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pp60$^{c\text{-}src}$ Binding to Polyomavirus Middle T-Antigen (MT) Requires Residues 185 to 210 of the MT Sequence

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Interaction with the *src* family of tyrosine kinases is crucial to the transforming action of polyomavirus middle T-antigen (MT). Association with MT activates the tyrosine kinase activity of pp60$^{c\text{-}src}$ and, through subsequent MT phosphorylation, creates binding sites for signalling molecules whose stimulation culminates in cell transformation. Despite this importance, and many studies, little is known of the mechanisms by which pp60$^{c\text{-}src}$ binds to MT. We report here isolation of the first MT mutants that disrupt pp60$^{c\text{-}src}$ binding without affecting the interaction between MT and protein phosphatase 2A (PP2A). Through deletion analysis we established that interaction with pp60$^{c\text{-}src}$ requires the sequences between amino acids 185 and 210 of MT, but these residues have no effect on PP2A binding. Cells expressing these mutants showed few altered properties, indicating that the PP2A-MT interaction alone has little influence on cell phenotype. Subcellular location of these mutant MT molecules was indistinguishable by immunofluorescence analysis from that of wild-type MT but was altered markedly on loss of PP2A binding. This suggests a possible role for PP2A in specifying subcellular distribution.

Expression of the middle T-antigen (MT) encoded by polyomavirus is sufficient to alter established rodent fibroblasts to a fully transformed and tumorigenic phenotype (43). MT is a protein of approximately 55 kDa that associates with cellular membranes and has no known enzymatic properties. Instead, MT promotes its effects by interacting with molecules that regulate cell growth and division (13, 24). So far, MT is known to associate with the 60-kDa regulatory (A) and 35-kDa catalytic (C) components of protein phosphatase 2A (PP2A) (34, 47), the *src* family tyrosine kinases, pp60$^{c\text{-}src}$ (12), pp62$^{c\text{-}yes}$ (26) and pp59$^{c\text{-}fyn}$ (7, 23, 28), the 85-kDa regulatory component of phosphatidylinositol 3′ OH kinase (PI3K) (10, 25), the 52- and 66-kDa forms of Shc (4, 14), Grb2 (4, 14), phospholipase C-γ1 (PLC-γ1) (40), and some members of the 14-3-3 family of proteins (32).

The 60-kDa A component of PP2A probably binds directly to MT, leading to an indirect association between MT and the 35-kDa C component but not the B subunits (3, 36). As PP2A reacts with both MT and small t, the binding site must lie within their shared N-terminal 191 amino acids. Mutations in a number of sites within this sequence, notably a cluster of cysteine residues, disrupt the interaction (3, 18, 19, 29).

Association between MT and PI3K is mediated via the SH2 domains of the PI3K 85-kDa subunit binding to phosphorylated tyrosine 315 of MT (42, 48). As a consequence, the concentration of PI3K products increases within cells (20, 39). PLC-γ1 binding to MT is also mediated by the SH2 domain binding to a phosphotyrosine residue in MT, in this case phosphorylated tyrosine 322, and this can cause an increase in the tyrosine phosphorylation state of PLC-γ1 in MT-expressing cells (40). Shc association occurs through a related mechanism: the novel PTB domain of Shc binds to phosphorylated tyrosine 250 of MT (2a, 4, 14). Once bound, Shc itself becomes tyrosine phosphorylated, providing a binding site for the SH2 domain of the adapter molecule Grb2 (4, 14). This, via the Grb2-associated guanine nucleotide exchange factor mSos, may be sufficient to promote accumulation of p21^{ras} in its GTP-bound active state and so stimulate the mitogen-activated protein kinase pathway (38). The similarity of these interactions to those occurring during growth factor receptor signalling has led us to propose that MT be considered as a permanently active homolog of a growth factor receptor (13). Disrupting the binding to MT of either PI3K, Shc, or, to a lesser extent, PLC-γ1, reduces MT's transforming ability (4, 10, 14, 25, 40).

MT binding to either PI3K, PLC-γ1, or Shc, then, requires prior tyrosine phosphorylation of MT. This phosphorylation is probably catalyzed by the *src* family tyrosine kinases also associated with MT. Therefore, MT binding to pp60$^{c\text{-}src}$ is a prerequisite to the formation of any further signalling complexes. Despite this importance, little is known of the molecular processes involved in forming the MT-pp60$^{c\text{-}src}$ interaction. It has been shown to be extremely robust, dissociating only under strong denaturing conditions (11), and only a single *src* family kinase is thought to be bound to each molecule of MT (6). Within the MT-pp60$^{c\text{-}src}$ complex the regulatory tyrosine residue of pp60$^{c\text{-}src}$, Tyr 527, is not phosphorylated and kinase activity is consequently activated (2, 5, 9). It is still not clear whether this dephosphorylation of pp60$^{c\text{-}src}$ occurs before interaction with MT or as a consequence of binding, perhaps by the coassociated PP2A. Similar to the association with PP2A, the sequences in MT required for the interaction with pp60$^{c\text{-}src}$ lie in the N-terminal region of the molecule. All of the MT mutations studied that disrupt PP2A binding also prevent pp60$^{c\text{-}src}$ association (3, 18, 19, 29), suggesting that these interactions may be linked.

We have reported previously that monoclonal antibodies which react with amino acids 200 to 219 of MT fail to bind MT associated with pp60$^{c\text{-}src}$, yet do react with the MT-PP2A complex (16). To study this further, we have now constructed several mutants within this region. Using a series of overlapping deletions, we demonstrated that the region of MT between amino acids 185 and 210 is required for pp60$^{c\text{-}src}$ binding but does not affect association with PP2A. This sequence does

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not appear to have similarities with any known regulators of pp60$^{c\text{-}src}$, and as such, probably represents a novel means of controlling its kinase activity. Disrupting the interaction with pp60$^{c\text{-}src}$ seemed to have little gross effect on MT's subcellular distribution, but lack of binding to PP2A caused a marked alteration in the location of mutant MT molecules. This suggests that association with PP2A may control the subcellular distribution of the MT signalling complex.

MATERIALS AND METHODS

Deletion mutagenesis. All mutations were made to one of two plasmids. pEMT is vector pEMBL8 containing the *Bam*HI-*Eco*RI fragment of polyomavirus lacking the MT intron. pUCMT consists of the same fragment of MT cloned into the vector pUC19. All plasmid preparations were isolated by Qiagen columns (Qiagen) according to the manufacturer's protocols. DNA manipulations were achieved by standard techniques (37). MT deletion mutants were made using a PCR-based technique (22). Two PCR fragments were isolated initially. The first was made with a primer 5′ to the MT region and an oligonucleotide containing the deletion. The other fragment was generated using a deletion-containing primer on the opposite strand and a 3′ oligonucleotide. These DNA pieces were then combined by PCR using the two external primers. An *Sph*I-*Cel*II restriction enzyme fragment containing the deletion was then isolated from the resulting DNA and used to replace the same segment excised from pEMT. The whole region was then sequenced with a Sequenase 2 plasmid sequencing kit (U.S. Biochemicals) to confirm both the presence of the deletion and the absence of any other mutations.

Cell culture, focus assays, and cell line isolation. All cell culturing was carried out by standard methods, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, followed by incubation at 37°C in the presence of 10% CO_2.

The ability to form foci was assayed on the Rat2 cell line. Barely subconfluent cells on 10-cm-diameter petri dishes were transfected overnight with 10 μg of plasmid DNA, using the calcium phosphate technique. The cells were then kept in culture for 14 days, changing the medium every 3 days. After this time the medium was removed and the cells were stained with Leishmann's solution (Merck).

Cell lines expressing each mutant MT were isolated by cotransfecting 5 μg of mutant DNA into Rat2 cells together with 1 μg of the plasmid pSVneo. At 24 h after transfection, the cells were subcultured into medium containing 700 μg of G418 (Gibco) per ml. After 7 days, individual colonies were isolated, checked by Western blotting for MT synthesis, and then recloned by limiting dilution.

Lysate preparation, in vitro kinase assays, and immunoprecipitations. Lysates were prepared, immunoprecipitated, labelled by an in vitro kinase reactions, separated on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels, and subjected to autoradiography as described previously (16). Monoclonal antibody PAb 762 (2a) was used to precipitate MT, and [γ-^{33}P]ATP was used to label proteins. Immunoprecipitations for Western blotting were also performed in the same manner, using PAb 762. For each mutant, the amount of lysate used was adjusted to provide equivalent amounts of MT. The resulting precipitates were then separated by SDS-polyacrylamide gel electrophoresis and transferred onto 0.2-μm nitrocellulose (Merck). Unreacted protein binding sites were blocked by incubation for 1 h in 5% powdered skimmed milk in phosphate-buffered saline (PBS). MT-associated proteins were then detected with specific antisera and horseradish peroxidase-labelled protein G (Zymed) and revealed by enhanced chemiluminescence reagent (Amersham) according to the manufacturer's instructions. The antibodies used were: for detection of pp60$^{c\text{-}src}$, an antipeptide serum raised in sheep (Affiniti Labs), an anti-Shc SH2 domain raised in rabbits (Transduction Labs), and an anti-PI3K SH2 domain raised in rabbits (Transduction Labs); and for detection of PP2A, antipeptide sera raised in rabbits (45) (a kind gift of Emin Ulug). For MT detection, PAb 762 antibody was purified on Sepharose-protein A columns and biotinylated with a commercially available biotinylation kit (Amersham). This was then used to probe blots and detected with horseradish peroxidase-labelled streptavidin (Amersham).

Immunofluorescence assays. Cells expressing mutant MT molecules were seeded onto glass coverslips and allowed to grow for 24 h. The medium was then removed, and the coverslips were washed with DMEM at 37°C. The DMEM was then removed, and the cells were fixed by the addition of 3.7% formaldehyde in PBS, followed by incubation for 10 min at room temperature. The cells were then washed twice with PBS and permeabilized by incubation with 1% Nonidet P-40 in PBS for 5 min. MT was detected by incubation with tissue culture fluid containing PAb 762 for 1 h at room temperature, followed by washing with PBS for 30 min. Biotinylated antimouse antibody (Amersham) was then added and incubated for a further 1 h, followed by PBS washes for 30 min. MT location was then revealed by incubation with Cy3-labelled streptavidin (Amersham) and incubation for 45 min, followed again by 30-min washes with PBS, and the coverslips were mounted on Aquamount (Merck). Photomicrographs were taken through a Nikon Optiphot microscope with a ×40 objective, using TMax 400 film (Kodak). All exposure times were similar.

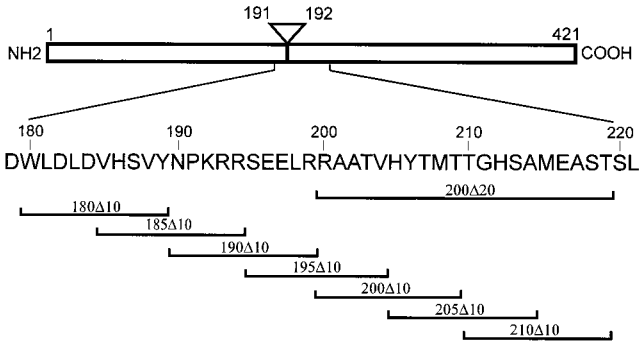

FIG. 1. Amino acids deleted by MT mutants. MT is represented schematically at the top, and the regions deleted from MT mutants are indicated underneath. The amino acid numbers of the N- and the C-terminal ends and the splice position are indicated. The amino acid sequence encoded by RNA sequences surrounding the splice point is shown in the single-letter code in the center.

RESULTS

Amino acids 180 to 210 are required for MT-induced transformation. Our finding that monoclonal antibodies which bind to amino acids 200 to 219 of MT do not immunoprecipitate the MT-pp60$^{c\text{-}src}$ complex suggested that this region was involved in the interaction with pp60$^{c\text{-}src}$ (16). As no mutations have been isolated in this area previously, we initially constructed a mutant DNA encoding an MT species lacking the whole sequence between amino acids 200 and 219 inclusive (Fig. 1; 200Δ20). This mutant DNA was then transfected into Rat2 fibroblasts to determine whether recipient cells could form foci on adherent cell monolayers (Fig. 2). Figure 2C shows that the 200Δ20 mutant was completely deficient in this assay. To examine whether the entire 20 amino acids is required to induce transformation, we constructed a series of overlapping 10-amino-acid deletion mutants (Fig. 1) and transfected them into Rat2 cells. Removing amino acids 210 to 219 (mutant 210Δ10) had no effect on the transforming action of MT (Fig. 2D), but 205Δ10 (panel E) induces less than 5% of the colonies formed by the same amount of wild-type DNA. However, MT with the 10 residues between amino acids 200 and 210 deleted was completely transformation defective (Fig. 2F). Therefore, the sequences of MT required to induce transformation extend only as far as amino acid 210 on the C-terminal side, and those between 210 and 220 are not necessary.

To determine how far this region extends in an N-terminal direction, more overlapping deletion mutants were constructed back to amino acid 180, well within the sequence common to MT and small t antigen (Fig. 1), and the DNA was again assayed for the ability to form foci (Fig. 2). Mutants 195Δ10 and 190Δ10 (Fig. 2G and H) showed no transforming activity in this assay. 185Δ10 induced a small number of colonies (Fig. 2I), suggesting a limit may have been approached, similar to the behavior of mutant 205Δ10. However, mutant 180Δ10, which extends further yet toward the N terminus, had no transforming activity (Fig. 2J). The whole region between amino acids 180 and 210 is therefore required for MT to induce transformation.

pp60$^{c\text{-}src}$, PI3K, and Shc binding to MT correlates with transformation. To examine the biochemical properties of these mutant MT species, we isolated stable cell lines expressing each protein by cotransfection of the mutant DNA with a *neo* gene-containing plasmid, followed by selection with G418. Each line was characterized to ensure expression of MT, and then lysates were prepared for immunoprecipitation studies.

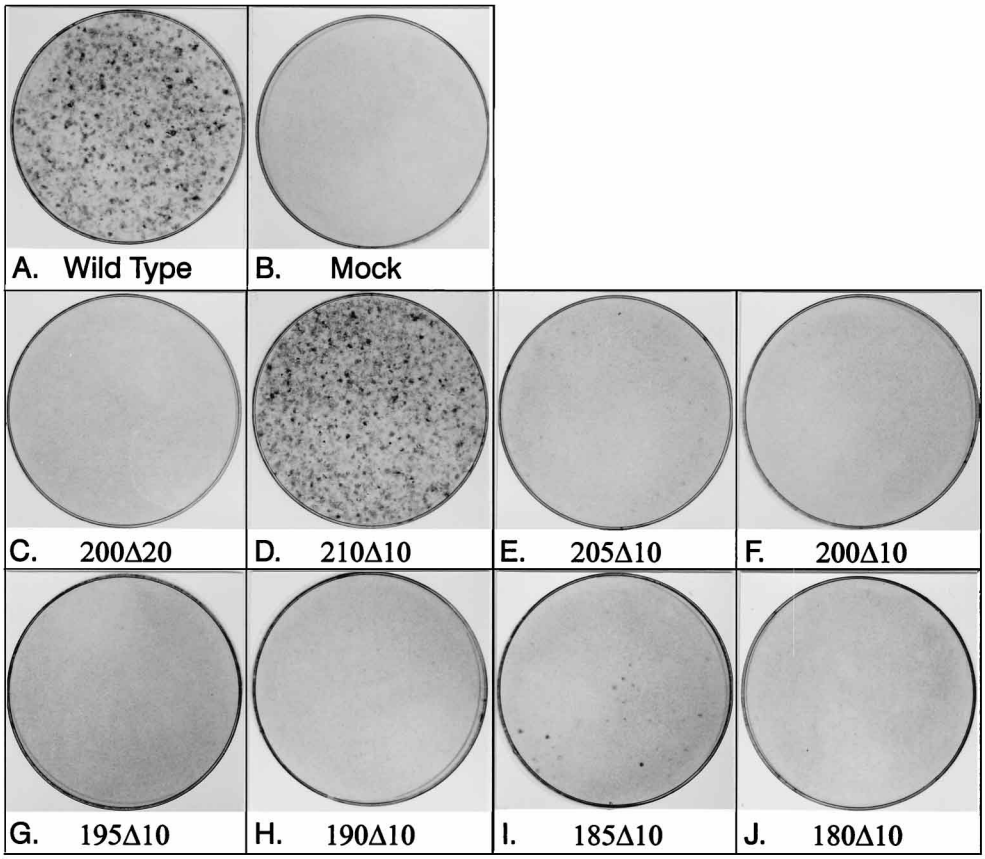

FIG. 2. The focus-inducing properties of MT deletion mutants. Ten micrograms of an MT cDNA-containing plasmid was transfected into Rat2 cells. After 14 days the cells were stained with Leishmann's reagent. (A) Foci induced by a plasmid containing wild-type MT cDNA. (B) Foci induced by a control plasmid. The deletion mutant used is indicated for the other panels.

To detect *src* family kinase activity associated with MT, an anti-MT monoclonal antibody, PAb 762, was used to immunoprecipitate MT and the resulting complex was incubated with [γ-^{33}P]ATP. Figure 3 shows the polypeptides in each MT mutant immunoprecipitate that became phosphorylated during this reaction. The *src* family kinases associated with wild-type MT phosphorylated MT, themselves, and the 85-kDa PI3K component (lanes 2 and 8), as reported previously. Mutant 210Δ10, which has wild-type levels of transforming activity, also exhibited similar phosphorylation patterns (lane 6). All of the mutants that have no focus-inducing capacity showed no MT-associated kinase activity (lanes 3, 4, 9, 11, and 12). The mutants that generate low numbers of foci (205Δ10 and 185Δ10) exhibited small amounts of kinase activity on longer exposures of the autoradiograph (data not shown). This may provide an explanation for their ability to induce only low numbers of foci. During calcium phosphate-mediated DNA transfection, the amount of DNA integrated into the host genome and expressed varies between individual cells. If pp60$^{c\text{-}src}$ has to be activated above a certain threshold to transform recipient cells, mutants that poorly activate pp60$^{c\text{-}src}$ will transform only those cells that express high levels of MT. This means that the number of foci observed reflects the proportion of cells expressing this large amount of protein. Therefore, there is an exact correlation between transforming capacity and MT-bound *src* family kinase activity in these mutants.

To identify the other proteins that are bound to each mutant MT, we employed an approach involving immunoprecipitation with the monoclonal antibody PAb 762, followed by Western blotting and finally detection of the associated proteins using other antibodies. Figure 4 shows the results of these experiments. We first probed the MT immunoprecipitates with a polyclonal anti-pp60$^{c\text{-}src}$ antibody (panel A). (As it is likely that pp60$^{c\text{-}src}$, pp62$^{c\text{-}yes}$, and pp59$^{c\text{-}fyn}$ bind to MT in an analogous manner, only pp60$^{c\text{-}src}$ was examined in these studies). pp60$^{c\text{-}src}$ was easily detected in wild-type MT immunoprecipitates (lanes 2 and 8) and was observed associated with 210Δ10 MT (lane 6), which also transforms cells and shows in vitro kinase activity. However, no pp60$^{c\text{-}src}$ was observed associated with the transformation-deficient mutant MT polypeptides (lanes 3, 4, 5, 9, 10, 11, and 12). The absence of kinase activity in immunoprecipitates containing defective MT protein is, therefore, due to a lack of binding to pp60$^{c\text{-}src}$, rather than to an inability to activate the kinase. Interaction of MT with both Shc and PI3K is dependent on MT's prior association with pp60$^{c\text{-}src}$. Probing duplicate blots with anti-Shc (panel B) or anti-PI3K 85-kDa subunit antibodies (panel C), therefore, showed similar results. Both proteins bound to wild-type (lanes 2 and 8) and 210Δ10 (lane 6) MT but did not associate with MT molecules that fail to bind pp60$^{c\text{-}src}$ (lanes 3, 4, 5, 9, 10, 11, and 12). Mutants 205Δ10 and 185Δ10, which exhibit a low level of focus-inducing capacity, were found to bind very small amounts of pp60$^{c\text{-}src}$, Shc, and PI3K when more sensitive experiments were performed (data not shown). There is, then, a complete correlation between the ability to bind pp60$^{c\text{-}src}$, Shc, and PI3K and transformation.

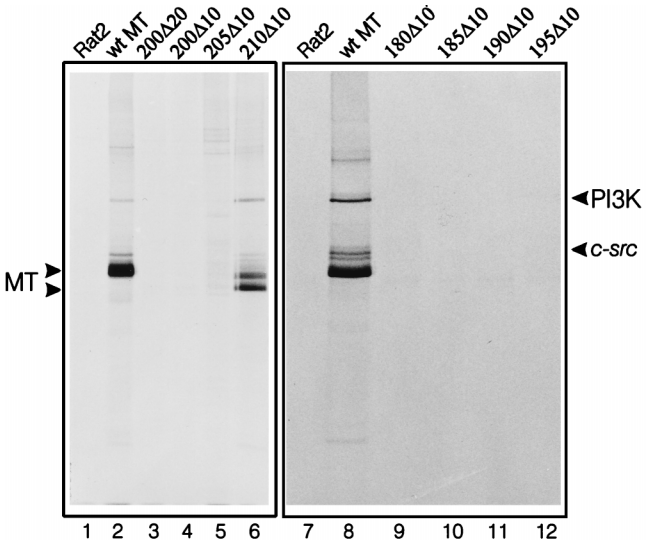

FIG. 3. In vitro kinase assay of mutant MT polypeptides from G418-selected cell lines. Lysates were prepared from cell lines expressing each MT deletion mutant, immunoprecipitated with monoclonal antibody PAb 762, incubated with [γ-^{32}P]ATP, and then separated on a polyacrylamide gel containing SDS. The deletion mutant expressed is indicated above each lane. The separate panels contain results from two different experiments, run on separate gels. Each experiment included a control using a lysate from the parental Rat2 cell line (lanes 1 and 7) and a wild-type MT-containing cell lines (lanes 2 and 8). The migration positions of wild-type and mutant MT polypeptides are indicated on the left, and those of the 85-kDa component of PI3K and pp60$^{c\text{-}src}$ are on the right.

When similar immunoprecipitates were probed with antibodies against the A and C components of PP2A, however, a different result was obtained (Fig. 4D and E). All of the MT polypeptides bound normal levels of both PP2A components, except the most N-terminal mutant, 180Δ10, which associated with neither (lane 9). This may explain why 185Δ10 retains some transforming activity, but 180Δ10 does not. The 185 position probably delineates the end of the region required to bind pp60$^{c\text{-}src}$ only, whereas the sequences between 180 and 184 are part of a region involved in associating with both PP2A and pp60$^{c\text{-}src}$. Probing similar blots with an anti-MT antibody demonstrated that similar levels of MT were expressed in each line (Fig. 4F), and the polypeptides were truncated relative to wild-type MT as predicted by the size of the deletion mutant used. Thus, the MT region required to bind pp60$^{c\text{-}src}$ lies between amino acids 185 and 210. To determine whether this region binds to pp60$^{c\text{-}src}$ directly, we have constructed a glutathione *S*-transferase fusion protein containing amino acids 180 to 220 of MT, but so far, we cannot detect any interaction with pp60$^{c\text{-}src}$ in cell lysates (2). This suggests that the association may also depend on additional factors, such as posttranslational modifications.

Phenotypic properties of cell lines expressing MT mutants. Deletion mutants 190Δ10, 195Δ10, 200Δ10, and 200Δ20 are the first MT species to be isolated that bind PP2A but not pp60$^{c\text{-}src}$. Cells expressing these mutants do not form foci (Fig. 2) or grow in soft agar (data not shown). To examine whether any other phenotypic changes in cells expressing these mutant MTs could be observed, cell lines expressing each polypeptide were allowed to grow to similar levels of confluency and then photographed under phase-contrast conditions (Fig. 5). The parental Rat2 cell line exhibited a flattened, fibroblast morphology, with no tendency to overgrow other cells, and a clear contact inhibition of growth (Fig. 5A). Expression of wild-type MT caused these cells to lose this contact inhibition; the cells overgrew one another in a random fashion, and individual cells appeared more rounded and refractile (Fig. 5B). Cells expressing focus-inducing mutant 210Δ10 showed a phenotype similar to that of wild-type MT-expressing cells (Fig. 5C), whereas those synthesizing nontransforming mutants exhibited few, if any, changes to the parental cells (Fig. 5D, E, and F). Therefore, the ability to bind PP2A in the absence of pp60$^{c\text{-}src}$ and any effects this may have on phosphatase activity have few overt

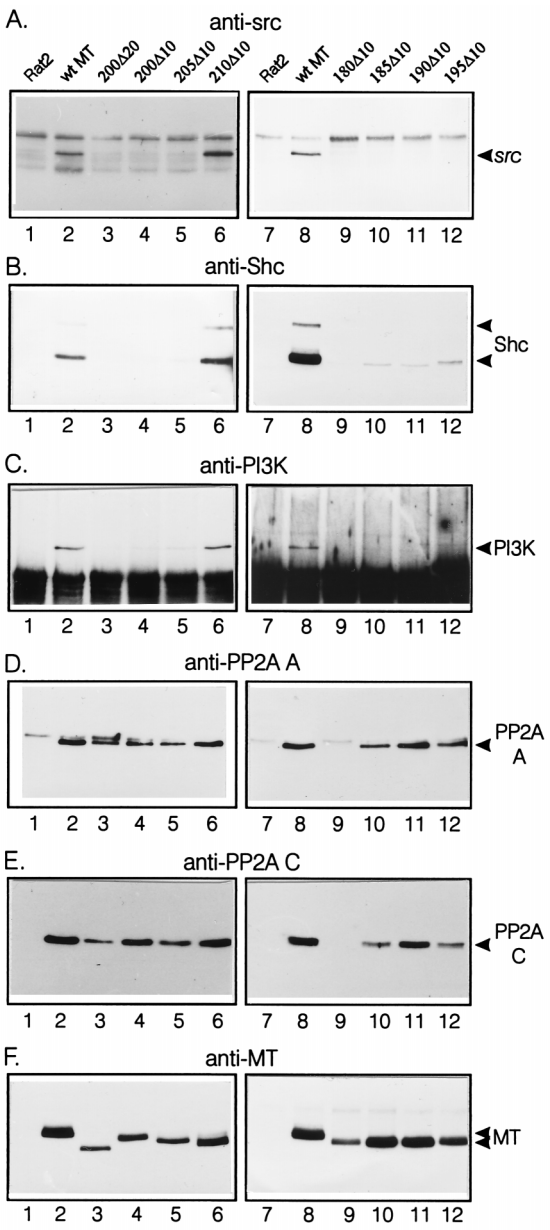

FIG. 4. Western blot analysis of immunoprecipitated proteins associated with deletion mutants of MT. Lysates of cell lines expressing mutant MTs were immunoprecipitated with PAb 762, electrophoretically separated, and then transferred onto nitrocellulose filters. These were then probed with the appropriate antibodies to detect MT-associated pp60$^{c\text{-}src}$ (A), Shc (B), the PI3K 85-kDa component (C), the PP2A A subunit (D), the PP2A C subunit (E), and MT itself (F). The deletion mutant analyzed in each case is indicated above the lanes in panel A. A negative (Rat2) and a positive (wt MT) control is included in each gel. The migration positions of the associated polypeptides detected are shown to the right of each panel.

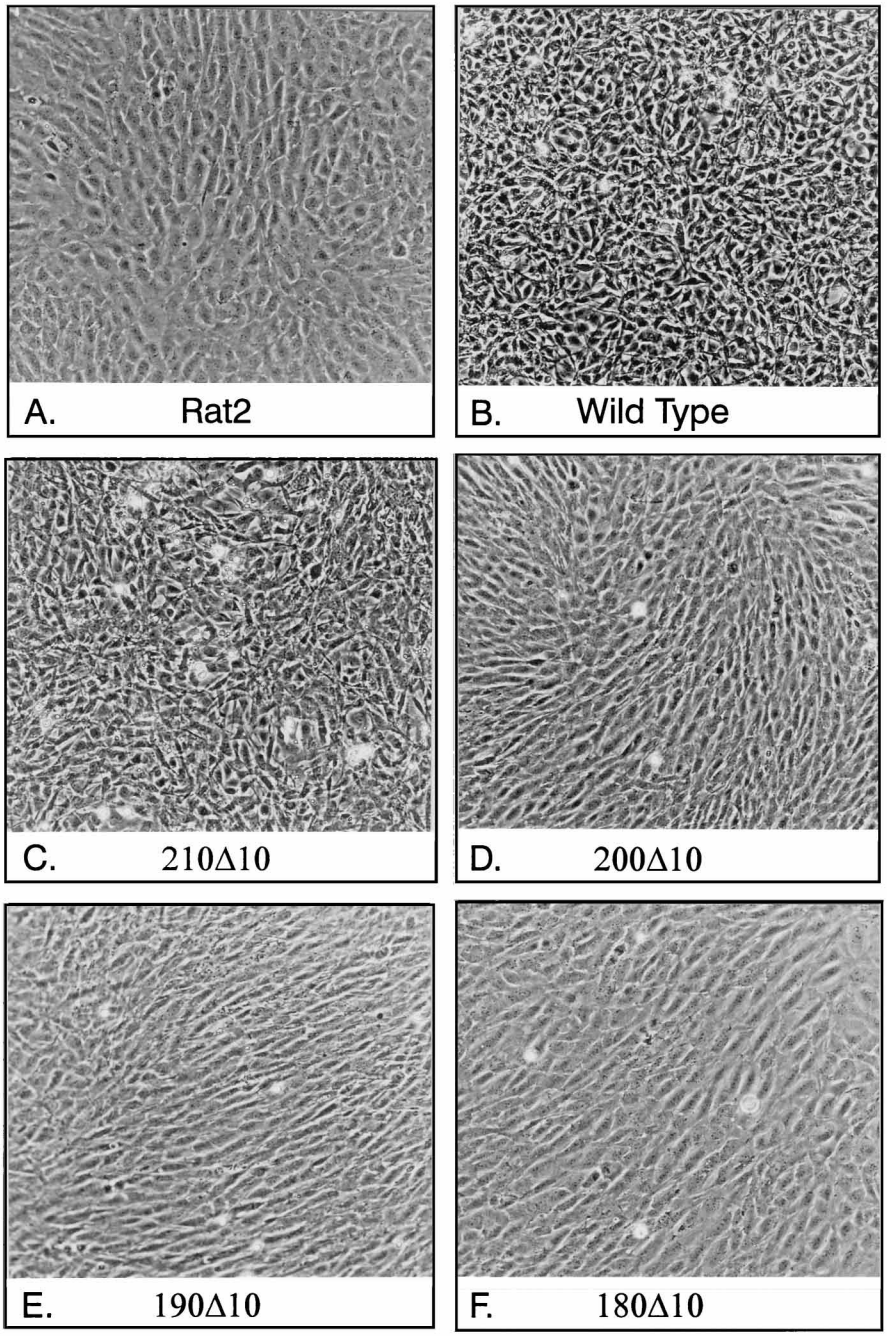

FIG. 5. Phase-contrast photomicrographs of Rat2 cell lines expressing selected mutants of MT. The parental Rat2 cell line (A), a wild-type MT-expressing cell line (B), and MT deletion mutant-expressing cell lines were grown in DMEM with 10% fetal calf serum until just confluent and then photographed under phase-contrast illumination. The mutant expressed is indicated under each panel.

effects on cell phenotype. Mutant cells expressing the partially transforming mutants (185Δ10 and 205Δ10) exhibited phenotypes between these two extremes, with the degree of alteration depending on the amount of MT expressed (data not shown).

Mutant MT subcellular location. It has been suggested that MT mutants that are defective in both PP2A and pp60$^{c\text{-}src}$ binding have an altered subcellular distribution (27, 31). To determine whether pp60$^{c\text{-}src}$ association alters the location of MT, mutant-expressing cells were subjected to immunofluorescence analysis using PAb 762 to detect MT (Fig. 6). Figure 6B shows the wild-type distribution found in transformed Rat2 cells, in which MT was located in grainy structures throughout the cytoplasm but was concentrated around the nucleus and in discrete patches at the cell periphery. An identical MT distribution was observed in mutant 210Δ10-transformed cells (Fig. 6C) and in cells expressing MT mutants that fail to associate with pp60$^{c\text{-}src}$ (Fig. 6D and E) (2a). The deletion mutant that lacks pp60$^{c\text{-}src}$ and PP2A interaction, however, showed a dramatic alteration in location (Fig.

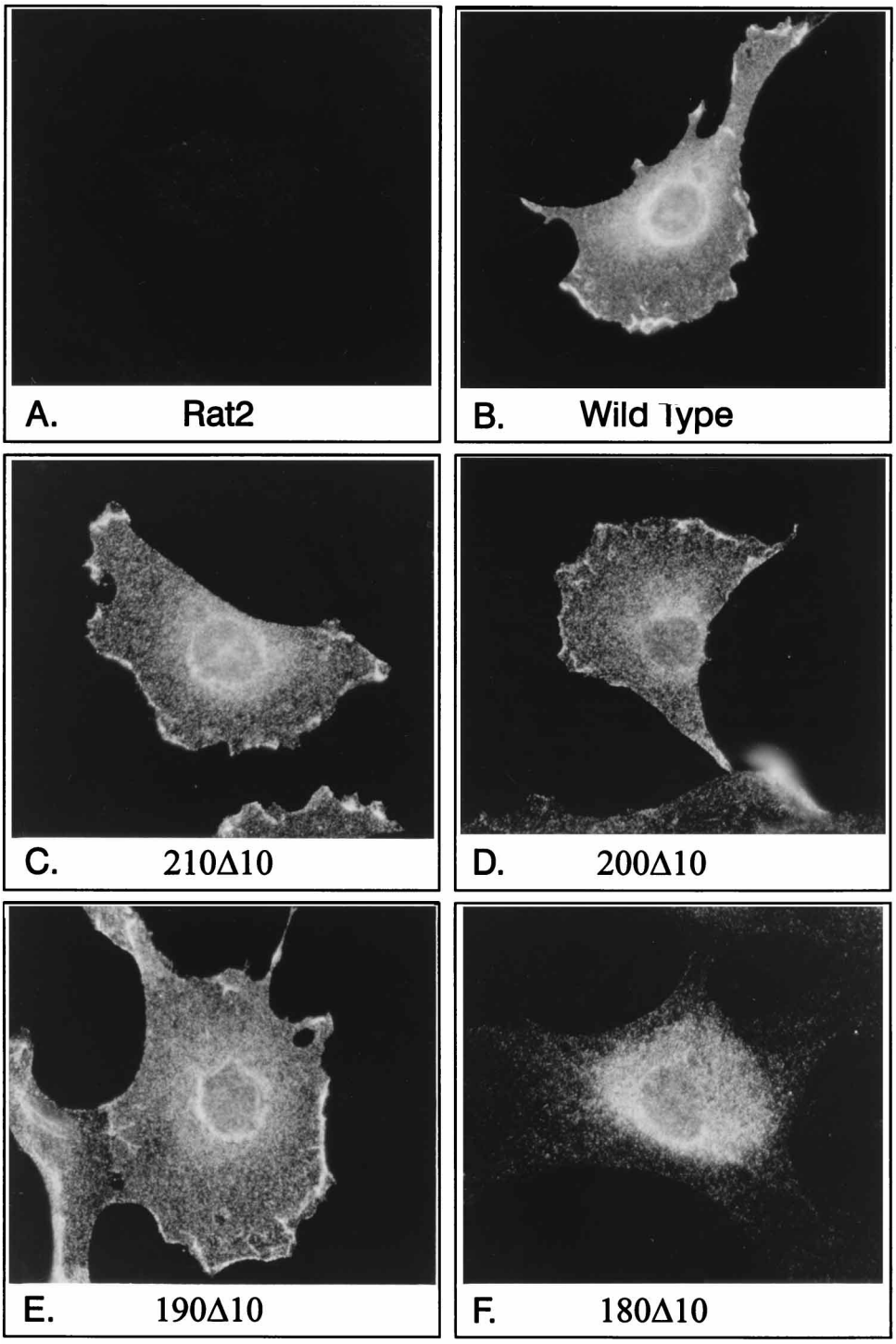

FIG. 6. Immunofluorescence analysis of MT present in Rat2 cell lines. Cell lines expressing various mutants of MT were seeded onto glass coverslips and stained with fluorescently labelled anti-MT antibodies. Representative cells are shown in each panel, together with a control Rat2 cell and a wild-type-expressing cell treated in a similar manner. The mutant expressed in each cell is indicated beneath each panel.

6F). 180Δ10 MT was still found in grainy cytoplasmic structures, but the accumulation in a perinuclear location and at the plasma membrane appeared to be absent. This suggests strongly that the subcellular distribution of MT observed by immunofluorescence is influenced by binding to PP2A rather than by association with pp60$^{c\text{-}src}$. It is not clear at present whether this is a consequence of the phosphatase activity or whether PP2A itself relocates the MT complex to a precise subcellular location. Therefore, the subcellular distribution of MT seen in immunofluorescence studies is grossly unaffected by association with pp60$^{c\text{-}src}$ but is influenced by binding to PP2A.

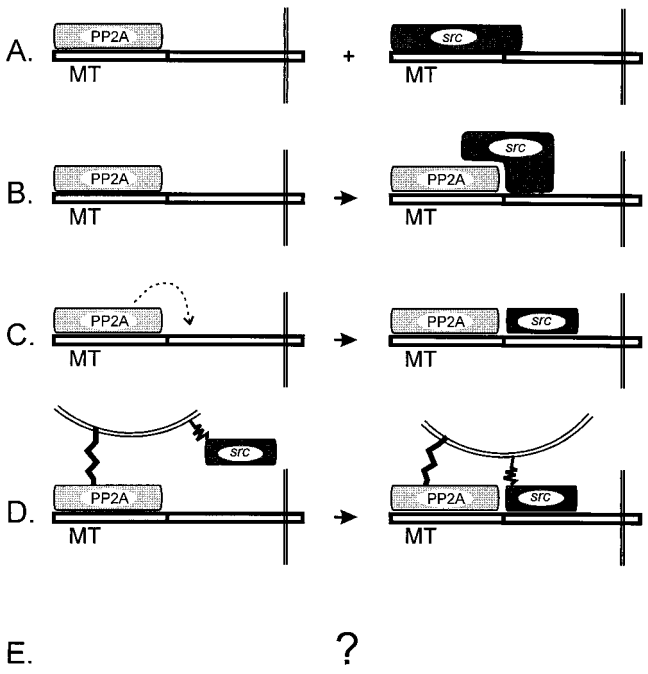

FIG. 7. Potential models for the relationship between pp60$^{c\text{-}src}$ and PP2A binding to MT. MT is represented in a linear fashion, and the sequences potentially recognized by PP2A or pp60$^{c\text{-}src}$ are represented schematically above. Fine double lines at the C-terminal end of MT represent membrane binding, and the rounded lines in model D signify a specific membrane bilayer to which PP2A and pp60$^{c\text{-}src}$ are attached. The broken arrow in model C represents the enzymatic action of PP2A.

DISCUSSION

The association between MT and pp60$^{c\text{-}src}$ has been known for some time to be crucial to the transforming properties of MT. Despite this, it has proven impossible to establish the molecular basis for the interaction, due in part to difficulties in recreating the association in vitro. Nearly all knowledge concerning the interaction, therefore, has been derived from studies of mutant molecules and by using monoclonal antibodies. It appears that only the kinase domain and a few C-terminal amino acids of pp60$^{c\text{-}src}$ are required for interaction with MT (8, 17), so the SH2 and SH3 domains normally responsible for pp60$^{c\text{-}src}$ regulation (41) are probably not involved in this case. This makes the mechanism behind this interaction particularly interesting, because it currently represents a unique means of influencing *src* family kinase activity.

The interaction with pp60$^{c\text{-}src}$ requires the N-terminal region of MT, as monoclonal antibodies that bind in this area fail to precipitate the complex and all previously isolated transformation-defective MT mutants with alterations in this region disrupt both MT-PP2A and MT-pp60$^{c\text{-}src}$ interactions. Here, for the first time, we have separated these associations and have isolated mutants that bind PP2A but not pp60$^{c\text{-}src}$. No mutant has so far been isolated that does not bind PP2A yet still associates with pp60$^{c\text{-}src}$. As others have suggested (3, 18, 19), then, it seems likely that MT, pp60$^{c\text{-}src}$, and PP2A are linked in some way. The mutants reported here eliminate the possibility that pp60$^{c\text{-}src}$ binds directly only to PP2A, as this is unlikely to be affected by MT mutants outside the PP2A binding region, and suggest a contact between MT and pp60$^{c\text{-}src}$ at one location, at least. This still leaves a number of possible models for these interactions, however (Fig. 7). Model A proposes that pp60$^{c\text{-}src}$ and PP2A recognize similar regions of MT. The observation that PP2A and pp60$^{c\text{-}src}$ can be detected in the same complex (45) suggests that this model is unlikely to be correct. Model B suggests that pp60$^{c\text{-}src}$ binding is a bipartite mechanism, with pp60$^{c\text{-}src}$ contacting both MT and PP2A. Model C offers the idea that the phosphatase action of PP2A is required for pp60$^{c\text{-}src}$ binding. Model D suggests the intriguing possibility that PP2A is required to alter the subcellular location of MT to a membrane site at which pp60$^{c\text{-}src}$ binding can occur. Finally, model E acknowledges that other, as yet unknown, mechanisms may be involved. Of course, all of these models are not mutually exclusive, and a combination of any of the suggestions may actually occur. The isolation of these new mutants that identify a region which is required, although possibly not sufficient, for interaction with pp60$^{c\text{-}src}$ at last provides us with some means of distinguishing between these possibilities.

Through the use of deletion analysis, we have limited the area of MT required to bind pp60$^{c\text{-}src}$, but not PP2A, to amino acids 185 to 210. Within this sequence are a number of putative phosphorylation sites (Fig. 1). Previous studies have suggested that phosphorylation may be required to promote the MT-pp60$^{c\text{-}src}$ interaction (30), so these sites are of particular interest. Analyzing this region with the Prosite database suggested that Ser195 is a strong candidate for a cyclic AMP-dependent protein kinase phosphorylation site but produced no strong matches for any of the other residues. Point mutagenesis is now being performed to determine whether any of these residues are required for binding to pp60$^{c\text{-}src}$.

The new MT mutants that lack pp60$^{c\text{-}src}$ binding ability, but still associate with PP2A, had no transforming activity (Fig. 2), and cell lines expressing the mutated polypeptides exhibited few altered morphological properties on initial observations (Fig. 5), although we cannot yet exclude more subtle effects on cell characteristics. The MT-PP2A interaction alone, then, seems to have little effect on cell phenotype. However, a different picture emerged when the subcellular locations of the mutant MT molecules were studied by immunofluorescence (Fig. 6). MT in Rat2-transformed cells exhibited a reticular staining pattern that stretches throughout the cytoplasm, with a strong accumulation in a juxtanuclear location and at discrete patches at the cell periphery. Much of this is similar to the distribution observed in lytically infected cells (15), with the exception of the plasma membrane location. This difference is probably a consequence of the use in the current study of transformed cells which contain a much higher percentage of complexed, rather than free, MT than do lytically infected cells (2a) and of the availability of more sensitive techniques and reagents. This distribution, observed by immunofluorescence, is unaffected by pp60$^{c\text{-}src}$ association but is altered dramatically by the loss of PP2A binding (Fig. 6F). In the absence of a PP2A interaction, MT no longer accumulates around the nucleus and, interestingly, is no longer associated with the cell periphery. These plasma membrane patches resemble areas of membrane ruffling observed in motile fibroblasts, so it will be interesting to examine whether these regions colocalize with polymerized actin molecules. Related observations confirming these results were reported previously (31), whereby MT mutants lacking both pp60$^{c\text{-}src}$ and PP2A binding were shown to exhibit a more diffuse MT location. Mutants that still associate with PP2A were not available to those authors, however, so they were unable to assess the contribution of each interaction. All of the MT mutants described here retain a functional membrane binding sequence at the C-terminal end of the protein, and the immunofluorescent staining patterns are still consistent with a membrane-bound state. The results shown sug-

gest that PP2A influences the final location of the MT complex rather than MT controlling the distribution of its associated proteins, as is usually envisaged. However, we cannot yet formally exclude a more indirect correlation between PP2A association and MT distribution. For example, the MT-PP2A association may stimulate membrane ruffling in cells, thus increasing the amount of MT detected in such structures. Alternately, in the absence of PP2A binding, another molecule may associate with MT, such as HSP70 (33, 46), and this may be responsible for the more diffuse location of MT. In this case, the diffuse 180Δ10 MT staining represents an altered location and the usual distribution of MT is an accumulation in juxtanuclear and cell periphery regions. Studies are now under way to investigate these and other possibilities. If, as we feel is more likely, PP2A binding does cause an MT location change, it could exert this effect through its phosphatase activity exposing sites in MT or through PP2A itself being targeted to this subcellular site. In this regard, it is interesting to note that although PP2A is generally considered to be a soluble, cytoplasmic protein, a number of recent reports have identified alternative locations, including membrane sites (1, 21, 35, 44, 45). Clearly, redistribution of MT may influence greatly how signals produced by MT complexes during cell transformation are generated and act and could also alter the PP2A activity at this specific location. Further experiments are under way to study these exciting possibilities. Finally, it has been suggested from biochemical studies that pp60$^{c\text{-}src}$ may control other aspects of MT's subcellular distribution (27). Although we have observed no evidence for this in our immunofluorescence analysis, this may merely reflect the fact that not all subcellular changes can be observed using one technique and some cytoskeleton association-mediated relocations are beyond the resolving power of immunofluorescence.

The interactions between MT and other signalling molecules are analogous to those occurring on activated tyrosine kinase-associated growth factor receptors. However, there appears to be no analogy between the interaction of MT and pp60$^{c\text{-}src}$ with any other pp60$^{c\text{-}src}$-binding molecules. A search of the protein database with the 185 to 210 region of MT produced no clear homologies, but it seems unlikely that MT is employing a totally unique mechanism of regulating pp60$^{c\text{-}src}$. Instead, MT might mimic the action of an as-yet-undiscovered regulator of pp60$^{c\text{-}src}$ activity. Such a regulator is now actively being sought.

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