Simian Virus 40 (SV40) Small t Antigen Inhibits SV40 DNA Replication In Vitro

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We describe a biochemical function of simian virus 40 small t antigen, the inhibition of simian virus 40 large T antigen-mediated viral DNA replication in an in vitro replication system. Our results suggest that in this system, small t antigen prevents protein phosphatase 2A-mediated activation of large T antigen.

The transforming region of the simian virus 40 (SV40) genome encodes two proteins: the large tumor antigen (large T) and the small tumor antigen (small t). SV40 large T is a multifunctional protein of approximately 90 kDa which is essential for the replication and expression of the viral genome in permissive (primate) cells as well as for the establishment and maintenance of cell transformation and tumor induction (24). The function of the 17-kDa small t is still uncertain (24). This protein shares 82 amino acids at its amino terminus with large T, while the remaining 92 amino acids are unique (24). The function of the sequence common to large T and small t is necessary for large T transforming activity, and small t can provide this function in *trans* (13). Furthermore, the inability of small t mutants to transform resting cells (2, 10, 20–22), the ability of small t to overcome the cell growth-arresting effect of theophylline (17), the ability of small t to enhance the transforming activity of limiting quantities of large T as well as to cooperate with large T in the transformation of some cell types (1), and the different oncogenic spectrum of SV40 mutants which do not express small t (3, 4, 11) all suggest that small t plays an important role in viral transformation and possibly in lytic infection. However, the biochemical basis for these biological effects remains elusive.

The small t protein interacts with two cellular proteins (16, 30) recently identified as the regulatory and catalytic subunits of protein phosphatase 2A (PP2A) (14, 27). This observation suggested to us the possibility that by binding PP2A, small t could influence its ability to dephosphorylate large T, thus modulating large T-mediated DNA replication (5, 8, 12, 15, 19, 25). To test this hypothesis, we have investigated, in an in vitro system (9), the ability of large T to support DNA replication in the presence of small t. In such a system derived from primate cells, the replication of plasmids that contain the SV40 origin of replication is completely dependent on added large T. We have utilized the pZ189 shuttle vector (6), which contains the SV40 origin of replication. Replication reaction mixtures contained 50 ng of pZ189 DNA; 30 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5); 7 mM MgCl₂; 0.5 mM dithiothreitol; 4 mM ATP; 200 μM each CTP, GTP, and

UTP; 100 μM each dATP, dGTP, and dTTP; 50 μM dCTP; 10 μCi of [α-³²P]dCTP; 10 μg of creatine phosphokinase; 40 mM creatine phosphate; 24 μl of HeLa cell extract (prepared as described in reference 9); and 4 μg of SV40 large T (Molecular Biology Resources, Milwaukee, Wis.) in a reaction volume of 60 μl. Reaction mixtures were prepared on ice, and, where indicated, SV40 small t was also added to the reaction mixture. (Small t was prepared as described elsewhere [29]; briefly, recombinant SV40 small t was expressed in pTR865-transformed *Escherichia coli* cells and purified from an insoluble complex by urea denaturation and column chromatography. The small t preparations were more than 95% homogeneous.) As a control, an equal volume of the buffer used to elute small t was added to the mixtures lacking small t. Reaction mixtures were incubated at 37°C, and, at the times indicated, 5-μl aliquots were withdrawn and the radioactivity in trichloroacetic acid (TCA)-precipitable material was determined as described previously (6). Gel electrophoresis and DNA purification were as described previously (6). Briefly, reactions were stopped by adjusting to 0.015 M EDTA and 0.2% sodium dodecyl sulfate and incubating the mixture for an additional 60 min at 37°C in the presence of 200 μg of proteinase K per ml.

When DNA replication extracts prepared from HeLa cells (a human carcinoma cell line) were used, the incorporation of radioactivity into TCA-precipitable material increased linearly for up to 4 h, after a lag time of about 30 min. These kinetics are similar to those observed previously with extracts from other cell lines and other SV40 origin-containing plasmids (6, 9, 23, 28). When purified SV40 small t was added to the reaction mixture at the beginning of incubation, incorporation of radioactivity was inhibited. This inhibition appeared to become more pronounced as a function of time of incubation, and the magnitude of inhibition was dependent on the amount of small t added (Fig. 1a). Analysis of the products of these in vitro DNA replication reactions on agarose gels revealed that the addition of small t did not appear to alter the normal structure of the replicated products (Fig. 1b). We observed the usual ladder of partially supercoiled plasmid molecules along with higher-molecular-weight molecules, which presumably include replicative intermediates. Linearization of the DNAs with *Eco*RI and densitometry of the single bands confirmed that the amount of label detected in DNA reflected the amount detected by TCA precipitation (Fig. 1c). The products obtained were all resistant to *Dpn*I digestion, indicating that the label incor-

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FIG. 1. Analysis of pZ189 DNA replication in vitro in the presence of SV40 small t. (a) Incorporation of [α - 32 P]dCTP into DNA. Replication reactions were carried out in the absence (■) and in the presence of 200 (○), 400 (▲), or 800 (●) ng of SV40 small t. Reaction mixtures were incubated at 37°C, and, at the times indicated, 5- μ l aliquots were withdrawn and the incorporation of radioactivity into TCA-precipitable material was determined as described previously (6). (b) Autoradiogram of the products of in vitro DNA replication (panel a). pZ189 was replicated in the presence of 200 ng (lanes 1 and 5), 400 ng (lanes 2 and 6), or 800 ng (lanes 3 and 7) of small t and in the absence of small t (lanes 4 and 8) (ng.t. indicates nanograms of small t used). Following incubation at 37°C for 240 min, the reactions were stopped as indicated in the text and the DNAs were purified as described previously (6). After electrophoresis on a 1% agarose gel, either without further treatment (lanes 1 to 4) or after treatment with 0.1 U of *DpnI* for 60 min at 37°C (lanes 5 to 8), the labelled products of in vitro replication were visualized by autoradiography. *DpnI* digests fully methylated (unreplicated) input DNA, while DNA which has undergone one or more rounds of replication in vitro is hemimethylated or unmethylated and resistant to *DpnI*. The positions of covalently closed circular DNA (Form I), relaxed circular DNA (Form II), and replicative intermediates (RI) are indicated. In parallel control reactions, a similar amount of *DpnI* digested 50 ng of unlabelled methylated pZ189 to completion. (c) The products of in vitro DNA replication (panel a) were linearized with 1 U of *EcoRI* for 60 min at 37°C and analyzed as described for panel b. The position of the single labelled band is shown. Lanes 1 to 4, minus *DpnI* and plus *EcoRI*; lanes 5 to 8, plus *DpnI* and plus *EcoRI*. Laser scanning densitometry analysis indicated that the amount of labelled DNA was the same in the presence or absence of *DpnI*.

porated was the result of complete replication of the plasmid rather than the result of nick translation or repair-type synthesis. It is unlikely that inhibition was due to a contaminating bacterial product in the small t preparation; comparable preparations from bacteria carrying the expression plasmid but lacking the small t insert did not inhibit the reaction. Furthermore, when the small t preparations and the control bacterial preparations were run on acrylamide gels and silver stained, the minor contaminating bands were also present in the control preparations. Thus, it seems unlikely that a contaminant in the preparation or a contaminant which binds to small t is responsible for the observed inhibitory effect on DNA replication. The addition of the same amount of albumin also failed to inhibit replication, suggesting that inhibition was not a nonspecific effect of changes in protein concentration.

The inhibitory effect of small t on SV40 DNA replication was reproducibly observed in several additional experiments in which different large T preparations were used (Fig. 2a).

This inhibitory effect was also observed with three independent preparations of HeLa cell extract as well as extracts from COS or GMO637 cells (Fig. 2b). When small t was added to the replication mixtures, the inhibitory effect of small t was apparent after a lag time of 30 to 60 min (Fig. 1a). This lag time was observed even when small t was added during the linear portion of the replication reaction (data not shown). The fact that there appeared to be a delay in the inhibitory action of small t on DNA replication suggested to us that time was required for altering the activity of some component of the replication complex (presumably large T). To test this possibility, we carried out reactions in which cell extracts were preincubated (at 37°C for 30 min) with large T in the presence and in the absence of small t. Preincubation was carried out in the absence of DNA, deoxyribonucleotides and deoxynucleotide triphosphates, and [α - 32 P]dCTP. As observed previously (26), preincubation of extracts with large T alone reduced the 30-min lag time for the onset of DNA replication, but otherwise DNA replication occurred at

FIG. 2. Inhibitory effect of small t on DNA replication. Two different large T preparations were used, large T lot 81003 (hatched bars) and large T lot 81046 (solid bars). (a) Inhibition of DNA replication induced by 400 ng of small t in different experiments with HeLa cell extracts. Bars 1 to 8 represent eight independent experiments, each with incorporation measured at 30, 60, 120, and 180 min. Values shown are for the 180-min time point. Percent inhibition of DNA replication was calculated as the ratio of label incorporated into TCA-precipitable material in reactions with or without added small t. (b) Inhibition of DNA replication induced by 800 ng of small t in different experiments with various cell extracts as follows: bars 9 and 10, HeLa cell extracts; bar 11, COS (an SV40-transformed African green monkey kidney cell line) extract; bar 12, GMO637 (an SV40-transformed normal human fibroblast cell line) extract. Values shown are for the 180-min time point. (c) Inhibition of DNA replication with HeLa extracts in different experiments preincubated (at 37°C for 30 min) in the presence of 4 μ g of large T and 400 ng of small t. The values shown are for the 180-min time point in experiments in which incorporation was measured at 30, 60, 120, and 180 min. Bars 13 to 17 represent five independent experiments. (d) Effect of preincubation with small t on pZ189 replication in vitro. HeLa cell extracts were preincubated with all of the components of the replication reaction mixture (see text), except [α - 32 P]dCTP, nucleotide mix, and pZ189 DNA, and with 4 μ g of large T plus 400 ng of small t (▲), neither large T nor small t (○), 4 μ g of large T alone (■), or 400 ng of small t alone (●). After preincubation of the mixture for 30 min at 37°C, reactions were started by the addition of [α - 32 P]dCTP, nucleotide mix, and pZ189 DNA. Large T (4 μ g) was also added to those reaction mixtures preincubated in the absence of large T. Aliquots were withdrawn at the times indicated, and incorporation of radioactivity into TCA-precipitable material was determined.

essentially the normal rate (Fig. 2d). However, when small t was present along with large T during this preincubation period, there was a dramatic inhibition of DNA replication, which was observed from the time of onset of DNA replication (Fig. 2c and d). This dramatic inhibition by small t depends on the presence of large T during the preincubation period; when extracts were preincubated with small t alone and large T was added at the beginning of the DNA replication reaction, the level of inhibition by small t was comparable to that observed in the absence of preincubation (Fig. 2d). Inhibition of DNA replication following preincubation of extracts with both small t and large T could also be partially overcome by further addition of large T at the beginning of the DNA replication reaction (data not shown).

These results suggest that addition of small t is not merely toxic to the reaction but rather that small t, in order to exert its inhibitory effect on DNA replication, must interact with some component present in the reaction mixture which is required for complete activation of large T. Three separate lines of evidence suggest that this occurs by small t inhibition of PP2A. First, small t associates with PP2A (7, 16, 29), and this association was also observed in our reaction mixtures by immunoprecipitation studies (data not shown). Second, experiments in which we incubated the reaction mixtures with [γ - 32 P]ATP and then immunoprecipitated the mixtures indicated that large T is phosphorylated during DNA replication in vitro, possibly making replication dependent on activation of large T by PP2A. Finally, the addition of 200 ng of the purified catalytic subunit of PP2A partially restored replication in the presence of 800 ng of small t (Fig. 3), and when only 400 ng of small t was used, the addition of 200 ng

of PP2A completely reversed the inhibitory effect of small t on DNA replication (Fig. 3). It has been shown that small t binds the regulatory (A) subunit of PP2A (7) and that there is no direct binding to the catalytic subunit (29). Thus, the addition of the free catalytic subunit would overcome small t inhibition, as observed in our experiments.

The results of this study indicate that SV40 small t inhibits SV40 DNA replication in vitro. Our observation that the inhibition of DNA replication can be overcome by the addition of the catalytic subunit of PP2A to the system suggests that the inhibition of DNA replication by small t is mediated through an inhibition of PP2A function. It was shown previously that dephosphorylation of SV40 large T at positions 120 and 123 by PP2A (8, 19) activated large T for in vitro DNA replication. We observed that SV40 large T becomes phosphorylated during incubation in our DNA replication extracts. In the presence of small t, preincubation of SV40 large T in the cell extracts leads to a loss of DNA replication activity which can be overcome by the addition of untreated SV40 large T. However, DNA replication occurs at essentially the normal rate if large T is preincubated in the cell extracts without small t. These results are consistent with the hypothesis that, in the in vitro DNA replication system, there is a dynamic process of phosphorylation and dephosphorylation of SV40 large T and SV40 small t inhibits large T-dependent in vitro DNA replication through inhibition of the PP2A-mediated dephosphorylation of large T. Whether small t inhibits large T-mediated SV40 DNA replication in vivo at any point in the lytic cycle is currently under study. It is also possible that proteins involved in cell DNA replication are targets for PP2A and

FIG. 3. Effect of the addition of the purified catalytic subunit of PP2A on the inhibition of pZ189 replication by small t. (a) Replication reaction mixtures contained 4 μ g of large T alone (□), 4 μ g of large T plus 200 ng of PP2A (●), 4 μ g of large T plus 800 ng of small t (▲), 4 μ g of large T plus 800 ng of small t plus 200 ng of PP2A (○), or 4 μ g of large T plus 400 ng of small t plus 200 ng of PP2A (---○---). Reaction mixtures were not preincubated, and the time course of incorporation of radioactivity was determined as described in the text. The effect of addition of PP2A to reaction mixtures containing both large T and small t was confirmed in three additional experiments in which three different large T preparations were used. (b) Autoradiogram of the products of the in vitro DNA replication experiment shown in panel a, at the 180-min time point. The DNAs were prepared as described in the text. Lanes: 1, large T alone; 2, large T plus small t; 3, large T plus small t (800 ng) plus PP2A; 4, large T plus PP2A. (c) The products of in vitro DNA replication (panel a, 180-min time point) were linearized with 1 U of *Eco*RI for 60 min at 37°C and analyzed as described previously. The DNA from the reaction mixture containing large T, PP2A, and 400 ng of small t was similar to that in lane 4 (data not shown).

that small t inhibition of PP2A plays a role in the transformation of growth-arrested cells by SV40. Interestingly, small t has been shown, in vitro, to inhibit the ability of PP2A to dephosphorylate the cellular myelin basic protein and myosin light chain (29) as well as p53 and large T (18).

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