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Control of Protein Phosphatase 2A by Simian Virus 40 Small-t Antigen

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Soluble, monomeric simian virus 40 (SV40) small-t antigen (small-t) was purified from bacteria and assayed for its ability to form complexes with protein phosphatase 2A (PP2A) and to modify its catalytic activity. Different forms of purified PP2A, composed of combinations of regulatory subunits (A and B) with a common catalytic subunit (C), were used. The forms used included free A and C subunits and AC and ABC complexes. Small-t associated with both the free A subunit and the AC form of PP2A, resulting in a shift in mobility during nondenaturing polyacrylamide gel electrophoresis. Small-t did not interact with the free C subunit or the ABC form. These data demonstrate that the primary interaction is between small-t and the A subunit and that the B subunit of PP2A blocks interaction of small-t with the AC form. The effect of small-t on phosphatase activity was determined by using several exogenous substrates, including myosin light chains phosphorylated by myosin light-chain kinase, myelin basic protein phosphorylated by microtubule-associated protein 2 kinase/ERK1, and histone H1 phosphorylated by protein kinase C. With the exception of histone H1, small-t inhibited the dephosphorylation of these substrates by the AC complex. With histone H1, a small stimulation of dephosphorylation by AC was observed. Small-t had no effect on the activities of free C or the ABC complex. A maximum of 50 to 75% inhibition was obtained, with half-maximal inhibition occurring at 10 to 20 nM small-t. The specific activity of the small-t/AC complex was similar to that of the ABC form of PP2A with myosin light chains or histone H1 as the substrate. These results suggested that small-t and the B subunit have similar qualitative and quantitative effects on PP2A enzyme activity. These data show that SV40 small-t antigen binds to purified PP2A in vitro, through interaction with the A subunit, and that this interaction inhibits enzyme activity.

Neoplastic transformation by polyoma-, papilloma-, and adenoviruses involves complex formation between their transforming proteins and cellular proteins involved in regulating cell proliferation (20). For example, the transforming protein of simian virus 40 (SV40), large T antigen (large T), binds to and presumably inactivates the growth-suppressing proteins p53 (see reference 30 for review) and the product of the retinoblastoma gene (RB) (17, 18). The transforming proteins of some human papillomaviruses also form complexes with p53 and the RB protein, suggesting a similar mechanism of transformation. On the other hand, the principal transforming protein of polyomavirus, medium-T antigen (medium T), does not bind to p53 or the RB protein. Instead, medium T associates with pp60$^{c\text{-}src}$, the product of the c-*src* proto-oncogene (14–16), and strongly activates its protein-tyrosine kinase activity (4, 9, 14). Genetic evidence indicates that the activation of pp60$^{c\text{-}src}$ plays a role in transformation by polyomavirus (3, 16). Medium T also binds to the pp62$^{c\text{-}yes}$ (27) and pp59$^{c\text{-}fyn}$ (10, 28) proteins, two pp60$^{c\text{-}src}$-related kinases, and phosphatidylinositol-3 kinase (43).

Polyomavirus medium T and both polyomavirus and SV40 small-t antigens also form complexes with the serine/threonine-specific protein phosphatase 2A (PP2A) (34, 42). As in the case of pp60$^{c\text{-}src}$, experiments with medium-T mutants suggest that complex formation plays a role in transformation (21). Although the small-t antigens are not transforming by themselves, they support or enhance transformation under certain conditions (1, 6, 11, 19, 31, 35, 37), possibly through an effect on PP2A.

PP2A constitutes a significant portion of the total serine/threonine-specific protein phosphatase activity in many tissues. The major PP2A holoenzyme consists of three subunits, the 37-kDa catalytic subunit (C) and two additional subunits of 61 and 55 kDa, termed A and B, respectively (12). The A and B subunits do not have phosphatase activity but they appear to regulate the activity of C. Analysis of the primary amino acid sequence has shown that the catalytic subunit is highly conserved between species and is a member of a gene family that includes the catalytic subunits of type 1 and 2B protein phosphatases (13). We have isolated and sequenced a human cDNA clone corresponding to a 61-kDa protein associated with polyomavirus middle-T antigen (41) and shown that it is the A subunit of PP2A (42). The deduced amino acid sequence revealed that it consists of 15 nonidentical repeats 38 to 40 amino acids long. cDNA clones encoding the A subunit were also reported by Hemmings et al. (22). PP2A has a broad substrate specificity in vitro, and estimates of activity in vivo indicate that it plays a role in a number of cellular signaling pathways (12).

Type 2A protein phosphatase is the only cellular protein known to associate with SV40 small-t during infection and transformation. All of the cellular effects of small-t, including transformation helper activity and transcriptional activa-

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tion, may be mediated through PP2A. During lytic infection of permissive cells, small-t is in excess and all of the PP2A is present as the small-t/PP2A complex (36). To determine the effects of SV40 small-t on PP2A, we have developed an in vitro reconstitution system with purified proteins. In this report we demonstrate that small-t associates directly with PP2A, through interaction with the A subunit, and inhibits the activity toward a number of substrates. In the accompanying report (36b), we demonstrate that small-t also inhibits the dephosphorylation of SV40 large-T antigen and the p53 growth suppressor protein.

MATERIALS AND METHODS

Materials. Histone H1 (type III-S), myelin basic protein, L-α-phosphatidyl-L-serine, 1,2-dioleoyl-*sn*-glycerol, phorbol 12-myristate 13-acetate (PMA), and Sephadex G-25 were obtained from Sigma. Sephadex G-50 and G-200 were purchased from Pharmacia.

Purification of proteins. The catalytic subunit (C), the two-subunit (AC), and the three-subunit (ABC) forms of PP2A were purified from bovine cardiac muscle as described previously (33). Purified phosphatases were stored and diluted in 25 mM Tris-HCl (pH 7.4)–1 mM EDTA–1 mM dithiothreitol (DTT)–50% glycerol (buffer A). The purity of the preparations was 60% for C, 90% for AC, and 50% for ABC.

Purified A subunit of PP2A was prepared from *Spodoptera frugiperda* cells which had been infected with a recombinant baculovirus containing the cDNA (41) encoding the human protein. Details of the preparation of recombinant baculovirus and purification of the expressed A subunit will be presented elsewhere (30a). Briefly, recombinant virus was prepared and used to infect *S. frugiperda* cells by standard procedures (38). At 60 h postinfection, cells were harvested and lysed by homogenization in hypotonic buffer. The homogenate was centrifuged and the A subunit was purified from the supernatant by chromatography on aminohexyl-Sepharose and Mono-Q columns. The purified subunit was stored at −20°C in 25 mM Tris-HCl (pH 7.4)–1 mM EDTA–1 mM DTT–10% glycerol.

Recombinant SV40 small-t antigen was expressed in pTR865-transformed *Escherichia coli* (2) and purified from an insoluble complex by urea denaturation and column chromatography. Cultures (50 ml) in logarithmic phase were induced for 2 h with 5 mM isopropyl-β-D-thiogalactopyranoside, incubated with lysozyme and EDTA, and lysed with Triton X-100. Small-t, which was present at 5-10% of total bacterial protein, was isolated along with bacterial debris after centrifugation at 18,000 rpm for 20 min in a Sorvall SS-34 rotor. The pellet was incubated with DNase and $MgCl_2$ to reduce viscosity and centrifuged again. The pellet was incubated with 10 M urea in 20 mM Tris-HCl (pH 8)–80 mM NaCl–2 mM DTT–0.25 mM $ZnCl_2$–0.02% Triton X-100 for 1 h at room temperature before centrifugation in a microfuge to remove insoluble material. Soluble proteins were pumped onto an upward-flow Sephadex G-200 column (2.5 by 40 cm) in the same buffer without zinc and urea, and 5-ml fractions were collected. About 40 to 50% of the total small-t was found in monomeric regions of the column (fractions 36 to 42). These were pooled and passed through a DEAE-cellulose column (2 by 5 cm) to remove the few contaminating bacterial proteins. As shown previously, monomeric small-t does not bind to DEAE-cellulose (5). The material that flowed through the column was then concentrated by application to a hydroxylapatite column (2 by 3 cm), and small-t was eluted with 100 mM potassium phosphate (pH 7.6)–80 mM NaCl–10% glycerol–0.02% Triton X-100. The pooled protein was then dialyzed against 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.25)–100 mM NaCl–0.1 mM DTT–10% glycerol–0.02% Triton X-100 (buffer B). From 300 to 400 μg of monomeric small-t was obtained from 50 ml of bacterial culture. Control fractions from *E. coli* transformed with the expression vector alone (no small-t insert) were prepared in an identical fashion.

Protein kinase C, purified as described by Kitano et al. (26), and microtubule-associated protein 2 kinase/ERK1 (MAP-2 kinase) (7) were kindly provided by Melanie Cobb, University of Texas Southwestern Medical Center. Protein concentrations were determined as described by Bradford (8).

Preparation of ^{32}P-labeled substrates. Bovine cardiac myosin light chains were phosphorylated as described previously (32). ^{32}P-histone H1 was prepared by a modification of the method of Jakes and Schlender (24). Histone H1 (500 μg) was incubated at 30°C for 45 min with protein kinase C (20 μg) in a 500-μl reaction volume containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EGTA (ethylene glycol tetraacetic acid), 2 mM $CaCl_2$, 33 μg of phosphatidylserine, 3.3 μg of diacylglycerol, 80 nM PMA, and 100 μM [γ-^{32}P]ATP (1,000 cpm/pmol). The stoichiometry of phosphorylation was 1 mol of ^{32}P per mol of histone H1. Phosphorylation of myelin basic protein (500 μg) was carried out for 1.5 h at 30°C with MAP-2 kinase (3 μg) in a 900-μl reaction volume containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine, 100 μg of bovine serum albumin (BSA) per ml, and 50 μM [γ-^{32}P]ATP (6×10^4 cpm/pmol). The reaction was terminated with 100 μl of 100 mM EDTA–100 mM $NaPP_i$ (pH 7.0). Sephadex G-50 equilibrated with 50 mM HEPES (pH 8.0) and 10% glycerol was used to remove unincorporated ATP. Incorporation of phosphate was 0.1 mol/mol of myelin basic protein.

Phosphatase assays. The effects of small-t on PP2A activity were determined by phosphatase assays after preincubation with small-t. Preincubations were carried out at 4°C unless indicated otherwise. Preincubation mixtures contained 5 μl of protein phosphatase diluted in buffer A and 10 μl of small-t diluted in buffer B, or of buffer B alone, for control incubations. These mixtures were incubated for 30 min, at which time 5 μl of 100 mM MOPS (morpholinepropanesulfonic acid, pH 7.0)–5 mM DTT–2.5 mg of BSA per ml was added, except for histone H1 assays, for which 5 μl of 250 mM imidazole-HCl (pH 7.4)–25 mM EGTA–5 mM DTT–1 mg of BSA per ml was added. The phosphatase assays were initiated by the addition of 5 μl of ^{32}P-labeled substrate. The reactions were terminated by the addition of 10% trichloroacetic acid, and the $^{32}P_i$ released was determined as described previously (32). The amounts of PP2A and small-t present are indicated in the figure legends. The concentrations of the various substrates used, expressed as the concentration of ^{32}P, were 2 μM myosin light chains, 1 μM histone H1, and 0.8 μM myelin basic protein. Protein phosphatase activities are expressed as turnover (picomoles of $^{32}P_i$ released per nanomole of enzyme) with molecular masses of 156 kDa for ABC, 101 kDa for AC, and 38 kDa for C and were corrected for the purity of the preparations.

Gel electrophoresis. Formation of complexes between PP2A and small-t was examined by nondenaturing polyacrylamide gel electrophoresis with a Pharmacia PhastGel System. These gels consisted of 8 to 25% acrylamide gradients and were run according to the manufacturer's instructions.

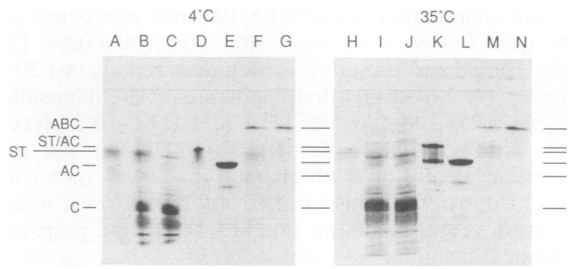

FIG. 1. Formation of complexes between small-t and PP2A. SV40 small-t and various forms of PP2A were incubated for 30 min at 4°C (lanes A to G) or 35°C (lanes H to N), and the mixtures were applied to a nondenaturing gel containing an 8 to 25% acrylamide gradient. The gels were run for 220 V-h and stained with Coomassie blue. The 4°C incubation mixes contained 2.5 μg of C (lanes B and C), 2.5 μg of the AC form (lanes D and E), 1.6 μg of the ABC form (lanes F and G), and 1.6 μg of small-t (lanes A, B, D, and F). The 35°C incubation mixes contained 5 μg of C (lanes I and J), 2.5 μg of the AC form (lanes K and L), 1.6 μg of the ABC form (lanes M and N), and 0.7 μg of small-t (lanes H, I, K, and M). The mobilities of the various species are indicated as follows: C, major staining band of the free C subunit; AC, AC form of PP2A; ST, purified small-t; ST/AC, small-t/AC complex; ABC, ABC form of PP2A.

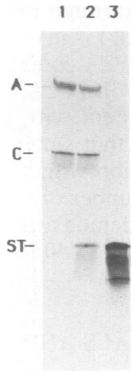

FIG. 2. Analysis of the small-t/AC complex by SDS-polyacrylamide gel electrophoresis. Small-t was incubated with the AC form of PP2A at 4°C. Mixtures containing 1.6 μg of small-t and 2.5 μg of AC or AC alone were applied to a 12.5% nondenaturing gel. Electrophoresis was carried out for 120 V-h, and the gel was stained with Coomassie blue. The protein bands corresponding to the AC form of PP2A or the small-t/AC complex were cut out of the gel, and the pieces (2 by 4 mm) were incubated overnight in SDS sample buffer. The eluted material was applied to an SDS-polyacrylamide gel and run as described in the text. The gel was stained with silver. Lane 1 contained the material eluted from the gel piece containing the AC form of PP2A. Lane 2 contained the piece of gel containing the small-t/AC complex. Lane 3 contained 1.2 μg of purified small-t. The migration positions of the A and C subunits of PP2A and the 17-kDa small-t (ST) are indicated. The band migrating below small-t corresponds to the 14-kDa truncated form of small-t.

From 2 to 3 μl of PP2A or isolated subunits, diluted in buffer A, was incubated for 30 min with 1 to 2 μl of small-t diluted in buffer B and applied to the gel. After electrophoresis, gels were stained with Coomassie blue. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (29) with a 10 to 15% acrylamide gradient.

RESULTS

Small-t forms a complex with the AC form of PP2A. Soluble, monomeric small-t was used for these studies because this form of the protein was shown to interact specifically with the 37- and 61-kDa cellular proteins corresponding to the C and A subunits of PP2A (5). Aggregated forms of small-t associate with several other cellular proteins, not believed to be biologically relevant because they are not detected in immunoprecipitates of infected cells. SV40 small-t antigen produced in bacteria is largely aggregated (5, 39), but monomeric small-t could be obtained when zinc was present during urea denaturation and renaturation. Only the monomeric fraction of small-t, as determined by Sephadex G-200 chromatography, was used for the studies described below. The small-t preparations were greater than 95% homogeneous when analyzed by SDS gel electrophoresis (data not shown). In addition to the 17-kDa small-t antigen, the 865i construct expresses a 14-kDa truncated form by internal initiation of translation from a sequence which resembles the bacterial ribosome-binding site (2). The 17-kDa and 14-kDa proteins were the only proteins detected in SDS gels of the purified protein.

PP2A was incubated with small-t and analyzed by nondenaturing polyacrylamide gel electrophoresis. As shown previously (33), the relative mobilities of the different forms of PP2A vary depending on the complex (Fig. 1). The purified preparation of C subunit showed a number of bands on these gels (lanes C and J). The major Coomassie blue-staining band (C) corresponded to the C subunit, as determined by immunoblotting of the gels with a monoclonal antibody directed against the C subunit (data not shown). These gels also resolved forms of the C subunit that migrated as a broad band just below the major C subunit band and as two sharp bands of higher mobility. The nature of these species is not known, but they could be due to sulfhydryl-disulfide exchange occurring during electrophoresis. The more slowly migrating species were contaminants in the preparation, as deduced from failure to bind antibodies during immunoblot analysis. The mobility of purified recombinant small-t (lanes A and H) was intermediate between those of the ABC and AC forms of PP2A. Although this seems slow for such a small protein, small-t has a basic isoelectric point (pI 8.6) and would have a low net charge in this gel system (pH 8.8) relative to the A (pI 5.0) and C (pI 5.3) subunits of PP2A. The broadness of the small-t band in this gel system, which does not contain reducing agents, is likely due to heterogeneity arising from sulfhydryl-disulfide exchange during electrophoresis. Inclusion of DTT in similar nondenaturing gels sharpens the small-t band (data not shown).

Incubation of the AC form of PP2A with small-t caused a shift in mobility to a more slowly migrating form (Fig. 1, lanes D and K). The new band had a mobility distinct from that of AC, ABC, and small-t. The appearance of this new band corresponded to the disappearance of the AC band (compare lanes D and E). The mobilities of the ABC form and free C were unaffected by incubation with small-t. The constituents of the newly migrating species, generated from incubation of AC with small-t, were determined by excision of the band from the native gel and analysis by SDS gel electrophoresis. Only three proteins were present in this band, corresponding to A, C, and small-t (Fig. 2). The silver-staining pattern indicated that stoichiometric amounts of the three proteins were present in this band. Only the 17-kDa form of small-t was detected, suggesting that the truncated 14-kDa form was not present in the complex isolated from the native gel. These data demonstrate that

small-t forms a complex with AC that has altered mobility during nondenaturing electrophoresis.

The mass of the B subunit of PP2A is 55 kDa, while that of small-t is 17 kDA. Since the mobility of the small-t/AC complex was intermediate between those of the AC and ABC forms, the complex probably contains 1 or 2 mol of small-t. This is consistent with the amounts of silver-stained protein present in the complex (Fig. 2). This also indicates that AC is not interacting with aggregated small-t. The incubation mixes for complex formation assays contained high concentrations of proteins (17 μM small-t and 2.5 μM AC). These concentrations are 500- to 1,000-fold above the estimated dissociation constant (see below), and the interaction of small-t with AC should be essentially complete, as observed in Fig. 1 (compare lanes D and E). Only part of the AC complex was converted to the small-t/AC complex during incubation with small-t at 35°C (Fig. 1, lane K). This could be due to the elevated temperature or to the lower ratio of small-t to PP2A used in this incubation (8 μM small-t, 2.5 μM AC).

No complex formation was seen when small-t was incubated with the ABC form of PP2A. This suggests that the presence of the B subunit inhibits the interaction of small-t. It is possible that small-t can exchange with the B subunit under the appropriate conditions. It has been shown previously that exogenous small-t will form complexes with the 61- and 37-kDa proteins, corresponding to the A and C subunits of PP2A, in cell extracts incubated at 35°C but not at 4°C (36a). To determine whether exchange of small-t with the B subunit requires incubation at elevated temperature, incubations were also done at 35°C (Fig. 1, lanes H to N). The results were essentially the same as observed at 4°C. No significant interaction of small-t with the ABC form or the free C subunit was detected. These data indicate that exchange of small-t for the B subunit in purified PP2A does not occur readily even at 35°C.

Previous evidence has indicated that small-t interacts primarily with the A subunit of PP2A (25). This is based on the selective release of the C subunit from immunoprecipitates of the small-t/AC complex by treatment with *N*-ethylmaleimide. To test whether the A subunit of PP2A is sufficient for interaction, we examined the interaction of small-t with the purified A subunit. Incubation of the A subunit with small-t resulted in the formation of a small-t/A complex which migrated slightly faster than small-t (Fig. 3, lane D). The faint bands present in lanes D and E were minor contaminants in the A subunit preparation. Formation of this complex coincided with a loss of staining intensity of the A subunit band. No complex was formed between small-t and the purified C subunit (lane B). The purified A and C subunits used were both competent for interaction, as demonstrated by the formation of the AC complex (lane G). In addition, incubation of purified A, C, and small-t resulted in the formation of the small-t/AC heterotrimeric complex (lane F). This demonstrates that small-t can interact directly with the A subunit and that the C subunit is not required.

Effect of small-t antigen on the phosphatase activity of PP2A. The effects of small-t on the phosphatase activities of different forms of PP2A were determined by preincubation of small-t with PP2A under conditions in which complexes form. Preincubation with small-t resulted in specific inhibition of the phosphatase activity of the AC form when myosin light chains, phosphorylated by Ca^{2+}- and calmodulin-dependent myosin light-chain kinase, were used as the substrate (Fig. 4). Small-t had no effect on the activities of ABC or free C toward myosin light chains, even in the presence of

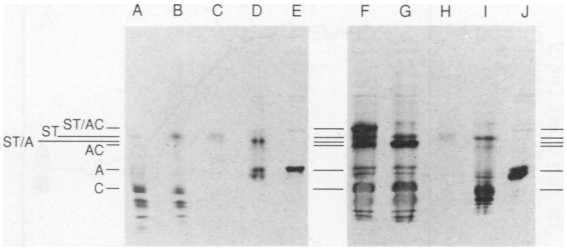

FIG. 3. Formation of complexes between small-t and the A subunit of PP2A. SV40 small-t was incubated with various subunits of PP2A for 30 min at 4°C and applied to an 8 to 25% nondenaturing polyacrylamide gel. The gels were run for 205V-h and stained with Coomassie blue. The incubation mixes contained 2.5 μg (lanes A and B) or 5 μg (lanes F, G, and I) of C subunit; 2.5 μg of purified A subunit (lanes D, E, F, G, and J); and 1.4 μg of small-t (lanes B, C, D, F, and H). The mobilities of the various species are indicated as follows: C, free C subunit of PP2A; A, free A subunit of PP2A; AC, AC form of PP2A; ST/A, small-t/A subunit complex; ST, purified small-t; ST/AC, small-t/AC complex.

a large molar excess of small-t (0.5 nM PP2A and up to 600 nM small-t). The lack of an effect on the activities of ABC or C corresponds to a lack of complex formation between small-t and these forms (Fig. 1).

The inhibition of the myosin light-chain phosphatase activity of AC by small-t exhibits a typical dose-response curve, with a 50% inhibitory concentration (IC_{50}) of 14 nM small-t monomer (Fig. 4A). The maximum extent of inhibition (48%) was reached at 150 nM small-t. The assays for small-t inhibition of the AC form have been repeated with four different preparations of small-t. In each case, a maximal inhibition of 40 to 75% was observed. Consistent with published data (33), the turnover of AC in the absence of small-t was two- to threefold higher than those of ABC and C. The turnover of AC in the presence of saturating small-t was identical to that of ABC and C. Divalent metal ions, particularly manganese, can affect the different forms of PP2A in a substrate-specific manner (12). The basal enzyme activity of the AC form was stimulated two- to threefold by 1 mM $MnCl_2$ when myosin light chains were used as the substrate (Fig. 5). While the total activity was increased in the presence of $MnCl_2$, the relative inhibition by small-t was the same as observed in its absence. In contrast to the AC form, 1 mM $MnCl_2$ inhibited the activity of both the free catalytic subunit and the ABC form of PP2A with myosin light chains (data not shown). Purified preparations of small-t alone had no phosphatase activity in the presence or absence of Mn^{2+} (data not shown).

Evidence that inhibition of phosphatase activity was due to small-t and not to a contaminant in the preparation was obtained with protein fractions from control bacteria. These bacteria were transformed with the expression vector that lacked a cDNA insert and did not produce small-t. Identical fractions, used for purification of small-t, were prepared in parallel from control bacteria and bacteria expressing small-t. The control and small-t-containing fractions were then assayed for their inhibitory effects on the AC form of PP2A. Since the protein concentration of the control fraction was much lower, due to the absence of small-t, equal volumes of the two fractions were used. Small-t concentrations ranging from 1 to 330 nM produced a progressive decrease in AC phosphatase activity, as shown in Fig. 4. Equal volumes of the control fraction had no effect on the activity of the AC form, even at volumes corresponding to

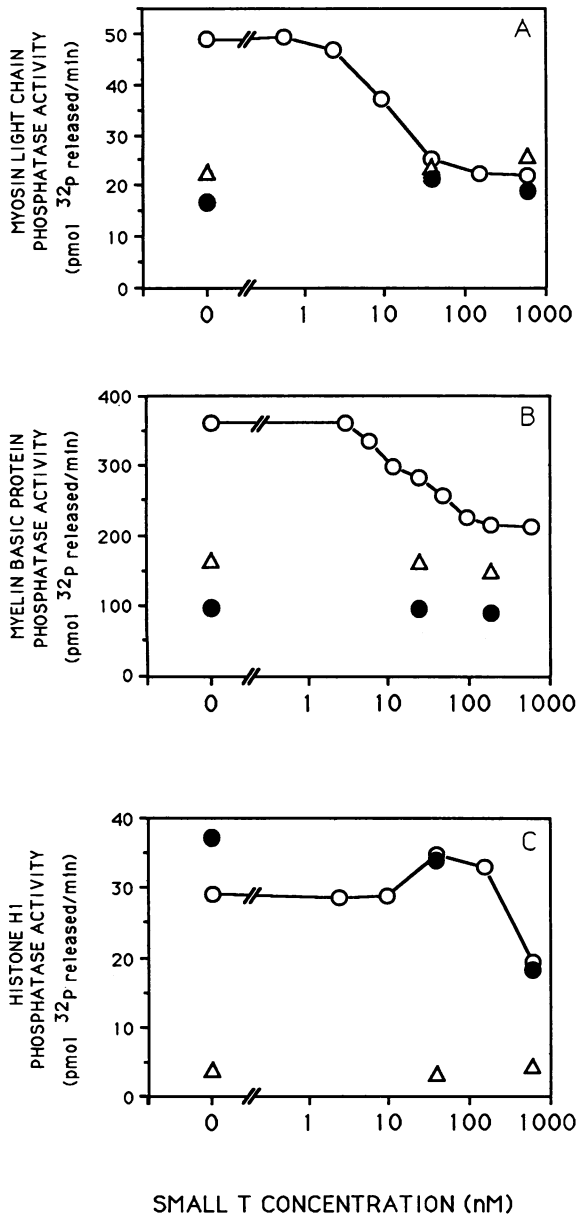

FIG. 4. Effects of preincubation with small-t antigen on the phosphatase activities of various forms of PP2A. The various forms of PP2A were preincubated for 30 min on ice with the indicated concentrations of small-t. Each form of PP2A was present at a final concentration of 0.5 nM. These mixtures were then assayed for protein phosphatase activity with myosin light chains (A), myelin basic protein (B), or histone H1 (C) as the substrate. Symbols: ●, ABC form; ○, AC form; △, free C subunit. The points at the far left are assays of control preincubations which did not contain small-t.

330 nM, at which small-t caused 75% inhibition of enzyme activity (data not shown). Since any contaminating proteins should be identical in these two fractions, we concluded that the inhibition of phosphatase activity was due to the presence of small-t.

The effect of small-t on dephosphorylation of myelin basic protein by PP2A resembled the effects on myosin light chain. Myelin basic protein was phosphorylated by MAP-2 kinase, which is an insulin-responsive protein kinase structurally

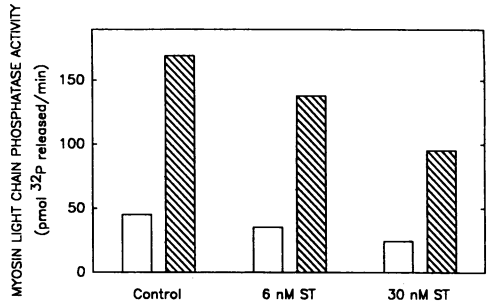

FIG. 5. Effects of Mn^{2+} on the activity of the AC form of PP2A and inhibition by small-t. AC (1 nM) was preincubated in the absence (control) or presence of 6 or 30 nM small-t (ST) as described in the text. Phosphatase activity was then determined in the absence (open bars) or presence (hatched bars) of 1 mM $MnCl_2$ with myosin light chains as the substrate.

related to cell cycle control protein kinases (7). The activity of AC with this substrate was higher than that of the ABC form, while free C had intermediate activity (Fig. 4B). The activity of AC decreased as a function of increasing small-t, while the activities of the other forms of PP2A were not affected. The IC_{50} of small-t inhibition of myelin basic protein phosphatase activity was 10 nM. Unlike myosin light-chain phosphatase activity, the activity of the small-t/AC complex was distinct from that of free C and the ABC form.

Consistent with previous observations (40), the turnover of ABC with histone H1 phosphorylated by protein kinase C was higher than that of AC, while that of free C was 8- to 10-fold lower (Fig. 4C). In contrast to the effects on myosin light-chain and myelin basic protein phosphatase activity, lower concentrations of small-t had a stimulatory effect on the AC form. No stimulation of free C or the ABC form was observed. At higher concentrations, inhibition of both AC and ABC was observed. This effect was only seen at the highest concentration of small-t tested (590 nM). This concentration is 40-fold higher than the IC_{50} for the effect on myosin light-chain or myelin basic protein phosphatase activity and appears to be nonspecific. The specific stimulation of AC was observed at concentrations (10 to 30 nM) which were in the same range as the IC_{50}s for the other substrates. This stimulation was moderate (20%) and dependent on the amount of small-t added. At a maximally stimulating concentration of small-t, the activity of AC was identical to that of the ABC form.

The data described above suggest a specific effect of small-t on the phosphatase activity of the two-subunit form of PP2A through formation of a small-t/AC complex. The IC_{50}s suggest a high-affinity interaction of small-t with AC. In addition to 0.5 nM AC, we have also determined the small-t concentration-response relationship at 1.0 nM AC with myosin light chains as the substrate. A similar IC_{50} (14 nM) was observed, which is consistent with an apparent equilibrium dissociation constant of 14 nM. A dissociation constant of 14 nM is also consistent with maximal inhibition requiring 150 nM small-t. While the kinetics have not yet been investigated, complex formation appears to be rapid, since the extent of inhibition of myosin light-chain phosphatase activity did not vary with preincubation times varying from 1 to 60 min (data not shown).

DISCUSSION

PP2A, a protein serine/threonine phosphatase, has been shown to be complexed to the middle- and small-t antigens of polyomavirus and the small-t antigen of SV40 during both lytic infection and transformation by these viruses (34, 42). Unlike middle T, the only cellular proteins known to interact with small-t antigens are the 61- and 37-kDa proteins, corresponding to the A and C subunits of PP2A. This suggests that all of the effects of small-t are mediated by interaction with PP2A. The known cellular effects of SV40 small-t include a helper function in the establishment and maintenance of the transformed phenotype, transactivation of transcription from certain promoters, dissolution of actin cables, and decreased sensitivity to inhibitors of DNA synthesis (36b). It is important to determine whether and how small-t effects the activity of PP2A. This study shows that SV40 small-t antigen binds specifically to the heterodimeric form of PP2A in vitro and that the primary effect of this interaction is inhibition of enzyme activity.

The primary interaction between SV40 small-t and PP2A is through the A subunit of the enzyme. The interaction of small-t with PP2A is specific for the heterodimeric AC form of PP2A, since no binding or altered enzyme activity was detected with the free C subunit or the ABC heterotrimeric form. Thus, the presence of the A subunit is necessary, whereas the B subunit blocks the interaction of small-t. This indicates that the B subunit and small-t bind to the same or mutually exclusive sites in the AC complex. This explains why the 55-kDa B subunit of PP2A is not present in small-t immune complexes (21, 34, 42). A direct interaction of small-t with the A subunit was demonstrated by formation of a complex between small-t and purified recombinant A subunit (Fig. 3). This confirms previous evidence for the interaction of small-t antigen with the A subunit (25). It is possible that small-t also interacts with the C subunit in the small-t/AC complex; however, these data demonstrate that the C subunit is not required for binding. In this regard, small-t is similar to the B subunit of PP2A. It has been shown with urea-dissociated subunits that the B subunit cannot interact directly with the C subunit (23). The B subunit does bind to the AC complex and alters the enzyme activity. It is not known if the B subunit interacts directly with the A subunit or if the C subunit is also required.

Small-t does not readily exchange with the B subunit present in purified ABC in vitro. This was true even at 35°C (Fig. 1), at which temperature formation of a complex between exogenous small-t and PP2A in extracts of CV-1 cells occurs readily (5). This suggests that the B subunit dissociates slowly from the ABC complex in vitro and/or that the B subunit has a much higher affinity for AC than small-t. It will be important to determine whether some cellular factor is required for efficient exchange of small-t for B or whether there are appreciable amounts of the AC form of PP2A in infected cells. Most evidence indicates that the three-subunit forms of PP2A are the major forms present in vivo (12). However, there is also evidence that in some cell types, the AC form is predominant (40).

Small-t and the B subunit, present in cardiac PP2A, have quantitatively and qualitatively similar effects on the activity of PP2A in vitro. Small-t interacted in a saturable manner with high apparent affinity (Fig. 4) to alter the phosphatase activity of the AC form. While small-t inhibited the activity of AC toward myosin light chains and myelin basic protein, it caused a mild stimulation of the activity toward histone H1. This is consistent with previous reports showing that the B subunit also stimulates activity toward histone H1 (23, 40). The inhibition of histone H1 phosphatase activity at higher concentrations of small-t appeared to be nonspecific and could be due to the direct interaction of small-t with this substrate. The major effect of small-t on the dephosphorylation of SV40 large T antigen and the p53 growth suppressor protein is inhibition of enzyme activity (36b). Although the maximal extent of inhibition by small-t was moderate (40 to 75%), this level of inhibition is consistent with the effect of the B subunit, the only other protein known to interact with AC. Small-t had effects on the dephosphorylation of myosin light chains and histone H1 that appeared to be identical to those of the cardiac B subunit. However, when myelin basic protein was used, the activity of the small-t/AC complex was higher than that of the ABC complex. This demonstrates that small-t and the B subunit have differential effects with some substrates and suggests that the effects of small-t may be dependent on the substrate. Small-t could act by selectively altering some cellular pathways while not affecting others. This selective effect would be consistent with the known cellular functions of small-t.

Multiple heterotrimeric forms of PP2A, composed of AC complexed to distinct B subunits (B, B′, or B″) have been identified in a number of different tissues and cell types (12). Like small-t, these B subunits differentially regulate the activity and substrate preference of the AC complex. For example, three forms of PP2A isolated from human erythrocytes (AC, ABC, and AB″C) have V_{max} values of 86, 9, and 198 mol of P_i released per min per mol of enzyme, respectively, with histone H1 as the substrate (40). The two heterotrimeric forms differ in the B subunit present; B is similar to the cardiac 55-kDa subunit, while B″ is 74 kDa. Since small-t appears to replace the B subunit in SV40-infected cells, the effect of small-t may be dependent on the type of B subunit present. In cells in which small-t plays a role in transformation, there may be a difference in the type or quantity of the B subunit present compared to cells in which small-t function is not required for transformation (36b).

In summary, the data presented here show that SV40 small-t interacts with PP2A in vitro through direct binding to the A subunit. The binding of small-t alters the activity of PP2A in a substrate-specific fashion primarily by inhibition of enzyme activity. This indicates that one effect of small-t expression during lytic infection of permissive cells or transformation of nonpermissive cells by SV40 is likely to be an alteration of PP2A activity. The control of PP2A by small-t would be expected to alter signaling through phosphorylation-dephosphorylation pathways regulated by PP2A. This alteration in protein dephosphorylation could account for the effects of small-t antigen on cell growth and gene transcription. While numerous viral oncogenes act through alterations in protein kinase activity, SV40, polyomavirus, and other papovaviruses are the first examples of transforming viruses that utilize alteration of a protein phosphatase in transformation. A complete understanding of the role of small-t antigens in transformation will require identification of cellular phosphoproteins whose dephosphorylation is altered by small-t. In the accompanying paper (36b), we show the effects of small-t on the dephosphorylation of two candidate proteins, SV40 large-T antigen and the p53 growth suppressor protein.

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