Novel Monoclonal Antibodies That Differentiate between the Binding of pp60^{c-src} or Protein Phosphatase 2A by Polyomavirus Middle T Antigen

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Fourteen pGEX plasmids that express defined regions of polyomavirus middle T antigen in bacteria have been constructed. These polypeptides have been used to generate 18 new monoclonal antibodies directed against the unique portion of middle T and to map the approximate position of the antibody recognition sites onto the protein sequence. All of the antibodies effectively immunoprecipitate middle T and the associated 60- and 35-kDa components of protein phosphatase 2A. Four of the antibodies, however, do not react with middle T when it is bound to pp60^{c-src}. These four probably bind to amino acids 203 to 218 of the middle T protein sequence, which are encoded by the mRNA immediately 3' to the splice junction that creates the C-terminal unique region. This suggests that additional middle T sequences are required for middle T's interaction with pp60^{c-src} than are needed for its binding to protein phosphatase 2A. The antibodies localize this extra region and provide a means of distinguishing between these two associations.

The early region of polyomavirus encodes three known proteins that are required both for the lytic growth of the virus and for its ability to transform fibroblasts in culture (reviewed in references 6, 13, 18, 42, and 56). The largest gene product (or large-T antigen [LT]) has a molecular mass of approximately 100 kDa, is found predominantly within the cell nucleus (15), is intimately involved in the replication of the viral genome, and has the ability to immortalize primary rodent fibroblasts (44). Middle-T antigen (MT) has a molecular mass of approximately 55 kDa, is found associated with cellular membranes (15), and is able to induce the expression of a fully transformed phenotype when expressed in established rodent fibroblasts (43, 58). The smallest gene product (small-T antigen [ST]) has a molecular mass of approximately 22 kDa, is found in both the cell cytoplasm and nucleus (36, 64), and has potentiating activity for both lytic growth and transformation (34, 37, 43). The sequence of the N-terminal 79 amino acids is the same for all three T antigens. Differential mRNA splicing creates a 112-amino-acid region of shared sequence between MT and ST and a unique sequence at the C-terminal end of each T-antigen species. This unique sequence comprises 706 amino acids for LT, 230 for MT, and 4 for ST. The subject of this study is MT.

MT seems to exert its transforming actions through its ability to bind to, and possibly alter the activities of, cellular proteins involved in cell growth regulation. It binds to the gene products of a number of tyrosine kinase proto-oncogenes, most notably pp60^{c-src} (9, 10), pp62^{c-yes} (27), and pp59^{c-fyn} (4, 22, 30). This binding stimulates the tyrosine kinase activity of pp60^{c-src} (2, 5), which may then phosphorylate tyrosine 315 and possibly tyrosine 250 of MT (21, 23, 48). This phosphorylation is thought to provide a binding site for an 80-kDa polypeptide (54) that has been identified as a component of a phosphatidylinositol 3'-OH kinase (PI3K) activity (7, 24, 38). In addition, MT binds polypeptides of 60

and 35 kDa (46), which have been identified as subunits of protein phosphatase 2A (PP2A) (41, 60). Other polypeptides of unknown function have also been reported to associate with MT (19, 39). Mutations in the MT sequence that disrupt any of these interactions abolish the transforming activity of the molecule. However, mutant MT molecules that associate with these polypeptides but fail to transform have been isolated (for example, see reference 16). Therefore, the interactions are probably necessary, but not on their own sufficient, for the transforming action of MT. The N-terminal region of MT is required for its interaction with both pp60^{c-src} and PP2A. Consequently, monoclonal antibodies (MAbs) directed against the first 79 amino acids common to all three T antigens fail to immunoprecipitate the MT-pp60^{c-src} (with or without PI3K) (12, 40, 53) and the MT-PP2A complexes (28). In addition, a number of mutants with alterations within this region that disrupt both MT-pp60^{c-src} (reviewed in reference 32) and MT-PP2A (59) interactions have been isolated. To date, since no mutant that distinguishes between the two associations has been found, it is not possible to determine the relative contributions made to the transformation process by each complex. ST can bind PP2A (39, 41, 59) but not pp60^{c-src} and cannot initiate transformation (34, 36). Therefore, this interaction with PP2A is not alone sufficient to establish the transformed phenotype.

A considerable amount of information has been derived in the past from the use of specific MAb probes to MT (1, 12, 14, 15, 40, 53). These enable the structure of MT and its associated proteins to be examined without perturbing the native conformation of the molecule. Alternative approaches to investigating the structure of MT, such as mutating the primary sequence, may alter the higher-order conformation of the molecule at other areas in addition to that changed by the mutation. This complication is unlikely to occur when MAb probes are used, but unlike alterations caused by mutations, if antibody-induced conformational changes are encountered they are readily apparent and easily studied. However, there are numerous regions of the MT molecule to

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which MAb probes do not exist, and the sequences recognized by the available antibodies have not been defined in detail. In an attempt to remedy this situation, we have constructed a set of bacterial plasmids that express defined regions of the MT sequence. These have been used both to produce new MAbs and to map the antigenic site recognized by these and previously isolated antibodies onto the MT linear amino acid sequence. With the aid of this antigenic map, the areas of MT masked by the binding of pp60^{c-src} and PP2A have been investigated. In addition to obscuring the N-terminal region of MT, it was found that pp60^{c-src} binding masked an area between amino acids 203 and 218 of MT, sequences not previously known to be involved in this association. Binding of PP2A to MT does not conceal this area, providing for the first time a clear distinction between these two interactions.

MATERIALS AND METHODS

Bacterial expression. Fragments of the polyomavirus MT sequence were expressed in bacteria by using the pGEX system (49). To facilitate subcloning from other vectors, we constructed three new pGEX plasmids by replacing the *Bam*HI-*Eco*RI cloning region of pGEX1, pGEX2T, and pGEX3X with the oligonucleotide

5'-GATCGAATTTCGTCGACGGATCC
3'-CTTAAGCAGCTGCCTAGGTTAA.

This creates a reversed *Bam*HI-*Eco*RI orientation relative to the glutathione *S*-transferase gene and adds a *Hinc*II restriction enzyme site. Fragments encoding MT were excised from pEMT (52) with restriction enzymes, purified by gel electrophoresis and electroelution, and blunt-end ligated into *Sma*I-cut pBT or pBS (Stratagene), and recombinants were selected. After the orientation was established by restriction enzyme analysis, an *Eco*RI-*Bam*HI DNA fragment was subcloned into the appropriate pGEX vector. The cloned piece was confirmed by DNA sequencing with a Sequenase 2 kit (Stratagene). The exact MT amino acids expressed were deduced from this sequence. The restriction enzymes used to create MT fragments were *Bst*XI, *Dde*I, *Hae*III, *Hpa*II, *Pst*I, *Pvu*II, *Rsa*I, and *Sac*I. All cloning procedures followed conventional protocols (47).

Protein expression was conducted as described previously (49). Proteins were purified by a modification of the procedures described previously (33). This is described briefly as follows. The bacteria were suspended in NET buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris Cl [pH 8.0]) plus 50 µg of lysozyme per ml and incubated for 10 min at room temperature. They were pelleted by centrifugation at 10,000 × *g* for 20 min and resuspended in 10 volumes of NET containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 2.5 mM dithiothreitol, and 0.3 TIU of aprotinin per ml. After being freeze-thawed twice, the bacteria were sonicated for 30 s at a power setting of 14 µm and then centrifuged for 10 min at 10,000 × *g*. For soluble proteins the supernatant was extracted with glutathione agarose as described previously (49). For insoluble proteins, the pellet was resuspended in 8 M urea in NET buffer, sonicated to ensure resuspension, diluted 10-fold with 50 mM NaCl-5 mM KH₂PO₄ (pH 10.7), and then incubated for 30 min at room temperature. The pH was adjusted to 7.8 with 0.5 M KH₂PO₄, and the solution was centrifuged. The supernatant was then extracted with glutathione agarose.

Cells and viruses. (i) **Growth.** The mouse fibroblast line 3T6 (American Type Culture Collection) was grown in

Dulbecco modified Eagle medium (high glucose) supplemented with 10% fetal calf serum, penicillin, and streptomycin. The mouse plasmacytoma line Sp2/0-Ag14 and all hybridomas were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Polyomavirus A2 was grown on primary whole mouse embryo cells and stored frozen at -20°C at a titer of approximately 10⁹ PFU per ml.

(ii) **Infection, metabolic labeling, and cell lysis.** We infected 75% confluent cultures of mouse 3T6 cells with 50 PFU of polyomavirus A2 per cell for 90 min at 37°C (or mock infected them without virus) and then fed them with Dulbecco modified Eagle medium-5% fetal calf serum. Unlabeled cells were harvested at 30 h postinfection. Metabolic labeling was performed by removing the medium at 24 h postinfection and incubating the cells for 4 h at 37°C in 6 ml of Dulbecco modified Eagle medium without methionine per 90-mm dish plus 1 mCi of [³⁵S]methionine (>1,000 Ci/mmol; Amersham International). The medium was then removed, and the cells were lysed. To do this, we washed the monolayer with ice-cold phosphate-buffered saline (PBS; 170 mM NaCl, 3.4 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄), lysed the cells by adding 1 ml of ice-cold extraction buffer (100 mM NaCl, 100 mM Tris-Cl [pH 8.5], 0.5% Nonidet P-40, 0.3 TIU of aprotinin per ml [Sigma]) per 90-mm dish, and incubated the mixture with constant rocking for 30 min at 0°C. The lysate was removed and centrifuged for 5 min at 15,000 × *g*, and the supernatant was stored at -70°C.

Electrophoresis and Western immunoblotting. Gel electrophoresis was conducted as described previously (31), using 10 or 12.5% separating gels. Electrophoretic transfer to nitrocellulose (MSI) (57) was performed by using a Hoefer miniblot cell for 1 h at a constant current of 300 mA. Unreactive sites were blocked by incubation with 5% powdered skim milk, and the immunoreactive proteins were detected by incubation of the filter successively with MAB containing tissue culture fluid, horseradish peroxidase-labeled anti-mouse immunoglobulin G (IgG; Amersham International), and then ECL Reagent (Amersham International) as specified by the manufacturer.

Immunoprecipitation and protein kinase assays. Lysates were cleared prior to use by incubation with protein A-Sepharose (Fermentech Ltd.) for 20 min at 0°C followed by isolation of the supernatant. Polypeptides were immunoprecipitated by reacting the lysate from 5 × 10⁵ cells on ice for 1 h with 40 µl of MAB containing tissue culture fluid. Either 2 µg of affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) or 1 µg of anti-rat IgG MAB OX12 (Sera Labs), as appropriate, was added, and incubation was continued for 30 min. Then 15 µl of a 50% suspension of protein A-Sepharose was added, and incubation was continued for a further 30 min with constant mixing. The protein A-Sepharose was isolated by centrifugation at 15,000 × *g* for 10 s, and the supernatant was removed. The Sepharose was washed four times by resuspension in 1 ml of NET-N buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl [pH 8.5], 0.05% Nonidet P-40, 0.2 TIU of aprotinin per ml) and reisolation by centrifugation. Following the final wash, bound polypeptides were eluted from the protein A-Sepharose by the addition of 35 µl of Laemmli sample buffer and incubation for 15 min at room temperature. The supernatant was then isolated, heated at 80°C for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In vitro protein kinase assays were performed by repeat-

FIG. 1. SDS-PAGE analysis of the MT sequence/GEX fusion proteins. pGEX plasmids encoding defined MT sequences were introduced into bacteria, and expression was induced as described previously (46). The fusion proteins were isolated as in Materials and Methods, separated by SDS-PAGE (12.5% acrylamide), and stained with Coomassie brilliant blue. The MT amino acids expressed are indicated above each lane, and the measured apparent molecular mass of the arrowed band is shown below each lane. The migration position of markers is indicated on the right in kilodaltons.

ing the above with unlabeled lysate and omitting the EDTA from the wash buffers. After washing, the protein A-Sepharose pellet was resuspended in 25 μ l of 25 mM Tris-Cl (pH 7.5)–5 mM $MnCl_2$ containing 5 μ Ci of [γ - ^{32}P]ATP (>5,000 Ci/mmol; Amersham International) and incubated at 30°C for 15 min. The protein A-Sepharose was washed once with NET-N buffer, and the bound polypeptides were eluted with Laemmli sample buffer as above.

Immunoprecipitation with two MABs was achieved by adding the test antibody to the lysate, incubating the mixture for 60 min, adding the second positive control antibody, continuing the incubation for a further 60 min, and then adding protein A-Sepharose. Cascade immunoprecipitations were performed by immunoprecipitating as above, isolating instead of discarding the cleared lysate, and repeating the procedure three times with the initial MAB. Any remaining antibody was removed by a further treatment with protein A-Sepharose, and the resultant lysate was immunoprecipitated by addition of the second MAB.

After electrophoresis the gels were stained with Coomassie blue R250, dried, and exposed to Hyperfilm β -Max (Amersham International).

MAB production. F_1 crossed mice (either CFA $\times$ BALB/c or B6BR $\times$ BALB/c), 8 weeks old, were immunized four times at 2-week intervals by intraperitoneal injection of 50 μ g of bacterial fusion protein mixed with Freund's adjuvant. After resting for 2 months, the mice received 50 μ g of fusion protein intraperitoneally 6 and 3 days prior to sacrifice. Hybridoma lines were established by polyethylene glycol fusion and selection in hypoxanthine-azaserine by usual procedures (20). Screening for antibody production was performed by a modification of a dot-blot procedure. Briefly, nitrocellulose sheets were incubated overnight at 4°C with a PBS solution containing 5 μ g of fusion protein per ml. Unreacted sites were blocked by incubation with 5% powdered skim milk and subsequent washing with PBS. Approximately 1 μ l of test tissue culture fluid was spotted on a grid and allowed to react for 30 min at room temperature. The filters were washed and reacted with horseradish peroxidase-labeled anti-mouse IgG; and antibodies were detected with ECL reagent.

Antibody subtypes produced were determined with a MAB typing kit (Amersham International).

RESULTS

Bacterial expression and MAB production. Fourteen polyomavirus DNA fragments encoding defined pieces of the MT sequence were isolated by restriction enzyme digestion and cloned into an appropriate pGEX vector, chosen so that the MT sequence was fused to the C terminus of glutathione S-transferase (49). The cloning procedure was checked, and the ends were confirmed, by direct DNA sequencing of the pGEX plasmid. Following isopropyl- β -D-thiogalactopyranoside (IPTG) induction, the fusion proteins were isolated on glutathione agarose beads as described in Materials and Methods. Figure 1 shows an SDS-PAGE analysis of the polypeptides produced in this manner, together with the apparent molecular masses of the fusion proteins and the amino acid numbers of the MT sequences encoded. Protein fragment yields were variable but frequently poor, with less than 100 μ g of soluble protein often being isolated from 1 liter of bacterial culture. This appears to be a reflection of the MT sequences synthesized, since similar problems were not encountered with the same clones expressing pieces of LT from the alternate reading frame (data not shown). The poor yields were not a consequence of low expression but were due to the location of the fusion protein within inclusion bodies. To improve the yield of soluble protein, we developed procedures to isolate the inclusion bodies following lysis of the bacteria and to solubilize the polypeptides by treatment with 8 M urea. After centrifugation, reduction of the urea concentration 10-fold by dilution with buffer produced a fusion protein able to bind to glutathione agarose for purification. Elution at high pH resulted in fusion protein preparations with yields between 3 and 10 times those obtained initially.

Two fusion proteins, covering amino acids 203 to 380 and 327 to 419 of the MT protein sequence, were used to immunize mice. Hybridoma production was then performed by conventional procedures and resulted in the isolation of 18 new MABs directed against these sequences. A PAB

TABLE 1. Binding of PAb antibodies to MT fragments

PAb no.	Binding to MT fragment (amino acid nos.):													
	1-104	1-201	5-70	90-128	92-144	106-201	106-304	199-240	203-304	203-380	219-314	282-345	305-380	327-419
701	+	+	+	-	-	-	-	-	-	-	-	-	-	-
721	-	-	-	-	-	-	-	-	-	+	-	+	+	-
722	-	-	-	-	-	-	+	-	+	+	+	-	-	-
725	-	-	-	-	-	-	-	-	-	+	-	+	+	-
741	-	-	-	-	-	-	-	-	-	+	-	+	+	+
742	-	-	-	-	-	-	-	-	-	+	-	+	+	+
743	-	-	-	-	-	-	-	-	-	+	-	+	+	+
744	-	-	-	-	-	-	-	-	-	+	-	-	+	+
745	-	-	-	-	-	-	-	-	-	+	-	+	+	-
746	-	-	-	-	-	-	-	-	-	+	-	+	+	+
747	-	-	-	-	-	-	-	-	-	+	-	+	+	+
748	-	-	-	-	-	-	-	-	-	+	-	+	+	+
749	-	-	-	-	-	-	-	-	-	+	-	+	+	+
750	-	-	-	-	-	-	-	-	-	+	-	-	+	+
751	-	-	-	-	-	-	-	-	-	+	-	+	+	+
752	-	-	-	-	-	-	-	-	-	+	-	+	+	+
753	-	-	-	-	-	-	+	+	+	+	+	-	-	-
754	-	-	-	-	-	-	+	-	+	+	+	+	-	-
755	-	-	-	-	-	-	+	+	+	+	-	-	-	-
756	-	-	-	-	-	-	+	+	+	+	-	-	-	-
757	-	-	-	-	-	-	-	-	-	+	-	-	+	+
758	-	-	-	-	-	-	+	+	+	+	-	-	-	-
759	-	-	-	-	-	-	+	+	+	+	-	-	-	-
760 ^a	+	+	-	-	-	-	-	-	-	-	-	-	-	-

^a The recognition site appears to be denaturation sensitive; it was therefore screened against native protein.

number (11) was assigned to each of these new antibodies. In addition, two further MAbs were isolated by using either MT isolated from a baculovirus expression system (17) (PAb 745) or a fusion protein containing MT sequences from amino acids 1 to 201 (PAb 760). The sites recognized by these antibodies were located by performing Western blot analysis of each MAb against electrophoretically separated bacterial lysate containing an expressed fusion protein. It was necessary to use Western blots for this screening to ensure that the reaction was to the fusion protein and not to a bacterially encoded contaminant or the polypeptide expressed from the alternate open reading frame of polyomavirus DNA fragments. Table 1 contains data obtained from these studies, together with a similar examination of previously isolated MAbs (PAb 701, PAb 721, PAb 722, and PAb 725 [14]) and demonstrates that the antibodies recognize a number of different antigenic sites. In addition, the sites recognized by these new antibodies are different from most of those recognized by previously isolated MAbs. Figure 2 indicates schematically the location of the binding site deduced from these experiments for each antibody on the linear MT amino acid sequence. The overall box represents the sequences that have been shown by a positive reaction to contain the antigenic site, and the hatched area represents sequences that do not react with the MAb. This suggests that the probable region of binding is within the solid box, but, since this is based on an absence of binding, it is a more tentative assignment. Table 2 lists the MT amino acid numbers of the PAb antibody recognition sites derived from Table 1 and used to construct Fig. 2. The defined site sequences correspond to the overall box in Fig. 2, and the probable site corresponds to the solid box. These data show that the antigenic sites recognized by these MAbs are spaced along the unique sequence of MT, for the first time providing probes throughout this area. The Ig subclass secreted by each of these new MAbs was also determined. All secrete a

κ light chain, and most secrete an IgG1 heavy chain. The exceptions are PAb 746, PAb 748, and PAb 749, which synthesize IgG2a, and PAb 757 and PAb 759, which make IgG2b antibodies.

Polypeptides immunoprecipitated by new MAbs. The polypeptides recognized by these new antibodies were then characterized. [³⁵S]methionine-labeled lysates were prepared from polyomavirus-infected or mock-infected mouse 3T6 fibroblasts and divided into aliquots, and each aliquot was immunoprecipitated with a single antibody. Polypeptides were separated by SDS-PAGE and detected by autoradiography. The results of such an experiment are shown in Fig. 3. All of the new MAbs directed against the unique region of MT (PAb 741 to PAb 759) recognize and immunoprecipitate MT at least as effectively as do the previously isolated antibodies (PAb 722 and PAb 725). In addition, all of these MAbs precipitate two polypeptides of apparent molec-

FIG. 2. Schematic representation of the location of the amino acid sequences recognized by MAbs. The linear amino acid sequence of MT is represented at the top, with the amino acid numbers of the N and C termini and the mRNA splice junction marked. The boxes shown represent the MT regions demonstrated to contain a MAb-binding site by reaction with fusion proteins, together with the PAb number of the antibody. The hatched areas represent regions which have shown no antibody binding. Therefore, the antigenic site probably lies within the solid box; however, since this is based on the lack of a reaction, the assignment is more circumstantial.

TABLE 2. MT amino acid sequences recognized by PAb antibodies

PAb no.	Defined site	Probable site
701	5-70	5-70
721	305-345	314-327
722	219-304	241-281
725	305-345	314-327
741	327-345	327-345
742	327-345	327-345
743	327-345	327-345
744	327-380	346-380
745	305-345	314-327
746	327-345	327-345
747	327-345	327-345
748	327-345	327-345
749	327-345	327-345
750	327-380	346-380
751	327-345	327-345
752	327-345	327-345
753	219-240	219-240
754	282-304	282-304
755	203-240	203-218
756	203-240	203-218
757	327-380	346-380
758	203-240	203-218
759	203-240	203-218
760 ^a	1-104	1-5

^a Assignments are doubtful as a consequence of the denaturation-sensitive nature of the site.

ular masses 60 and 35 kDa. Western blot analysis with a specific antipeptide serum (data not shown) identified the 35-kDa polypeptide as the catalytic subunit of PP2A previously found associated with MT (41, 59, 60). It is probable, therefore, that the 60-kDa polypeptide is the regulatory subunit of PP2A that has been shown to bind directly to MT (45). A MAb to the N-terminal region of MT (PAb 701) did not immunoprecipitate these two polypeptides despite reacting with MT (Fig. 3A, lane 22). Because these PP2A components also bind to ST (39, 41, 59), the MT region involved in interacting with these species is within the N-terminal sequences common to M and ST, and so it is likely that their binding masks the antigenic site recognized by this MAb. No polypeptides with the same apparent molecular mass are precipitated from uninfected cell lysates (Fig. 3B; data not shown).

In addition to the polypeptides described above, a number of other species are seen in these immunoprecipitates. Some are found in most lanes and so are candidates for MT-associated proteins (or breakdown products of MT [18]), whereas others are precipitated by only a few MAbs and are present when uninfected (Fig. 3B; data not shown) and infected (Fig. 3A) cell lysates are used. Most prominent are a high-molecular-mass protein immunoprecipitated by all the MAbs directed to the region from 327 to 345 (PAb 741 to PAb 743, PAb 746 to PAb 749, PAb 751, and PAb 752); 150-, 53-, and 52-kDa polypeptides recognized by PAb 753; and a 69-kDa species precipitated with PAb 746 and PAb 753. The last two MAbs do not recognize the same sequences on MT, so this 69-kDa polypeptide may share more extensive sequence similarity with MT. Lack of recognition of the high-molecular-mass polypeptide species by any PAb to

FIG. 3. SDS-PAGE analysis of the [³⁵S]methionine-labeled polypeptides immunoprecipitated by MAbs from cell lysates. Polyomavirus-infected (A) or mock-infected (B) cells were labeled with [³⁵S]methionine and lysed, and equal volumes of lysate were immunoprecipitated with MAbs, as in Materials and Methods. Only three representative lanes are shown in panel B. The lane numbers are indicated below each lane, and the PAb number of the antibody used to effect precipitation is shown above each lane. The migration position of markers, together with their molecular masses in kilodaltons, are indicated between the panels. The migration position of the T antigens and the associated 60- and 35-kDa components of PP2A are indicated on the left in panel A, and those of a number of host-encoded polypeptides recognized by these antibodies are indicated on the right in panel B. These include a high-molecular-mass protein recognized by PAb 741 and PAb 746 (and PAb 742, PAb 743, PAb 747, PAb 748, PAb 749, PAb 751, and PAb 752 [data not shown]); 150-, 53-, and 52-kDa polypeptides recognized by PAb 753; and a 69-kDa species recognized by PAb 746 and PAb 753.

FIG. 4. SDS-PAGE analysis of the polypeptides labeled with ^{32}P following incubation of immunoprecipitates with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Equal volumes of lysates from polyomavirus-infected (A) or mock-infected (B) 3T6 cells were immunoprecipitated with different MAb and then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of Mn^{2+} . The lane numbering is as in Fig. 3. The migration positions of LT, MT, and the 80-kDa component of PI3K (80) are indicated on the left in panel A. The migration position of the high-molecular-mass polypeptide phosphorylated in precipitates with PAb 741 and PAb 746 (and PAb 742, PAb 743, PAb 747, PAb 748, PAb 749, PAb 751, and PAb 752 [data not shown]) and the three polypeptides phosphorylated in PAb 753 precipitates are indicated on the right in panel B.

other regions of MT may indicate that only limited sequence homology between the two polypeptides exists and that the cross-reaction could be fortuitous (see Discussion).

Protein kinase activities immunoprecipitated by MAbs. Polypeptides immunoprecipitated by the above antibodies were subjected to an *in vitro* protein kinase assay, and the results are shown in Fig. 4. MAbs that react with the C-terminal portion of MT (PAb 741 to PAb 754 and PAb 757) precipitate a protein kinase activity that phosphorylates both 55- and 57-kDa forms of MT (48), similar to that previously described for PAb 725 (12). PAb 722 precipitates show phosphorylation of only the smaller form (12). Polypeptides precipitated by MAbs to the central region of MT (PAb 755, PAb 756, PAb 758 and PAb 759), however, showed little or no protein kinase activity. The lack of reaction in precipitates with these new MAbs was unexpected, because few antibodies to the MT unique sequences have shown this property previously. All of these novel MAbs immunoprecipitate MT effectively, coisolate the subunits of PP2A bound to MT (see above), and react with amino acids 203 to 240 (probably 203 to 218) of the molecule. As described previously (12), precipitates formed with MAbs to the N-terminal region of MT also show little or no protein kinase activity. All immunoprecipitates possessing MT-phosphorylating activity also showed phosphorylation of an 80-kDa polypeptide, which is probably the component of PI3K previously identified (7, 24). In addition, some of the cross-reacting species identified above incorporate small amounts of phosphate. However, these are independent of the presence of MT (Fig. 4B) and so represent the action of other protein kinases.

The lack of protein kinase activity in an immunoprecipitate containing MT could be caused by the properties of the antibody in one of two ways. Either the MAb used inhibits

the kinase activity by some means or, alternatively, the antibody fails to precipitate the kinase. To determine which of these possibilities is operative here, we performed two experiments. First, immunoprecipitates were formed with two MAbs, one showing a negative reaction and the other showing a positive reaction, and assayed for kinase activity. If the enzyme in the precipitates without activity were inhibited by the first antibody, it would also inhibit the kinase reaction precipitated by the second. Figure 5, containing the results of such an experiment, shows clearly that addition of an antibody that does not precipitate a kinase activity (PAb 701, PAb 755, PAb 756, PAb 758, or PAb 759) does not inhibit the activity seen in immunoprecipitates formed with one that does (PAb 750; chosen to recognize an antigenic site as far away from that of the other antibodies as possible to minimize any effects of steric hindrance on the binding). Second, cascade immunoprecipitations were performed and analyzed for protein kinase activity. In this experiment all the polypeptides bound by one MAb are removed from a lysate and those remaining are then immunoprecipitated by another antibody that shows kinase-precipitating activity (Fig. 6). Control reactions with a positive MAb (PAb 757) show that all of the kinase-active MT is removed by this antibody, leaving little to be precipitated by PAb 750 (Fig. 6, lanes 3 and 4). Immunoprecipitation with the four novel kinase-negative MAbs isolated here (PAb 755, PAb 756, PAb 758, and PAb 759) does not remove the MT-associated kinase activity (Fig. 6, lanes 5 to 12). This is similar to the immunoprecipitation previously reported for PAb 701 (Fig. 6, lanes 1 and 2) (12). From these two experiments we conclude that PAb 701, PAb 702 (data not shown), PAb 755, PAb 756, PAb 758, and PAb 759 are unable to react with the kinase-active form of MT rather than inhibiting its activity by some means. The protein kinase

FIG. 5. SDS-PAGE analysis of the polypeptides labeled with ^{32}P in vitro following immunoprecipitation with two antibodies. Equal volumes of lysate from polyomavirus-infected 3T6 cells were immunoprecipitated with either a single antibody (lanes 1 to 6) or two antibodies (lanes 7 to 12) and then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The PAb numbers of the antibodies used are indicated above each lane. The migration positions of molecular mass markers are indicated on the right, and those of LT, MT, and the 80-kDa PI3K component (80) are indicated on the left.

activity in MT immunoprecipitates has been shown previously to be mainly that associated with the $\text{pp60}^{\text{c-src}}$ bound to MT (9, 10). Therefore, it seems likely that these MABs recognize MT when it is bound to subunits of PP2A but not when it is bound to $\text{pp60}^{\text{c-src}}$.

DISCUSSION

A number of bacterial expression plasmids have been constructed and used to produce a series of new MABs directed against the polyomavirus MT. Sites recognised by these and other MABs have been mapped onto the linear amino acid sequence of MT. The 20 MABs described here, together with some previously reported MABs (14), provide novel probes to all areas of the unique region of MT. Antibodies to most of these sites have not previously been isolated, and some areas have not been studied by mutational analysis. Therefore, experiments performed with these MABs provide the first investigation of these regions of MT. Only a few MABs reacting with the sequences common to ST and MT but not LT have been described (40), so this area still lacks adequate probes. The availability of the GEX fusion proteins should enable this to be remedied soon.

All of the MABs directed against the unique C-terminal region of MT effectively immunoprecipitate MT (Fig. 3). Since these antibodies recognize sites along the molecule, this suggests that most of this region is readily accessible in vivo. In addition, the 60- and 35-kDa components of PP2A, which have previously been shown to bind to MT (41, 46, 60), are precipitated by these MABs, which indicates that PP2A binding has no masking effect on these MT unique regions. MABs to the first 69 amino acids of MT, however, are not able to precipitate PP2A, probably as a consequence of the bound polypeptides masking the antigenic site recognized by these antibodies (Fig. 3) (28). This, together with studies of mutations within this area (19, 26, 39, 59) and the observation that PP2A also effectively binds ST (39, 41, 59),

FIG. 6. SDS-PAGE analysis of polypeptides phosphorylated in vitro following a cascade immunoprecipitation. Equal volumes of a lysate of polyomavirus-infected 3T6 cells were immunoprecipitated three times with a MAB, and the depleted lysate was reacted with PAb 750. The first and last precipitates were then incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and the phosphorylated polypeptides were separated by SDS-PAGE. The first and second antibodies used are indicated above each lane; e.g., lane 1 was precipitated with PAb 701, and the remaining lysate was reacted with PAb 750 and loaded in lane 2. The migration positions of molecular mass markers are indicated on the right, and those of LT, MT, and the 80-kDa component of PI3K (80) are indicated on the left.

suggests that the 60- and 35-kDa species probably interact with the region common to MT and ST.

With regard to immunoprecipitation of a protein kinase activity with MT, as with PP2A binding, MABs to the C-terminal region of MT effectively precipitate an MT-kinase complex and those to the N-terminal end do not (Fig. 4). Immunoprecipitates formed with MABs reacting with amino acids 203 to 218, however, also do not show kinase activity, despite containing the PP2A species associated with MT. Mixing experiments and cascade immunoprecipitations have demonstrated that this is not a consequence of antibody inhibition of the kinase by direct steric hindrance of the enzyme, an induced conformational change in MT, or displacement of the kinase from the MT molecule, since all of these would also inhibit the activity shown in coprecipitates and after cascade reactions. Rather, it reflects the inability of the MAB to recognize the kinase-active fraction of MT. The kinase responsible for most of this activity has previously been shown to be the $\text{pp60}^{\text{c-src}}$ bound to MT (9, 10). Therefore, these MABs recognize MT when it is bound to PP2A but not when it associates with $\text{pp60}^{\text{c-src}}$. This identifies for the first time a property that differentiates between MT binding to PP2A and to $\text{pp60}^{\text{c-src}}$. Whether $\text{pp60}^{\text{c-src}}$ actually contacts residues within this region or merely prevents access by the antibody is not clear. It is possible that lack of reaction is due to a conformational change of MT induced by the binding of $\text{pp60}^{\text{c-src}}$. However, the observation that four separate MABs reacting within this region demonstrate the same response makes this possibility less likely. $\text{pp60}^{\text{c-src}}$ must also interact with other sequences in the unique portion of MT, particularly the region around tyrosine 315, since this is the major phosphate-acceptor site of the kinase reaction (21, 23, 48). However, that association

does not inhibit access by antibodies that react close to Tyr-315 (PAb 721, PAb 725, and PAb 745). Together, these observations suggest that masking of MT in the region from 203 to 218 by pp60^{c-src} is a consequence of a close interaction and probably involves direct binding. To date, no mutations have been reported within this region, but their properties would clearly be interesting. A requirement for these MT unique sequences in pp60^{c-src} binding may explain the absence of an interaction between ST and pp60^{c-src} (39, 59), as well as the clear association seen with mutant 82JF3 that lacks the C-terminal 86 amino acids of MT (8). The reason for the failure of mutations in the hydrophobic domain of MT, that still retain the sequences between 203 and 218, to bind pp60^{c-src} (3, 55) remains unclear, although the MT cellular location might well influence binding in some circumstances. It has been reported that MT, pp60^{c-src}, and PP2A can be found within the same complex (26, 41, 59). It has been noted, however, that considerably more PP2A than pp60^{c-src} associates with MT (59), so an MT-PP2A complex probably exists without any associated pp60^{c-src}. We have shown here that this inference is correct, and we have provided a direct means of isolating this complex. It remains unproven, however, whether an MT-pp60^{c-src} complex exists without PP2A, although there are some arguments to suggest that it does (19, 59). The data we have presented here show for the first time that the two interactions are different to some degree and identify an additional region of MT that is probably involved in binding pp60^{c-src} only.

The isolation of a new range of MAbs spanning the unique region of MT and the ability to produce others and define their specificity provide important tools for future work. Such site-specific probes are extremely useful when studying the binding of host proteins to the MT molecule and when determining the role of each interaction in inducing transformation. This not only includes *in vitro* biochemical studies as presented here but also will allow the effects of the antibodies on cell phenotype following microinjection, and hence the roles of these complexes *in vivo*, to be investigated. Such an approach has already proven successful in studies of the action of the Ras protein (29, 35, 50, 51, 63), another cytoplasmic, membrane-associated oncogene product.

The cross-reactions observed with these MAbs may also provide information about parts of MT that are similar to host protein sequences. Since the sites recognized by these MAbs have been localized to a small region of MT, it has been possible to search a protein data base for corresponding amino acid sequences. The most significant homology found was between positions 327 to 344 of MT (recognized by PAb 741, PAb 742, PAb 743, PAb 744, PAb 746, PAb 747, PAb 748, PAb 749, PAb 751, and PAb 752) and the *Notch* gene of *Drosophila melanogaster* (amino acids 2160 to 2177). Identical amino acids are marked with *, and those of similar character are marked with .

	327		344
MT	P E E Q V P Q L I P P P I I P R A G		
	* . * . * . * . * .		
<i>Notch</i>	P G Q Q Q P Q L I T Q P T V I S A G		
	2160		2177

Notch encodes a protein of approximately 300 kDa (25, 62), similar to the large polypeptide recognized by these MAbs (Fig. 3). In addition, *Notch* protein has similarities to epidermal growth factor and a cytoplasmic domain that contains a number of putative phosphorylation sites (25, 62). Interestingly, the high-molecular-mass polypeptide recog-

nized by these MAbs incorporates phosphate in an *in vitro* protein kinase assay (Fig. 4). However, the sequences in *Drosophila Notch* identified in this search are missing from the sequences of rat and *Xenopus* homologs (61). It is possible that another unidentified but related protein which retains this homologous region is present in mouse fibroblasts. Other cross-reactions are also observed in the immunoprecipitates shown here. These may be fortuitous sequence homologies if confined to a small site but may also reflect a similarity of actions if more widespread. Further studies are required to elucidate these intriguing possibilities.

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