J. Biochem. Mol. Biol. Vol. 28, No. 4 pp. 331~335 July 31, 1995

Analysis of the Stoichiometry and the Domain for Interaction of Simian Virus 40 Small-t Antigen with Protein Phosphatase 2A

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(Received December 2, 1994)

Abstract: Simian virus 40 (SV40) small-t antigen (small-t) has been known to regulate the activity of a cellular enzyme, protein phosphatase 2A (PP2A), composed of A, B, and C subunits, via binding to the A subunit. In the study presented here, the stoichiometry of the binding of small-t to PP2A was determined to be 1 : 1. It was also shown that small-t binds to the AC form of PP2A with a higher apparent affinity than it binds to the free A subunit. We also characterized the interaction of PP2A with wild-type and various mutant small-ts. A single-point mutant (Val134Met) and a double-point mutant (Trp147Gly,Leu152 Pro) of small-t exhibited 3-fold and 5-fold lower potencies in inhibiting PP2A activity, respectively. This suggests that the region around amino acids between 134 and 152 of small-t might be important in regulating the enzyme activity of PP2A.
Key words: protein phosphatase 2A, simian virus 40 small-t, stoichiometry.

Simian virus 40 (SV40) small-t antigen (small-t) exerts several effects on cells infected with SV40. For instance, it helps SV40 large T antigen (large T) in the transformation of permissive cells (Bikel *et al.*, 1987) and when expressed, it induces the proliferation of CV-1 cells (Sontag *et al.*, 1993). Small-t binds a cellular enzyme, protein serine/threonine phosphatase 2A (PP2A) in virally infected or transformed cells (Pallas *et al.*, 1990; Walter *et al.*, 1990). In uninfected cells, PP2A exists as a heterotrimer composed of structural (A), catalytic (C), and regulatory (B) subunits. Small-t has been found to be complexed only with the heterodimeric form (AC) of PP2A in SV40-infected cells. Hence it is believed that upon infection of cells with SV40, small-t replaces the B subunit of PP2A. The B subunit of PP2A and small-t are similar to each other in that they readily form a complex with and regulate the activity of the purified AC form of PP2A *in vitro* (Yang *et al.*, 1991; Kamibayashi *et al.*, 1992). They have similar inhibitory effects on the enzyme activity of the AC form of PP2A against the majority of substrates including myosin light chain and myelin basic protein (Yang *et al.*, 1991; Kamibayashi *et al.*, 1994), while towards a small subset of substrates such as MAP kinase and MAP kinase kinase (Sontag *et al.*, 1993; Kamibayashi *et al.*, 1994), they have differential regulatory effects. Since small-t mutants that do not bind to PP2A lose the ability to induce cell proliferation (Sontag *et al.*, 1993), interaction with PP2A is thought to be crucial for mediating small-t's cellular functions.

Small-t is composed of 174 amino acids. While its N-terminal 82 amino acids are identical to large T (Berk and Sharp, 1978), the remaining C-terminal portion is unique to small-t and contains two cysteine-rich clusters (Cys-X-Cys-X-X-Cys) at amino acids 111 to 116 and 138 to 143 (Friedman *et al.*, 1978). Consistent with the fact that large T is not capable of binding PP2A (Yang *et al.*, 1979), the N-terminal 82 amino acids of small-t are not required for binding PP2A (Sontag *et al.*, 1993). The domain directly involved in binding PP2A has been determined to be the amino acid residues between 96 and 106 within the small-t-unique region of the molecule (Mungre *et al.*, 1994). The two cysteine clusters are thought to affect the protein stability (Jog *et al.*, 1990).

In this report, we determined the stoichiometry of the binding of small-t to PP2A to understand the structural basis for small-t being functionally similar to the B subunit of PP2A. We also characterized the domain of small-t important in regulating PP2A activity using various small-t mutant proteins whose gene contains point mutations at sites corresponding to amino acids beyond residue 106 and outside the cysteine clusters.

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Materials and Methods

Materials

A monoclonal antibody against SV40 small-t (Pab 419) was purchased from Oncogene Science (Uniondale, USA).

Source of proteins

The N-terminally truncated forms of wild-type small-t and point mutant proteins were obtained from Dr. Kathleen Rundell (Northwestern University). They had been produced by localized random chemical mutagenesis and bacterial expression according to the procedures of Shortle and Nathans (1978) and Myers *et al.* (1985). The two-subunit (AC) form of PP2A was purified from bovine cardiac muscle as described previously (Mumby *et al.*, 1987). The A subunit was purified from Spodoptera frugiperda 9 cells (Yang *et al.*, 1991; Kamibayashi *et al.*, 1992).

Analysis of small-t binding to PP2A

Formation of complexes between the AC form or the A subunit of PP2A with small-t was examined by nondenaturing polyacrylamide gel electrophoresis with a Pharmacia phast gel system (Uppsala, Sweden) and immunoblotting with a monoclonal antibody against small-t (Pab419) or antisera against the A subunit of PP2A as described previously (Yang *et al.*, 1991; Kamibayashi *et al.*, 1992). Briefly, the gels containing 20% acrylamide were pre-run for 10 volt hours (Vh) and then subjected to the sample loading and separation at 10 mA for 200 Vh. After electrophoresis, proteins were electrophoretically transferred from polyacrylamide gels onto nitrocellulose membranes. The membranes were probed with the appropriate antibodies, and proteins were detected by Chemiluminescent method as recommended by Amersham Co. (Buckinghamshire, UK).

Preparation of ^{32}P-labelled substrate and protein phosphatase assay

Myosin light chain (MLC) was phosphorylated with MLC kinase and used as a substrate for PP2A, whose phosphatase activity was determined as described previously (Yang *et al.*, 1991). Briefly, 0.5 nM (final concentration) of the AC form of PP2A was preincubated with varying amounts of wild-type or mutant small-t at 4℃ for 30 min. ^{32}P-labelled MLC (final concentration of 2 μM) and the 5 x reaction buffer (100 mM MOPS, pH 7.0, 5 mM DTT, 2.5 mg/ml BSA) were then added to this mixture in a final reaction volume of 25 μl and incubated at 30℃ for 10 min. After stopping the reaction with 10% trichloroacetic acid, the amount of $^{32}P_i$ released was measured by scintillation counting.

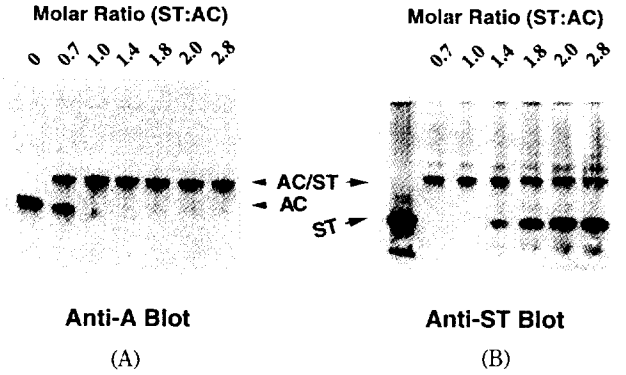

Fig. 1. A stoichiometric binding of small-t to the AC form of PP2A. The AC form of PP2A was preincubated with varying molar ratios of wild-type small-t for 30 min on ice. The formation of the complex was analyzed by nondenaturing gel electrophoresis using Pharmacia's PhastGel system and immunoblotting using the ECL detection system with anti-A subunit antiserum (panel A) or anti-small-t antiserum (panel B). The gels contained 20% polyacrylamide and the final concentration of AC was 1.9 μM. The arrows indicate the positions where AC/ST complex, free AC, or free small-t (ST) migrated.

Results

The stoichiometry of the interaction between small-t and PP2A

The stoichiometry of the binding of small-t to the AC form of PP2A was determined by measuring the molar ratio of AC to small-t at the point at which binding could be considered saturated (Fig. 1). A fixed amount (final concentration of 1.9 μM) of the AC form of PP2A was incubated with varying amounts (a molar ratio of small-t to AC of from 0 to 2.8) of small-t and the resulting complex formation was assayed by nondenaturing gel electrophoresis followed by immunoblot analysis with anti-A subunit (panel A) or anti-small-t (panel B) antibodies. When the added amount of small-t was lower than that of AC (that is, when the molar ratio of small-t to AC was 0.7), there was some free AC (panel A), while all of the small-t was in the complex (panel B). When the molar ratio of small-t to AC was 1, all of AC and small-t were in the complex. With small-t in excess (molar ratios above 1), all of AC was in the complex (panel A), while some free small-t was present (panel B), indicating that the extent of complex formation reached a plateau. These data indicate that 1 molecule of small-t binds to 1 molecule of AC.

In Fig.2, the A subunit of PP2A was used instead and the binding of small-t to the A subunit of PP2A was assayed using a fixed concentration (4 μM) of the A subunit of PP2A and varying amounts of small-t.

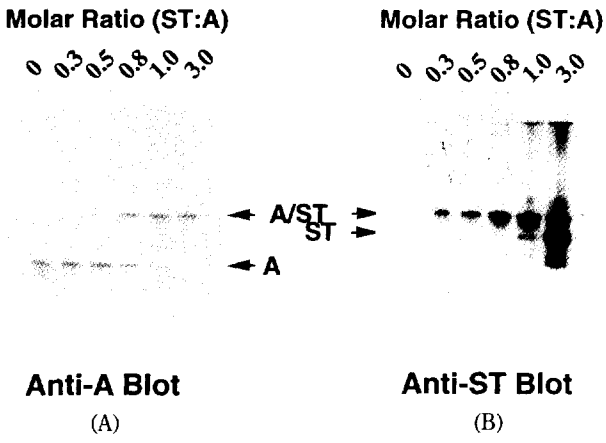

Fig. 2. Higher apparent binding of small-t to AC than to the A subunit of PP2A. Assays were performed in the same manner as in Fig. 1, except that free A subunit (4 μM) was used instead. The arrows indicate the positions of A/ST complex, free ST, and free A subunit.

When small-t was limiting, free A was observed (panel A), while all of small-t was present in the complex (panel B). However, when the molar ratio was 1, there were both free A and free small-t. This is different from the results of the above experiments in Fig. 1 using the AC form. One possibility is that there were inactive proteins present in the A subunit preparations. However, this possibility could be ruled out, because when small-t was provided three-fold in excess, all of the A subunit was in the complex (panel A), while free small-t was present (panel B). This indicates that the binding affinity of small-t for free A is lower than that for the AC form.

Effect of point mutations of small-t on its ability to regulate PP2A

Binding of small-t to PP2A has been shown to occur through direct binding to the A subunit of PP2A (Yang *et al.*, 1991). The domain in small-t involved in this binding has been assigned to the region of amino acids 96 to 106 in a recent study employing several small-t mutants, most of which had point mutations at sites corresponding to the amino acids between 96 and 106 (Mungre *et al.*, 1994). To localize the domain involved in regulating PP2A in the present study, we examined several other small-t mutant proteins which had been generated by point mutations at sites encoding amino acids beyond residue 106. Single point mutant proteins had the amino acid substitutions from lysine to asparagine at residue 126 (Lys126Asn), or from valine to methionine at 134 (Val134Met), while a double point mutant had the amino acid changes from tryptophan to glycine at residue 147 and from leucine to proline at residue 152 (Trp147Gly;Leu152Pro). For negative and positive comparisons, another point mutant (Cys97 Ser), produced by site-directed mutagenesis, and wild-type small-t were also included in the assays. In addition to the point mutation, each mutant had a truncation from the N-terminal to the 51st amino acid. Due to a Shine-Dalgarno sequence (13 base pairs upstream of a methionine codon at amino acid 52), a truncated protein (14 kDa) is expressed in addition to the full length protein (Bikel *et al.*, 1983). Since this truncated form of small-t binds and inhibits PP2A *in vitro* as efficiently as the full-length small-t (our own unpublished data; Mungre *et al.*, 1994), we used the truncated forms of each mutant and wild-type, which were expressed by using the expression vector, pKK223-3 (Pharmacia; Uppsala, Sweden).

Using these mutants and wild-type, we investigated their abilities to regulate the phosphatase activity of the AC form of PP2A (Fig. 3). The wild-type small-t inhibited the dephosphorylation of myosin light chain (MLC) by AC in a concentration-dependent manner with a 50% inhibitory concentration (IC_{50}) of approximately 33 nM. On the other hand, the point mutant, Cys97Ser showed almost no ability to inhibit PP2A activity, consistent with the findings by others who used the full-length mutant with the same mutation at this position (Mungre *et al.*, 1994). This mutant is believed to be defective in the domain responsible for binding to PP2A (Mungre *et al.*, 1994). The profile of PP2A inhibition by the Lys126Asn mutant was very similar to that by wild-type. When mutation resulted in the change of amino acid at 134 from valine to methionine (Val134Met), the ability to inhibit the enzyme activity of the AC form of PP2A was 2~3 fold diminished, compared to the wild-type small-t. The double point mutant protein, Trp147Gly;Leu152Pro had even further diminished potency to inhibit the activity of PP2A, as manifested by a 5~6 fold higher IC_{50} of this mutant protein than the IC_{50} of wild-type. Taken together, these results suggest that amino acid 126 of small-t has little effect on the ability of small-t to regulate PP2 A, while regions around amino acid 134 and amino acids 147 to 152 of small-t might be important in efficiently regulating the enzyme activity of PP2A.

Discussion

Since small-t (17 kDa) and the B subunit (55 kDa) of PP2A behave similarly in regulating the activity of PP2A towards many substrates, we explored the possibility that two or more molecules of small-t might bind to PP2A. However, in the studies presented here, the stoichiometry of binding of small-t to PP2A rather turned out to be 1 : 1, indicating that one molecule of

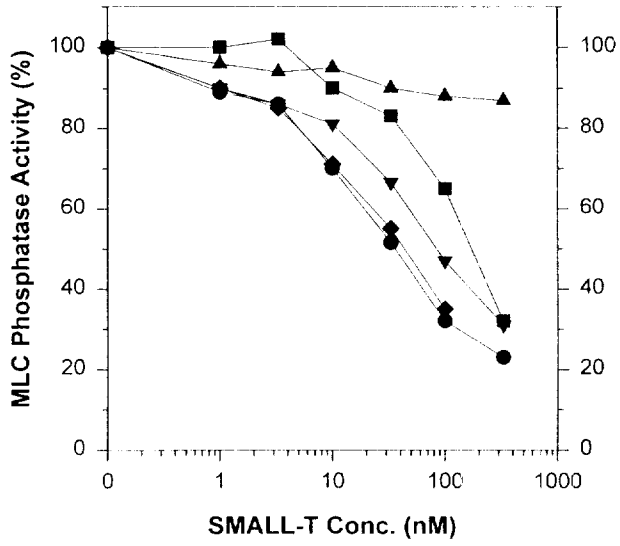

Fig. 3. Effect of point mutations of small-t on its inhibitory activity of PP2A. Varying concentrations of the truncated forms of wild-type small-t (●) and various point mutants of small-t (Cys97 Ser : ▲, Lys126Asn : ◆, Val134Met : ▼, Trp147Gly:Leu152Pro : ■) were preincubated with the AC form of PP2A for 30 min on ice. These mixtures were then assayed for protein phosphatase activity using ^{32}P-labelled MLC as described under Materials and Methods. 100% activity indicates the activity of PP2A in the absence of small-t.

small-t is enough to mimic the B subunit in regulating the activity of the AC form of PP2A towards many substrates. This suggests that small-t and the B subunit share some common three-dimensional structural motifs, even though the primary sequences of these proteins have no homology to each other. In addition, since small-t possesses transformation-helping activity (Bikel *et al.*, 1987), while the B subunit of PP2A does not, the two proteins probably also have some domains structurally unique to each other. These unique domains might allow them to have different regulatory effects on the dephosphorylation of some substrates by the AC form of PP2A. Alternatively, since the B subunit has a larger molecular weight than small-t, it can be proposed that the B subunit has all of the domains that small-t has plus some extra domains, and the lack of certain domains could confer the transformation-helping activity to small-t.

The extent of binding of small-t to AC was shown to be higher than to the free A subunit, indicating that the affinity of small-t for the free A subunit is lower (roughly 10 to 50 times) than the affinity for the AC form of PP2A. Higher apparent affinity of small-t for AC than for A can be achieved if additional binding sites for small-t are provided on the AC form. These additional binding sites could be on either the A or the C subunit. The additional small-t binding sites in the A subunit may be hidden in the free A subunit but undergo a conformational change when the C subunit is bound. If the additional binding sites are on the C subunit, these sites might become available for binding when the C subunit is complexed with the A subunit, because small-t can not bind to the free C subunit (Yang *et al.*, 1991). It is also possible that the binding sites for small-t are always present in the C subunit but are recognized by small-t only when small-t itself undergoes a conformational change through binding first to the A subunit.

Studies using point mutants of small-t revealed several residues of small-t important for regulating the enzyme activity of PP2A. Mutation resulting in the amino acid change from lysine to asparagine at residue 126 had no effect, while mutations at sites corresponding to amino acid(s) of residue 134 or between residues 147 and 152 caused a 2~6 fold decrease in the potency of small-t to inhibit the enzyme activity of the AC form of PP2A. From these results, it can be proposed that amino acids 134 to 152 of small-t might be involved in regulating PP2A activity. It is not known, however, whether this domain is directly involved in regulating the activity of PP2A or whether it is the domain responsible for binding PP2A. If the latter possibility is true, amino acids 134 to 152 of small-t might be involved in binding to the additional sites present in the A or the C subunit of PP2A as discussed above, because the domain of small-t for direct binding to the A subunit of PP2A resides between amino acids 96 and 106 (Mungre *et al.*, 1994). However, it can be also imagined that this region of amino acids 134 to 152 of small-t has no functions at all, but point mutations at this region affect the conformations and thus the functions of the domains directly involved in either binding or regulating PP2A. Nonetheless, these data indicate that the integrity at regions around amino acids 134 to 152 is important in maintaining the ability of small-t to regulate the enzyme activity of PP2A.

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