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# Identification of Regions in Polyomavirus Middle T and Small t Antigens Important for Association with Protein Phosphatase 2A

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**Two subunits of protein phosphatase 2A (PP2A) have been shown previously to bind to the small t and middle T antigens (ST and MT, respectively) of polyomavirus. To determine sequences important for binding of PP2A to ST and MT, we first constructed a series of ST mutants in regions known to be important for biological activity of ST and MT. Several mutations in two small regions just amino terminal to the Cys-X-Cys-X-X-Cys motifs of ST and MT abolished PP2A binding to ST in vitro. Parallel mutations were constructed in MT to investigate the role of PP2A binding in the function of polyomavirus MT. Wild-type and mutant MT proteins were stably expressed in NIH 3T3 cells and analyzed (i) for their ability to induce transformation and (ii) for associated cellular proteins and corresponding enzymatic activities previously described as associating with wild-type MT. A number of the mutant MTs were found to be defective in binding of PP2A as assayed by coimmunoprecipitation. In contrast, a deletion of the highly conserved stretch of amino acids 42 to 47 (His-Pro-Asp-Lys-Gly-Gly) in the ST-MT-large T antigen common region did not affect PP2A binding to MT. MT mutants defective for PP2A binding were also defective in transformation, providing further evidence that association with PP2A is important for the ability of MT to transform cells. All mutants which were impaired for PP2A binding were similarly or more dramatically impaired for associated protein and lipid kinase activities, supporting the possibility that PP2A binding is necessary for the formation and/or stability of an MT-pp60$^{c\text{-}src}$ complex.**

Middle T antigen (MT), the primary transforming protein of polyomavirus (40, 50, 53), carries out its transforming function by associating with and modulating the activities of cellular proteins involved in control of cell proliferation. These proteins include pp60$^{c\text{-}src}$ (6), c-*yes* protein (23), c-*fyn* protein (4, 18, 24), phosphatidylinositol 3-kinase (PI 3-kinase) (57), Shc (2, 8), Grb2 (2, 8), 14-3-3 proteins (35), and the 36-kDa catalytic subunit and the 63-kDa regulatory subunit of protein phosphatase 2A (PP2A) (38, 56). The small t antigens (STs) of polyomavirus, simian virus 40 (SV40), and other papovaviruses complex with the PP2A subunits but not with the other MT-associated proteins.

The role of PP2A association in polyomavirus MT and ST functions is not well defined. Consistent with a potential role in MT-ST stimulation of cell proliferation, PP2A has been implicated in control of cell cycle progression in several systems (11, 14, 16, 21, 26, 27, 31). However, because all MT mutants examined to date that are defective in PP2A binding are also defective in binding pp60$^{c\text{-}src}$ (13, 15, 34, 44, 46), the contribution of PP2A is difficult to assess. One possible explanation for this observation is that these mutations disrupt PP2A binding by affecting the structural integrity of MT. Alternatively, PP2A binding to MT may be necessary for complex formation with pp60$^{c\text{-}src}$. The role of PP2A binding in SV40 ST function was recently examined (48). Overexpression of SV40 ST in CV-1 cells resulted in the activation of the mitogen-activated protein kinase kinase Mek1 and the mitogen-activated protein kinase Erk1, presumably by inhibition of PP2A's activity (48). In these experiments, SV40 ST was also capable of stimulating cell proliferation (48). Similar experiments have not been reported in the case of polyomavirus ST, and it should be noted that the expression level of ST in the SV40 experiments was much greater than the expression of polyomavirus MT and ST normally seen during viral infection.

The fact that the two PP2A subunits associate with ST and MT but not with large T antigen (LT) indicates that the 112-amino-acid stretch common only to ST and MT is important for binding to this enzyme. This stretch, extending from amino acids 80 to 191 in ST and MT, contains a number of residues that are highly conserved in the STs and MTs of different papovaviruses, including two conserved cysteine motifs (Cys-X-Cys-X-X-Cys) (12) important for MT function (28). Two mutants with yet unexplained phenotypes are located in the conserved sequences flanking these motifs. MT mutant BP3, which contains three point mutations just amino terminal to the first cysteine motif, was reported to be transformation defective yet associated with protein kinase activity (28). The mutant bc1075, with a mutation in a region just amino terminal to the second cysteine motif of ST and MT, was reported to be competent for MT transformation; however, the ST version of this mutant was found to be defective in its ability to stimulate the replication of viral DNA and the maturation of virus particles (29). Because PP2A is the only cellular protein known to associate with ST, it is possible that bc1075 ST, and therefore bc1075 MT, association with this enzyme is defective. Because of the interesting phenotypes of these mutants, we targeted these regions for genetic analysis of PP2A association with ST and MT.

In the present study we first analyzed a series of mutants in

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ST for their effect on PP2A binding in vitro. The same series of mutants in an MT context were then examined for association with all known associated cellular proteins detectable in vivo and for their corresponding enzymatic activities, if known. The results reported here demonstrate that the two small regions amino terminal to each of the two cysteine motifs in MT (the regions containing mutants BP3 and bc1075) are important for ST and MT association with PP2A. All mutations that decrease PP2A binding to MT also impair the ability of MT to associate with $pp60^{c\text{-}src}$ and PI 3-kinase and to transform mouse fibroblasts.

## MATERIALS AND METHODS

**Construction of mutants.** All mutants except for three were constructed originally in a polyomavirus ST vector (pBluescript pyst) based on Stratagene's pBluescript plasmid. Mutants bc1075, bc1073, and *dl*HPDKGG were constructed originally in a pGem 3Zf(+) (Promega)-based polyomavirus ST vector, pDP100. The details of these constructions are presented below. Subsequently, the mutations were transferred to the MT cDNA vector pBD15-MT (10), by recloning the *Bam*HI-*Sph*I fragment of each of the pBluescript or pGem 3Z(+) mutant pyst plasmids, which contains sequences shared between ST and MT, into the similarly digested pBD15-MT. Finally, each mutant MT cDNA was transferred into the retroviral vector pLJ (22) as previously described (10). All plasmid constructions were performed by standard molecular cloning techniques (42).

To facilitate the construction and sequencing of the mutants, the plasmid pBluescript pyst was first constructed as follows. The *Sau*3AI fragment from the plasmid pTR841 (43) which contains polyomavirus ST coding sequences 173 to 808 was cloned into the *Bam*HI site of pGem 3Zf(+) (Promega) under the control of the SP6 promoter to generate the plasmid pDP100. This plasmid was then used as a template in PCR with the following set of primers: primer a, 5′-ATATGCGGCCGCGGATCCACCATGGATAGAGTTCTGAGCA GAGC-3′; primer b, 5′-ATATCTCGAGGATCTTAGGGGGAGAGCCTTGG ATTATACACGCTGTGCACATC-3′. The ST start ATG codon and the antisense version of the ST stop codon are underlined in the respective primers. To facilitate cloning of the PCR product, the 5′ primer included *Not*I, *Bam*HI, and *Nco*I sites and the 3′ primer included an *Xho*I site. PCR with pDP100 as the template was performed using *Taq* (Perkin-Elmer Cetus) as instructed by the supplier. The resulting PCR fragment was cut with *Not*I and *Xho*I and ligated into the correspondingly prepared pBluescript SK− (Stratagene) under the control of the T3 promoter. The resulting plasmid, designated pBluescript pyst, has an improved eukaryotic translation initiation signal.

Plasmids pyst(R106S), pyst(P108A), and pyst(C111S) (see Fig. 1B) were generated by PCR. In all three reactions, primer b (described above) was used as the 3′ primer and the 5′ primer varied. The 5′ primers in each case corresponded to the ST region targeted for mutation and contained the desired nucleotide change. The sequences of the mutagenic 5′ primers are as follows (the underlined nucleotides represent nucleotide changes):

R106S, 5′-ATATCTGCAGCATGCCTCTTACCTGC-3′
P108A, 5′-ATATCTGCAGAATGGCTCTTACC-3′
C111S, 5′-ATATCTGCAGAATGCCTCTTACCTCCC-3′

Each oligonucleotide also contained a *Pst*I site at the 5′ end that corresponds to a unique *Pst*I site in the ST cDNA. The fragments produced by PCR were digested with *Pst*I and *Xho*I and cloned into the corresponding *Pst*I and *Xho*I sites of pBluescript pyst.

To construct plasmid pyst(R99K) (see Fig. 1B), a mutagenic 3′ primer containing a *Pst*I site (5′-GGCATTCTGCAGAACCGCTGGTAGTATTTATCA CC-3′) was used in combination with the 5′ primer (primer a) described above. The PCR product was digested with *Not*I and *Pst*I and cloned into the corresponding sites of pBluescript pyst.

Additional plasmids (see Fig. 1B), pyst(M107I/L109R), pyst(*dl*107-109), pyst(*dl*108-109), and pyst(*ins*107AL), were created by replacing ST wild-type sequences between the *Pst*I and *Bsp*MI sites of pBluescript pyst with specifically designed pairs of oligonucleotides:

M107I/L109R, 5′-GAATACCTAGGACCTGCCTAG-3′
3′-ACGTCTTATGGATCCTGGACGGATCATTT-5′

*dl*107-109, 5′-GAACCTGCCTAG-3′
3′-ACGTCTTGGACGGATCATTT-5′

*dl*108-109, 5′-GAATGACCTGCCTAG-3′
3′-ACGTCTTACTGGACGGATCATTT-5′

*ins*107AL, 5′-GAATGGCTCTTCCTCTTACCTGCCTAG-3′
3′-ACGTCTTACCGAGAAGGAGAATGGACGGATCATTT-5′

To construct pyst(*ins*144ALEQ), pBluescript pyst was partially digested by *Sca*I and blunt end ligated to synthetic *Xba*I linker 1082 (5′-TGCTCTAG AGCA-3′) from New England Biolabs (NEB). The resulting mutation introduces an insertion of 4 amino acids between valine 144 and leucine 145 (see Fig. 1B).

MT plasmid pBP3 (28), generously provided by Bill Markland and Alan Smith, was used to generate the corresponding ST mutant by using oligonucleotides a and b for PCR. The resulting *Not*I-to-*Xho*I-digested PCR product was subcloned into *Not*I-to-*Xho*I-digested pBluescript SK−, creating plasmid pyst(BP3).

A variant of plasmid pDP100 with trimmed sequences 3′ to ST, designated pDP101, was used in the construction of the bc1075 ST mutant plasmid. pDP101 was made by excising the approximately 315-bp fragment of pDP100 extending from the polyomavirus *Xmn*I site to the polylinker *Sma*I site and inserting NEB *Bam*HI linker 1021.

The bc1075 mutant of polyomavirus (29) consists of Cys-142–to–Tyr substitution of the MT and ST proteins (see Fig. 1B). This mutant was generously provided by G. Magnusson in the form of a *Bam*HI-to-*Eco*RI fragment of the polyomavirus genome cloned into the plasmid pAT153 (52). The mutant DNA obtained also contained a silent G-to-A mutation at nucleotide 595 which remains in our final pLJ construct. The bc1075 mutation was transferred into an ST background in two cloning steps to create plasmid pDP101.1075. First, the *Sac*I (ST internal site)-to-*Sac*I (3′ polylinker site) fragment of pDP100 (see above) was replaced with the *Sac*I fragment from the pAT153/bc1075 plasmid containing the bc1075 mutation. The *Dra*III (ST internal site)-to-*Dra*III (plasmid backbone) fragment in this plasmid was replaced with a similarly digested fragment from pDP101, restoring the 3′ end of the ST cDNA. To create a *Bam*HI site on the 5′ end of the ST cDNA in pDP101.1075 for use in transferring the mutation to a pBD15-MT, a unique 5′ *Hin*cII site in pDP101. 1075 was digested and NEB *Bam*HI linker 1021 was ligated in.

The polyomavirus mutant bc1073 (29), generously provided by G. Magnusson in the form of a *Bam*HI-to-*Eco*RI fragment of the polyomavirus genome cloned into the plasmid pAT153 (52), consists of a change that results in a Thr-76–to–Ile substitution. The *Bsm*I fragment from the pAT153/bc1073 plasmid containing the bc1073 mutation was cloned into the similarly digested DP100. To transfer the ST mutation into pBD15-MT, plasmid pDP100.1073 was digested with *Hin*cII (5′ polylinker site) and ligated to NEB *Bam*HI linker 1021.

To create pyst(*dl*HPDKGG), pDP100 was digested with *Hin*dIII and *Eco*RI and the ST-containing fragment was ligated into the *Hin*dIII and *Eco*RI sites of a modified pGem 3Zf(+) plasmid which lacks an *Alw*NI site. The resulting plasmid, pDP100(*Alw*NI−), was digested with *Alw*NI, and ST wild-type sequences were replaced with an oligonucleotide cassette:

5′-CTGCTACTGAGCCATGCCTTAATGCAGGAATTGAACAGTCT-3′
3′-AGTGACGATGACTCGGTACGGAATTACGTCCTTAACTTGTC-5′

The resulting plasmid, pyst(*dl*HPDKGG), with the mutant ST under the control of the SP6 promoter, creates a deletion of amino acids His-42 to Gly-47. To transfer the ST mutation into pBD15-MT, plasmid pyst(*dl*HPDKGG) was digested with *Hin*cII (5′ polylinker site) and ligated to NEB *Bam*HI linker 1021.

Plasmid pG2HPR60α3-11 contains the human α isoform cDNA of the 63-kDa subunit of PP2A inserted into the *Eco*RI site of pGem-2 (Promega) (17). pG2HPR60α3-11 was cleaved with *Eco*RI, and the resulting fragment containing PP2A 63-kDa subunit coding sequences and 5′ untranslated sequences was ligated into the *Eco*RI site of pGem 3Zf(+) (Promega). The resulting plasmid, designated pG260, was digested with *Bam*HI (5′ polylinker site) and *Eag*I (PP2A 63-kDa subunit internal site) and ligated to an oligonucleotide cassette to remove 5′ untranslated sequences and create a new *Nco*I site at the initiation codon:

5′-GATCCACCATGGCGGC-3′
3′-GTGGTACCGCCGCCGG-5′

This modification created plasmid pKC63 with the PP2A 63-kDa subunit under the control of the SP6 promoter.

**Expression of ST and PP2A in vitro.** To assay complex formation between mutant STs and PP2A, proteins were transcribed and translated in vitro. Briefly, 5 μg of wild-type and mutant STs was linearized and transcribed with the appropriate RNA polymerase. pKC63 (5 μg) was digested with *Eco*RI and transcribed with 40 U of SP6 RNA polymerase. [$^{35}$S]methionine-labeled proteins were synthesized according to a standard translation protocol (Promega) by incubating 7 to 10 μg of cRNA with 50 μl of nuclease-treated rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine (1,200 Ci/mmol; NEN; 0.5 μl for ST and 5 μl for the 63-kDa subunit of PP2A). Two microliters of the total sample was suspended in sample buffer, heated for 3 min at 95°C, and analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) to normalize for the amount of ST prior to mixing.

**Complex formation in vitro.** Following translation, lysates containing [$^{35}$S]methionine-labeled ST were mixed with lysates containing [$^{35}$S]methionine-labeled PP2A. The mixture was diluted with 100 μl of Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 10% [vol/vol] glycerol, 137 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride, aprotinin at 0.03 U/ml), supplemented with 1 mM dithiothreitol, and incubated with rocking at 4°C for 2 h. After addition of 2 μl of polyclonal rabbit antiserum that recognizes ST (rabbit anti-t serum) (37) and 400 μl of NP-40 lysis buffer, complexes were incubated with rocking for an additional 1 h and then for 45 min with protein A-Sepharose beads

(Pharmacia). Immune complexes were washed once with NP-40 lysis buffer, twice with 0.5 M LiCl–20 mM Tris (pH 8.0), and finally with ice-cold phosphate-buffered saline (PBS). Washed immunoprecipitates were suspended in sample buffer, heated for 3 min at 95°C, and analyzed by SDS–12.5% PAGE.

**Cells and tissue culture.** NIH 3T3 cells and BALB/3T3 clone A31 (both from C. D. Sher) and Psi-2 cells (gift from C. Cepko) (3) were maintained at 37°C in Dulbecco modified Eagle's medium supplemented with 10% calf serum.

**Retrovirus infections and establishment of NIH 3T3 clones and pooled populations.** Transfection of Psi-2 cells, infections with virus supernatants, drug selection, and isolation of G418-resistant clones and pooled populations were carried out as previously described (5).

**Transformation assays.** Focus formation assays were performed as previously described (32). At the end of the assay period (2 to 3 weeks), cells were fixed with 3.7% formaldehyde in PBS and stained with methylene blue as previously described (5).

**Radiolabeling of cells and immunoprecipitations.** For metabolic labeling with methionine, subconfluent 100-mm-diameter dishes of cells were labeled for 4 h with [$^{35}$S]methionine (275 μCi/ml) in 3 ml of Dulbecco modified Eagle's medium minus methionine supplemented with 2% dialyzed calf serum. Cells were washed twice with ice-cold PBS and lysed with rocking for 10 min at 4°C in 1.0 ml of NP-40 lysis buffer supplemented with 1 mM sodium orthovanadate. Lysates were scraped and cleared at 13,000 × *g*. Prior to immunoprecipitation, extracts were normalized for MT by immunoblotting. Lysates were incubated with rocking at 4°C for 2 h with rabbit anti-t serum (37) in the presence of 2% low-fat milk (Bio-Rad). Following a 45 min incubation with protein A-Sepharose beads, immune complexes were washed once with NP-40 lysis buffer, once with PBS, twice with 0.5 M LiCl–20 mM Tris (pH 8.0), and finally with ice-cold distilled $H_2O$. Washed immunoprecipitates were suspended in sample buffer, heated for 3 min at 95°C, and analyzed by SDS–10.5% PAGE.

**Gel electrophoresis and autoradiography.** SDS-PAGE was performed according to the method of Laemmli (25). All exposures were on XAR-5 film (Kodak). For quantitation of bands, gels were analyzed with a Molecular Dynamics PhosphorImager, model 400E.

**Protein kinase assays.** MT immunoprecipitations were performed as described for immunoprecipitation of radiolabeled cells except that lysis buffers contained 2 mM sodium orthovanadate. Protein amounts were determined by the Bradford assay, and immunoprecipitations were performed with extract amounts normalized to give approximately equal amounts of MT as determined by immunoblotting or by immunoprecipitation of metabolically labeled MT. Immunoprecipitates were incubated in 50 μl of kinase buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MnCl_2$) containing 10 μCi of [γ-$^{32}$P]ATP. Reaction mixtures were incubated for 25 min at room temperature with occasional shaking, washed once with 1% NP-40 lysis buffer, and analyzed by SDS–9.5% PAGE. Protein kinase activity was also assayed by using enolase as an exogenous substrate as previously described (20).

**Immunoblotting.** Immunoblotting was performed by standard procedures (51) essentially as described elsewhere (39) using a 1:2,500 dilution of rabbit anti-t serum (37). Alkaline phosphatase-conjugated second antibody (Promega) was used at a dilution of 1:7,500. Proteins were visualized by incubation of the blots with color development substrates.

**Phosphatase activity assays.** Phosphatase activity associated with MT immunoprecipitates was assayed as described previously (30), except for isolation of the phosphopeptide. Kemptide (Bachem California) was incubated for 30 min at 30°C with cyclic AMP (cAMP)-dependent protein kinase catalytic subunit (Promega) in a 1.6-ml reaction mixture containing 0.25 mM peptide, 0.375 mM ATP, 5 mM $Mg(C_2H_3O_2)_2$, 50 mM morpholineethanesulfonic acid (MES) (pH 7.0), 0.2 mg of fatty-acid-free bovine serum albumin (BSA) (Sigma) per ml, 100 μCi of [γ-$^{32}$P]ATP, and 10 U of cAMP-dependent protein kinase catalytic subunit per ml. Phosphopeptide was isolated by first passing the reaction mixture twice through a Sep-Pak $C_{18}$ column (Waters), then washing the column extensively with distilled deionized $H_2O$ until the level of radioactivity in the wash fractions stopped decreasing rapidly, and finally eluting the phosphopeptide in 5 ml of 60% high-pressure liquid chromatography-grade methanol (1-ml fractions) and drying by vacuum centrifugation. The extent of phosphorylation was typically 20 to 50%. Phosphopeptide was resuspended in 50 mM Tris–0.1 mM EDTA, pH 7.5, at a concentration of approximately 180 μM $^{32}$P-peptide. Immunoprecipitates of MT were prepared as described above except that immune complexes were washed four times with PBS and once with enzyme dilution buffer (50 mM Tris, 150 mM NaCl, 1 mg of BSA per ml, 0.1% 2-mercaptoethanol, 0.1 mM EDTA, pH 7.5). Following the addition of 10 μl of enzyme dilution buffer and 10 μl of assay buffer (enzyme dilution buffer minus saline), 5 to 10 μl of phosphopeptide substrate was added to the immunoprecipitates and the reaction mixtures were incubated for 30 min at 30°C with agitation. The reactions were stopped by addition of 467 μl of 5 mM silicotungstate dissolved in 1 mM $H_2SO_4$. Then 93 μl of 5% ammonium (hepta)molybdate dissolved in 2 M $H_2SO_4$ was added to each tube, and then 700 μl of a 1:1 mixture of isobutanol-toluene was added to extract the phosphomolybdate complex (45). The tubes were vortexed for 15 s and centrifuged for 1 min at 13,000 × *g*. The organic (upper) phase (500 μl) was combined with 5 ml of scintillation fluid and counted to determine the amount of $^{32}$P released by the immune complexes. The reaction was linear in the range used.

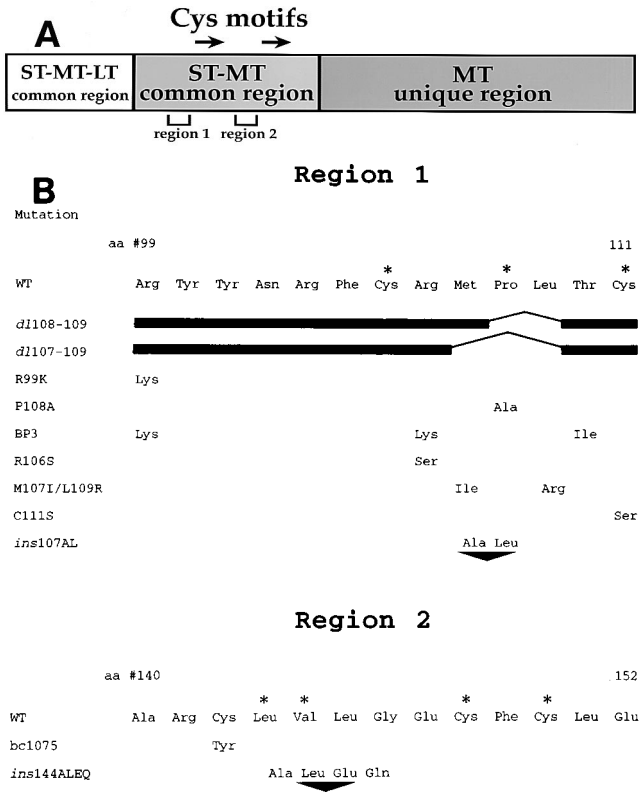


FIG. 1. (A) Schematic representation of polyomavirus MT. Cys-X-Cys-X-X-Cys motifs (Cys Motifs) are indicated by arrows. The locations of regions 1 and 2 are indicated. The portions of MT in common with ST and LT or unique to MT are indicated. (B) Summary of amino acid alterations in the various MT mutants used in this study. Region 1 (amino acids 99 to 111) and region 2 (amino acids 140 to 152), two small regions of MT amino acid sequence that contain the mutations analyzed in this study, are shown. Residues conserved among all papovavirus family members are denoted by asterisks. Amino acid substitutions introduced into MT are indicated below the corresponding wild-type (WT) sequence. Amino acids inserted between existing residues are centered beneath their insertion point. Mutants *dl*HPDKGG (deletion of amino acids 42 to 47) and bc1073 (Thr-76–to–Ile substitution), which contain mutations amino terminal to region 1 in the ST-MT-LT common region, are not shown.

**PI 3-kinase assays.** Polyphosphoinositide kinase activity associated with MT immunoprecipitates was assayed as described previously (1) with the following mixture of lipids: PI, PI-4-phosphate, PI-4,5-bisphosphate, and phosphatidylserine. The reaction mixture was 20 μM ATP with 10 μCi of [$^{32}$P]ATP (3,000 Ci/mmol) and was incubated at room temperature for 10 min. The extracted samples were cochromatographed with nonradioactive standards (PI, PI-4-phosphate, and PI-4,5-bisphosphate) in an *n*-propanol–2 M acetic acid solvent system and visualized by $I_2$ vapor. Thin-layer chromatography plates were exposed in PhosphorImager cassettes (Molecular Dynamics) for detection and quantitation of the radioactive products.

## RESULTS

**Construction of mutants in regions 1 and 2.** ST mutants were constructed in two small regions, amino acids 99 to 111 and 142 to 145 (Fig. 1), which lie just to the amino-terminal side of each of the two highly conserved cysteine motifs. The mutants constructed include the two previously reported mutants located in these regions, BP3 and bc1075, and four types of mutants designed to probe the importance of these regions for binding to PP2A. Mutants R99K, R106S, P108A, and C111S have single amino acid changes, while the mutant M107I/L109R has two amino acid changes (see the legend to Fig. 1 for nomenclature). In mutants *dl*108-109 and *dl*107-109, 2 and 3 amino acids were deleted, respectively. Mutants

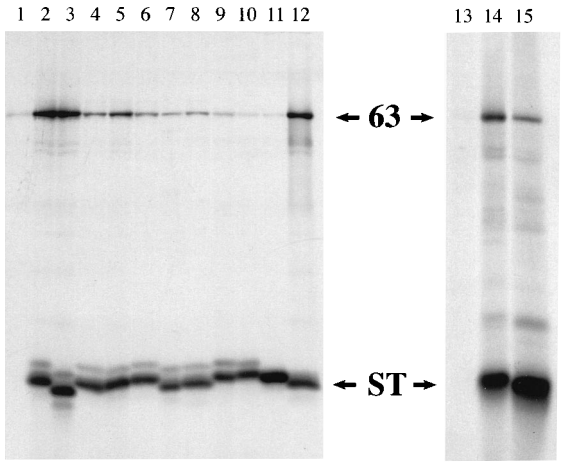


FIG. 2. Association of the 63-kDa subunit of PP2A with various STs in vitro. The 63-kDa subunit of PP2A and wild-type and mutant STs were translated in vitro in the presence of [$^{35}$S]methionine. Following translation, lysates containing [$^{35}$S]methionine-labeled PP2A 63-kDa subunit were mixed with various [$^{35}$S]methionine-labeled STs or no ST, incubated, and immunoprecipitated with rabbit anti-t serum as described in Materials and Methods. Proteins in the immune complexes were resolved by SDS-PAGE and visualized by autoradiography. The positions of migration of ST and the 63-kDa subunit of PP2A are indicated. Lane 1, no ST present; lane 2, wild-type ST; lane 3, pyst(P108A); lane 4, pyst(R106S); lane 5, pyst(BP3); lane 6, pyst(M107I/L109R); lane 7, pyst(*dl*107-109); lane 8, pyst(*dl*108-109); lane 9, pyst(*ins*107AL); lane 10, pyst(C111S); lane 11, pyst (bc1075); lane 12, pyst(bc1073); lane 13, no ST present; lane 14, ST; lane 15, pyst(*dl*HPDKGG). Lanes 13 to 15 are from a separate experiment. The 63-kDa band seen in lanes 1 and 13 represents a small amount of the PP2A 63-kDa subunit which binds nonspecifically even in the absence of ST.

*ins*107AL and *ins*144ALEQ contain insertions of 2 and 4 amino acids, respectively.

**Association of ST mutants with PP2A in vitro.** To analyze the association between the ST mutants and PP2A, mutant STs were translated in vitro in the presence of [$^{35}$S]methionine and then mixed individually with [$^{35}$S]methionine-labeled, translated PP2A 63-kDa regulatory subunit. After incubation, ST-PP2A complexes were immunoprecipitated with rabbit anti-t serum and analyzed by SDS-PAGE. As shown in Fig. 2, the 63-kDa subunit of PP2A is coimmunoprecipitated with wild-type ST (lane 2). However, several STs mutated in region 1 showed a defect in PP2A binding. An ST mutant with a deletion of Pro-108 and Leu-109 [pyst(*dl*108-109)] was partially reduced in PP2A binding (lane 8). A greater defect was seen when this deletion was extended to include Met-107 [pyst (*dl*107-109)] (lane 7). Another mutant with an insertion of 2 amino acids at the same position [pyst(*ins*107AL)] was also defective in PP2A binding (lane 9). Three additional region 1 ST mutants containing single or multiple point mutations [pyst (R106S), pyst(BP3), and pyst(M107I/L109R)] were partially defective in PP2A binding (lanes 4, 5, and 6). A mutant ST with a single amino acid change at Cys-111 [pyst(C111S)] completely abolished PP2A binding (lane 10). Finally, mutants with a Pro-108–to–Ala substitution [pyst(P108A)] and an Arg-99–to–Lys substitution [pyst(R99K)] bound wild-type levels of the 63-kDa subunit of PP2A (lane 3 and data not shown). Therefore, some but not all residues of region 1 are important for efficient complex formation with PP2A.

To test if residues in region 2 are important for mediating binding to PP2A, ST mutants containing either a mutation in Cys-142 [pyst(bc1075)] or an insertion between Val-144 and Leu-145 [pyst(*ins*144ALEQ)] were examined. Neither bound detectably to PP2A (lane 11 and data not shown).

**Mutations in the ST-MT-LT common region.** Two additional regions were targeted for mutational analysis. The sequence HPDKGG (amino acids 42 to 47) is highly conserved among all papovavirus members. Mutations in the region HPDKGG have been previously reported to affect the transactivation function of SV40 ST (33, 47). In addition, the sequence DKGG is found in the 55-kDa regulatory subunit of PP2A, which normally binds to the 63-kDa subunit. Because ST and MT bind to PP2A in place of the 55-kDa regulatory subunit (39), the sequence HPDKGG represented a potential region in the ST-MT-LT common region that might assist in complex formation with PP2A. To determine if this was the case, a deletion of residues 42 to 47 [pyst(*dl*HPDKGG)] was constructed. Mutant pyst(*dl*HPDKGG) was partially impaired for PP2A binding in vitro (Fig. 2, lane 15). ST mutant bc1073, containing a Thr-76–to–Ile substitution, was reported to be defective in virus production (29). In contrast to mutant bc1075, mutant bc1073 retained the ability to stimulate viral DNA synthesis (29). To determine if there is a correlation between PP2A binding and ST's ability to stimulate viral DNA synthesis and maturation of virus particles, mutant bc1073 was assayed for PP2A binding. As shown in Fig. 2, lane 12, pyst(bc1073) binds wild-type levels of PP2A.

**Amino acid residues in region 1 and region 2 of MT are important for MT-mediated transformation.** To study the effect of these mutations on MT-mediated transformation, mutant ST sequences were transferred into an MT background, subsequently cloned into the retroviral vector pLJ, and assayed for focus formation on NIH 3T3 cells (see Table 1). Transformation efficiency was measured as the ratio of focus-forming units to G418-resistant CFU. Four types of transforming behavior were observed. First, three of the mutants, R99K, R106S, and P108A, had essentially wild-type transforming characteristics. Second, BP3 was wild type in its ratio of foci to G418-resistant colonies but gave noticeably smaller foci than did the wild type. Third, three of the mutants, M107I/L109R, *dl*108-109, and bc1075, retained a very low but reproducible level of transforming activity. Finally, the remaining mutants, C111S, *ins*107AL, *dl*107-109, and *ins*144ALEQ, were negative for transformation. Similar results were obtained when the assays were performed using BALB/3T3 clone A31 fibroblasts (data not shown). The MT mutants *dl*HPDKGG and bc1073 were wild type for transformation.

**Mutations of MT residues in regions 1 and 2 affect binding of PP2A and other host cell proteins.** To verify that each mutant was stably expressed, immunoblotting was carried out on lysates from pooled populations of G418-resistant NIH 3T3 clones (data not shown). While all lines expressed stable MTs, the levels of expression for the various mutants differed. To compensate for differences in expression, the amount of MT was normalized in all subsequent experiments (see Materials and Methods).

To analyze the ability of each mutant protein to associate with PP2A and other host proteins, MT immunoprecipitates were prepared from lysates of cells metabolically labeled with [$^{35}$S]methionine. As shown in Fig. 3A, a gradient of PP2A association (see the bands at 36 and 63 kDa) which paralleled the transforming activity of the mutants was observed. The mutants with wild-type transforming activity (R99K, R106S, and P108A) bound near wild-type levels of the 36- and 63-kDa proteins (lanes 3, 4, and 6). Somewhat surprisingly, the partially transformation-defective mutant BP3 also retained binding of the 36- and 63-kDa proteins (lane 5). Two mutants that retained a very low level of transforming ability, M107I/L109R and *dl*108-109, were partially defective in binding PP2A (lanes 7 and 8). The remaining transformation-defective mutants, C111S, *ins*107AL, *dl*107-109, *ins*144ALEQ, and bc1075, bound

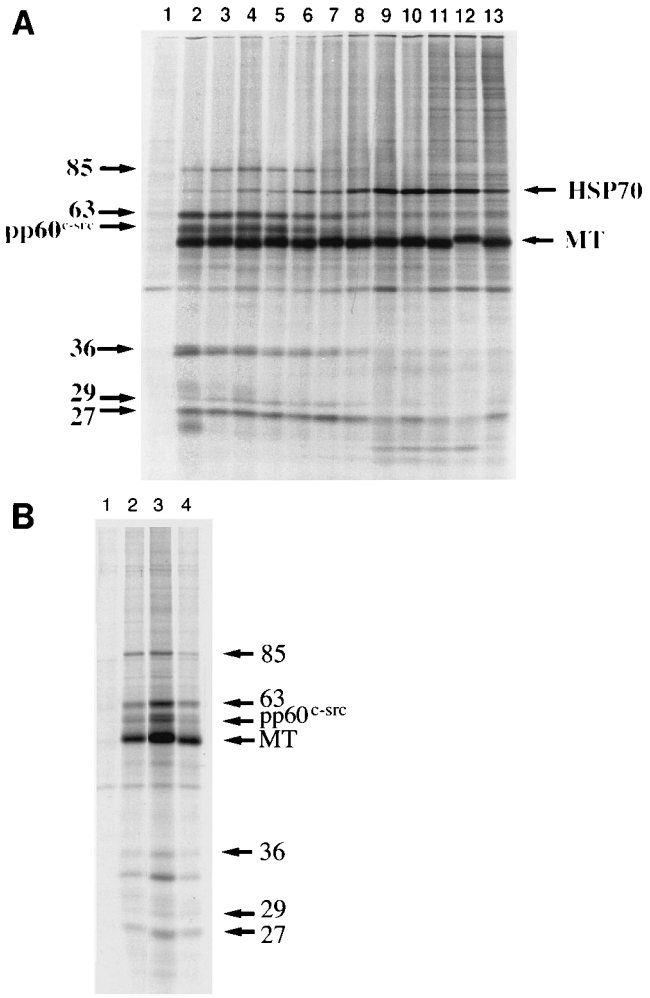


FIG. 3. Association of cellular proteins with MT mutants. (A) NIH 3T3 cells expressing only G418 resistance (pLJ), wild-type MT, or the various MT mutants were labeled with [$^{35}$S]methionine for 4 h prior to lysis. Lysates were immunoprecipitated with rabbit anti-t serum as described in Materials and Methods. Proteins in the immune complexes were resolved by SDS-PAGE and visualized by autoradiography. The positions of migration of wild-type MT, the 36- and 63-kDa subunits of PP2A, the 85-kDa subunit of the PI 3-kinase, the 73K heat shock protein (HSP70), pp60$^{c\text{-}src}$ (and *src* kinase family members), and the 27- and 29-kDa proteins are indicated. Lane 1, pLJ; lane 2, wild-type MT; lane 3, R99K; lane 4, P108A; lane 5, BP3; lane 6, R106S; lane 7, M107I/L109R; lane 8, *dl*108-109; lane 9, C111S; lane 10, *ins*107AL; lane 11, *dl*107-109; lane 12, *ins*144ALEQ; lane 13, bc1075. (B) Association of cellular proteins with MT mutants bc1073 and *dl*HPDKGG. Lysates were prepared and immunoprecipitated as described for panel A. Associated proteins are indicated as described above. Lane 1, pLJ; lane 2, wild-type MT; lane 3, bc1073; lane 4, *dl*HPDKGG. The overall level of labeling seen in lane 3 (bc1073) is higher than that seen on average. In panel B, the PP2A catalytic subunit migrated as two bands of 34 and 36 kDa.

even lower levels of PP2A (lanes 9 to 13). As shown in Fig. 3B, the mutants bc1073 and *dl*HPDKGG bound wild-type levels of the 36- and 63-kDa proteins (lanes 2 and 3). Therefore, regions 1 and 2 of MT are important for binding PP2A.

When the other MT-associated proteins were examined, several points emerged. First, most mutants defective in binding PP2A still bound the 27- and 29-kDa-associated proteins (previously described as 14-3-3 proteins [35]) at near wild-type levels. These results indicate that at least a portion of the tertiary structure of these MT mutants is still intact. However, at least three of the mutants, C111S, *dl*107-109, and *ins*144ALEQ, did bind lower levels of the 27- and 29-kDa proteins (Fig. 3A, lanes 9, 11, and 12). Second, the bands at approximately 60 kDa, which likely represent pp60$^{c\text{-}src}$ and related kinases, decreased when PP2A binding decreased. Third, the level of the 85-kDa band, which represents the 85-kDa subunit of PI 3-kinase, also decreased when PP2A binding decreased. In each case, the level of the 85-kDa protein varied in parallel with the presumed pp60$^{c\text{-}src}$ band. Finally, the amount of a 70-kDa protein, 73K heat shock protein (36, 55), increased as levels of the other bound proteins decreased.

TABLE 1. Transforming abilities and associated activities of MT mutants[a]

| Mutant | Transformation efficiency[b] | PP2A activity[c] | Protein kinase activity | PI 3-kinase activity |
|---|---|---|---|---|
| *dl*HPDKGG | ++++ | NA | +++ | +++ |
| bc1073 | ++++ | ++++ | ++++ | NA |
| R99K | ++++ | ++++ | ++++ | ++++ |
| P108A | ++++ | +++ | ++++ | ++++ |
| BP3 | ++++[d] | ++++ | ++++ | ++++ |
| R106S | ++++ | +++ | ++++ | ++++ |
| M107I/L109R | −[e] | + | + | + |
| *dl*108-109 | −[e] | + | + | + |
| C111S | − | − | − | − |
| *ins*107AL | − | + | + | − |
| *dl*107-109 | − | + | − | − |
| *ins*144ALEQ | − | − | − | − |
| bc1075 | −[e] | + | + | + |

[a] For all assays: ++++, >75% of the wild-type level; +++, 50 to 75% of the wild-type level; ++, 25 to 50% of the wild-type level; +, 5 to 25% of the wild-type level; −, <5% of the wild-type level; NA, not assayed.
[b] As measured by ratio of focus-forming units to G418-resistant colonies.
[c] As measured by $^{32}$P counts released and presented relative to wild-type levels. The average value of counts released for the wild type was 1,068.
[d] Foci were smaller than wild-type foci.
[e] A few small foci and a small amount of protein and lipid kinase activities were detectable.

**Transformation function of wild-type and mutant MTs correlates with associated phosphatase activity.** To measure the amount of phosphatase activity associating with each of the MT mutants, MT immunoprecipitates prepared from each cell line were assayed with a peptide substrate (Table 1). Mutants which are transformation competent, R99K, P108A, BP3, and R106S, had near (>50%) wild-type levels of associated phosphatase activity. The three mutants that retained a very low level of transforming activity, M107I/L109R, *dl*108-109, and bc1075, retained 15 to 25% of wild-type levels of associated phosphatase activity. Mutants C111S, *ins*107AL, *dl*107-109, and *ins*144ALEQ, which are completely defective for transformation, retained an even lower level, 4 to 11%, of associated phosphatase activity. These results closely parallel those obtained by quantitating the amount of associated PP2A from [$^{35}$S]methionine-labeled MT immunoprecipitates (data not shown; see also Fig. 3).

**PP2A binding and associated phosphatase activity correlate with associated kinase activities.** To assay for associated protein kinase activity, immunoprecipitates of each MT mutant were incubated in vitro with [$\gamma$-$^{32}$P]ATP in the absence (Fig. 4) or presence (Fig. 5) of an exogenous substrate. Mutants that bound wild-type levels of both associated PP2A and the p60 band (Fig. 3A) had wild-type levels of associated protein kinase activity (R99K, R106S, BP3, and P108A; Fig. 4 and 5, lanes 3 to 6). Mutants defective for PP2A and p60 binding were defective in associated protein kinase activity (Fig. 4 and 5, lanes 7 to 13). Mutants M107I/L109R, *dl*108-109, and bc1075,

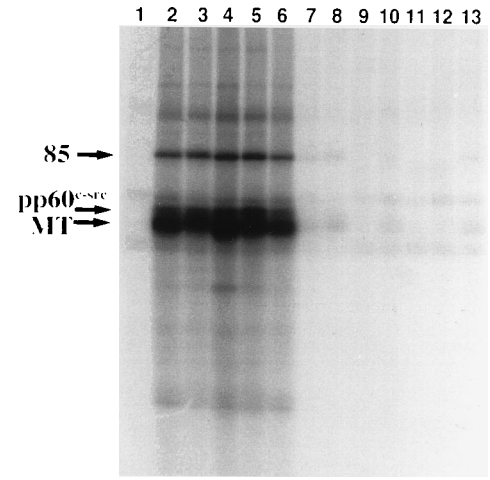


FIG. 4. Protein kinase activity associated with MT mutants assayed in the absence of an exogenous substrate. Lysates from the same set of cells described for Fig. 3A were normalized for MT expression and immunoprecipitated with rabbit anti-t serum. Immune complexes were incubated with [$\gamma$-$^{32}$P]ATP and analyzed by SDS-PAGE. Phosphoproteins were visualized by autoradiography. The positions of the p85 subunit of PI 3-kinase, pp60$^{c\text{-}src}$, and MT are shown. The order of lanes is the same as for Fig. 3A.

which retained 15 to 25% of associated phosphatase activity, retained a low level of associated protein kinase activity. This associated activity was more easily detectable when enolase was used as an exogenous substrate (Fig. 5, lanes 7, 8, 10, and 13). In the particular experiment shown in Fig. 4, the level of associated protein kinase activity for *ins*107AL (lane 10), which was markedly reduced in PP2A binding, was higher than that seen on average. Mutants C111S, *dl*107-109, and *ins*144ALEQ, which were defective for PP2A binding, failed to coimmunoprecipitate an associated protein kinase activity (Fig. 4 and 5, lanes 9, 11, and 12). The MT mutants *dl*HPDKGG and bc1073 demonstrated wild-type levels of associated kinase activity (data not shown). Thus, the amount of p60 band associated with the various MT mutants (Fig. 3) correlated well with the level of associated protein kinase activity (Fig. 4 and 5). Similar

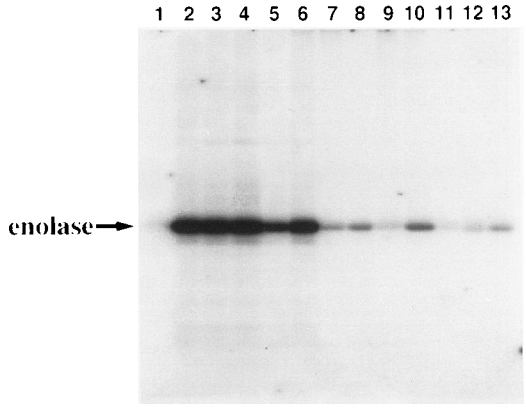


FIG. 5. Protein kinase activity associated with MT mutants assayed with enolase as an exogenous substrate. Lysates from the same set of cells described for Fig. 3A were normalized for MT expression and immunoprecipitated with rabbit anti-t serum antibody as described in Materials and Methods. Immune complexes were incubated in a kinase reaction mix containing enolase as described in Materials and Methods and analyzed by SDS-PAGE. Phosphoproteins were visualized by autoradiography. The position of migration of enolase is indicated. The order of lanes is the same as for Fig. 3A.

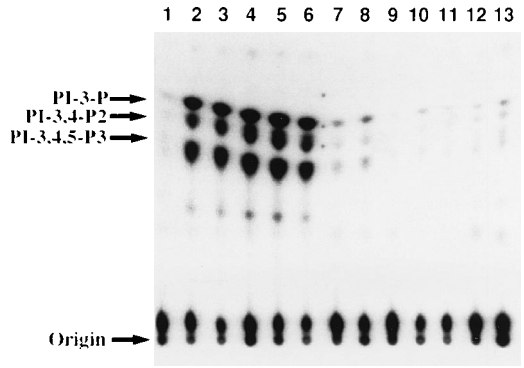


FIG. 6. PI 3-kinase activity associated with MT mutants. Lysates from the same set of cells described for Fig. 3A were normalized for MT expression and immunoprecipitated with rabbit anti-t serum. Immune complexes were analyzed for their ability to phosphorylate phosphoinositides as described in Materials and Methods. Positions of reaction products are indicated: PI-3-P, PI-3-phosphate; PI-3,4-P2, PI-3,4-bisphosphate; PI-3,4,5-P3, PI-3,4,5-trisphosphate. The order of lanes is the same as for Fig. 3A.

results were obtained when parallel immunoprecipitates were analyzed for PI 3-kinase activity (Fig. 6); associated PI 3-kinase activity correlated very well with the level of bound 85-kDa protein seen in Fig. 3.

## DISCUSSION

We have performed a mutational analysis of MT in several regions that were potentially important for PP2A binding. Two of these regions are located just amino terminal to each of the two cysteine motifs that lie within the portion of MT in common with ST but not with LT. Initially, we constructed a set of ST mutants and assayed for their ability to bind PP2A in vitro. Mutations in both regions affected PP2A binding. In region 1, Leu-109 appears to be important for PP2A binding, as indicated by the phenotypes of the two small deletions (*dl*108-109 and *dl*107-109) and a double point mutation (M107I/L109R). In addition, Cys-111 is likely to be important for mediating binding to PP2A, as is the spacing between residues (see mutants C111S and *ins*107AL). In region 2, where a smaller number of mutations were made, Cys-142 appears critical for binding. It is possible that residues Val-144 and Leu-145 are also important; an insertion between these residues is defective (*ins*144ALEQ). Most of the corresponding mutations in MT affect the binding of PP2A. All mutant MTs that are defective for PP2A binding are also defective for transformation, providing additional evidence that PP2A association with MT is important for MT-mediated transformation. In addition, mutations that affect the binding of PP2A concomitantly affect the binding of pp60$^{c\text{-}src}$ and PI 3-kinase without grossly affecting MT structure.

Deletion of the hexamer HPDKGG in the ST-MT-LT common region did not affect MT's ability to transform or to associate with PP2A and other host cell proteins. The sequence HPDKGG was targeted for mutational analysis for two reasons. First, the sequence DKGG is present in the 55-kDa regulatory subunit of PP2A, which like ST and MT binds the 63-kDa subunit of PP2A (39). Second, mutations in this sequence (amino acids 42 to 47), which is conserved among all papovavirus family members, have been reported to affect the transactivation function of SV40 ST (33, 47). If data on MT in vivo can be extrapolated to SV40 ST, our results suggest that the conserved hexamer helps bind another protein, other than PP2A, which is necessary for ST-mediated transactivation.

However, the corresponding HPDKGG deletion in polyomavirus ST produced a partial defect in PP2A binding in vitro. This may simply reflect inefficient folding in vitro. Two additional mutant ST proteins (R106S and BP3) translated in vitro also show a more dramatic effect on binding to PP2A than is observed in vivo in an MT background. Whether the differences in PP2A binding seen between ST in vitro and MT in vivo are due to differences in assays or intrinsic differences in the two proteins remains to be seen. MT translated in vitro fails to form a complex with PP2A (unpublished data). Therefore, it will be important to examine the transactivation function and PP2A binding in vivo of the ST mutants.

We have observed that MT mutations that affect PP2A binding lead to increased levels of a coimmunoprecipitated 70-kDa protein. We have identified this protein by immunoblotting as the constitutive 73K heat shock protein (data not shown). Walter et al. previously reported that the 73K heat shock protein associates with another PP2A-defective MT mutant, NG59, but not with wild-type MT (55). It is possible that this binding is due to improper folding of the mutant proteins. Alternatively, the availability of a heat shock protein binding site may be exposed when other MT-associated proteins are not bound to MT (36). Consistent with the latter possibility, wild-type MT associates with the 73K heat shock protein when MT levels are in excess of PP2A levels (36). In addition, we have preliminary evidence to indicate the existence of a specific binding site on MT for the 73K heat shock protein (unpublished data). Further evidence to suggest that the structural integrity of the mutant MTs is intact comes from zinc binding studies. Several defective MTs (C111S, bc1075, *ins*107AL, and *dl*107-109) were analyzed for zinc binding, and all bind wild-type levels of zinc (40a).

Our results both extend and contrast with previously published data. The importance of residues in region 1 (amino acids 99 to 111) is consistent with previous data on SV40 ST. Mungre et al. report that amino acids 97 to 103 in SV40 ST, which correspond to polyomavirus residues 105 to 111, are important for mediating binding of PP2A to SV40 ST (33). In addition, a peptide with SV40 ST sequences, which corresponds to residues 104 to 114 of MT, inhibits the ability of SV40 ST to associate with PP2A (33). Although SV40 ST amino acid Pro-101 has been implicated in PP2A binding (33), the corresponding mutation in polyomavirus ST and MT does not affect PP2A binding. Differences such as this are not unexpected, given that previous analysis has shown that PP2A sequences necessary for binding to the various T antigens, although similar, are not identical (41). Consistent with our results for region 2, mutation of Val-134 in SV40 ST, which corresponds to Val-144 of MT, affects the binding of PP2A and the ability to cotransform rat cells (19). In addition, this valine residue is absolutely conserved among all papovavirus members. Finally, it has been reported that bc1075, which has a point mutation in region 2, is defective in stimulation of viral DNA synthesis and maturation of virus particles, while bc1073 is wild type in viral DNA synthesis but defective in virus particle production. In our hands, bc1075 is defective for PP2A binding when assayed in both MT and ST backgrounds, while bc1073 is wild type. These results suggest that the inability of bc1075 to induce viral DNA synthesis (29) is due to a defect in PP2A binding and that PP2A binding alone does not account for the defect in maturation of virus particles.

The data presented here, together with previous data on mutants of MT that fail to bind PP2A (13, 34, 46, 54), are consistent with a model for ordered formation of the MT-cellular protein complex in which PP2A association is required for pp60$^{c\text{-}src}$ association. Subsequent phosphorylation of MT on tyrosines 250 and 315 by pp60$^{c\text{-}src}$ would then provide binding sites for Shc (2, 8) and the p85 subunit of PI 3-kinase (20, 49). Some previously identified MT mutants are completely defective in binding to PP2A and pp60$^{c\text{-}src}$ (13, 15, 34, 44, 46). Other mutants, including several presented in this study, bind an intermediate level of PP2A (34). For these mutants, the binding of pp60$^{c\text{-}src}$ is always equally affected as or more severely affected than the binding of PP2A, consistent with PP2A association being required for binding of pp60$^{c\text{-}src}$. Previous results from immunoprecipitation studies using monoclonal antibodies specific for the region in common between MT and ST are also consistent with this model of ordered assembly. Most of these antibodies immunoprecipitate only MT molecules not in complex with either PP2A or pp60$^{c\text{-}src}$ (7, 9, 37). A few antibodies immunoprecipitate only an MT-PP2A complex with no associated pp60$^{c\text{-}src}$ (9). However, no antibodies that precipitate an MT-pp60$^{c\text{-}src}$ complex without associated PP2A have been found, suggesting that MT-pp60$^{c\text{-}src}$ complexes do not exist in the absence of associated PP2A. The apparent dependency of pp60$^{c\text{-}src}$ binding on PP2A binding may be due to a structural, nonenzymatic role for PP2A in assembly of the MT complex. Alternatively, PP2A phosphatase activity may be required. Despite the fact that this model of ordered assembly of PP2A and pp60$^{c\text{-}src}$ explains all the relevant published data, most of the evidence at this point is correlative in nature. We are presently attempting to reassemble the MT complex step by step in vitro to more directly test this model of an ordered assembly of these enzymes.

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