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NOTES

Simian Virus 40 Large T-Antigen-Dependent DNA Replication Is Activated by Protein Phosphatase 2A In Vitro

R. LAWSON,[1]* P. COHEN,[2] AND D. P. LANE[1]

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Herts EN6 3LD,[1] and Biochemistry Department, Medical Research Council Protein Phosphorylation Group, The University, Dundee DD1 4HN,[2] United Kingdom

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The simian virus 40 large T antigen (T) is a multifunctional phosphoprotein. We found that T-dependent simian virus 40 DNA replication is substantially inhibited by okadaic acid. This result suggests that DNA replication is activated by dephosphorylation in vitro. We show here that the target activated by dephosphorylation, which stimulates DNA replication, is T and that the phosphatase involved is protein phosphatase 2A.

The simian virus 40 (SV40) large T antigen (T) is a multifunctional protein encoded by the early region of the SV40 genome. In an infected cell, it regulates viral gene expression, initiates viral DNA replication, and stimulates host cell replication (19, 22). Several biochemical properties of T have been identified. The protein binds DNA with a marked preference for two sites within the viral origin of replication (15). It exhibits both helicase (31) and ATPase (2, 12) activities, which are involved in local unwinding at the origin before DNA replication (32). T also interacts with at least five cellular proteins: p53 (16, 18), DNA polymerase α (11, 26) the retinoblastoma protein (6), a related protein designated 107K (7, 9), and a cellular transcription factor (20).

Regulation of T must be complex in order that so many functions may be carried out by one protein. One possible mode of regulation at the posttranslational level is phosphorylation since T is known to be a phosphoprotein. Indeed, the turnover of phosphate, particularly serine-bound phosphate on T, is faster than the turnover of the protein itself (8), suggesting that regulation by phosphorylation is important. Two clusters of phosphorylation sites on T have been mapped (23, 29), one near the N terminus and one near the C terminus. Each cluster contains one phosphothreonine and four phosphoserine residues. The first evidence that phosphorylation of T changed its biochemical properties came from the observation that incubation with alkaline phosphatase removed all serine-bound phosphate (24), increasing the binding of T to site 2 at the origin of replication and stimulating its ability to support in vitro DNA replication (21). It has also been shown that the monomeric and dimeric forms of T are less highly phosphorylated than the tetrameric form (10). Thus, the phosphorylation of T may affect its structure as well as its function.

To study the regulation of T by phosphorylation, we used an SV40 in vitro DNA replication system. This system is a useful model for eucaryotic DNA replication because the viral minichromosome has a nucleoprotein structure analogous to cellular chromatin, and because replication is initiated at a single origin of replication and proceeds bidirectionally. In vitro, the SV40 system replicates a plasmid containing the origin of replication and the reaction depends on the addition of a eucaryotic cell extract, an ATP-regenerating system, ribonucleotides and deoxyribonucleotides (including [α-^{32}P]dATP), and one viral protein, namely, T. Replication is quantified by the incorporation of radioactively labeled dAMP into DNA. This model system allowed us to investigate the regulation of T in a functional assay under conditions which mimic DNA replication in vivo and in particular to study the effect of protein phosphatases (PPs) and their inhibitors.

The SV40 in vitro DNA replication assay was performed essentially as described by Li and Kelly (17) in multiples of a basic 50-μl reaction mixture. Each 50 μl contained 30 μl of HeLa S100 extract (12 to 15 mg of protein per ml), 300 ng of pSV40 (13), 0.5 μCi of [α-^{32}P]dATP (3,000 Ci/mmol), and between 0.6 and 1.5 μg of T. At certain time points, samples were removed from the reaction mixture, added to 1 ml of ice-cold trichloroacetic acid (10%) containing 0.1 M sodium PP_i, and mixed. After 15 min on ice, the precipitate was collected on a Whatman GF-C filter and washed twice with 5% trichloroacetic acid and twice with ethanol. Radioactivity was detected by liquid scintillation counting. The T used in the assays was purified from adenovirus type 5-SVR111-infected 293 cells by immunoaffinity chromatography on PAb 419 columns exactly as described previously (25). To directly compare phosphorylated and partially dephosphorylated T, half of the T preparation was treated with alkaline phosphatase while still bound to the antibody column and the column was washed to remove the phosphatase before elution (14). The other half of the preparation was treated in a similar manner, except that alkaline phosphatase was omitted. Equal amounts of phosphorylated and partially dephosphorylated T were then tested for their abilities to support in vitro DNA replication. As expected, alkaline phosphatase-treated T had an increased ability to support DNA replication, especially at early time points (Fig. 1). The high levels of dAMP incorporation seen in this experiment were due to the use of an unusually active S100 extract (80 to 100 pmol/h). dAMP incorporation values of around 30 to 50 pmol/h are more commonly observed with our HeLa S100 extracts.

* Corresponding author.

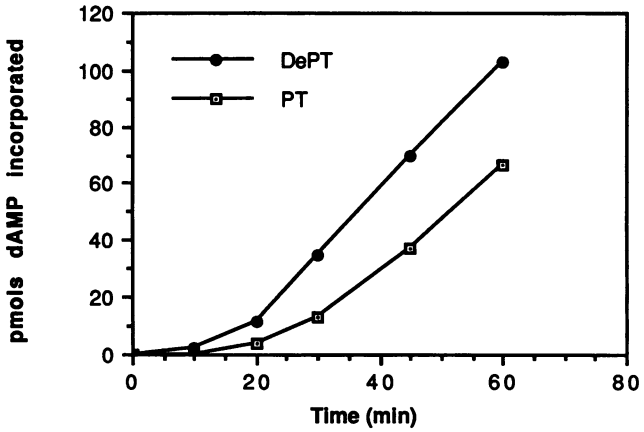

FIG. 1. Dephosphorylation of T increases its activity in the DNA replication assay. DePT, T treated with alkaline phosphatase; PT, untreated T.

Using the above assay, we investigated the effect of okadaic acid on SV40 in vitro DNA replication. This compound has been shown by two-stage mouse skin carcinogenesis to be a potent tumor promoter (28). Unlike other tumor promoters (e.g., 12-*O*-tetradecanoylphorbol-13-acetate), it does not act via stimulation of protein kinase C but will specifically inactivate PP1 and PP2A in vitro (1). Okadaic acid has recently been used to identify and quantify PPs in tissue extracts (5). Our preliminary results showed that okadaic acid, isolated from the black sponge *Halichondria okadaii*, could significantly inhibit SV40 DNA replication in vitro. Titration of okadaic acid concentration showed that this toxin at 0.1 μM partially inhibited T-dependent replication, with maximal effects (≈65% inhibition) being obtained at 1 μM (Fig. 2).

Having established that okadaic acid had an effect on replication, we investigated the effect in the presence of T which had been treated with alkaline phosphatase. The results are shown in Fig. 3. We observed no significant inhibition of DNA replication with 1 μM okadaic acid, although a slight inhibition was detected at late time points. This slight inhibition may have been due to some rephosphorylation of T by kinase(s) in the S100 fraction, further dephosphorylation not being possible owing to the presence

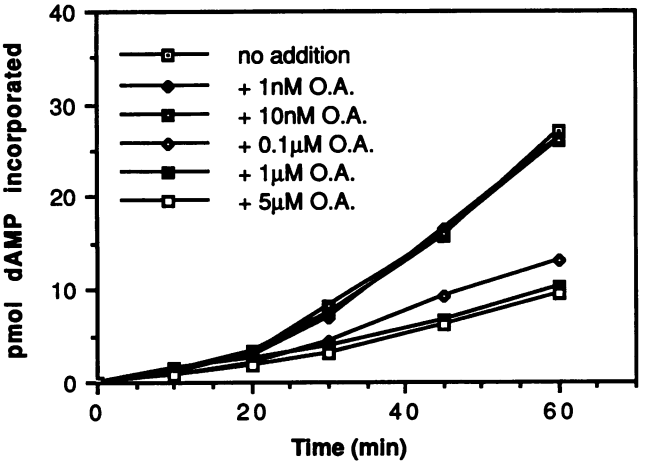

FIG. 2. Effect of okadaic acid (O.A.) concentration in the DNA replication assay.

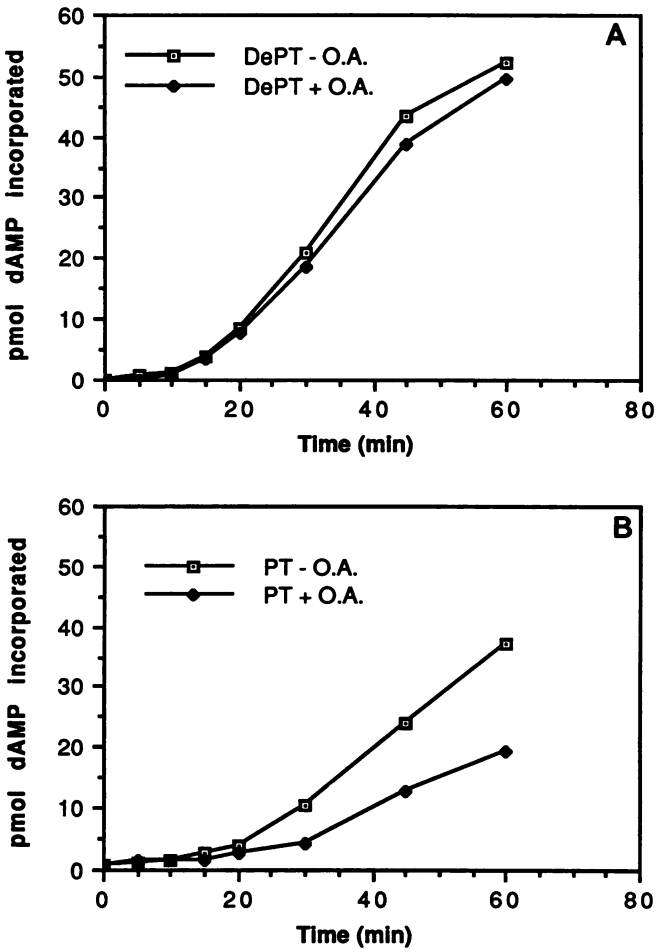

FIG. 3. Effect of okadaic acid (O.A.) on replication in the presence of T treated with alkaline phosphatase (DePT) (A) and untreated T (PT) (B).

of okadaic acid. As the only difference between this and earlier experiments was the phosphorylation state of T, we conclude that okadaic acid must inhibit the phosphatase present in the extract which normally dephosphorylates T during the SV40 DNA replication reaction. The specificity of okadaic acid allows us to predict that the phosphatase involved is PP1 and/or PP2A.

Although PP2A is inhibited much more potently than PP1 by okadaic acid in dilute solutions (5), this toxin could not be used here to unequivocally identify which enzyme was responsible for dephosphorylating T in the concentrated S100. This is because the intracellular concentrations of PP1 and PP2A are both ≈1 μM, so that similar concentrations are required for maximal inhibition of either enzyme in concentrated extracts. This problem has been discussed previously (5). Two further approaches were therefore adopted. First, the extracts were supplemented with the purified catalytic subunits of PP1 and PP2A from rabbit skeletal muscle (4) to increase the endogenous concentrations of these enzymes in the S100 (i.e., 19 ± 2 mU of PP1 per ml and 16 ± 2 mU of PP2A per ml when assayed at a 300-fold final dilution as described previously [4]). These experiments showed that T-dependent replication was markedly stimulated at early time points by the addition of PP2A to a theoretical final concentration of 68 mU/ml but not by the addition of PP1 to

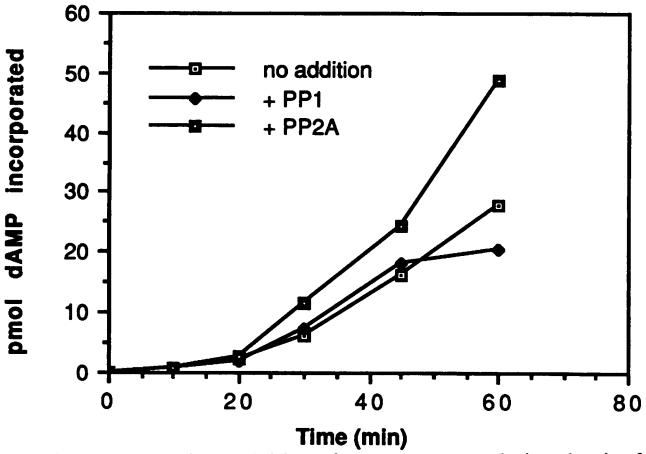

FIG. 4. Effect of the addition of excess pure catalytic subunit of PP1 or PP2A in the DNA replication assay.

a theoretical concentration of 170 mU/ml (Fig. 4). We use the term theoretical because assays of phosphatase activity in these supplemented extracts showed evidence for partial inactivation of the added enzymes. Nevertheless, in all cases PP2A addition resulted in a greater stimulation than that seen with PP1. Second, addition of inhibitor 2 (1 μM), a thermostable protein that inhibits PP1 specifically (reviewed in reference 3), had no inhibitory effect on DNA replication at either early or late time points (Fig. 5). Control experiments done at the same dilution of the S100 fraction (i.e., 30 μl of S100 diluted to 50 μl in 50 mM Tris [pH 7.0] containing 0.1% [vol/vol] 2-mercaptoethanol) with ^{32}P-labeled phosphorylase kinase (27) showed that 1 μM inhibitor 2 inhibited (by at least 70%) the initial rate of dephosphorylation of the β subunit (catalyzed by PP1), whereas dephosphorylation of the α subunit (catalyzed by PP2A) was not inhibited (Fig. 6). Together, these experiments demonstrate that PP2A, but not PP1, is the okadaic acid-sensitive activity in the S100 which stimulates the ability of T to promote replication.

The inhibition of replication by 1 μM okadaic acid varied from T preparation to T preparation and ranged from 50 to 65%. Increasing the concentration of okadaic acid to 5 μM had no further effect (Fig. 2). This variation, and the failure of okadaic acid to block replication completely, are probably explained by different T preparations containing different

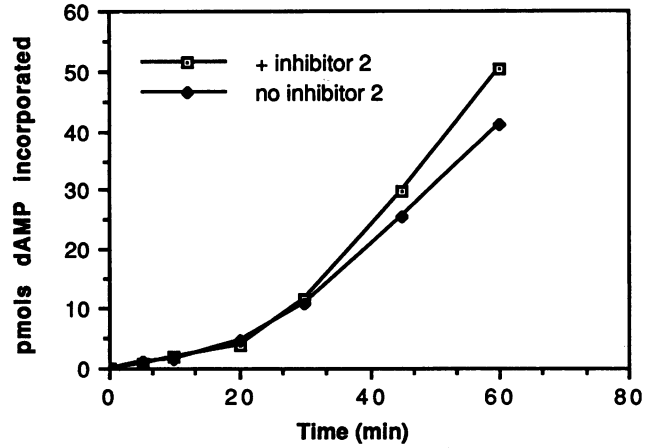

FIG. 5. Effect of the specific PP1 inhibitor, inhibitor 2, on replication.

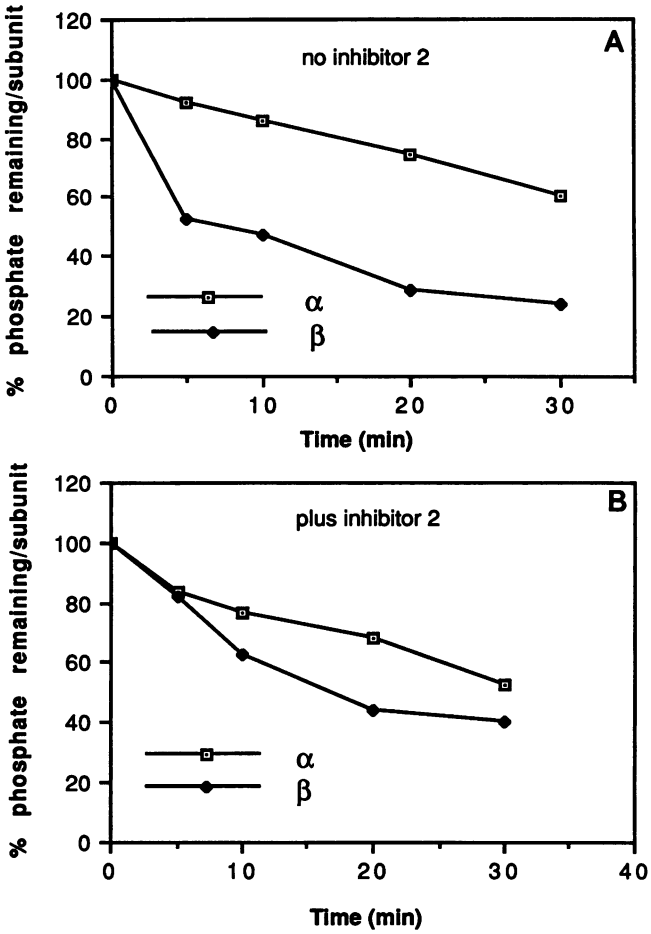

FIG. 6. Effect of inhibitor 2 on the dephosphorylation of the α and β subunits of phosphorylase kinase by phosphatases present in the S100 replication extract.

proportions of phosphorylated and dephosphorylated protein. Alternatively, phosphorylated T may be less active (but not inactive) in stimulating replication. A third possibility is that T is not only dephosphorylated by PP2A but also by an additional (okadaic acid-insensitive) PP, such as Ca^{2+}-calmodulin-stimulated PP (PP2B) or the Mg^{2+}-dependent PP (PP2C). Involvement of the former can be excluded, however, because addition of EGTA (2 mM) to the S100 had no effect on replication. The contribution of PP2C could not be assessed because Mg^{2+} is required for replication and because specific inhibitors of this enzyme are not yet available. Further work is required to answer these questions.

Recently, Virship and Kelly (30) described the purification of replication protein C, a cellular protein required for efficient DNA replication in vitro. They tryptically digested replication protein C, sequenced several of the fragments, and showed that the amino acid sequences obtained were identical to sequences of mammalian PP2A. They conclude that replication protein is PP2A and suggest that PP2A can stimulate SV40 DNA replication in vitro by dephosphorylation of T and other replication proteins.

We showed here that SV40 T is indeed a target for PP2A in vitro and that the dephosphorylation of T is sufficient to stimulate T-dependent SV40 DNA replication. If this should prove to be the case in vivo, then phosphorylation-dephosphorylation may be a key mechanism involved in the regulation of DNA synthesis.

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