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Identification of Binding Sites on the Regulatory A Subunit of Protein Phosphatase 2A for the Catalytic C Subunit and for Tumor Antigens of Simian Virus 40 and Polyomavirus

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Protein phosphatase 2A is composed of three subunits: the catalytic subunit C and two regulatory subunits, A and B. The A subunit consists of 15 nonidentical repeats and has a rodlike shape. It is associated with the B and C subunits as well as with the simian virus 40 small T, polyomavirus small T, and polyomavirus medium T tumor antigens. We determined the binding sites on subunit A for subunit C and tumor antigens by site-directed mutagenesis of A. Twenty-four N- and C-terminal truncations and internal deletions of A were assayed by coimmunoprecipitation for their ability to bind C and tumor antigens. It was found that C binds to repeats 11 to 15 at the C terminus of A, whereas T antigens bind to overlapping but distinct regions of the N terminus. Simian virus 40 small T binds to repeats 3 to 6, and polyomavirus small T and medium T bind to repeats 2 to 8. The data suggest cooperativity between C and T antigens in binding to A. This is most apparent for medium T antigen, which can only bind to those A subunit molecules that provide the entire binding region for the C subunit. We infer from our results that B also binds to N-terminal repeats. A model of the small T/medium T/B-A-C complexes is presented.

The transforming proteins of small DNA tumor viruses form multiple complexes with cellular proteins involved in signal transduction and growth control. These interactions play an important role in virus-induced tumorigenesis. Simian virus 40 (SV40) large T binds to the tumor suppressor proteins p53 (40, 42) and Rb (16) and presumably inactivates their function. Polyomavirus medium T associates with pp60$^{c\text{-}src}$ and activates its protein-tyrosine kinase activity (4, 12, 14). It also binds to other members of the c-*src* family (9, 36, 39). In addition, medium T binds to and activates phosphatidylinositol-3 kinase (13, 32). Moreover, medium T forms a complex with two cellular proteins of approximately 61 and 37 kDa (23, 24, 35, 50, 59, 60, 64). The two proteins are associated with each other in uninfected cells (23). The medium T antigen of nontransforming *hrt* mutants (3) does not form a complex with the 61-kDa and 37-kDa proteins but binds to the 73-kDa heat shock protein instead (23, 24, 35, 50, 60, 64, 70). These data suggest that complex formation between medium T and the 61-kDa and 37-kDa proteins might be necessary for transformation. SV40 small T forms a complex with two cellular proteins of approximately 56 and 32 kDa (76). These proteins are also associated with polyomavirus and BK virus small T (55, 56). They are identical to the medium-T-associated 61-kDa and 37-kDa proteins, respectively (50, 71).

The 61-kDa protein was purified and partially sequenced, and its cDNA was cloned from a human cDNA library (72). Its predicted amino acid sequence revealed a protein consisting of 15 imperfect repeats, most of which are 39 amino acids long. It had no resemblance to known proteins in data banks. The 37-kDa protein was also purified, partially sequenced, and found to be identical to the catalytic C subunit of protein phosphatase 2A (PP2A) (51), which had been cloned and sequenced previously (15, 22, 34, 66). It was also shown that the 61-kDa protein is the regulatory A subunit of PP2A (51, 73). The amino acid sequence of the A subunit is highly conserved between species, as demonstrated by comparison of the human and bovine proteins (73). In addition, two homologous cDNAs corresponding to α and β isoforms of the A subunit of PP2A were isolated from a porcine kidney library (26), and the predicted amino acid sequences of these proteins are highly related to the two human Aα and Aβ isoforms, respectively (72).

PP2A is a member of the family of serine/threonine-specific protein phosphatases that includes protein phosphatases 1 and 2B. It is the major soluble form of protein serine/threonine phosphatase in most tissues and cells. The holoenzyme is composed of the catalytic subunit C and two regulatory subunits, A and B. Multiple heteromeric forms of PP2A consisting of A and C complexed to different B subunits (Bα, Bβ, B′, and B″) have been identified in different tissues and cell types (10, 63). The nucleotide sequences of cDNAs for the B subunits from human (45), rabbit (25, 45), *Saccharomyces cerevisiae* (25), and rat (49) have been reported recently.

SV40 small T binds the purified A subunit (75) as well as the A-C form of PP2A but does not bind the free C subunit or the holoenzyme (75). Because the B subunit also binds to the A subunit, it probably prevents small T from binding. Likewise, polyomavirus medium T appears to compete with the B subunit for binding to A because complexes of medium T with A-C but not with A-B-C have been isolated from cells (23, 24, 35, 50). It has also been demonstrated that, in cell extracts, SV40 small T is bound to the A-C form through the A subunit (29).

SV40 small T inhibits the phosphatase activity of the A-C

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form of PP2A when myosin light chains, myelin basic protein, SV40 large T antigen, or p53 is used as an exogenous substrate (46, 61, 75). This inhibitory effect of small T is similar but not identical to that of the B subunit. Inhibition of PP2A might explain the biological function of small T as a cofactor in cell transformation, and it is reminiscent of the action of the tumor promoter okadaic acid (67), which also inhibits PP2A (10). The targets of okadaic acid and small T are different, however, because okadaic acid binds to the C subunit and inhibits phosphatase activity completely, whereas small T binds to the A subunit and inhibits phosphatase activity up to a maximum of 50% in vitro (27, 30, 52, 75). The finding that the inhibition of PP2A stimulates cell growth indicates that PP2A plays a negative role in growth control, as proposed by Cohen and Cohen (11). Several other studies support this notion (17, 19, 33, 41, 74). In yeast (33) and mammalian (54) cells, the expression and activity of PP2A was found to be constant throughout the cell cycle. Therefore, PP2A may not be subject to cell cycle control like other growth-regulatory proteins.

PP2A stimulates SV40 large-T-dependent SV40 DNA replication in vitro by dephosphorylating Ser-120 and Ser-123 of large T (62, 68). The phosphorylation of these sites has a negative effect on binding of large T to the origin of replication, resulting in replication inhibition. Addition of SV40 small T to the in vitro replication system is inhibitory (6), presumably because small T inhibits dephosphorylation of Ser-120 and Ser-123 by PP2A. In vivo, SV40 small T probably stimulates DNA replication early in infection (55a), as does polyomavirus small T (43, 47, 48). How the seemingly opposite in vitro and in vivo effects of small T can be reconciled is an open question.

To better understand how T antigens interact with PP2A and influence its proposed role in growth control, we characterized binding sites for the T antigens and the C subunit on the A subunit of PP2A. The results demonstrate that all tumor antigens bind in the N-terminal half of the A subunit, whereas the C subunit binds to the C terminus. Although the binding regions for different tumor antigens overlap, they are not identical. Furthermore, the data suggest cooperativity in binding between tumor antigens and the C subunit.

MATERIALS AND METHODS

Mutants of PP2A-Aα. Site-directed mutagenesis was performed by the method of Kunkel (38) with the Muta-Gene kit from Bio-Rad. Briefly, CJ236 bacteria were transformed with pBluescript M13+ (Stratagene) containing cDNA encoding the human Aα subunit of PP2A (plasmid p16) (72, 73). The bacteria were infected with helper phage VCSM13 (Stratagene). Single-stranded uracil-containing (U-DNA) was isolated and stored at −70°C. Mutagenic oligonucleotides were annealed to the U-DNA, the second strand was synthesized, and competent XL1Blue bacteria (Stratagene) were transformed. Mutants were identified by restriction patterns, cDNA fragment sizes, and sizes of in vitro-translated proteins.

To synthesize the C-terminal truncation mutants, mutagenic oligonucleotides were made that contained a stop codon and an overlapping *Bam*HI site: TAGGATCC. The 5′ and 3′ annealing stretches were 16 to 19 nucleotides long and ended on at least one G or C. The *Bam*HI site was used for screening. The N-terminal truncation mutants and the single-repeat deletion mutants were created by loop-out mutagenesis. The oligonucleotides were 55 to 63 nucleotides long and complementary to the regions immediately upstream and downstream of the loops. N-terminal truncation mutant 181 was made by cutting plasmid p16 with *Eag*I in the multicloning site upstream of the insert and with *Nco*I at nucleotide 536 within the insert (before Met-180). The larger piece was purified from a low-melting-point agarose gel. To religate, a linker with *Eag*I and *Nco*I sites was made so that the Kozak sequence (37) of p16 was conserved as far as the *Nco*I site permitted: GGC CGG GCG GGG GAA AGG GAC GGA GCC AC.

Wild-type and mutant plasmids were transcribed as described previously (72). Briefly, the plasmids were linearized with *Cla*I and transcribed with T7 RNA polymerase (Promega). The RNA was translated in the presence of [^{35}S]cysteine (NEN) with a reticulocyte translation kit (Promega).

Confirmation of the mutations by DNA sequencing was not considered necessary for the following reasons. (i) C-terminal truncations were constructed by inserting a new restriction site at the site of mutation. This site was used for selection. (ii) All truncation and deletion mutant plasmids were digested with different restriction enzymes and yielded fragments of expected sizes. (iii) All proteins encoded by truncation mutants had the apparent molecular weights on polyacrylamide gels that were expected from the locations of the mutations. (iv) Proteins encoded by single-repeat deletion mutants were slightly smaller than the wild-type protein, as one would expect. (v) Very similar results for the locations of binding sites were obtained independently from truncation and deletion mutants. (vi) Mutations in adjacent repeats provided a consistent picture of the length of binding sites. For example, deletion of neighboring repeats resulted in a steady loss and subsequent gain of binding.

Tumor antigens. (i) SV40 small T. Recombinant SV40 small T was a gift from Kathy Rundell. It was purified from bacteria as described previously (20).

(ii) Polyomavirus small T. The baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) and its derivative AcYMpst (see below) were propagated in *Spodoptera frugiperda* cells (Sf9) at 27°C in TNM-FH medium (GIBCO) supplemented with 10% fetal calf serum. Polyomavirus small T cDNA from plasmid pST1 (77) was placed behind the polyhedrin promoter in transfer vector pAcYM1 (44). pST1 was cleaved with *Bst*XI at nucleotides 174 and 1702, and the ends were blunt-ended with T4 DNA polymerase (Promega). The transfer vector pAcYM1 was linearized with *Bam*HI and also blunt-ended. The small-T-encoding fragment was subsequently ligated with pAcYM1, resulting in transfer plasmid pYMpst.

Sf9 cells were cotransfected with pYMpst and with the DNA of virus Ac*M*NPV by a modified version of the calcium phosphate precipitation technique (5). The medium was screened first for recombinant viruses by dot blotting (53). The expression of small T was verified by immunoprecipitation. Finally, the recombinant baculovirus, AcYMpst, was plaque purified. Sf9 cells were infected with recombinant baculovirus AcYMpst. After 37 h, the cells were washed two times with Tris-buffered saline and lysed on ice with 50 mM Tris-HCl (pH 8.0)–150 mM NaCl–2% Nonidet P-40 (NP-40)–1 mM EDTA–2 μg of aprotinin per ml–100 μg of phenylmethylsulfonyl fluoride per ml. The nuclei were pelleted at 11,000 × *g* and 4°C for 15 min. The supernatant was mixed with 0.5 volume of 50 mM Tris-HCl (pH 7.5)–50 mM NaCl–40% glycerol. Finally, the extract was centrifuged at 100,000 × *g* and 4°C for 30 min, and the supernatant was frozen. The extracts contained approximately 10 ng of

polyomavirus small T per μl, as determined by Western immunoblotting.

(iii) Polyomavirus medium T. Human 293 cells (21) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. They were infected with hybrid adenovirus type 5 containing the cDNA for polyomavirus medium T under control of the late adenovirus promoter (59). Cell extracts were prepared as described previously (72) and contained approximately 30 ng of polyomavirus medium T per μl, as determined by polyacrylamide gel electrophoresis (PAGE) and silver staining.

Complex formation. (i) SV40 small T. Wild-type and mutant A subunit RNAs were translated, and 10 μl of each reaction mixture was incubated on ice for 30 min with 0.3 μl (200 ng) of SV40 small T or 0.3 μl of buffer. After addition of 5 μl of hamster antitumor serum (a gift from Kathy Rundell), the incubation on ice was continued for 2 h. Then, 10 μl of settled protein A-Sepharose CL-4B (Pharmacia) in 20 μl of RIPA (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100) was added. The samples were kept on ice for 1 h and mixed every 10 min. For gel analysis of total translation products, an aliquot of the supernatant was dissolved in sodium dodecyl sulfate (SDS)-PAGE sample buffer (final concentrations: 10% glycerol, 60 mM Tris-HCl [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 10 mM dithiothreitol, 0.01% bromophenol blue). The pellets were washed three times with 1 ml of ice-cold RIPA, mixed with 20 μl of two-times-concentrated sample buffer, and boiled for 5 min. The supernatants were analyzed on 0.5-mm-thick SDS–12% polyacrylamide gels. The gels were treated with acetic acid and 2,5-diphenyloxazole for fluorography.

(ii) Polyomavirus small T. The conditions described for SV40 small T were used with the following modifications for polyomavirus small T. Translation reaction mixes were incubated with an estimated 200 ng of polyomavirus small T in 20 μl of infected Sf9 cell extract. As a control, extract from uninfected Sf9 cells was used. For immunoprecipitation, 1 μl of rat antipolyomavirus tumor ascites was used.

(iii) Polyomavirus medium T. The conditions described for SV40 small T were used for polyomavirus medium T with the following modifications. Translation reaction mixes were incubated with approximately 500 ng of polyomavirus medium T in 15 μl of infected 293 cell extract. As a control, extract from uninfected 293 cells was used. For immunoprecipitation, 1 μl of monoclonal antibody (ascites) against the medium T peptide GluGlu was used (24). Instead of RIPA, TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.2% Tween 20, 0.02% sodium azide) was used.

(iv) C subunit of PP2A. The conditions described for SV40 small T were used for the PP2A subunit C with the following modifications. There was no need to add external C subunit to the translation mixtures because reticulocyte lysate contains approximately 5 ng of endogenous C subunit per μl, as determined by Western blotting. This amounts to 50 ng of C subunit per assay, which is sufficient for complex formation. Further addition of 80 ng of pure exogenous C subunit had no effect. Reticulocyte lysate also contains approximately 8 ng of endogenous A subunit per μl, as determined by Western blotting. We raised rabbit antibodies against the peptide KVTRRTPDYFL (KL), corresponding to the C terminus of PP2A-Cα. The peptide was coupled to bovine serum albumin with glutaraldehyde. After the second boost injection, the antiserum was isolated (69). For immunoprecipitation, 0.5 μl of anti-KL serum was used. Controls were carried out with 6.6 μg of peptide KL. TBST was used instead of RIPA.

(v) Immunoprecipitation under mild or stringent conditions. The term mild conditions indicates that translation mixtures were not diluted after complex formation and that all incubations were performed at 4°C, as described above. Stringent means that translation mixtures were diluted after complex formation and that all incubations thereafter were performed at 20°C. For example, after translation of A subunit RNA and incubation on ice to allow association of A with C (present in the reticulocyte lysate) or with added SV40 small T, the reaction mixture was diluted 10- to 50-fold with TBST or RIPA, as indicated in the figure legends, and kept at 20°C throughout all following incubations and washes. Protein A-Sepharose was suspended in the same buffer that was used for dilution and washing.

Two buffers, TBST and RIPA, were used for immunoprecipitations. For SV40 and polyomavirus small T, both buffers were used with similar results. For medium T and the C subunit, TBST was used because these proteins bind only weakly to the A subunit if RIPA is present. Note that the descriptions mild and stringent do not refer to the type of buffer used.

Quantification of complex formation. Immunoprecipitates were quantified by scanning X-ray films with a Hewlett Packard Scan Jet II C and by subsequently analyzing the data with the Scan Analysis 68,000 program (Biosoft Inc.). When there were major differences in intensity between bands on the same film, several autoradiograms from different exposure times were scanned. In order to increase the linearity of the signals, the Hyperfilm-MP films (Amersham) were flashed before exposure. The percentages of immunoprecipitated proteins were calculated after subtraction of the control bands that were obtained in the absence of T antigens or in the presence of KL peptide. Mutant-protein immunoprecipitation was expressed relative to wild-type-protein precipitation. Adjustments were made for differences in the amount of synthesis of wild-type and mutant proteins. All experiments were repeated at least twice. The maximum variation of values obtained from separate experiments was ±50%. However, in most cases, the variation was much lower.

RESULTS

Rationale for construction of A subunit mutants. The amino acid sequence and repeat structure of the A subunit of PP2A are shown in Fig. 1. It has been demonstrated previously that this protein has a rodlike shape, with an axial ratio of 10.5 to 1 (8, 27). This suggests that the 15 repeats are arranged in a linear fashion, thereby forming a rod. Such a structure is well suited for the study of protein-binding sites by deletion mutant analysis. One might expect that deletions, spread over the polypeptide from one end to the other, would permit the determination of binding sites that are arranged in a linear fashion on the rod-shaped protein. Based on this assumption, we created three types of mutants: (i) truncations from the C terminus, (ii) truncations from the N terminus, and (iii) internal deletions. Most mutants represent deletions of one or several repeats. The exact beginning and end of each repeat were chosen based on a molecular model of the A subunit. This model, which will be presented in detail elsewhere (72a), was developed through the use of helical-wheel projections of each repeat. Briefly, all repeats form two short amphipathic helices paired in an antiparallel fashion by their hydrophobic sides and connected by a loop (Fig. 2). Consecutive repeats are connected by loops and stacked to form a rod structure. The end points of N- and

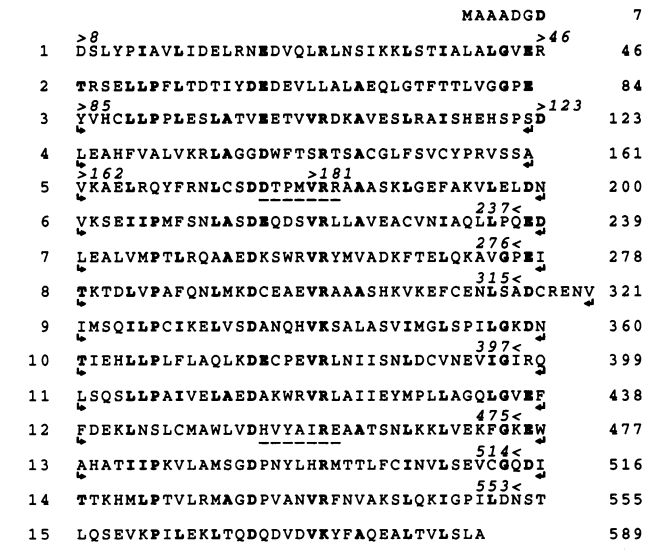

FIG. 1. Amino acid sequence and location of mutations in PP2A-Aα. N- and C-terminal truncations are indicated by arrows, > and <, respectively. The position of an arrow for N-terminal truncations indicates the first amino acid following the initiating methionine. The position of a C-terminal arrow indicates the last amino acid of the mutant protein. The number next to an arrow gives the position of the amino acid under the arrow. Numbers on the left indicate repeats, and numbers on the right indicate amino acids. Conserved amino acids are shown in boldface. The stretches of amino acids removed in mutants Δ3 to Δ13 are indicated within the repeats 3 to 13 by bent arrows under the first (↳) and last (↲) amino acid deleted. The underlined amino acids in repeat 5 were substituted by the underlined amino acids in repeat 12 for mutant SUB (the underlined amino acids in repeat 12 were not changed).

C-terminal truncations and the sites for removal of individual repeats are located in loops between repeats, as shown in Fig. 1 and 3.

Binding sites for the three tumor antigens and the C subunit, which have all been shown to be associated with the A subunit, were determined. Plasmids encoding the mutant A subunits were transcribed with T7 RNA polymerase and translated in vitro in the presence of [^{35}S]cysteine in a reticulocyte lysate. To allow complex formation with tumor antigens, the in vitro-synthesized A subunits were incubated with purified SV40 small T or with cell extracts containing either polyomavirus small T or polyomavirus medium T. For complex formation with the C subunit, it was not necessary to add exogenous C subunit, as reticulocyte lysate contains sufficient endogenous PP2A. We have determined by Western blotting that the reticulocyte lysate contains approximately equimolar amounts of the A and C subunit (5 to 10 ng/μl) (data not shown). We are uncertain about the amount of the B subunit. Newly synthesized, radioactive A subunit associates with C subunit from the reticulocyte lysate, since anti-C subunit antibodies coprecipitate radioactive A subunit. This means either that radioactive A subunit binds to free C subunit or that it exchanges with unlabeled A subunit from A-C or A-B-C complexes. Addition of T antigens after synthesis of radioactive A subunit allows complex formation with radioactive A bound to C. Presumably, this trimeric complex is then precipitated with antibodies to tumor antigens.

Binding of SV40 small T. The binding site for SV40 small T was determined by immunoprecipitation of the A subunit-small T complex with hamster anti-SV40 tumor serum. As shown in Fig. 4a, the mutant proteins with C-terminal truncations, designated 553 to 237, form complexes with SV40 small T. Similar amounts of the radioactive mutant and wild-type proteins were assayed (Fig. 4e), and similar amounts of complexes were formed in each case. These data suggest that the C terminus of the A subunit is not involved in the binding of small T and that the binding site is located within the six N-terminal repeats. Some A subunit was found in the precipitates without added small T. This is due to nonspecific binding to the protein A-Sepharose. A similar background was observed with control serum (data not shown). The amount of radioactive protein complexed to small T was quantitated by scanning (Materials and Methods).

Deletion of the first two N-terminal repeats had no effect on the binding of SV40 small T (Fig. 4a, mutants 8, 46, and 85). However, a 3-fold reduction in binding was observed when three repeats were removed (mutant 123), and a 10-fold reduction occurred with the removal of four repeats (mutant 162). When 4.5 repeats were deleted (mutant 181), binding fell below 5% of the wild-type level. These data indicate that the binding site for SV40 small T involves repeats 3 to 5. The quantitation of complex formation is summarized in Fig. 3.

The results obtained with C- and N-terminal truncation mutants were confirmed and extended by the use of single-repeat deletions. As shown in Fig. 5a, mutant proteins with a deletion of repeat 4, 5, or 6 abolished binding completely, while deletion of repeat 3 or 7 resulted in reduced binding compared with the wild-type protein. Therefore, the binding site for SV40 small T starts with repeat 3 and extends through repeat 7, whereby the core of the binding site involves repeats 4, 5, and 6.

When the binding studies were carried out under more stringent conditions, i.e., at a higher dilution and higher temperature (Materials and Methods), an effect of C-terminal truncations on small T binding was observed. As shown in Fig. 6a, the mutant proteins 553 to 276 showed fourfold-reduced binding compared with the wild-type protein. When the C-terminal truncation included repeat 7 (mutant 237), a drastic reduction of binding (30-fold) was observed. As discussed below, the effect of C-terminal deletions on small T binding is probably due to an indirect effect of the C subunit.

Binding of polyomavirus small T. Studies similar to those described for SV40 small T were carried out with polyomavirus small T. Because purified polyomavirus small T was not available, we used extracts from insect cells (Sf9) infected with a baculovirus vector encoding polyomavirus small T. Approximately 200 ng of polyomavirus small T was contained in the 20-μl extract used for each assay. Extract from uninfected Sf9 cells served as a control. Radioactive A subunit complexed to polyomavirus small T was precipitated with rat antipolyomavirus tumor serum.

Only deletion of amino acids 2 to 7 from the N terminus (mutant 8) had no effect on the binding of polyomavirus small T. All other mutations reduced binding in comparison to the wild-type A subunit. As shown in Fig. 4b and Fig. 3, truncation of the first N-terminal repeat (mutant 46) reduced binding 4-fold, truncation of repeats 1 and 2 (mutant 85) reduced binding 30-fold, and truncation of three or more repeats from the N terminus abolished binding completely. Truncation of up to seven repeats from the C terminus (mutants 553 to 315) reduced binding approximately 10-fold compared with the wild type. Further truncations including

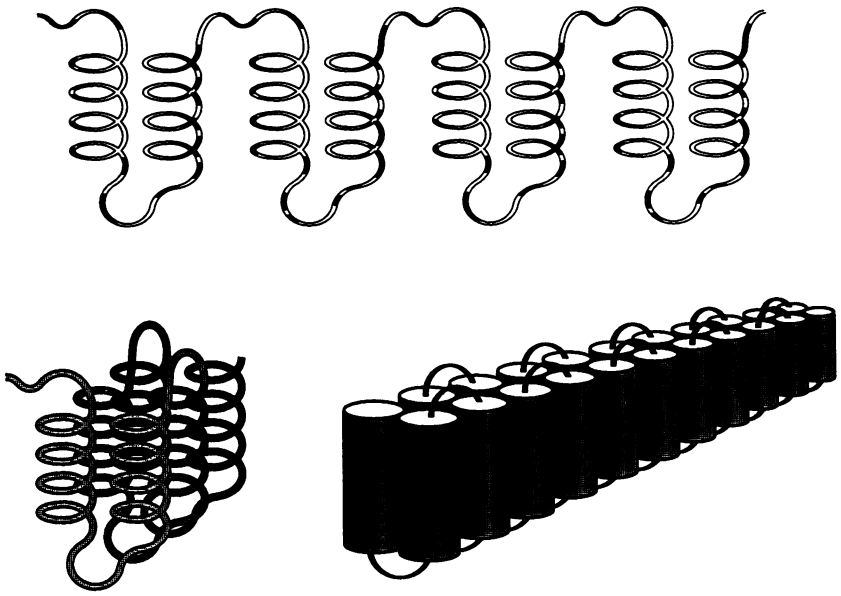

FIG. 2. Three-dimensional model of the A subunit of PP2A. The upper figure illustrates four repeats, each consisting of two amphipathic helices approximately 14 amino acids in length, which are paired with their hydrophobic (light) sides and connected by a loop of approximately seven residues. The repeats are folded (lower left) to form a rod-shaped molecule (lower right). The folding is stabilized by hydrophobic interactions between adjacent repeats. The straight arrangement of the rod is an oversimplification. In reality, neighboring helix barrels are probably tilted towards each other. The loops between alpha helices are presumed to play a role in the interaction with the B and C subunits as well as with tumor antigens. Truncations and deletions were constructed by cuts within presumed loop regions. Mutant SUB was made by substituting seven amino acids from the loop in repeat 5 with seven equivalent amino acids from the loop in repeat 12.

repeats 8 (mutant 276) and 7 (mutant 237) resulted in less than 5% of wild-type binding.

As shown in Fig. 5b, deletion of repeat 3, 4, or 5 resulted in no detectable binding of polyomavirus small T to the mutant protein. Deletion of repeat 6, 7, or 8 resulted in weak binding (less than 5%), and deletion of repeat 9 or 10 reduced binding approximately threefold. Furthermore, removal of repeat 11, 12, or 13 reduced binding 10- to 30-fold.

Our data demonstrate that the binding characteristics of SV40 and polyomavirus small T show both similarities and differences. Both antigens are affected much more by N-terminal than by C-terminal deletions, but polyomavirus small T binds over a wider range of the N terminus (repeats 2 to 8) than SV40 small T (repeats 3 to 6).

Binding of polyomavirus medium T. For the medium T studies, an extract from 293 cells infected with an adenovirus-polyomavirus hybrid containing the cDNA for polyomavirus medium T under the control of the late adenovirus promoter (59) was used as a source of medium T. Control extracts were prepared from uninfected 293 cells. Medium T-A subunit complexes were precipitated with a monoclonal antibody against medium T. As shown in Fig. 4c and Fig. 3, deletion of the first repeat from the A subunit had no effect on medium T binding (mutants 8 and 46). Truncations of two or more repeats from the N terminus reduced binding to undetectable levels (mutants 85 to 181). As also shown in Fig. 4c, all C-terminal truncations (553 to 237) were negative for binding of medium T. For these C-terminal mutants, binding of polyomavirus small T was reduced to approximately 10% of the wild-type level (Fig. 4b), whereas the binding of SV40 small T was unaffected (Fig. 4a). Both the N-terminal repeats 3 to 8 (Fig. 5c) and the C-terminal repeats 11 to 15 (Fig. 5c and 4c) were essential for binding of medium T. Deletion of repeat 9 or 10 resulted in 3- to 10-fold-reduced binding (Fig. 5c). Thus, binding of medium T to the A subunit is more affected by deletions than binding of either small T antigen.

When using TBST buffer for the medium-T-binding assays, nonspecific binding was frequently observed. This can be seen with mutants 123 to 181 (Fig. 4c). The background was reduced when immunoprecipitates were washed with RIPA instead of TBST. However, this also reduced the specific signal and required very long exposure times for fluorography (Materials and Methods). It should be pointed out that binding of medium T to A was considerably weaker than binding of small T, as indicated by the stability of the medium T and small T complexes in RIPA and TBST. Whereas the yield of small T-A subunit complexes was the same in both buffers, the yield of medium T-A as well as the yield of C-A was approximately fivefold lower in RIPA than in TBST. That the medium T-A-C complex is labile in RIPA buffer has been observed previously (23). Under optimal conditions, i.e., in RIPA or TBST for SV40 and polyomavirus small T and in TBST for medium T and the C subunit, between 3 and 6% of synthesized radioactive wild-type A subunits were coimmunoprecipitated.

Binding of the C subunit. A-C complexes were immunoprecipitated with a peptide antibody directed against the C terminus of the C subunit (designated KL− in Fig. 4d). Control precipitations were performed in the presence of excess competing peptide (designated KL+ in Fig. 4d) and with preimmune serum (not shown). As shown in Fig. 4d, the C subunit did not bind to any C-terminal truncation mutants of the A subunit (mutants 553 to 237). In addition, deletion of internal repeat 11, 12, or 13 also abolished C subunit binding (Fig. 5d1). Neither N-terminal truncations nor deletions of single N-terminal repeats had an effect on binding under mild conditions. These data suggest that the C

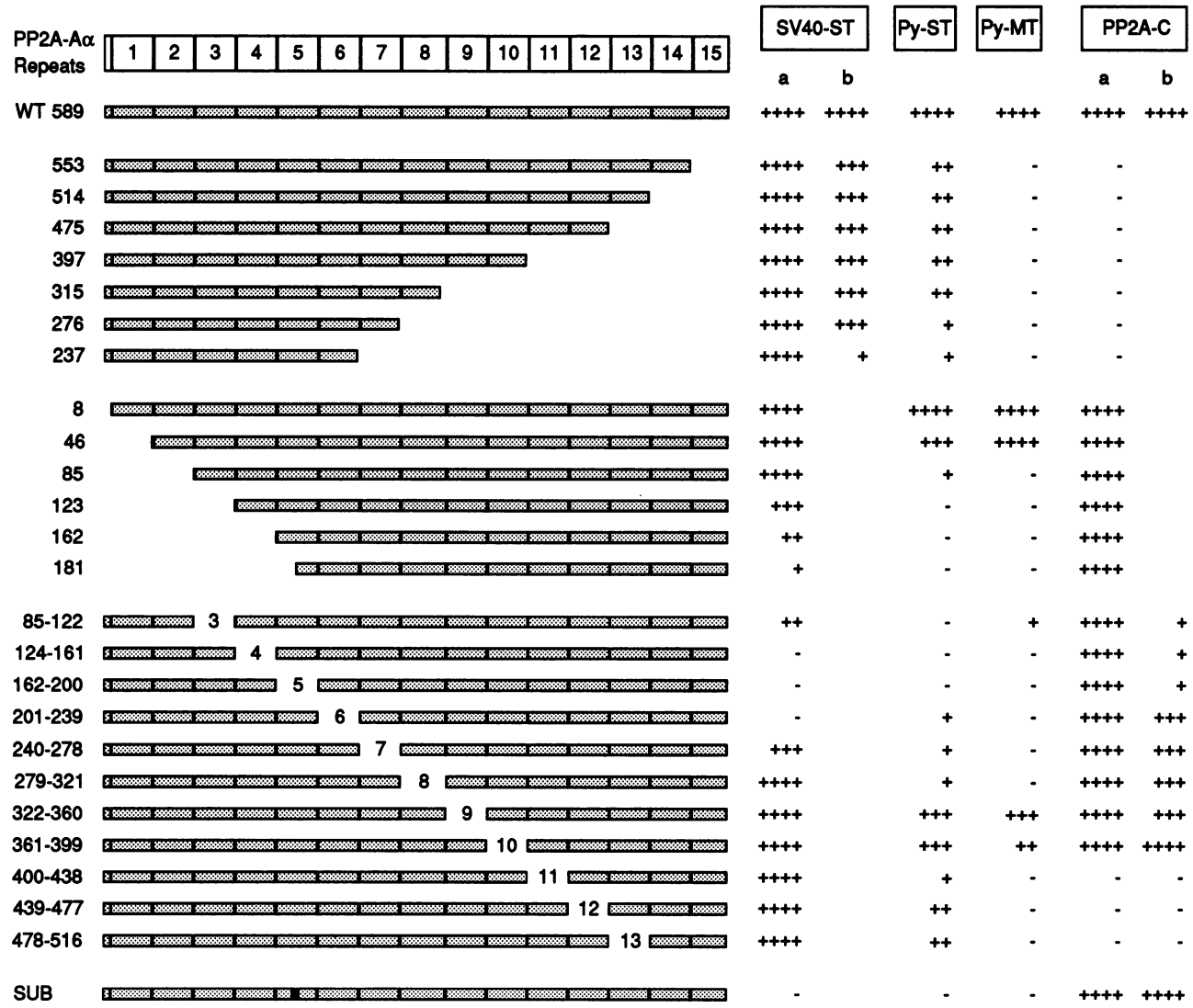

FIG. 3. Scheme of PP2A-Aα mutant proteins; summary of complex formation with tumor antigens and PP2A-C. The top bar symbolizes PP2A-Aα in a linear arrangement of its 15 repeats. The shaded bars represent the repeats present in the wild-type (WT) protein, C- and N-terminal truncations, and single-repeat deletions. The terminology of the mutants is as follows: for C-terminal truncations, the numbers indicate the last amino acid; for N-terminal truncations, the numbers indicate the first amino acid after the initiating methionine (e.g., mutant 8 has amino acids 2 to 7 deleted); for single-repeat deletions, the numbers indicate the stretch of amino acids deleted. In the substitution mutant SUB, the amino acid sequence DTPMVRR in the middle of repeat 5 has been substituted by the sequence HVYAIRE. The latter sequence is identical to a sequence in the middle of repeat 12 (see Fig. 1). The results of complex formation are summarized on the right. SV40-ST, SV40 small T; Py-ST, polyomavirus small T; Py-MT, polyomavirus medium T; PP2A-C, catalytic subunit of PP2A; mild (a) and stringent (b) conditions for complex formation and washing of complexes are described in Materials and Methods. Scoring: −, nonspecific or nondetectable binding; +, 1 to 5% of wild-type coimmunoprecipitation; ++, 5 to 15%; +++, 15 to 50%; ++++, 50 to 150%.

subunit binds to the C-terminal five repeats of the A subunit. Under the more stringent conditions (Materials and Methods), removal of repeats 3 to 9 resulted in reduced binding (Fig. 5d2). This might be an indirect effect of the B subunit (see Discussion).

Binding of T antigens and the C subunit to a loop substitution mutant. Our experiments have demonstrated that binding of all three T antigens was completely abolished by deletion of repeat 4 or 5, suggesting that these repeats are located in the center of overlapping binding sites. Based on the model presented in Fig. 2, one might suspect that the loops between amphipathic helixes are involved in binding. To test this idea, we replaced seven consecutive amino acids (DTPMVRR) in the loop of repeat 5 (Fig. 1, underlined sequence) with seven different amino acids (HVYAIRE) from the corresponding loop of repeat 12 and assayed the effect of this alteration on the binding of T antigens and C subunit. The sequence HVYAIRE was selected because repeat 12 is not directly involved in T-antigen binding and because loop 12 is very different in sequence from loop 5. Another reason for mutating DTPMVRR in loop 5 was that an almost identical sequence, DTPVVRR, was found in the fission yeast *Schizosaccharomyces pombe cdc25*$^+$ gene product (57), a tyrosine- and serine/threonine-specific phosphatase that plays an important role in controlling mitosis (18, and references therein). A comparison of the A subunit

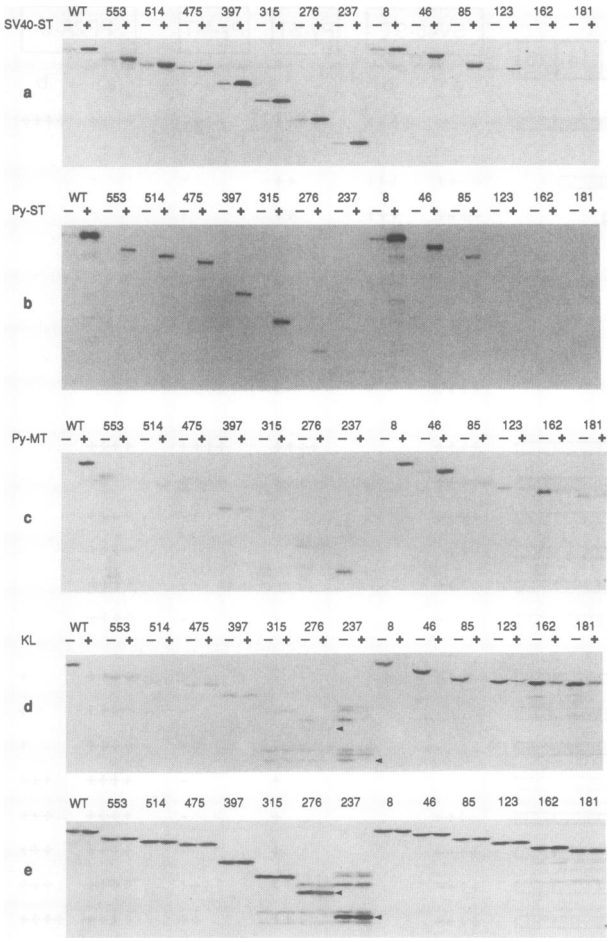

FIG. 4. Complex formation of C- and N-terminally truncated PP2A-Aα with tumor antigens and PP2A-C. [^{35}S]cysteine-labeled translation mixtures were used for immunoprecipitations as described in Materials and Methods. Lanes +, addition of SV40 small T (a), polyomavirus small T (b), polyomavirus medium T (c), or competing peptide KL (d); lanes −, addition of solutions without T antigens (a to c) or without peptide KL (d). (e) Aliquots of the translation mixtures used in panel d. Arrowheads in panels d and e point to mutant proteins 276 and 237. The largest protein on the gels is the 65-kDa wild-type A subunit (WT); the smallest protein is mutant 237, with a molecular mass of 26 kDa. Fraction of immunoprecipitates loaded: a, 0.2; b, 0.2; c, 0.75; d, 0.75. (e) Fraction of translation mixtures loaded, 0.05. Exposure times: a, 2.5 days; b, 8 days; c, 1.5 days; d, 12 h; e, 12 h.

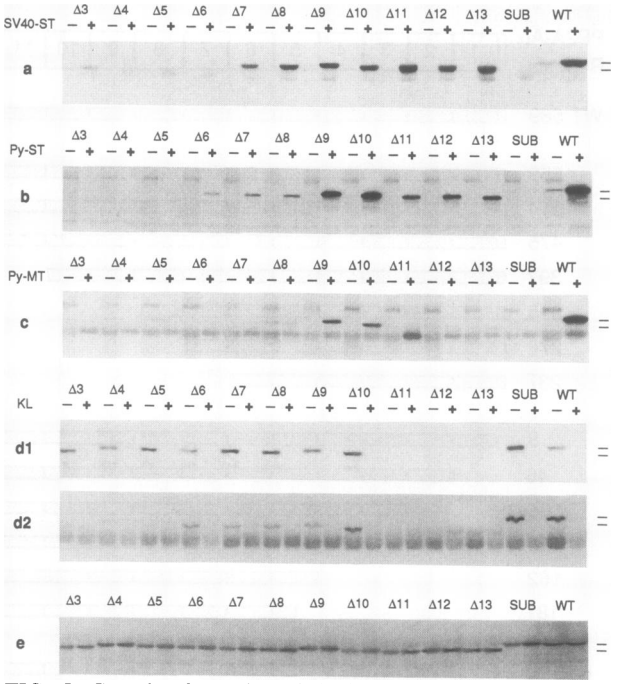

FIG. 5. Complex formation of single-repeat deletion mutants of PP2A-Aα with tumor antigens and PP2A-C. [^{35}S]cysteine-labeled translation mixtures were used for immunoprecipitations as described in Materials and Methods. Δ3, Δ4, etc., represent deletion of repeat 3, 4, etc. (see Fig. 1 and 3). Lanes + and −, same as in Fig. 4. (d1) Mild conditions and (d2) stringent conditions (20-fold dilution with TBST) for complex formation and washing of complexes, as described in Materials and Methods. Dashes on the right indicate the size difference between full-length proteins (wild type [WT] and SUB) and proteins missing 1 repeat; slight differences in migration between different deletion mutants have also been observed, e.g., between Δ9 and Δ10 (c) and between Δ3 and Δ4 (e). Fraction of immunoprecipitates loaded: a, 0.25; b, 0.25; c, 0.25; d1, 0.25; d2, 0.8. (e) Fraction of translation mixtures loaded, 0.025. Exposure times (in days): a, 4; b, 32; c, 41; d1, 14; d2, 6; e, 4.

and the Cdc25$^+$ protein shows a low degree of sequence homology (25% identity) over a stretch of 61 amino acids (identical residues are shown in boldface):

Cdc25$^+$ 332 **S**P**S**P**M**A**F**A**M**Q**E**D**A**E**Y**D**E**Q**D**T**P**V**V**R**R**T**Q**S**M**F**L**
PP2A-Aα 159 **S**S**A**V**K**A**E**L**R**Q**Y**F**R**N**L**C**S**D**DTPMVRRA**A**A**S**K**L**

Cdc25$^+$ 363 **N**S**T**R**L**G**L**F**K**S**Q**D**L**V**C**V**T**P**K**Q**S**T**K**E**S**E**R**F**I**S**
PP2A-Aα 190 **G**E**F**A**K**V**L**E**L**D**N**V**K**S**E**I**I**P**M**F**S**N**L**A**S**D**E**Q**D**S**

As shown in Fig. 5a to c, the loop substitution mutant protein (SUB) did not yield any binding of SV40 small T, polyomavirus small T, or polyomavirus medium T. However, C subunit binding was unaffected by this mutation (Fig. 5d1 and d2). These results are consistent with the suggestion that the T antigens bind to the N terminus of the A subunit, whereas the C subunit binds to the C terminus. These findings also support our model for the three-dimensional structure of the A subunit, in which loops play an important role in protein interaction.

DISCUSSION

In this investigation, we determined regions on the A subunit of PP2A that are involved in binding of SV40 small T, polyomavirus small T, polyomavirus medium T, and the catalytic C subunit of PP2A. Significant differences in binding were observed between the different tumor antigens as well as between the tumor antigens and the C subunit. In each case, the binding region extended over several repeats. Binding of SV40 small T involved repeats 3 to 6, whereas binding of polyomavirus small T and medium T involved repeats 2 to 8 of the N terminus. Binding of the C subunit was strongly affected by deletions of repeats 11 to 15 at the C terminus of the A subunit and weakly affected by N-terminal deletions. C-terminal deletion of repeats 11 to 15 also reduced binding of the tumor antigens to the A subunit. This was most pronounced for medium T. Polyomavirus small T binding was less affected by these deletions, and SV40 small T binding was affected only under stringent binding condi-

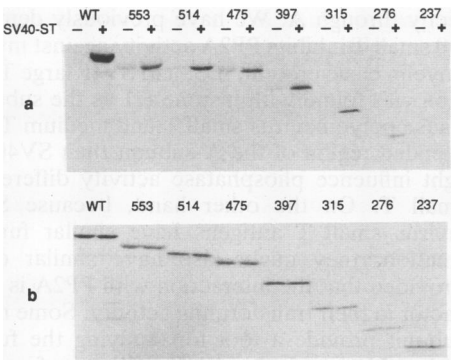

FIG. 6. Complex formation of C-terminally truncated PP2A-Aα with SV40 small T (SV40-ST) under stringent conditions. Translation mixtures were diluted 50-fold with TBST before addition of the first antibody, and incubations were performed at room temperature as described in Materials and Methods. (a) Coimmunoprecipitation with (+) or without (−) SV40-ST; (b) aliquots of the translation mixtures used in panel a. (a) Fraction of immunoprecipitates loaded, 1. (b) Fraction of translation mixtures loaded, 0.01. Exposure times: a, 2 days; b, 2 days.

tions. The only mutation that had no effect on the binding of any protein was a deletion of amino acids 2 to 7. A surprising result was that deletion of repeat 9 or 10 only slightly reduced binding of A to polyomavirus small T or medium T and had no effect on the binding of A to SV40 small T and C. These repeats might have another function than binding to proteins. For example, they might serve as a spacer between the N- and C-terminal domains.

The positions of regions of the A subunit involved in binding of T antigens and the C subunit and the degree of their involvement are illustrated in Fig. 7 (lower part). The locations of the proposed binding sites are shown in the upper part of the figure. The A subunit is drawn as a rod with an axial ratio of 10 to 1 (8). This shape, which presumably results from the linear arrangement of repeats, explains why the deletions that abolish binding are arranged in a contiguous manner. Our data suggest that all T antigens bind exclusively to N-terminal repeats and that the C subunit binds only to the C terminus. To explain the effects of C-terminal deletions on T-antigen binding, we propose that there is cooperativity between T antigens and the C subunit. Binding of polyomavirus medium T to the A subunit appears to depend completely on this interaction with the C subunit. If binding of the C subunit is abolished by C-terminal deletions, medium T cannot bind by itself to its N-terminal binding site on A. This suggestion is strengthened by the fact that the region of the C terminus that affects medium T binding coincides exactly with the binding site for the C subunit. If binding of medium T to A depends on the simultaneous binding of C to A, one might predict two binding sites on medium T, one for C and a different one for A. It should be possible to test this hypothesis by creating two types of mutants, one defective in binding to C and another defective in binding to A. We found that polyomavirus and SV40 small T antigens are less dependent on cooperation with C than is medium T. This could be due to their higher affinity for A.

As mentioned earlier, medium T and the small T antigens are associated in cells with A-C but not A-B-C complexes, and, in vitro, SV40 small T binds purified A and A-C but not holoenzyme. These data suggest that T antigens and the B subunit compete for the same or an overlapping site on the A subunit. Because we found that the T antigens bind to the N terminus of the A subunit, we conclude that the B subunit also binds to a region within the N terminus. The observation that N-terminal deletions of A inhibited C binding (Fig. 5d2) indicates that C and B, like C and T antigens, cooperate in binding to A. There were no T antigens present in the assays for C binding, but by Western blotting with antiserum to the purified B subunit from bovine heart, we detected B subunit in the reticulocyte lysate (unpublished). We assume that this B subunit participated when the interaction of C and A was assayed. Binding of C to A was reduced 30-fold when repeat 3, 4, or 5 was deleted and 3-fold when repeat 6, 7, 8, or 9 was deleted. Therefore, if our model is correct, repeats 3 to 9 should be involved in binding the B subunit. The recent results by Kamibayashi et al. (31) are in good agreement with our data. It was demonstrated that treatment of holoenzyme (A-B-C) with the bifunctional cross-linking reagent 1,6-bismaleimidohexane results in the formation of covalently linked A-B, A-C, and B-C heterodimers, whereas mixing A with B or B with C does not lead to the formation of A-B or B-C complexes, respectively. These findings not only prove that B and C make contact in the holoenzyme; they also indicate that the interaction of A, B, and C is stabilized in the heterotrimeric complex.

Because the binding site for SV40 small T antigen is spread over four repeats of the A subunit and the sites for polyomavirus small T and medium T are even larger, extending over approximately seven repeats, we conclude that the corresponding binding regions on the T antigens are equally large. As suggested previously (73), it is likely that these regions are located within the unique sequences of the tumor antigens that are not present in the corresponding large T antigens (amino acids 83 to 174 of SV40 small T, 80 to 195 of polyomavirus small T, and 80 to 191 of polyomavirus medium T). Because polyomavirus small T and medium T have almost identical sequences in the unique region except for four additional amino acids at the C terminus of medium T, it is not surprising that both proteins bind to the same repeats. SV40 small T, which has limited sequence homology with the polyomavirus T antigens, binds to fewer but overlapping repeats. One might suspect that the amino acids which are conserved between the SV40 and polyomavirus T antigens play a crucial role in binding to the A subunit. On the other hand, the three-dimensional structure may be more important for binding than sequence homology. Several mutants of medium T whose mutations are located in the unique region (*hrt* mutants) (3) are defective in binding to the A subunit. Some of these mutants, such as SD15 and NG18, have deletions of larger segments of the polypeptide. It is remarkable, however, that mutant NG59, which differs from the wild type only by the replacement of aspartic acid at position 179 with isoleucine and asparagine (7), is completely negative in binding (23, 35, 50). This suggests that this mutation gives rise to a conformational change involving a larger area. Several mutants of SV40 small T whose mutations are located in the unique region exhibit normal binding to the A subunit (28).

In view of the finding that the T-antigen-binding regions extend over a large part of the A subunit, we were surprised that a replacement of only seven amino acids in the loop of repeat 5 caused complete failure to show any T-antigen binding (mutant SUB). The finding that SUB has no apparent effect on C binding, even under stringent conditions, suggests that this mutation does not affect binding of the B subunit, assuming that binding of B is required for efficient binding of C. This indicates in addition that the binding

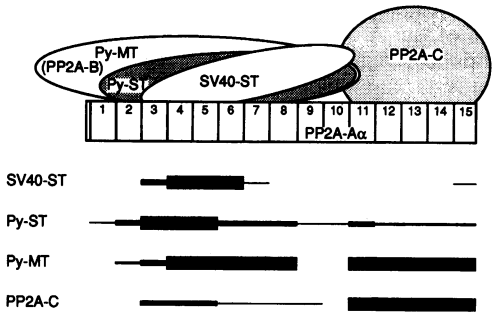

FIG. 7. Regions of PP2A-Aα involved in binding of tumor antigens and PP2A-C; model of different PP2A complexes. The bar symbolizes the A subunit in a linear arrangement of its 15 repeats. The thickness of the black lines under the bar reflects the importance of various repeats of PP2A-Aα for binding to T antigens and PP2A-C. The thickest line corresponds to − and the thinnest to +++ in Fig. 3. Each change in thickness corresponds to a threefold change in degree of binding. The thick line for polyomavirus medium T (Py-MT) under repeats 11 to 15 does not imply binding of medium T to the C terminus but indicates cooperativity with the C subunit. Similar considerations apply to the small T antigens. The line for PP2A-C under repeats 3 to 9 does not imply binding of the C subunit to the N terminus but indicates cooperativity with the B subunit. A model for the arrangement of subunits within PP2A is shown at the top. The tumor antigens, and presumably the B subunit, bind N-terminally, and the C subunit binds C-terminally. The major binding regions are repeats 3 to 6 for SV40 small T (SV40-ST), repeats 2 to 8 for polyomavirus small T (Py-ST) and Py-MT, and repeats 11 to 15 for PP2A-C. The overlap between the C subunit and tumor antigens (and B) symbolizes binding between these proteins.

specificity of B differs from that of the T antigens, although the binding region might be the same. It is noteworthy that the α and β forms of the B subunit show no sequence homology to the tumor antigens (25, 45, 49).

An alternative model could account for the result that binding of T antigens and the C subunit is affected by deletion of both N- and C-terminal repeats. If these proteins possess two domains each that bind simultaneously to N- and C-terminal regions of the A subunit, then deletion of N-terminal repeats would still permit binding of T antigens to the C terminus, and deletion of C-terminal repeats would permit some binding of the C subunit to the N terminus. The small T antigens could have a higher affinity to the N terminus, medium T could bind equally well to both ends, and the C subunit could bind more strongly to the C terminus. This model would require that the T antigens and the C subunit be rod-shaped molecules. It has been demonstrated, however, that the C subunit is spherical (27), and therefore cannot extend over the entire length of the A subunit. The shape of the T antigens has not been determined, but because of their small size, it seems unlikely that they can cover the entire length of A. Furthermore, since the C subunit occupies a large domain of the C terminus, it is difficult to imagine that the T antigens bind simultaneously to the same region. For these reasons, we do not favor this model.

In vitro, PP2A dephosphorylates a number of proteins which play an important role in growth control, such as Rap-1 (58), microtubule-associated protein 2 (MAP2) kinase (1), $p34^{cdc2}$ (41), S6 kinase (2), p53 (61), and histone H1 (65). With these substrates, it will be important to assay and compare the effects of T antigens and the B subunit on PP2A activity. They might affect activity by direct contact with C or indirectly through A. We have previously demonstrated that SV40 small T inhibits PP2A activity against myosin light chains, myelin basic protein, p53, and SV40 large T, whereas stimulation was found with histone H1 as the substrate (61, 63). Because polyomavirus small T and medium T bind to a more extended region of the A subunit than SV40 small T, they might influence phosphatase activity differently from SV40 small T. On the other hand, because SV40 and polyomavirus small T antigens have similar functions in transformation, they might also have similar effects on PP2A, provided that the interaction with PP2A is the determining factor in their transforming activity. Some mutants of the A subunit provide a tool for studying the function of PP2A in vivo. Mutants lacking the binding site for either B or C could be introduced into cells. These mutants might interfere with the normal function of PP2A, resulting in changed phosphorylation states of growth-regulatory proteins or in alterations of the growth phenotype.

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