Amino-Terminal Regions of Polyomavirus Middle T Antigen Are Required for Interactions with Protein Phosphatase 2A

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Polyomavirus middle T antigen (MT) is the major transforming protein of the virus. It functions through interactions with a number of cellular proteins involved in cell proliferation. MT forms complexes with protein phosphatase 2A (PP2A), pp60^{c-src}, phosphatidylinositol 3-kinase, and Shc. We introduced both deletion and point mutations into three regions of MT and examined their ability to associate with PP2A and pp60^{c-src}. The first 25 amino acid residues of MT are required for association with PP2A and pp60^{c-src}. Amino acids 105 to 111, comprising the sequence Cys-Arg-Met-Pro-Leu-Thr-Cys, is also required for complex formation between MT and PP2A. However, the sequence Asp-Lys-Gly-Gly (amino acids 44 to 47), also found in the B subunit of PP2A, is dispensable for complex formation between MT and PP2A. We find a strict correlation between the ability of MT to associate with PP2A and the ability of MT to associate with pp60^{c-src}. One mutant, L5E, associates with a phosphatase other than PP2A, pp60^{c-src}, and phosphatidylinositol 3-kinase in a manner similar to that of wild-type MT yet is reduced in its transforming ability on NIH 3T3 cells.

Polyomavirus middle T antigen (MT) is the major transforming protein of the virus. The gene encodes a protein of 421 amino acids which associates with cell membranes through a hydrophobic region near the carboxy terminus. MT has no known intrinsic biochemical activity; rather, it functions through interactions with a number of cellular proteins.

MT interacts with the catalytic (C) and regulatory (A) subunits of protein phosphatase 2A (PP2A), a protein serine/threonine phosphatase (32, 44). Interaction with these subunits is necessary for transformation by MT (18, 31, 38). The PP2A holoenzyme is a heterotrimeric protein complex consisting of the A (63-kDa) and C (36-kDa) subunits as well as a variable regulatory (B) subunit (54, 55, 72, or 74 kDa) (7, 29, 43). Although MT replaces the B subunit in the PP2A complex, MT shares only one sequence motif (the sequence DKGG) with the 55-kDa B subunit (33). The 74-kDa B subunit has no identifiable homology with MT (19). The small t antigens (STs) of polyomavirus, simian virus 40 (SV40), and BK virus also interact with PP2A. Thus, it is reasonable to expect that the region(s) important for association with PP2A will be found in both polyomavirus MT and ST. We previously reported that a cluster of cysteine residues (amino acids 120 to 153) is important for interactions between MT and PP2A (17). The cysteine cluster is also present in STs of polyomavirus, SV40, and BK virus.

MT interacts with pp60^{c-src}, a protein tyrosine kinase associated with the cytoplasmic surface of cell membranes (2, 11), and with the closely related protein tyrosine kinases, the *c-yes* and *fyn* proteins (5, 20, 23, 25). MT activates the enzymatic activity of the kinase in the complex (2, 4, 10). The interaction of MT with pp60^{c-src} is necessary but not sufficient for morphological transformation by MT (28).

Several tyrosine residues of MT, including tyrosine 250 and tyrosine 315, can be phosphorylated. Phosphorylation of both residues is necessary for full transformation by MT (27). Phos-

phorylation of tyrosine 250 allows MT to interact with Shc (3, 13), a Src homology 2 domain-containing protein which, when phosphorylated on tyrosine residue(s), interacts with the Src homology 2 domain of Grb2 (39). The Grb2-Shc complex associates with the mammalian homolog of SOS, a GTP exchange factor, leading to the activation of Ras (35). This series of interactions may account for the activation of Ras by MT (21, 34). Phosphorylation of tyrosine 315 is necessary for interactions with p85, the regulatory subunit of phosphatidylinositol (PI) 3-kinase (6). The interaction between MT and p85 results in the translocation of PI 3-kinase from the cytosol to the membrane (6).

Earlier mutagenesis studies suggested that the amino-terminal region of MT is important for association with pp60^{c-src} (reference 28 and references therein). However, these studies were carried out before it was known that MT associates with PP2A. More recent work suggests that pp60^{c-src} interacts with MT through a region in the middle of MT, amino acids 203 to 210, since a monoclonal antibody directed against this region blocks complex formation between MT and pp60^{c-src} (14) and deletion of amino acids 200 to 210 results in an MT protein which can associate with PP2A but not with pp60^{c-src} (13). MT appears to associate with both PP2A and pp60^{c-src} in the same complex, perhaps acting as a bridge between the phosphatase and the kinase (42). We decided to reassess the role of the amino terminus of MT to determine whether this region is important for interactions with PP2A. We show that several point mutations and two deletion mutations in the amino terminus of MT abrogate the ability of MT to associate with PP2A. One mutant appears to associate with a phosphatase other than PP2A and pp60^{c-src} in a manner similar to that of wild-type MT but is reduced in its transforming ability. We also show that a deletion of the amino acids CRMPLTC (amino acids 105 to 111), which are present in MT and ST but not large T antigen (LT), abrogates the ability of MT to associate with PP2A. A similar region in SV40 ST was shown to be necessary for interactions between SV40 ST and PP2A (30). Our results show a strict correlation between the association of MT with PP2A and the association between MT and pp60^{c-src}, lending further support to the hypothesis that MT-PP2A in-

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teractions are necessary for subsequent complex formation with pp60^{c-src}. They also suggest an additional role for MT-PP2A complexes in transformation by MT.

MATERIALS AND METHODS

Cells. NIH 3T3 cells were obtained from Marguerite Vogt (Salk Institute) and maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. 293 cells were also maintained in DME plus 10% calf serum.

Plasmids and mutagenesis. The polyomavirus MT coding region was cloned into Bluescript KS as a *XhoI-EcoRI* fragment from pMTneoMLV (15, 22) for subsequent mutagenesis. Oligonucleotide-directed mutagenesis was carried out according to the method of Kunkel et al. (24). Briefly, CJ236 bacteria were transformed with Bluescript containing the MT coding region, a positive clone was infected with M13KO7 helper phage, and uracil-containing single-stranded DNA was isolated. The mutagenic oligonucleotides were phosphorylated, annealed to the single-stranded DNA, filled in with T4 DNA polymerase and ligase, and used to transform MV1190 bacteria. Mutations were confirmed by DNA sequencing. The mutant MT genes were cloned into the expression vector, pRSV (12), in which the *HindIII* site was replaced by a *XhoI* site by ligation of a synthetic linker. The ligation reaction contained three DNAs: the *XhoI-BamHI* fragment of pRSV, the *EcoRI-BamHI* fragment from polyomavirus DNA containing the 3' untranslated region and the poly(A) addition site, and the *XhoI-EcoRI* fragment containing the mutated coding region of MT. Thus, MT is expressed from the Rous sarcoma virus promoter and utilizes the polyomavirus poly(A) addition site. FC-4, a plasmid consisting of the murine *c-fos* promoter driving expression of the bacterial chloramphenicol acetyltransferase (CAT) gene, pGem4, and pRSVβgal were used as described previously (17). pSV2neo (40) was used for G418 selection in transfection assays.

Transfections. Cells were seeded at 10⁵ in 6-cm-diameter plates 24 h prior to transfection. 293 cells were seeded on polylysine-coated plates. Cells were transfected with plasmid DNA by calcium phosphate-mediated precipitation as described previously (17). 293 cells were transfected with 10 μg of either pRSVMT or one of the vectors expressing a mutant MT as indicated. After 14 to 16 h, cells were washed with Tris-buffered saline (TBS) and refed with DME supplemented with 10% calf serum. NIH 3T3 cells were transfected with 2 μg of FC-4, 1 μg of the indicated MT-expressing plasmid, 5 μg of pRSVβgal, and 7 μg of pGem4 for transient CAT assays. After 14 to 16 h, cells were washed with TBS and refed with DME supplemented with 0.5% calf serum. NIH 3T3 cells were transfected with 1 μg of the indicated MT-expressing plasmid, 2 μg of pSV2neo, and 12 μg of pGem4 for transformation assays.

In vivo radiolabeling and immunoprecipitations. Transfected cells were radiolabeled with 100 μCi of ³⁵S-Translabel (Amersham, Arlington Heights, Ill.) per ml for 16 h in cysteine and methionine-free DME supplemented with 4% dialyzed calf serum. Nonidet P-40 lysates were prepared as described previously (17), and MT proteins were immunoprecipitated with 25 μl of PAb815, a rat monoclonal antibody directed against MT (provided by Joseph Bolen), followed by rabbit anti-rat immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) as a second layer. Preimmune and immune antisera directed against the carboxy-terminal 20 amino acids of the catalytic subunit of PP2A were a gift from Marc Mumby (University of Texas Southwestern Medical Center, Dallas). Immune complexes were collected with protein A-sepharose (Repligen, Cambridge, Mass.). Washed immunoprecipitates were fractionated on a 7% polyacrylamide gel (acrylamide-bis, 29:1). Gels of immunoprecipitates from ³⁵S-labeled lysates were dried and exposed, and MT bands were quantitated with a Molecular Dynamics PhosphorImager. Unlabeled lysates were prepared 24 h after transfection of 293 cells for immunoprecipitation of MT complexes and analysis of MT-associated phosphatase or kinase activity.

Phosphatase and kinase assays. MT immunoprecipitates were assayed for MT-associated phosphatase activity (i.e., PP2A) with ³²P-labeled casein as a substrate as described previously (17). Washed immune complexes were incubated in phosphatase buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7], 1 mM dithiothreitol, 100 μg of bovine serum albumin [BSA] per ml, 50 mM leupeptin, 0.05% Nonidet P-40, 1 mM MnCl₂) containing 50,000 cpm of ³²P-labeled casein at 30°C for 15 min. Reactions were terminated by precipitation with trichloroacetic acid, and after centrifugation, supernatants were counted by Cerenkov counting with an LS 6000SC liquid scintillation counter (Beckman) to determine counts released. Phosphatase activity was normalized to the amount of MT expressed in ³⁵S-labeled cells transfected in parallel. MT immunoprecipitates were assayed for MT-associated kinase activity (i.e., pp60^{c-src}) as described previously (17). Washed immune complexes were resuspended in kinase buffer [20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 7), 1 mM dithiothreitol, 10 mM MnCl₂] containing 20 μCi of [³²P]ATP, in either the presence or absence of 20 μM nonradioactive ATP, and incubated at 30°C for 15 min. Reaction mixtures were washed with Nonidet P-40 lysis buffer and resuspended in 2× sample buffer. Enolase was used as an exogenous substrate in some cases (9). Kinase activity was normalized to the amount of MT expressed as described above. MT immunoprecipitates were assayed for associated PI 3-kinase activity as described by Whitman et al. (45). Washed immune complexes were incubated in 25 μl of kinase buffer [20 mM HEPES (pH 7.1), 0.8 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-

tetraacetic acid (EGTA), 0.8 mM sodium phosphate]–9.5 μl of 20 mM HEPES (pH 7.1)–0.5 μl of PI (20 mg/ml in CHCl₃) at 4°C for 20 min. Reactions were started by the addition of MgCl₂ and nonradioactive ATP (final concentrations, 10 mM and 40 μM, respectively) and 20 μCi of [³²P]ATP in a total volume of 50 μl. Reaction mixtures were incubated at 25°C for 20 min, and reactions were stopped with 100 μl of 1 M HCl. Phospholipids were extracted and products were separated by thin-layer chromatography as described elsewhere (17). The plates were scanned with a Molecular Dynamics PhosphorImager.

Western blot (immunoblot) analysis. After fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), samples were transferred to Immobilon-P membranes (Millipore Co., Bedford, Mass.) with a semidry transfer apparatus. Filters were blocked for 1 h at room temperature in TBS (20 mM Tris [pH 8], 150 mM NaCl) containing 3% BSA (Sigma). Filters were then incubated in primary antibody in TBS containing 0.5% Tween 20 (TBST) for 1 h. Filters were washed five times in TBST, incubated with horseradish peroxidase-conjugated protein A (Amersham) in TBST for 1 h, washed five times in TBST, and then developed by using enhanced chemiluminescence reagents (Amersham) according to the manufacturer's protocol. Filters were exposed to Kodak XAR-5 film and developed.

Transformation assays. Cells were split at a ratio of 1:6 24 h after transfection. Three plates were fed with DME supplemented with 5% calf serum to assay for focus formation. Cells were refed every 3 to 4 days and stained with crystal violet after 2 to 3 weeks to visualize foci. G418 (400 μg/ml) was added to the remaining three plates maintained in DME supplemented with 10% calf serum. Cells from one of the plates were passaged as a mass culture; the other two plates were stained with crystal violet.

CAT assays. Lysates were prepared 24 to 48 h after transfection. CAT assays and β-galactosidase assays were carried out using standard protocols (17). CAT assays were carried out on lysates normalized to β-galactosidase activity to correct for differences in transfection efficiency.

RESULTS

Mutagenesis of polyomavirus MT. We introduced a series of small deletion or point mutations into three regions of MT, amino acids 1 to 25, 44 to 47, and 105 to 111, as illustrated in Fig. 1. The first two regions are shared among all three polyomavirus T antigens. The amino terminus of MT has been suggested to be important for interactions between MT and pp60^{c-src} (28). The first 25 amino acid residues of MT contain two small blocks of amino acids conserved among STs of polyomavirus, SV40, and BK virus (Fig. 1A). We deleted both conserved regions, removing amino acids 2 to 7 and 13 to 25. The point mutant Val4Asp, a nontransforming mutant of MT, was described previously (8). In addition, we mutated the adjacent conserved leucine 5 to a glutamic acid and the conserved arginine at position 7 to a methionine. We also changed leucines 13 and 14 to alanine residues, leucines 16 and 17 to alanines, leucine 19 to alanine, arginine 21 to methionine, and tryptophan 24 and glycine 25 to alanines.

The longest contiguous block of conserved amino acids found in the STs of polyomavirus, SV40, and BK virus, as well as in MT, is the sequence His-Pro-Asp-Lys-Gly-Gly (HPD KGG, amino acids 42 to 47 in polyomavirus). The sequence DKGG is found at a similar position in the cloned 55-kDa B subunit of PP2A (33) and is the only stretch of homology between MT and the B subunit. We introduced a series of point mutations into this region (Fig. 1B). Recent experiments on SV40 ST suggest that amino acids 97 to 103, comprising the sequence Cys-Lys-Gln-Trp-Pro-Glu-Cys (CKQWPEC) in the SV40 ST unique region, are important for complex formation between ST and the A subunit of PP2A (30). The proline residue at amino acid 101 in SV40 ST was shown to be critical for interaction with the A subunit, since mutation to alanine resulted in a 100-fold reduction in association. MT contains a similar sequence, Cys-Arg-Met-Pro-Leu-Thr-Cys (CRMPLTC), at amino acid residues 105 to 111. We made an analogous mutation of proline 108 in MT to alanine, as well as deleted the sequence CRMPLTC, to test the role of this region in complex formation between MT and PP2A (Fig. 1C). All mutants were cloned into pRSV to examine protein expres-

FIG. 1. Conserved regions of polyomavirus (PyV), SV40, and BK virus (BKV) STs and polyomavirus MT. Amino acid numbering is for polyomavirus MT. Identical amino acids are boxed. The mutations made in polyomavirus MT are shown below the conserved amino acid sequences.

sion, complex formation, and biological activities in mammalian cells.

Expression of mutant proteins and analysis of associated phosphatase activity. Plasmids encoding the wild-type and mutant MT proteins were transfected into 293 cells, and cells were labeled with ^{35}S -Translabel to examine protein expression. All the mutant proteins were expressed; thus, the plasmids were able to direct the synthesis of stable proteins in mammalian cells (Fig. 2).

To examine complex formation between PP2A and the various MT mutants, we assayed MT-associated phosphatase activity, using casein previously phosphorylated by cyclic AMP-dependent protein kinase as a substrate. Plasmids encoding the wild-type and mutant MT proteins were transfected into 293 cells, and MT-associated phosphatase assays were performed using immunoprecipitated complexes from cell lysates. Phosphatase activity was readily detectable in the transfected cells by this assay (Fig. 3A). Since the level of expression of the different mutants varied (Fig. 2), phosphatase activity was normalized to MT protein expressed in parallel transfections which were metabolically labeled with ^{35}S -Translabel. The amino-terminal deletion mutants *d12-7* and *d113-25* and the point mutants V4D, LL13,14AA, LL16,17AA, and WG24,25AA were defective for complex formation with PP2A as judged by this assay (Fig. 3A). In contrast, four other point mutants in this region, L5E, R7M, L19A, and R21M, possessed an associated phosphatase activity (Fig. 3A). Note that the phosphatase activity associated with MT was particularly high in the experiment in Fig. 3A (left panel); the phosphatase activity associated with the mutants L5E and R7M varied from 36 to 120% of the level associated with wild-type MT in other experiments (16). The association between the L5E mutant and PP2A appeared to be similar in stability to wild-type MT-

FIG. 2. Expression of polyomavirus MT mutant proteins. 293 cells were transfected with plasmid DNA encoding the indicated MT proteins as described in Materials and Methods, cells were labeled with ^{35}S -Translabel, and MT proteins were immunoprecipitated with a monoclonal antibody, MAb815. Proteins were fractionated by PAGE and visualized with a Molecular Dynamics PhosphorImager. The bands corresponding to MT and the regulatory (A) and catalytic (C) subunits of PP2A are indicated. The various MT mutants are described in Fig. 1.

FIG. 3. Phosphatase activity associated with the MT mutant proteins. 293 cells were transfected with plasmid DNA; 24 h after transfection, MT proteins were immunoprecipitated with MAb815 and assayed for associated phosphatase activity with ^{32}P -labeled casein as a substrate. MT-associated phosphatase activity was measured by release of free ^{32}P from labeled casein. The values have been normalized to the amount of MT protein expressed in parallel transfections labeled with ^{35}S -Translabel and immunoprecipitated with MAb815.

PP2A interactions since both were stable to washes in 0.5 M NaCl (16). Both complexes were sensitive to lysis in RIPA buffer (16). The three mutants of the DKGG sequence, D44K, K45D, and GG46,47AA, were similar to wild-type MT in associated phosphatase activity (Fig. 3B). Mutation of the conserved proline residue, P108A, had little effect on the level of associated phosphatase activity in this assay; however, deletion of amino acids 105 to 111 abolished associated phosphatase activity (Fig. 3C).

The ability of wild-type and mutant MT proteins to coprecipitate the regulatory (A) and catalytic (C) subunits of PP2A paralleled their ability to coprecipitate phosphatase activity, with one notable exception. The L5E mutant, which possesses associated phosphatase activity (Fig. 3A), failed to coprecipitate the regulatory subunit of PP2A (Fig. 2A). The C subunit is more difficult to observe in the ^{35}S -labeled immunoprecipitations, so we detected the presence of the C subunit by immunoprecipitation for MT, followed by Western blot analysis using antisera directed against the C subunit. Wild-type MT clearly coprecipitated the C subunit of PP2A, as did the R7M mutant protein (Fig. 4). Both of these MT proteins possess an associated phosphatase activity (Fig. 3A). The V4D mutant protein did not coprecipitate the C subunit (Fig. 4) and did not possess an associated phosphatase activity (Fig. 3A). The L5E mutant protein also did not coprecipitate the C subunit of PP2A (Fig. 4), even though it clearly possesses an associated phosphatase activity (Fig. 3A). These four MT proteins were

FIG. 4. Coimmunoprecipitation of the C subunit of PP2A with MT. 293 cells were transfected with plasmid DNA encoding the indicated MT proteins, cell lysates were prepared, and MT complexes were immunoprecipitated (IP) with MAb815. Proteins were fractionated by PAGE and transferred to nitrocellulose, and the C subunit of PP2A was detected by immunoblotting using an antiserum directed against the C subunit (αC). The filter was incubated with horseradish peroxidase-conjugated protein A and detected by enhanced chemiluminescence. The filter was exposed to Kodak XAR-5 film for 15 s.

expressed at similar levels as determined by immunoprecipitation from ^{35}S -labeled cell lysates (16).

MT-associated kinase activity correlates with MT-associated phosphatase activity. We next studied the effects of the mutations on MT-associated kinase activity. 293 cells were transfected with plasmids encoding the various MT proteins, and immune complex kinase assays were carried out after isolation of MT from cell lysates. Kinase activity was normalized to MT protein expressed in parallel transfections (see above). The six amino-terminal MT mutants which were defective for associated phosphatase activity were also defective for associated kinase activity (Fig. 5A). The mutants L5E, R7M, L19A, and R21M, which possessed an associated phosphatase activity, likewise had an associated kinase activity. Note that the kinase activity associated with MT was high in the experiment shown in Fig. 5A (left panel); the kinase activity associated with the L5E and R7M mutants varied from 42 to 116% of the level of kinase activity associated with wild-type MT in other experiments (16). Kinase activity associated with the L5E mutant was examined both with and without nonradioactive ATP present in the kinase reaction and with enolase as an exogenous substrate. The mutant protein behaved similarly to wild-type MT under all conditions (16). Similar results were obtained for all of these mutants when enolase was used as an exogenous substrate in the kinase reactions. Similarly, the three mutants in the DKGG region had wild-type levels of associated kinase activity (Fig. 5B). The mutant at proline 108 also had wild-type levels of associated kinase activity, whereas the mutant deleted of amino acids 105 to 111 had no associated kinase activity (Fig. 5C). In summary, the seven mutants which had no associated phosphatase activity also lacked associated kinase activity, whereas the eight mutants which had associated phosphatase activity retained associated kinase activity.

The L5E mutant MT is defective for activation of the *c-fos* promoter. We tested the ability of the various mutant MT proteins to activate expression from the *c-fos* promoter. NIH 3T3 cells were cotransfected with a plasmid in which the murine *c-fos* promoter drives expression of the CAT gene and a plasmid expressing one of the various MT-expressing constructs. After transfection, cells were placed in 0.5% calf serum, and 24 to 36 h later extracts were prepared and CAT assays were performed. Saturating amounts of MT-expressing plasmids were used in these experiments; therefore, differences in activation of *c-fos* expression are unlikely to be due to variation in the level of expression of the different mutant

FIG. 5. Kinase activity associated with the MT mutant proteins. 293 cells were transfected with plasmid DNA; 24 h after transfection MT proteins were immunoprecipitated with MAb815 and assayed for associated kinase activity with [γ -³²P]ATP. Proteins were fractionated by PAGE and visualized with a PhosphorImager. Values have been normalized to the amount of MT expressed in parallel transfections labeled with ³⁵S-Translabel and immunoprecipitated with MAb815.

proteins. Cotransfection of wild-type MT activated expression from the *c-fos* promoter approximately 5.5-fold above the background (70% conversion of chloramphenicol to acetylated forms compared with 13%) (Fig. 6A). The two amino-terminal deletion mutants, *dl2-7* and *dl13-25*, and the V4D point mutant did not activate the *c-fos* promoter above background levels. In contrast, the R7M point mutant activated the *c-fos* promoter to nearly wild-type levels. Interestingly, the L5E mutant protein, which possesses associated phosphatase and kinase activities (Fig. 3A and 5A), did not activate expression from the *c-fos* promoter. All three mutants of the DKGG sequence activated *c-fos* expression to wild-type levels (Fig. 6B). The P108A mutant induced expression from the *c-fos* promoter similarly to wild-type MT (Fig. 6C). Thus, with the exception of the L5E mutant, the ability of MT mutants to activate the *c-fos* promoter correlated with their ability to associate with PP2A and pp60^{c-src}.

The L5E mutant is reduced in its ability to transform NIH 3T3 cells. The MT mutants were assayed for their ability to induce focus formation on NIH 3T3 cells. Wild-type MT produced foci on NIH 3T3 cells (Table 1). Mutants which were defective for association with PP2A and pp60^{c-src} were unable to induce focus formation. The amino-terminal mutants *dl2-7*, *dl13-25*, and V4D were unable to induce focus formation on NIH 3T3 cells (Table 1). Mutants which were not defective for association with PP2A and pp60^{c-src} (R7M, the three mutants of the DKGG region, and P108A) were similar to wild-type MT (Table 1). The L5E mutant, which has associated phos-

FIG. 6. Activation of the *c-fos* promoter by MT and various mutants. NIH 3T3 cells were transfected with plasmids encoding the CAT gene driven by the *c-fos* promoter, either wild-type or mutant MT, and Rous sarcoma virus β -galactosidase and then placed in DME containing 0.5% calf serum for 24 to 36 h. CAT assays were performed on lysates normalized for expression of Rous sarcoma virus β -galactosidase. [¹⁴C]chloramphenicol and acetylated products were separated by thin-layer chromatography and quantitated with a PhosphorImager. The percent conversion of [¹⁴C]chloramphenicol to acetylated forms was determined, indicating the level of activity of the *c-fos* promoter.

phatase and kinase activities, was reduced in its ability to form foci, producing 8 to 30% of the number of foci induced by wild-type MT (Table 1). Thus, as observed for the ability to activate *c-fos* expression, focus-forming ability of the MT mutants paralleled the ability to form complexes with PP2A and pp60^{c-src}, with the exception of the L5E mutant, which associated with a phosphatase and pp60^{c-src} but was reduced approximately sevenfold in its ability to form foci on NIH 3T3 cells.

TABLE 1. MT-induced focus formation in NIH 3T3 cells

Construct	No. of foci obtained ^a in:			
	Expt 1	Expt 2	Expt 3	Expt 4
None (vector alone)	0	0	2	0
MT	195	114	48	27
V4D	1	0	0	0
L5E	16	18	14	ND
R7M	80	90	86	ND
dl2-7	3	0	0	ND
dl13-25	3	0	0	ND
D44K	ND	ND	ND	17
K45D	ND	ND	ND	23
GG46,47AA	ND	ND	ND	19
P108A	ND	ND	ND	21

^a Per microgram of MT-expressing plasmid DNA. ND, not determined.

The L5E mutant retains associated PI 3-kinase activity. Since the L5E mutant showed wild-type levels of associated protein kinase activity, we tested its association with PI 3-kinase. Kinase assays were carried out with immune complexes isolated from transfected 293 cells with PI as a substrate. Lipids were extracted and separated by thin-layer chromatography, and products were quantitated with a Molecular Dynamics PhosphorImager. Wild-type MT coimmunoprecipitated with an activity which incorporated 79,796 counts into PI diphosphate, whereas immunoprecipitates from vector-transfected cells had an activity which incorporated 29,718 counts (37% of the level of wild-type MT). A similar activity was found in immunoprecipitates from cells transfected with the mutant V4D (35,961 counts incorporated into PI diphosphate, 45% of the wild-type level). The L5E mutant coimmunoprecipitated with levels of PI 3-kinase activity similar to those with which wild-type MT coimmunoprecipitated (67,117 counts incorporated, 84% of the wild-type level). The three MT proteins were expressed at similar levels (16). Thus, the L5E mutant appeared to be normal with regard to association with PI 3-kinase.

DISCUSSION

We analyzed three regions of MT to determine their importance for complex formation between MT and PP2A. We introduced both deletion and point mutations into the first 25 amino acid residues of MT, changing amino acids conserved among STs of polyomavirus, SV40, and BK virus. Deletion of amino acids 2 to 7 or 13 to 25 resulted in a mutant MT that was unable to associate with PP2A. Mutation of a conserved valine (V4D), two pairs of dileucines (LL13,14AA and LL16,17AA), or a conserved tryptophan-glycine (WG24,25AA) each resulted in a protein which could not associate with PP2A. The V4D mutant was previously shown to be transformation defective, presumably because of its failure to interact with pp60^{c-src} (8). The arginines at positions 7 and 21 and the single leucine at position 19 do not appear to be required for complex formation, although they are conserved among the three viruses.

Mutation of leucine 5 to glutamic acid produced an MT protein which has an associated phosphatase activity yet does not detectably associate with PP2A by coimmunoprecipitation. Moreover, this mutant appears to be similar to wild-type MT in its association with pp60^{c-src} and PI 3-kinase yet is reduced in its transforming ability. It will be important to determine whether other aspects of MT signaling through pp60^{c-src}, such as association with and phosphorylation of Shc, remain intact in the mutant MT. It is possible that the L5E mutant protein associates with another isoform of PP2A which is not recognized by the antiserum directed against the C subunit of PP2A. However, no band of the size expected for the A subunit was coimmunoprecipitated from lysates prepared from metabolically labeled cells. Alternatively, the L5E mutant protein could associate with another phosphatase. The apparent lack of association of the L5E mutant with PP2A suggests that association of MT with PP2A per se is not required for association with pp60^{c-src}. However, since the mutant retains an associated phosphatase activity, there could still be a requirement for such an activity for MT to associate with pp60^{c-src}. The L5E defect could suggest a role for the amino-terminal region of MT in transformation in addition to its presumed role in promoting complex formation between MT, PP2A (or another phosphatase), and pp60^{c-src}. An intriguing possibility is that the associated phosphatase activity is sufficient to promote association between MT and pp60^{c-src} but does not provide another

function(s) required for full transformation by MT. Alternatively, the mutation could disrupt the binding of another protein to MT in this region.

Collectively, these mutations define a region of MT important for association with PP2A and suggest that pairs of aliphatic amino acids, V-4-L-5, L-13-L-14, and L-16-L-17, and a W-24-G-25 are necessary for interactions. Dileucines have been implicated in endocytosis and lysosomal targeting of proteins (1, 26, 41). It will be important to determine whether mutation of the dileucines in MT affects direct interactions between MT and PP2A or intracellular localization of MT. These results do not suggest a mechanism by which the mutations affect association of MT with PP2A. The mutations could alter the tertiary structure of MT, leading to an inability of MT to associate with PP2A, or could affect a region of MT that binds PP2A.

The second region that we examined contains the longest contiguous stretch of amino acids conserved among ST antigens of polyomavirus, SV40, and BK virus. This sequence, HPDKGG, is found at amino acids 42 to 47 in MT. The sequence DKGG is also found at a similar location (amino acids 47 to 50) in the cloned B subunit of PP2A (33). In light of this conservation, it was somewhat surprising that this region was completely dispensable for complex formation between MT and PP2A and for transformation by MT. It is possible that this region is important functionally, perhaps in STs or LTs, which also contain this region. Alternatively, the conservation of this region could be fortuitous.

The first two regions that we mutated in MT are also shared with LT, which does not associate with PP2A. Therefore, they would not be expected to contain the sole determinants for interactions with PP2A. Recent work on SV40 ST suggests that two conserved cysteine residues and an intervening proline residue, in the sequence CKQWPEC (CRMPLTC in polyomavirus), found in the region shared by MT and ST but not LT, are important for interactions between SV40 ST and the A subunit of PP2A (30). Deletion of the sequence CRMPLTC (amino acids 105 to 111 of MT) resulted in a protein which was unable to associate with PP2A. However, mutation of proline 108 in MT resulted in a protein which was still able to associate with PP2A. Mutation of the analogous proline in SV40 ST (P-101) resulted in a 100-fold decrease in the association between the mutant ST and PP2A (30). In addition, the cysteine-rich cluster found in both MT and ST (amino acids 120 to 153 in polyomavirus) is required for complex formation between MT and PP2A (17).

The mutation of proline 108 in MT appears to be less deleterious than the analogous mutation (Pro-101) in SV40 ST. SV40 ST, polyomavirus ST, and polyomavirus MT have different requirements for their association with the A and C subunits of PP2A (37). The A subunit consists of a series of 15 repeats which probably form an extended rod-like structure (36, 44). The carboxy-terminal repeats (repeats 11 to 15) are involved in interactions between the A and C subunits (37). Deletion of these repeats has little effect on interactions between either polyomavirus or SV40 ST with PP2A but disrupts the interaction of MT with PP2A (37), suggesting that MT contacts the C subunit, perhaps stabilizing the complex. SV40 ST requires the smallest region of the A subunit for association (repeats 4 to 6). Polyomavirus ST interacts with a slightly larger region (repeats 3 to 6), while MT requires the largest region (repeats 3 to 8). It is possible that the region CXXPXXC is required for interactions between MT and PP2A, as suggested by the behavior of the deletion mutant *dl* CXXPXXC, but that a more subtle mutation (i.e., P108A) is more detrimental to SV40 ST-PP2A association than to MT-PP2A association. The

P108A mutant of MT may form a more stable complex with PP2A than the analogous mutant in SV40 ST because of the contacts between MT and the C subunit of PP2A.

Our results implicate three regions of MT as being required for complex formation between MT and PP2A: the first 25 amino acid residues, the CRMPLTC motif (amino acids 105 to 111), and the cysteine-rich cluster (amino acids 120 to 153) (17). These regions could be important for either direct protein-protein interactions between MT and PP2A or the conformation of MT, indirectly affecting interactions between MT and PP2A. It will be interesting to see whether similar features will be found in other B subunits of PP2A.

Finally, our results lend further support to the presumed role of complex formation between MT and PP2A in the association of MT with pp60^{c-src}. All mutants of MT which fail to associate with PP2A also fail to associate with pp60^{c-src}. MT, PP2A, and pp60^{c-src} are apparently present together in a complex (42). However, it is not clear whether MT-PP2A complexes are a structural determinant for association between MT and pp60^{c-src} (i.e., pp60^{c-src} would physically contact both MT and PP2A) or whether association of MT with PP2A results in a modified form of MT which can then interact with pp60^{c-src}. We are currently studying whether association of MT with PP2A affects the phosphorylation state of MT and, if so, whether altered phosphorylation affects the ability of MT to associate with pp60^{c-src}.

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