater extent than extracts prepared from S phase cells. This observation suggests that the activity of a cellular factor(s) that influences the net phosphorylation state of T antigen (and possibly other crucial replication proteins) is regulated in a cell cycle dependent manner.

Results

RP-C is identical to the catalytic subunit of protein phosphatase 2A

Purification of RP-C from 220 l of HeLa cells ($\sim 10^{11}$ cells) yielded ~ 200 μg of the purified protein (Virshup and Kelly, 1989). A sample containing 1.1 nmol (40 μg) was subjected to peptide sequencing by a modification of the method of Aebersold *et al.* (1987). Four tryptic peptides gave unambiguous sequence, including one containing 24 consecutive residues. No amino acid sequence matches were found in the NBRF protein databank (v. 19.0). However, comparison of the peptide sequences with the six frame translation of the GenBank v.58 nucleic acid databank revealed that the RP-C peptide sequences were completely identical to the predicted sequences of the catalytic subunit of protein phosphatase 2A ($PP2A_c$) from human liver, bovine adrenal gland, rabbit skeletal muscle and porcine kidney (Green *et al.*, 1987; Arino *et al.*, 1988). Human liver contains two closely related $PP2A_c$ mRNAs which differ in eight amino acids in the amino-terminal region of the protein (Arino *et al.*, 1988). From the peptide sequence obtained to date we are unable to state which form of $PP2A_c$ we have purified. In all preparations of RP-C we have observed two polypeptides of 32 and 34 kd which are virtually identical by two-dimensional chymotryptic peptide mapping. We initially attributed the presence of two polypeptide species to proteolysis or post-translation modification of the protein (Virshup and Kelly, 1989). However, these two polypeptides may be the products of two very closely related genes.

HeLa RP-C was compared with $PP2A_c$ purified from bovine heart (the generous gift of M.Mumby, University of Texas Health Science Center, Dallas). By SDS–PAGE, the slower migrating species of RP-C and $PP2A_c$ appear to have identical mobilities, while the faster migrating species of RP-C appears to have a slightly lesser mobility than the corresponding species of bovine $PP2A_c$ (Figure 1B). Immunoblotting experiments with a monoclonal antibody (PTC-1) raised against bovine $PP2A_c$ (Mumby *et al.*, 1985)

similarly demonstrated that the bovine and HeLa phosphatases are virtually identical in their immunoreactivity. Thus, by several criteria, HeLa RP-C appears to be identical to $PP2A_c$; we propose that the protein be referred to as $PP2A_c$ (Tung *et al.*, 1985) or as RP-C/$PP2A_c$, both to indicate its function and to distinguish it from the recently identified and unrelated replication protein RF-C (Tsurimoto and Stillman, 1989).

Purified RP-C/$PP2A_c$ was found to be active in the dephosphorylation of *p*-nitrophenyl phosphate (data not shown) and myosin light chain. Fractions from the final step in the RP-C/$PP2A_c$ purification (gel filtration over Superose 12 under dissociating conditions; Virshup and Kelly, 1989) were assayed for protein phosphatase activity using myosin light chain as substrate and for the ability to stimulate DNA replication in the reconstituted SV40 system (Figure 2). The protein phosphatase and DNA replication activities co-chromatographed exactly, consistent with the hypothesis that the phosphatase activity of RP-C/$PP2A_c$ is responsible for the stimulation of SV40 DNA replication.

RP-C/$PP2A_c$ dephosphorylates T antigen in a site-specific manner

SV40 large T antigen is a phosphoprotein with multiple sites of serine and threonine phosphorylation. To determine whether RP-C/$PP2A_c$ is capable of dephosphorylating the amino acids at any of these sites, we prepared immunoaffinity-purified T antigen labeled *in vivo* with ^{32}P. Incubation of increasing amounts of RP-C/$PP2A_c$ with [^{32}P]T antigen removed a maximum of 30–40% of the phosphoryl groups from T antigen in 1 h, while calf intestinal alkaline phosphatase removed 70% of the phosphoryl groups under the same conditions (Figure 3). The limited dephosphorylation of T antigen by RP-C/$PP2A_c$ could be due to the specific dephosphorylation of a limited subset of phosphoamino acids, or to the inefficient dephosphorylation of multiple phosphoamino acids. To distinguish between these possibilities [^{32}P]T antigen was incubated with purified RP-C/$PP2A_c$ or calf intestinal alkaline phosphatase, and the dephosphorylated products were compared by two-dimensional phosphopeptide analysis following digestion with trypsin and pronase (Figure 4) (Scheidtmann *et al.*, 1982). Proteolysis of untreated [^{32}P]T antigen produced ~14 distinct peptides (Figure 4A), only five of which remained after treatment with calf intestinal alkaline phosphatase (Figure 4C). In contrast, incubation of T antigen with 100 ng of RP-C/$PP2A_c$ for 60 min removed only two phosphopeptides (Figure 4B, solid arrows). We conclude that RP-C/$PP2A_c$ efficiently removes only a limited number of phosphoryl groups from T antigen in a site-specific manner. The sites dephosphorylated appear to be a subset of the sites dephosphorylated by calf intestinal alkaline phosphatase, which has been shown to be dephosphorylate phosphoserine but not phosphothreonine residues in T antigen (Shaw and Tegtmeyer, 1981; Grasser *et al.*, 1987; Klausing *et al.*, 1988). It thus appears likely that RP-C/$PP2A_c$ dephosphorylates one or two phosphoserine residues. Further studies are underway to determine which specific phosphoamino acid residues in T antigen are dephosphorylated, and to correlate the dephosphorylation with the previously demonstrated biological activities of RP-C/$PP2A_c$.

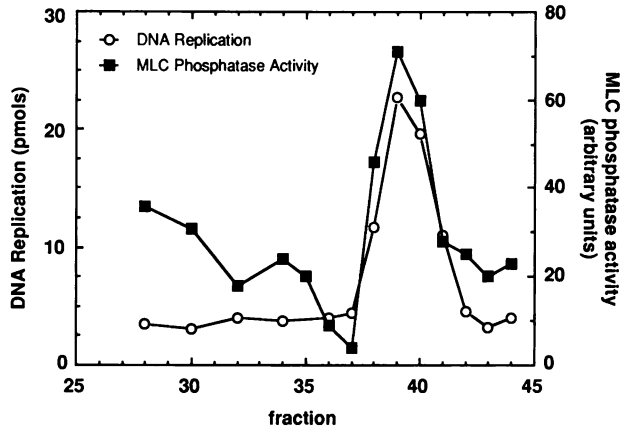

Fig. 2. Replication stimulation and phosphatase activity co-purify. Dialyzed fractions from the final step in RP-C purification were assayed for both stimulation of reconstituted SV40 DNA replication *in vitro* (Virshup and Kelly, 1989) and phosphatase activity using [^{32}P]myosin light chain as substrate.

RP-C/$PP2A_c$ stimulates DNA replication in extracts from early G_1 cells

Roberts and D'Urso have previously demonstrated that extracts from cells in G_1 are several-fold reduced in their ability to support SV40 DNA replication when compared to extracts from S phase and log phase cells (Roberts and D'Urso, 1988). In addition, the G_1 extracts were found to be deficient in their ability to unwind SV40-*ori*-containing DNA. Since we have previously shown that RP-C/$PP2A_c$

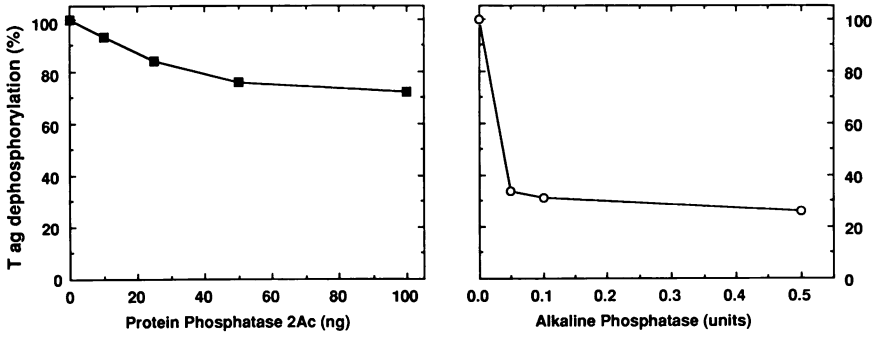

Fig. 3. $PP2A_c$ dephosphorylates SV40 large T antigen. T antigen labeled *in vivo* with ^{32}P was incubated with increasing amounts of RP-C/$PP2A_c$ or calf intestinal alkaline phosphatase (Boehringer Mannheim) for 60 min at 37°C. Dephosphorylation of [^{32}P]T antigen was quantitated by SDS–PAGE, excision of the Coomassie-stained T antigen band, and scintillation counting. All assays were performed in duplicate on at least two occasions with similar results.

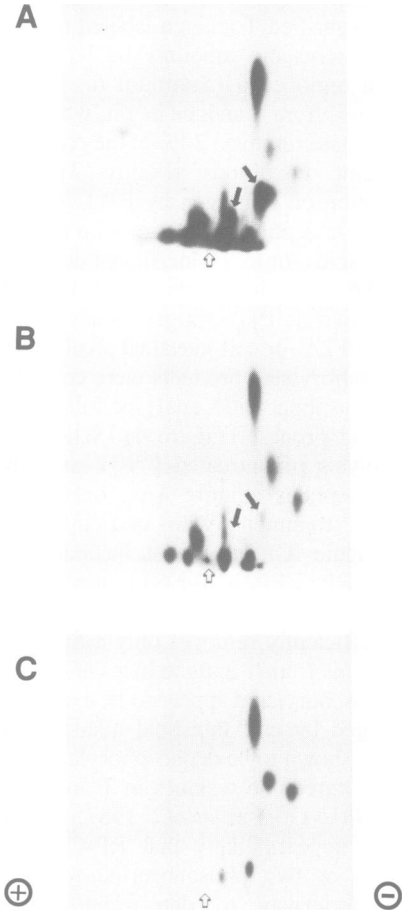

Fig. 4. $PP2A_c$ dephosphorylates a specific site(s) on T antigen. [^{32}P]T antigen (2.5 μg) was incubated either alone (**A**), with 200 ng RP-C/$PP2A_c$ (**B**), or with 0.5 U calf intestinal alkaline phosphatase (**C**) for 60 min at 37°C. After SDS–PAGE and Coomassie staining, the T antigen-containing gel slices were excised and digested sequentially with trypsin and pronase E. Two-dimensional peptide mapping was performed with the buffers of Scheidtmann (Scheidtmann *et al.*, 1982). Open arrows indicate the origin. Electrophoresis was performed in the horizontal direction and chromatography in the vertical direction.

stimulates the unwinding reaction, it was of interest to determine whether the replication activity of G_1 extracts could be rescued by addition of the purified protein. We fractionated both K562 and HeLa cells into G_1, S and G_2 populations by counterflow centrifugal elutriation, which separates cells on the basis of size. The DNA content of representative fractions, analyzed by flow microfluorimetry, is shown in Figure 5(A). The smallest cells are generally those immediately post-mitosis and are thus the earliest G_1 population. Replication assays in extracts prepared by standard methods (Wold *et al.*, 1989) confirmed the observation that, on a per μg protein basis, extracts from G_1 cells are significantly less efficient in supporting SV40 DNA replication *in vitro* than extracts from S phase cells (data not shown). The difference in replication efficiency between G_1 and S phase cell extracts ranged from 3- to 15-fold. Extracts from the smallest G_1 cells were the most deficient in the ability to support *in vitro* SV40 DNA replication, while extracts from later G_1 cells were approximately half as active as S phase cell extracts. G_2 cell extracts were generally half as active in replication as S phase cell extracts (data not shown).

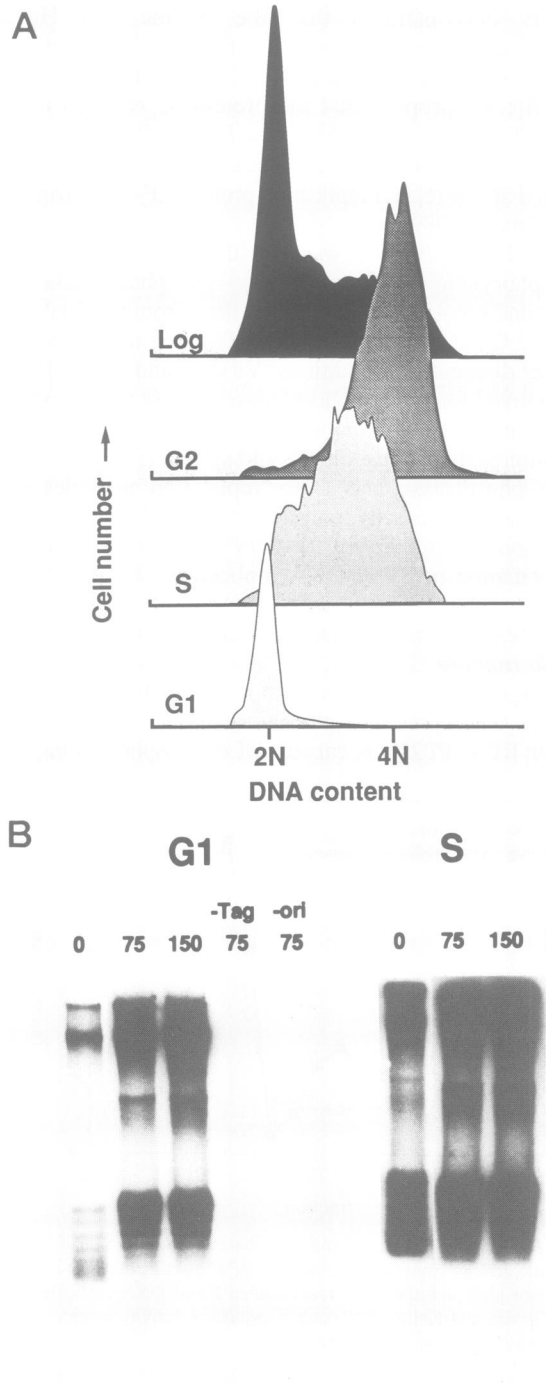

Fig. 5. RP-C/$PP2A_c$ stimulates SV40 DNA replication in extracts from G_1 cells. (**A**) DNA content of representative fractions derived from log-phase HeLa cells by counterflow centrifugal elutriation. Samples from log, G_1, S and G_2 phase populations were stained with ethidium bromide and analyzed by flow microfluorimetry. (**B**) Effects of RP-C/$PP2A_c$ on SV40 DNA replication in G_1 and S phase extracts. Purified RP-C/$PP2A_c$ was added as indicated above each lane (ng/reaction) to SV40 DNA replication reactions containing 45 μg extract from G_1 or S phase HeLa cells. T antigen was omitted from the indicated reaction, and in the reaction labeled -ori, a plasmid containing a 4 bp deletion in the SV40 minimal origin of replication (pUC.8-4) was used in place of pUC.HSO (which contains the wild-type *ori*). Reaction products were analyzed by electrophoresis in 1% agarose gels.

We next tested the ability of purified RP-C/$PP2A_c$ to stimulate replication in the G_1 extracts. Between 100 and 450 ng of RP-C/$PP2A_c$ restored the replication activity of

Table I. Effect of purified RP-C/PP2A$_c$, RP-A and PCNA on replication in G_1 cell extracts

Replication protein added		DNA synthesis (pmol)	
	(ng)	G1	S
PP2A$_c$	0	3.0	44.5
PP2A$_c$	40	7.1	–
PP2A$_c$	80	13.0	60.8
PP2A$_c$	120	15.8	–
PP2A$_c$	160	25.3	74.0
PP2A$_c$(minus T antigen)	120	0.9	–
PP2A$_c$(minus ori)	120	0.9	–
RP-A	200	4.0	61.8
PCNA	50	3.7	41.3

Purified RP-C/PP2A$_c$ RP-A or proliferating cell nuclear antigen (PCNA) were added to SV40 DNA replication reactions containing 50 μg extract from G_1 or S phase K562 cells. DNA replication was quantitated by measuring the incorporation of [α-^{32}P]dCTP into trichloroacetic acid-insoluble material.

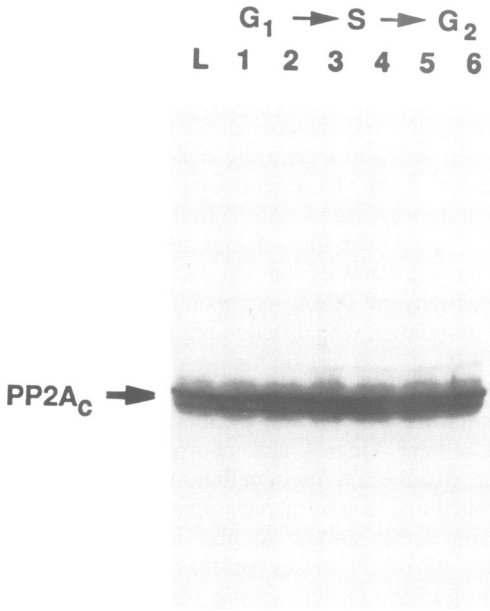

Fig. 6. PP2A$_c$ protein levels do not vary through the cell cycle. Fifty micrograms of extract from selected elutriation fractions of K562 cells were separated by SDS–PAGE and immunoblotted with monoclonal antibody PTC-1 raised against bovine heart PP2A$_c$ (Mumby *et al.*, 1985). Arrows indicate the position of purified RP-C/PP2A$_c$. L = log.

such extracts to near S phase levels (Figure 5B and Table I). The DNA synthesis observed in G_1 extracts supplemented with RP-C/PP2A$_c$ was due to authentic SV40 replication because it was completely dependent on both T antigen and a functional SV40 origin of replication. The stimulation of replication in G_1 extracts is specific to RP-C/PP2A$_c$; as previously reported (Roberts and D'Urso, 1988), RP-A and PCNA did not stimulate replication in G_1 extracts (Table I). RP-C/PP2A$_c$ stimulated replication in S phase extracts as well, although generally no more than 2-fold. The simplest interpretation of these results is that DNA replication with G_1 extracts is inefficient because a critical replication protein is in an inactive phosphorylated state. According to this hypothesis, RP-C/PP2A$_c$ restores replication activity by dephosphorylating this protein. It follows that the activity of a cellular factor that influences the phosphorylation state of the critical protein varies in a cell cycle dependent manner. Our data do not provide decisive information concerning the nature of the critical phosphoprotein. T antigen is a likely candidate given our previous results (and see Discussion), although we cannot rule out a role for a cellular protein at this time.

One obvious explanation for our data is that the level of RP-C/PP2A$_c$ may change during the cell cycle. To test this possibility, equal quantities of extracts prepared from cells at different stages of the cell cycle were subjected to SDS–PAGE and the levels of PP2A$_c$ were measured by immunoblotting. The absolute amount of RP-C/PP2A$_c$ was found to be relatively constant throughout the cell cycle (Figure 6). It remains possible that the specific activity of the enzyme is modulated in a cell cycle dependent manner. *In vivo* PP2A$_c$ is associated with regulatory subunits of 60, 55 and 54 kd (Imaoka *et al.*, 1983; Tung *et al.*, 1985; Mumby *et al.*, 1987; Chen *et al.*, 1989). Although it is known that these subunits modulate the activity of the phosphatase, it is not known whether the subunit composition of the enzyme varies during the cell cycle. An alternative explanation for our data is that the activity of a specific kinase(s) is increased in G_1 cells and/or extracts. Further work will be required to distinguish between these and other possibilities.

Discussion

This study demonstrates that RP-C, a cellular protein purified solely on the basis of its ability to stimulate SV40 DNA replication in a cell-free system, is identical to the catalytic subunit of PP2A. This identity was established by amino acid sequence analysis, and corroborated by electrophoretic mobility on SDS–PAGE, immunologic cross-reactivity and enzymatic activity. PP2A was identified as one of four phosphatase species in liver and skeletal muscle, and represents a major phosphatase component in many tissues (Alemany *et al.*, 1984). Three forms of the enzyme were identified by ion-exchange chromatography, and were shown to have the same catalytic subunit by immunoblotting. Homogeneous preparations of all three isozymes contained the catalytic subunit of PP2A (designated PP2A$_c$) along with one or two additional polypeptides, thought to regulate the phosphatase activity (Tung *et al.*, 1985). Here we have shown that purified RP-C/PP2A$_c$ dephosphorylates the viral initiator protein T antigen in a site-specific manner, and we propose that this accounts for the observed stimulation of SV40 DNA replication *in vitro*. While we cannot completely rule out the possibility that RP-C/PP2A$_c$ activates DNA replication by some mechanism other than dephosphorylation (e.g. binding to T antigen), this appears unlikely.

Previous studies have provided substantial evidence that RP-C/PP2A$_c$ directly interacts with T antigen in the initial stages of SV40 DNA replication *in vitro* (Virshup and Kelly, 1989). Pre-incubation of RP-C and T antigen substantially alters the initial kinetics of the *in vitro* replication reaction such that DNA synthesis is initiated more rapidly. Additionally, RP-C/PP2A$_c$ stimulates unwinding of *ori*-containing DNA in a reaction that requires T antigen and a ssDNA binding protein such as RP-A. While in principle the stimulation of unwinding could be due to dephosphorylation of RP-A rather than of T antigen, this is inconsistent with the observation that RP-C/PP2A$_c$ stimulates unwinding

when a heterologous SSB from *E.coli* is substituted for RP-A (Virshup and Kelly, 1989). It is possible that RP-C/PP2A$_c$ dephosphorylates other cellular proteins involved in replication; however, our data strongly suggest that RP-C/PP2A$_c$ activates SV40 DNA replication *in vitro* primarily through its effect on T antigen. Experiments aimed at demonstrating that RP-C/PP2A$_c$ stimulates replication solely by dephosphorylation of T antigen and not by additional non-covalent interactions have been undertaken, but have proven technically difficult due to the tendency of T antigen to become inactive when incubated at 37°C.

The concept that dephosphorylation of specific sites on T antigen enhances the activity of the protein in DNA replication is supported by previous studies (see Introduction). Mutant T antigens lacking carboxyl-terminal phosphorylation sites Ser 679, and to a lesser extent, Ser 677, appear to replicate more efficiently *in vivo*, suggesting that these sites are important in the regulation of the DNA replication activities of T antigen (Schneider and Fanning, 1988). However, to define the precise residues involved, it will be necessary to determine which phosphoamino acids in T antigen are dephosphorylated by RP-C/PP2A$_c$ and to test directly the effects of individual phosphorylation site mutations on the efficiency of DNA replication in the cell-free system.

What effect might dephosphorylation of T antigen have on its interaction with the SV40 origin sequence? Previous studies have demonstrated that dephosphorylation of T antigen by calf intestinal alkaline phosphatase has no significant effect on its helicase and ATPase activities but increases the extent of binding to the viral origin several-fold (Grässer *et al.*, 1987; Mohr *et al.*, 1987; Klausing *et al.*, 1988). Dephosphorylation of T antigen by RP-C/PP2A$_c$ appears to enhance binding of T antigen to the origin sequence <2-fold under replication conditions (our unpublished results), an effect that may not be sufficient to explain the 5- to 10-fold stimulation of *in vitro* replication of RP-C/PP2A$_c$ (Mohr *et al.*, 1987; Klausing *et al.*, 1988). Dephosphorylation of T antigen by RP-C/PP2A$_c$ stimulates the local unwinding of *ori*-containing dsDNA (Virshup and Kelly, 1989; Wold *et al.*, 1989). Taken with the previous results, this finding suggests the possibility that RP-C/PP2A$_c$ increases the efficiency with which T antigen melts the SV40 origin region. Dephosphorylation of T antigen could also facilitate its interaction with cellular proteins required for replication such as DNA polymerase α-primase complex (Smale and Tjian, 1986; Gannon and Lane, 1987).

Phosphorylation/dephosphorylation cycles may be a major mechanism for the regulation of T antigen activity during the viral multiplication cycle in infected cells. T antigen performs multiple functions during SV40 infection. Its known activities include repression of early SV40 gene expression, activation of late SV40 gene expression, activation of cellular genes, and initiation of viral DNA replication (Tooze, 1981). These multiple activities may be temporally controlled by differential phosphorylation of T antigen during viral infection. Consistent with this hypothesis, T antigen phosphoamino acids have been demonstrated to turn over with differing half-lives during infection (Van Roy *et al.*, 1983b; Scheidtmann, 1986). Studies of T antigen phosphorylation in infected cells have shown that recently synthesized T antigen is under-phosphorylated and nuclear in location, in contrast to older T antigen, which is more highly phosphorylated and cytoplasmic (Scheidtmann *et al.*, 1984). Additional studies have suggested that specific sites on T antigen are phosphorylated in a sequential manner by distinct kinases in different intracellular locations (Simmons, 1984; Grässer *et al.*, 1988).

RP-C/PP2A$_c$ markedly stimulates SV40 DNA replication in crude extracts from early G_1 phase cells. This suggests a critical replication protein is phosphorylated and thus inactivated in G_1 extracts. Given the results presented here, the most likely target of the added RP-C/PP2A$_c$ in the G_1 extract is T antigen. This conclusion is supported by preliminary studies (not shown) indicating that the dependence of DNA replication in G_1 extracts on RP-C/PP2A$_c$ is a function of the source of the T antigen. For example, when the T antigen is produced from insect cells infected with a recombinant baculovirus, the basal replication activity of G_1 extracts is markedly increased and the stimulatory effect of RP-C/PP2A$_c$ is greatly reduced. Presumably, T antigen from insect cells differs in its phosphorylation and perhaps other post-translational modifications from T antigen prepared from mammalian cells. The data presented here support the hypothesis that G_1 extracts are less active in SV40 DNA replication because their ability to specifically dephosphorylate T antigen is diminished, either by a decreased phosphatase activity, or through an increased kinase activity. It follows that one or both of these activities change as the cell progresses through the G_1 phase of the cell cycle. One possibility is that the activity of PP2A$_c$ is modulated through the cell cycle by association with different regulatory subunits (Tung *et al.*, 1985).

The finding that the activity of the viral initiator protein can be controlled by phosphorylation and dephosphorylation by cellular kinases and phosphatases suggests the possibility that the activity of cellular initiator proteins may be controlled in a similar manner. Phosphorylation/dephosphorylation cycles appear to play a crucial role in the regulation of other phases of the cell cycle, e.g. the dissociation of the nuclear lamina (Ottaviano and Gerace, 1985) and the initiation of mitosis (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). Alteration of the phosphorylation state of a cellular initiator protein might similarly control the onset of cellular DNA replication at the entry into S phase.

Materials and methods

Peptide sequencing

The amino acid sequence of RP-C was obtained by a modification of the method of Aebersold *et al.* (1987). Forty micrograms (~1.1 nmol) of purified protein was reduced with 10 mM DTT in 10 mM Tris, pH 8.0, 1% SDS for 60 min at 60°C. Iodoacetamide was then added to a final concentration of 4 μg/ml and allowed to react for 3 min at room temperature. The protein was concentrated by overnight precipitation in 90% ethanol, then separated by SDS–PAGE on a 10% minigel, and electrophoretically transferred to a 0.2 μm nitrocellulose membrane (Biorad). Protein bands were visualized by Ponceau S staining, partially destained in 1% acetic acid, and the membrane incubated with 0.5% polyvinylpyrolidone-40 in 100 mM acetic acid for 30 min at 37°C. The 32/34 kd bands were excised, minced, thoroughly rinsed in dH_2O, and incubated overnight in siliconized polypropylene tubes with 300 μl 100 mM Tris–HCl, pH 8.2 with 5% acetonitrile and 2 μg TPCK-trypsin (Sigma). The resultant peptides were separated by reverse-phase HPLC on a 2.1 mm × 22 cm Brownlee Bu-300 column in 0.06% trifluoroacetic acid with a gradient of 5–80% acetonitrile. Peptide sequencing was performed on a Applied Biosystems model 470A

gas-phase protein sequencer and model 120A PTH analyzer in the Biopolymers Laboratory of the Howard Hughes Medical Institute (Johns Hopkins). Peptide sequence data was compared with the GenBank v. 58.0 DNA databank on a VAX 8530 using the TFASTA program written by Dr W.Pearson.

Phosphatase assays

Fractions eluted from a Superose 12 gel filtration column (Virshup and Kelly, 1989) were dialyzed and then assayed for phosphatase activity by incubation of 2 μl aliquots with [^{32}P]myosin light chain phosphorylated by myosin light chain kinase (the generous gift of L.Satterwhite, Johns Hopkins University School of Medicine) in 30 mM HEPES, pH 7.5, 7 mM $MgCl_2$, 100 mM NaCl and 100 μg/ml BSA in a final volume of 100 μl. Phosphatase reactions were allowed to proceed for 3 min at room temperature and then stopped by the addition of 100 μl 0.1 M $NaPP_i$/1 mg/ml BSA and 100 μl 100% trichloroacetic acid. The acid-insoluble precipitate was collected by filtration through 934-AH filters (Whatman) and assayed for radioactivity in a scintillation counter. Phosphatase activity was expressed as the percentage of radioactivity made acid-soluble.

Stimulation of reconstituted SV40 DNA replication by Superose 12 fractions was assayed as described (Virshup and Kelly, 1989) in a reaction containing 50 ng pUC.HSO, 0.8 μg T antigen, 200 ng RP-A, 30 ng PCNA and 15 μg CF II.

In vivo labeling of T antigen with ^{32}P

Near-confluent 293 cells in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (four 175 cm^2 flasks) were infected with a recombinant adenovirus expressing the SV40 large T antigen from the major late promoter (kindly provided by Y.Gluzman) at a multiplicity of infection of ~10, as previously described (Wold *et al.*, 1989). Twenty-four hours post-infection the medium was removed and replaced with 10 ml/flask phosphate-free minimal essential medium (Gibco) supplemented with 5% dialyzed calf serum (Hazelton) and carrier-free $H_3{}^{32}PO_4$ (ICN), 0.5 mCi/ml. After 6 h incubation with $H_3{}^{32}PO_4$, the monolayers were washed once with phosphate-free medium and lysed with a total of 10 ml 0.5% NP-40, 20 mM Tris−HCl, pH 8.0, 200 mM LiCl, 1 mM EDTA, 1 mM DTT, 100 μg/ml PMSF. The ^{32}P-labeled T antigen was purified by immunoaffinity chromatography as previously described (Dixon and Nathans, 1985; Simanis and Lane, 1985; Wold *et al.*, 1989), yielding 160 μg [^{32}P]T antigen with 36 400 c.p.m./μg. The T antigen preparation additionally contains an ~17 kd phosphorylated polypeptide which is thought to represent an amino-terminal fragment of T antigen (Mohr *et al.*, 1987).

Dephosphorylation of T antigen

[^{32}P]T antigen (0.5 μg) was incubated with RP-C/PP2A_c (100 ng or as indicated) or calf intestinal alkaline phosphatase (Boehringer Mannheim) in 30 mM HEPES, pH 7.5, 7 mM $MgCl_2$ 100 mM NaCl, 100 μg/ml BSA in a final volume of 20 μl for 60 min (or as indicated) at 37°C. Reactions were terminated by the addition of 10 μl of buffer containing 10% SDS/625 mM Tris/50% glycerol. After heating to 65°C for 15 min the samples were subjected to SDS−PAGE. The gel was then lightly stained with Coomassie Brilliant Blue, the T antigen band excised and the associated ^{32}P determined by scintillation counting. All assays were performed in duplicate on at least two occasions with similar results.

Phosphopeptide maps

[^{32}P]T antigen (2.5 μg) was incubated as described above, with 0 or 200 ng RP-C/PP2A_c, or with 0.5 U calf intestinal alkaline phosphatase for 60 min at 37°C. After SDS−PAGE and Coomassie staining, the T antigen-containing gel slices were partially lyophilized, then incubated overnight at 37°C in siliconized glass tubes with TPCK-trypsin, 50 μg/ml, in 500 μl of 100 mM NH_4HCO_3. The samples were then boiled for 5 min to inactivate the trypsin, cooled, and pronase E (Sigma) added to a final concentration of 20 μg/ml. They were incubated for an additional 5 h at 37°C, then the supernatant was removed and lyophilized in a siliconized glass tube. High-voltage electrophoresis and ascending chromatography was performed with the buffers of Scheidtmann (Scheidtmann *et al.*, 1982) on 160 μm cellulose sheets (Kodak) which were then subjected to autoradiography for 24−48 h on Kodak XAR film at −80°C with an intensifier screen (Cronex, Du Pont).

Cell cycle fractionation and replication assays

Log-phase HeLa or K562 cells (4−6 × 10^5 cells/ml) were concentrated and loaded at 65 ml/min into a Beckman model JE 5.0 elutation chamber spinning at 3000 r.p.m. Fractions were collected at flow rates from 75 to 300 ml/min. Nuclear DNA content was assessed by standard flow cytometric analysis of ethidium bromide stained cells. Elutriation fractions were concentrated by centrifugation at 600 *g* for 5 min and hypotonic lysates made as previously described (Wold *et al.*, 1989) except that K562 cells were not washed in hypotonic buffer before lysis. Extracts generally contained between 4 and 7 mg/ml protein.

The ability of the extracts to support SV40 DNA replication was assayed as previously described for HeLa log-phase extracts (Wold and Kelly, 1988; Wold *et al.*, 1989) in a reaction containing cell extract (45−50 μg), 0.5 μg T antigen and 50 ng of plasmid containing the SV40 origin of DNA replication (pUC.HSO). Purified RP-C/PP2A_c, RP-A, and PCNA were added as indicated to SV40 DNA replication reactions containing 50 μg extract from G_1 or S phase cells. Reaction products were analyzed by electrophoresis in 1% agarose gels and quantitated by determination of acid-insoluble radioactivity as previously described.

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