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Transformation and Tumorigenic Properties of a Mutant Polyomavirus Containing a Middle T Antigen Defective in Shc Binding†

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Polyomavirus middle T antigen is phosphorylated on several tyrosine residues which act as binding sites for cellular proteins, including phosphatidylinositol 3-kinase, Shc, and phospholipase C-γ. In this report we describe the transforming properties and tumor-inducing ability of a polyomavirus that contains a single-site mutation in middle T antigen which changes a tyrosine residue at amino acid position 250 to serine. This mutation disrupts the association of middle T with the transforming protein Shc. The mutant virus is weakly transforming, inducing foci which are smaller and of different morphology than those of the wild type. Although the virus induced tumors in close to 100% of inoculated mice, the spectrum of tumors and their morphology were altered compared to those of wild-type virus. The mutant virus induced a reduced frequency of kidney and thymic tumors. Both the mammary gland and the thymic tumors that were induced were histologically distinct from those induced by wild-type polyomavirus. These results demonstrate that the signal transduction pathway that is deregulated by the middle T-Shc association is important for full transformation of cells in culture and for tumor induction in some target tissues in the mouse-polyomavirus system.

Polyomavirus transforms rat fibroblasts in culture and induces a broad spectrum of tumors when inoculated into newborn mice (30, 54). Biochemical and genetic studies in tissue culture systems have shown that middle T antigen is sufficient to induce full transformation in established cell lines (44, 55), while large T antigen is necessary for immortalization of primary cells (23, 37). The T antigens perform their functions by associating with and regulating a number of cellular proteins. Large T antigen associates with the retinoblastoma tumor suppressor gene product pRB (21, 23, 37). Viruses encoding mutant large T antigen that cannot bind pRB are unable to immortalize primary cells (23, 37) but transform established cells in culture and induce a variety of tumors in mice (23). Middle T antigen's ability to transform cells and induce tumors is dependent on its ability to associate with and activate members of the Src family of tyrosine kinases (3, 7, 9, 28, 36, 41). As a result of these associations, middle T antigen is phosphorylated on several tyrosine residues (50), which act as binding sites for the cellular proteins phosphatidylinositol 3-kinase (PI 3-kinase) (1, 34, 53, 58), Shc (6, 15), and phospholipase C-γ (PLC-γ) (52). PI 3-kinase binds to phosphorylated tyrosine at amino acid position 315 (34, 53), Shc binds to phosphorylated tyrosine at amino acid position 250 (6, 15), and PLC-γ binds to phosphorylated tyrosine at amino acid position 322 (52).

Under appropriate experimental conditions, polyomavirus induces tumors in more than a dozen distinct cell types in the mouse (13, 22, 30). High-tumor virus strains induce a broad array of tumors of both epithelial and mesenchymal origin when inoculated into newborn C3H/BiDa mice (13). Previous studies with the wild-type strain, PTA, have shown that this virus induces a high frequency of salivary gland, mammary gland, thymic, kidney, and hair follicle tumors (13, 25). Genetic determinants within VP1 (the major capsid protein), large T antigen, middle T antigen, and noncoding regulatory sequences are necessary for achieving a full spectrum and high frequency of tumor induction (24, 25, 27). To study the role of middle T antigen and its interactions with other cellular proteins in the induction of different tumor types, middle T mutations have been introduced into the PTA virus background, which contains all known genetic determinants necessary for tumor induction.

Studies with the mutant virus PTA-1387T, which encodes a truncated middle T that fails to associate with membranes and does not interact with pp60$^{c\text{-}src}$, showed that middle T association with pp60$^{c\text{-}src}$ is necessary for tumor induction in mice as well as transformation of cells in culture (7, 28). To begin to study the role of tyrosine phosphorylation of middle T antigen, a mutation that changes the tyrosine at 315 to phenylalanine was introduced into the virus (8). Biochemical studies showed that phosphorylation of this tyrosine promotes binding of the 85-kDa regulatory subunit of PI 3-kinase (53). Mutation at tyrosine 315 creates a middle T that cannot be phosphorylated at this site and thus cannot bind PI 3-kinase (34, 53). This mutant virus showed a reduced ability to transform rat fibroblasts in culture, and the foci induced by the mutant had a weakly transformed morphology compared to those induced by wild-type virus (8). When this mutant middle T was introduced into a high-tumor virus background, the virus, designated PTA-1178T, induced an overall lower frequency of tumors than wild-type PTA, and some tumor types were affected more dramatically than others (26, 53). Compared to wild-type virus, PTA-1178T induced very few salivary gland and kidney tumors, and the thymic and mammary gland tumors that it induced had morphological features different from those induced by wild-type virus (26). These results suggest that the association of middle T and PI 3-kinase is essential in obtaining full transformation and tumorigenesis and that deregulation of this sig-

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† Dedicated to Clyde Dawe, who died in July 1996 at the age of 75. His tireless research efforts and inspirational mentoring and guidance made this work possible.

nal transduction pathway is essential for transformation of some cell types and not others.

In this study, we report on the biological properties of a mutant virus (PTA-mt250YS) that encodes a middle T antigen containing serine in place of the tyrosine at amino acid 250. Previous studies have shown that phosphorylation of this site promotes the binding of Shc to middle T antigen (6, 15) and that middle T antigens containing substitutions of tyrosine 250 or surrounding amino acids within the NPXY domain fail to bind Shc (6, 10, 15) and are defective for transformation (10, 16, 17, 40). The Shc family of proteins, consisting of polypeptides of 46, 52, and 66 kDa (43), associate with phosphotyrosine-containing proteins through either their SH2 or phosphotyrosine binding (PTB) domains (4, 31, 35). Tyrosine-phosphorylated Shc proteins are thought to activate the *ras* signaling pathways through coupling with GRB2 and SOS, a Ras GDP/GTP exchange factor (38, 48). Here we show that the mutant virus PTA-mt250YS is partially defective for transformation of rat fibroblasts and has an altered tumor profile when assayed in mice. The virus induces a reduced frequency of kidney tumors and thymic tumors and an altered morphology of both thymic tumors and mammary gland tumors. Comparison of this tumor profile and the one induced by PTA-1178T, encoding a middle T antigen that does not associate with PI 3-kinase, indicates that deregulation of each signal transduction pathway is necessary for the induction of some tumor types and not for others and that there is some cross talk between the two signaling pathways.

MATERIALS AND METHODS

Cells and viruses. The wild-type high-tumor polyomavirus strain, PTA, has been previously described (13, 24, 25, 27). The polyomavirus mutant PTA-mt250YS was generated by site-directed mutagenesis on a PTA background (10). This mutation alters middle T antigen by replacing the tyrosine at position 250 with serine but does not affect large or small T antigen. Virus lysate was generated by transfecting NIH 3T3 cells as previously described (23), followed by plaque purifying on NIH 3T3 cells and expanding on primary baby mouse kidney (BMK) cultures (56, 59). BMK cells were prepared and maintained as described previously (59).

The F111 rat fibroblast cell line was routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere at 37°C. Cell lines expressing wild-type and mutant middle T antigen were generated by transfecting F111 cells with the *Bam*HI-to-*Eco*RI fragment of the appropriate viral genome that had been subcloned into pUC18. This viral DNA fragment includes the regulatory sequences necessary for transcription and the sequences encoding intact small and middle T proteins, as well as an amino-terminal fragment of large T antigen (amino acids 1 to 334). These plasmid DNAs were cotransfected with pSVneo (51) into F111 cells by the calcium phosphate method (49), and G418-resistant colonies were isolated and screened for expression of middle T antigen. Several independent clones expressing middle T from the following viruses were used in this study: PTA, PTA-1178T, PTA-mt250YS, and PTA-1387T. PTA encodes a wild-type middle T; PTA-1178T encodes a middle T antigen in which tyrosine 315 is replaced with a phenylalanine (8); PTA-mt250YS encodes a middle T with serine in place of tyrosine 250; and PTA-1387T encodes a truncated middle T antigen (7). Cell lines transfected with the subcloned DNA from the virus containing no middle T, Py808A (39), and with pSVneo (51) alone were used as controls.

Transformation assays. Transformation was measured by focus forming assays (8, 13). Thirty-five-millimeter-diameter dishes containing 2×10^5 F111 cells were infected with lysates containing high titers of virus. Approximately 24 h postinfection, cells were removed from the plate with trypsin and transferred to two 60-mm-diameter plates in DMEM supplemented with 10% calf serum. Every third day, the medium was changed. After the cells reached confluence, the medium was changed to DMEM supplemented with 2% calf serum. After 14 days, the macroscopic foci were scored and photographed.

Coimmunoprecipitation and immunoblotting. Cell and tumor extracts were made as previously described (11, 13, 23). Tumors that had been stored at −70°C were thawed and homogenized in lysis buffer containing protease and phosphatase inhibitors (0.137 M NaCl, 0.02 M Tris-HCl [pH 9.0], 0.001 M $MgCl_2$, 0.001 M $CaCl_2$, 10% glycerol, 1% Nonidet P-40, aprotinin [0.01 mg/ml], leupeptin [0.005 mg/ml], 0.1 mM sodium orthovanadate, phenylmethylsulfonyl fluoride [0.1 mg/ml]). Confluent cells from a 100-mm-diameter dish were washed in phosphate-buffered saline and lysed in 1 ml of the same lysis buffer. The lysates were cleared at 13,000 × *g*, and the protein concentration in the extracts was determined by the Bradford assay.

For coimmunoprecipition studies, 500 μg of total protein from each extract was incubated with 5 μl of an anti-middle T antibody, Pab762 (15), for 2 h. Immune complexes were collected on protein A-Sepharose, washed three times with lysis buffer, and eluted in sodium dodecyl sulfate (SDS) sample buffer. To assay total lysates, 50 μg of protein was suspended in SDS sample buffer.

Immunoblotting and washing were done by standard procedures (32). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes. The membranes were probed with a monoclonal antibody to polyomavirus T antigens, F4 (42), to detect middle T antigen, and with anti-Shc rabbit antibody (Upstate Biotechnology Inc.) to detect Shc. Antigen was visualized by the enhanced chemiluminescence method (Amersham).

Generation of tumor profiles. Newborn C3H/BiDa mice (less than 24 h old) were inoculated intraperitoneally with 50 μl of virus lysate (>2×10^6 PFU/ml). Mice were necropsied when moribund (13). Portions of tumors were either fixed in Bouin's fluid or frozen at −80°C. Upon necropsy, all overt tumors were recorded and confirmed histologically.

Virus replication in the mouse. Unstained sections of kidneys from 12- and 25-day-old infected mice were used for immunocytochemistry. The peroxidase-antiperoxidase procedure (Dako PAP kit) was used with rabbit anti-polyomavirus VP1 as the primary antibody (18). Positive reaction to the antigen was indicative of productive lytic infection.

DNA was extracted from kidneys of 12- and 25-day-old infected mice as described previously (18, 19). Briefly, kidneys were homogenized in STE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), SDS was added to 1%, and the homogenate was incubated with proteinase K (100 μg/ml). The solution was extracted with phenol-chloroform, incubated with RNase (100 μg/ml), and ethanol precipitated. To determine the levels of viral DNA, 1 μg of total DNA was digested with *Eco*RI, subjected to electrophoresis on a 1% agarose gel, transferred to a nylon membrane, and probed with cloned viral DNA. The probe DNA was labeled with digoxigenin (DIG), using the random primer labeling technique with DIG-coupled UTP. The DIG-labeled probe was hybridized to the membrane-bound DNA under standard conditions (49). Viral DNA was immunodetected with anti-DIG Fab fragments conjugated to alkaline phosphatase and visualized with a chemiluminescence substrate (Boehringer Mannheim).

Virus recovery from tumors and confirmation of genotype. Tumor tissue was homogenized in DMEM, frozen and thawed three times, and cleared by centrifugation. The supernatant was used to infect BMK cells. After visible cytopathic effect or approximately 1 week postinfection, the supernatant and cells were collected, frozen and thawed several times, and used as a crude virus lysate. To determine the nucleotide sequence of the recovered virus, 2 μl of the virus lysate was mixed with 20 μl of GeneReleaser (BioVentures, Inc.) and microwaved for 6 min, and the appropriate primers and reagents for PCR were added (2). The PCR, using as a 5′ primer the polyomavirus sequence CACAAGGGAATGGA ATG (nucleotide 900) and as a 3′ primer the polyomavirus sequence GGTGG AGTATACTAGAAATGCCGGG (nucleotide 1500), was performed under standard conditions using the GeneAmp kit (Perkin-Elmer). The 600-nucleotide fragment was cloned into the pCR2.1 cloning vector, using the TA cloning kit (Invitrogen). The cloned region was sequenced from three independently isolated plasmids by the Biopolymer Laboratory at the University of Maryland at Baltimore, using an Applied Biosystems automatic sequencer.

RESULTS

Mutant middle T 250YS does not associate with Shc. Studies have shown that phosphorylation of tyrosine 250 of middle T antigen is necessary for the association of Shc with middle T (6, 10, 15). To study the significance of this association in transformation and tumor induction, we analyzed a virus containing a single-site mutation which alters the tyrosine at position 250 in middle T antigen to serine but does not affect the large T open reading frame. To compare partially transforming and nontransforming middle T mutant to wild-type middle T, a series of cell lines expressing various middle T antigens was generated. An established rat fibroblast cell line was cotransfected with (i) a cloned fragment of the polyomavirus genome that expresses middle and small T antigen as well as the amino-terminal end of large T antigen and (ii) a plasmid expressing resistance to G418. We selected several independent G418-resistant clones that express wild-type middle T antigen as well as various middle T mutants. The clones were screened by Western blotting for approximately equal expression of middle T antigen (Fig. 1A). Figure 1B shows an immunoblot of anti-middle T immune complexes probed with anti-Shc antibodies.

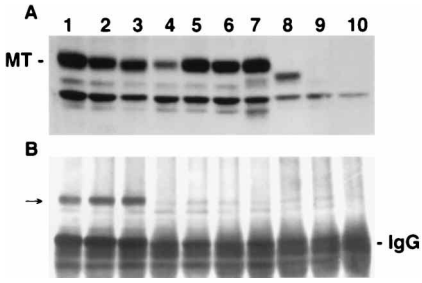

FIG. 1. Mutant middle T does not associate with Shc. (A) Immunoblot of middle T antigen, using 50 μg of total protein from cell lines expressing wild-type or mutant middle T. (B) Immunoblot of Shc in anti-middle T antibody Pab762 immune complexes, made with 500 μg of total protein from cell lines expressing wild-type or mutant middle T. Lanes 1 and 2, wild-type middle T; lane 3, 315YF middle T; lanes 4 to 7, 250YS middle T; lane 8, 1387T middle T; lane 9, 808A; lane 10, pSVneo. The arrow in panel B shows the migration of the Shc isoform. The migration of immunoglobulin G (IgG) and middle T antigen (MT) is indicated.

The results confirm that the mutant middle T containing serine in place of tyrosine 250 (middle T 250YS) did not associate with Shc, whereas both wild-type middle T antigen and middle T containing a mutation changing tyrosine 315 to phenylalanine (middle T 315YF) did associate with Shc. Further analysis of these immunoprecipitates showed that both wild-type and mutant middle T 250YS associate with the 85-kDa regulatory subunit of PI 3-kinase, while middle T 315YF does not (data not shown).

Polyomavirus encoding middle T 250YS induces fewer and morphologically altered foci. To investigate the ability of middle T 250YS to transform cells in culture, we compared foci induced by virus encoding the mutant middle T to those containing wild-type middle T. The mutant virus, PTA-mt250YS, induced approximately 1/10 the number of foci as were induced by wild-type virus, and the foci differed in appearance from wild-type foci (data not shown). Similar results have been previously reported (10). The foci induced by PTA-mt250YS were smaller than those induced by wild-type virus, and the transformed cells appeared elongated and tightly adherent, while cells transformed by wild-type virus were rounder and less adherent. These results suggest that the association of Shc with middle T antigen is necessary for full morphological transformation of established rat fibroblasts.

Virus growth in the mouse is unaffected by middle T 250YS. Efficient tumor induction in mice is associated with a sustained and disseminated viral infection (18). After inoculation of newborn C3H mice, polyomavirus goes through an acute phase of replication and widespread dissemination. Peak virus titers are found approximately 12 days postinfection, after which virus levels decrease coincident with the development of humoral immunity. Kidneys are one of the major sites of viral replication and persistence (18, 19, 47). Two approaches were used to compare the growth properties of PTA-mt250YS and wild-type PTA in mice. Mice were inoculated with each virus at birth and sacrificed at 12 or 25 days postinfection. We compared viral DNA levels in kidneys by Southern blotting and compared the numbers of lytically infected cells by immunocytochemistry. Total DNA was isolated from infected kidneys and digested with *Eco*RI. Figure 2 shows the levels of viral DNA in two mice infected with each virus at both 12 and 25 days postinfection. Figure 3 shows the immunoperoxidase staining for VP1 on sections of kidneys from 12- and 25-day-old mice infected with either wild-type PTA or mutant PTA-mt250YS virus. The expression of VP1 is indicative of productive lytic infection. The two approaches showed that the two viruses replicated in the animal during this initial period to similar extents and with similar kinetics.

Polyomavirus encoding middle T 250YS induces a limited spectrum of tumors. To compare the tumor-inducing ability of PTA-mt250YS to that of wild-type virus, we inoculated approximately 20 newborn C3H mice with each virus and recorded the number of macroscopic tumors present at necropsy. Table 1 shows the tumor profiles of both viruses. PTA-mt250YS induced tumors in 22 of the 23 inoculated mice. Despite the high frequency of mice bearing macroscopic tumors, compared to the wild-type virus, the mutant virus induced a low frequency of thymic tumors and failed to induce any overt kidney tumors. Although the mutant virus induced mammary tumors at a frequency similar to that for the wild-type virus, differences became apparent when the numbers of male and female mice with tumors were compared. All of the female mice inoculated with wild-type virus developed mammary tumors, and 10 of 11 females inoculated with mutant virus had tumors. Over 60% (10 of 16) of the male mice inoculated with wild-type virus had at least one mammary tumor, while only 1 (8%) of 12 males inoculated with PTA-mt250YS developed mammary tumors. The frequencies of hair follicle tumors were similar in the two groups of mice. In contrast to the other common targets of virally induced tumors, the frequency of salivary gland tumors induced by the mutant virus was sixfold higher than that of the wild-type virus. Histological evaluation of sections of salivary glands confirm this difference: 100% of mice inoculated with mutant virus developed salivary gland tumors that were visible macroscopically or microscopically, while 29% of mice inoculated with wild-type virus had overt or occult salivary gland tumors. The reason for this difference is not clear. The mean age of necropsy for mice inoculated with mutant virus was 110 days postinfection (dpi), which was slightly longer than that of wild-type virus, which was 76 dpi. This difference may reflect the lower incidence of the more lethal kidney and thymic tumors.

The morphological patterns of thymic and mammary tumors induced by PTA-mt250YS differ from those induced by PTA. PTA-induced thymic tumors are composed predominantly of epithelial cells (13, 14, 26). In these epitheliomas, the normal lymphoid component is displaced by the large mass of neoplastic epithelial cells. On the other hand, some thymic tumors induced by PTA-1178T were predominantly lymphoid and had a basic normal thymic architecture. These tumors were classified as organoid thymomas (26). The histological pattern of the thymic tumors induced by PTA-mt250YS was of both types, with a bias toward an organoid-like appearance. Three of the four overt tumors identified at necropsy were of the organoid type, while the fourth was an epithelioma. Only 3 of the 16 microscopic tumors were of the epithelial type, while the other 13 were predominantly organoid in appearance. The normal structure of the thymic medulla and cortex seen in uninfected mice is shown in Fig. 4A. The organoid-like histol-

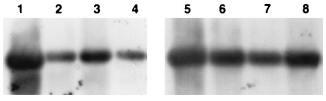

FIG. 2. Levels of viral DNA replication of wild-type and mutant virus in kidneys of infected mice are similar. Southern blot analysis of DNA isolated from the kidneys of 12-day-old mice (lanes 1 to 4) or 25-day-old mice (lanes 5 to 8) infected at birth with PTA (lanes 1, 2, 5, and 6) or with PTA-mt250YS (lanes 3, 4, 7, and 8) and probed with cloned viral DNA. Each lane represents the DNA from the kidney of a single mouse.

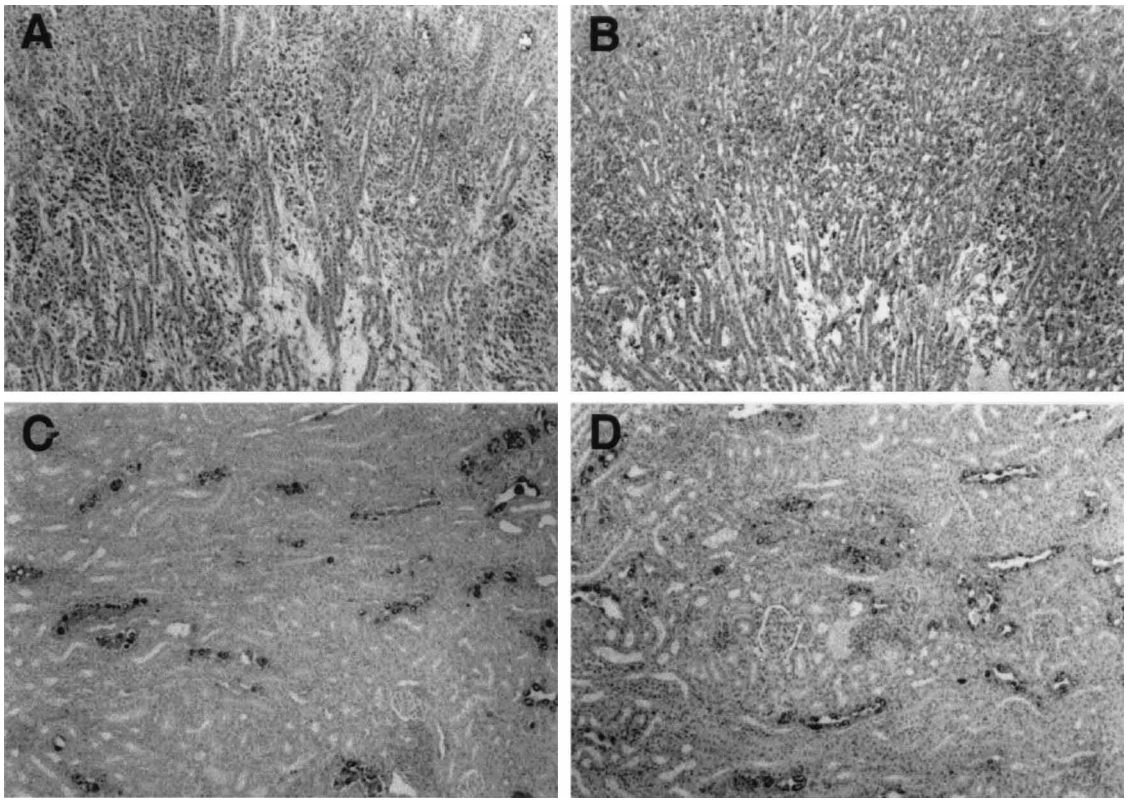

FIG. 3. Amounts of productively infected kidney cells in mice inoculated with wild-type and mutant virus are approximately equal. Immunocytochemistry for VP1 was performed on kidney sections from mice infected with PTA (12 days dpi [A] and 25 dpi [C]) or PTA-mt250YS (12 dpi [B] and 25 dpi [D]). Magnification, ×100.

ogy in both occult (Fig. 4B) and overt (Fig. 4D) tumors induced by PTA-mt250YS and the typical epithelial tumor induced by wild-type virus (Fig. 4C) are also shown.

Histological examination of mammary tumors induced by wild-type and mutant polyomavirus revealed a number of different morphological types of tumors. In this study as well as in our previous studies, we have found the predominant types of mammary neoplasm induced by wild-type virus to be the intraductal carcinoma of the comedo type (Fig. 5A) and the papillary intraductal carcinoma histotype (Fig. 5B). The two most common histotypes induced by PTA-mt250YS were a solid undifferentiated carcinoma (Fig. 5D) and an intraductal carcinoma (Fig. 5C). Although wild-type virus induces a high frequency of intraductal carcinomas, it rarely induces mammary tumors of the undifferentiated type (20, 26).

Tumors induced by PTA-mt250YS retain the mutant genotype and phenotype. Tumors induced by PTA-mt250YS did not result from reversion of the single-point mutation to wild type or from second-site mutations in which Shc associated with middle T through an alternative mechanism. We reisolated virus from two mammary and two salivary gland tumors induced by mutant virus and sequenced the nucleotides surrounding the mutated sites. In all four cases, the tyrosine-to-serine substitution was present and no additional mutations were seen. Additionally, we tested protein lysates from two mammary tumors and a salivary gland tumor induced by PTA-mt250YS for the presence of middle T-Shc complexes. Figure 6 shows that middle T present in tumors induced by mutant virus did not associate with Shc, while middle T from wild-type tumors did.

DISCUSSION

In this study, we assessed the transforming properties and tumor-inducing capabilities of a mutant middle T antigen that is unable to associate with the product of the oncogene *shc*. Middle T antigen, the major oncoprotein of polyomavirus, associates with $pp60^{c\text{-}src}$, activating its tyrosine kinase function, and serves as a substrate for $pp60^{c\text{-}src}$, with the major sites of phosphorylation being tyrosines 250, 315, and 322 (5, 9, 50). These phosphorylated sites act as binding sites for Shc, PI 3-kinase, and PLC-γ, respectively (1, 6, 15, 34, 52, 53, 58).

TABLE 1. Tumor profile induced by wild-type and mutant PTA-mt250YS viruses[a]

Virus	Fraction of mice with tumors	Mean age (days) at necropsy (range)	No. (%) of mice with tumors				
			Mammary gland	Salivary gland	Thymus	Hair follicle	Kidney
PTA	21/21	76 (63–84)	15 (71)	3 (14)	20 (95)	19 (90)	17 (81)
PTA-mt250YS	22/23	110 (60–137)	11 (50)	20 (87)	4 (17)	22 (96)	0

[a] Newborn C3H/BiDa mice were inoculated with the viruses indicated and sacrificed when moribund. Numbers refer to macroscopic tumors detected at necropsy and subsequently confirmed histologically.

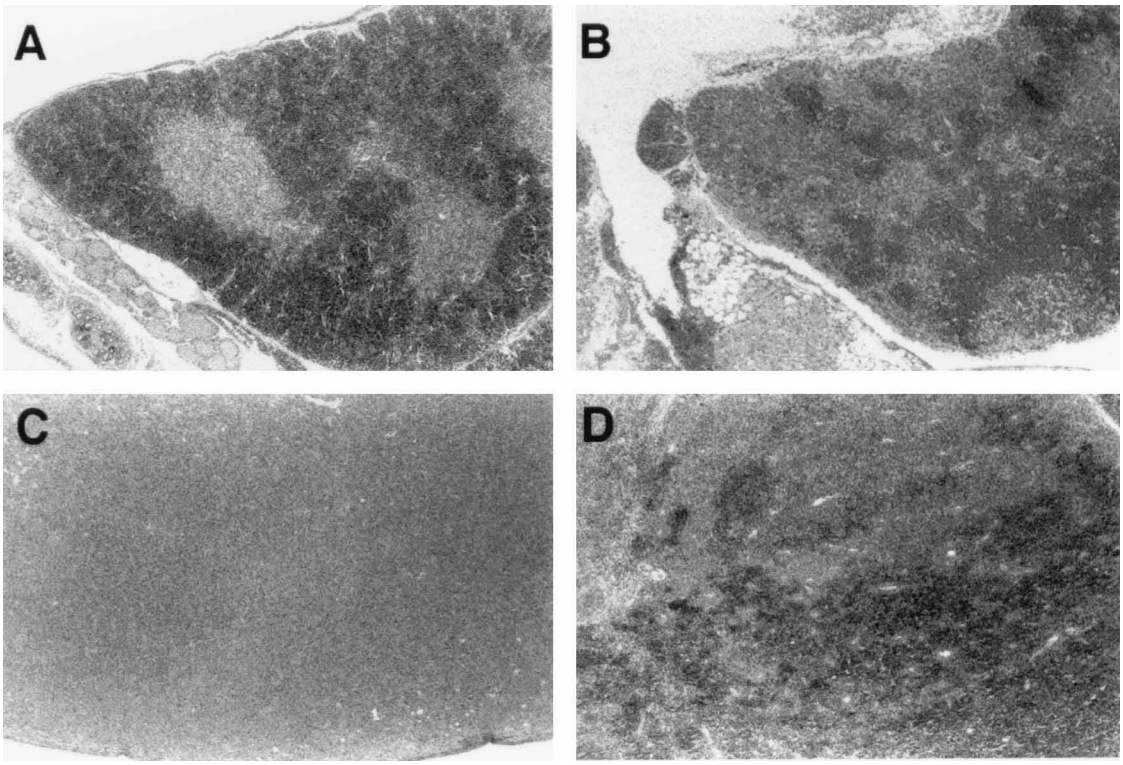

FIG. 4. Histological patterns of thymic tumors induced by wild-type and mutant polyomavirus are different. Shown are hematoxylin-and-eosin-stained sections of normal thymus (A), microscopic thymic tumor induced by PTA-mt250YS (B), and macroscopic tumors induced by PTA (C) and PTA-mt250YS (D). Magnification, ×100.

Using site-directed mutagenesis, a single amino acid change in middle T, serine for tyrosine 250, was generated. By altering this site, middle T antigen no longer binds to Shc, although it still associates with and activates the kinase activity of $pp60^{c\text{-}src}$ and still associates with PI 3-kinase and PLC-γ (6, 10, 15). Previous studies have shown that Shc acts as an adapter protein, associating with GRB2 and SOS. This complex is thought to lead to the activation of *ras* (38, 48). By comparing wild-type middle T antigen to this mutant middle T, we were able to determine the importance of the association of Shc with middle T antigen and thus assess the role of the *ras* signal transduction pathway in transformation and tumor induction.

We compared the transforming properties of polyomavirus encoding a mutant middle T that replaces tyrosine 250 with serine with those of wild-type polyomavirus in tissue culture. We found that the mutant virus is partially defective in transformation of rat fibroblasts, inducing a reduced number of foci as well as foci of altered, weaker morphology. These results are similar to those previously reported by several other investigators (10, 16, 40). Based on these results, we conclude that the association of Shc with middle T antigen is necessary to fully transform established rat fibroblasts. Furthermore, these results support the observation that abrogation of the *ras* signal transduction pathway disrupts transformation of fibroblasts by polyomavirus middle T antigen (33, 45).

To determine the role of the middle T-Shc association in the induction of neoplasia in cell types other than fibroblasts, we compared the tumor-inducing capabilities of wild-type and mutant virus in the mouse. Wild-type virus induces a high frequency of tumors in epithelial cells of the thymus, mammary gland, salivary gland, and hair follicle and in mesenchymal cells of the kidney (13). The mutant virus induces a high frequency of hair follicle and salivary gland tumors, suggesting that the middle T-Shc association is not necessary for development of those tumor types. The mutant virus does not induce kidney tumors and induces a low frequency of thymic tumors. Furthermore, the tumors derived from the mammary and thymic epithelium appear histologically different from those induced by wild-type virus. These differences in the frequency and appearance of tumors induced by the mutant virus compared with those of the wild-type virus indicate that the middle T-Shc association is important in neoplastic transformation of these cell types. The PTA-mt250YS-induced thymic tumors were mainly organoid in appearance, with lymphocytes as the predominant cell type in the tumor. The neoplastic epithelial cells within the tumor were not as highly proliferative as those in PTA-induced thymic epitheliomas, suggesting that activation of the *ras* pathway is necessary for cell proliferation in thymic epithelium. In the case of mammary tumors, it is not clear whether the mutant virus induces tumors in different cell types within the mammary gland, resulting in different morphologies, or whether the neoplastic cells originate from similar infected target cells but take on a different appearance after transformation. In either case, the difference in histological appearance of the tumors suggests that deregulation of the *ras* pathway is an important step in some mammary tumors.

The differences that we observe in tumor frequencies are not due to overall differences in growth of the two viruses, since the two viruses replicate to similar levels in the kidney at the early stages of infection. It is possible, though, that the wild-type and mutant viruses replicate to different levels in the various target tissues. This could explain the high level of salivary gland tumors and low frequency of kidney and thymic tumors induced by the mutant virus. In either case, the data suggest that

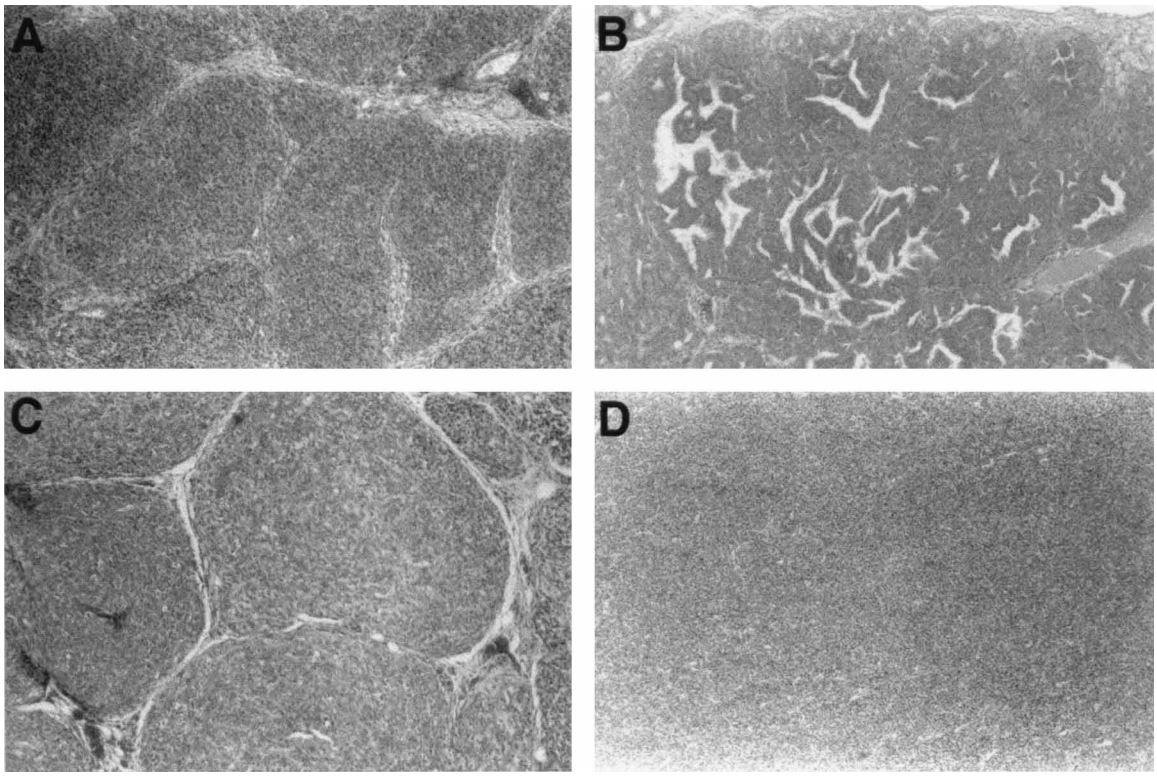

FIG. 5. Different histological patterns of mammary gland tumors are induced by wild-type and mutant polyomavirus. Shown are hematoxylin-and-eosin-stained sections of the predominant types of mammary gland tumors induced by PTA (A and B) and PTA-mt250YS (C and D). The two most frequent types induced by PTA are the intraductal (A) and papillary (B) carcinomas, while the major histotypes induced by PTA-mt250YS are the intraductal (C) and undifferentiated (D) carcinomas. Magnification, ×100.

the association of middle T antigen with Shc is necessary for the tumor profile that we observe with wild-type virus.

Some of the differences observed between the tumor profiles of wild-type virus and PTA-mt250YS are similar to those previously reported with the mutant PTA-1178T (26, 53). This virus encodes a middle T antigen in which the tyrosine at position 315 has been changed to phenylalanine. This mutation disrupts the association of middle T antigen with the 85-kDa regulatory subunit of PI 3-kinase (53). PTA-1178T induces tumors at an overall lower frequency, and with a delayed appearance, compared to the wild-type virus. The frequency of both salivary gland and kidney tumors is especially reduced, and the histological pattern of thymic and mammary tumors is altered (26). The changes in the tumor profile of PTA-mt250YS seem to be a subset of those altered by PTA-1178T. Neither virus induces kidney tumors, but PTA-mt250YS induces salivary gland tumors. Both viruses induce thymic tumors of the organoid type. Although both mutant viruses induce mammary tumors of altered morphology, the predominant type induced by PTA-1178T is described as having a biphasic or myoepithelial pattern (26), while PTA-mt250YS predominantly induces an undifferentiated type which is rarely induced by either PTA or PTA-1178T.

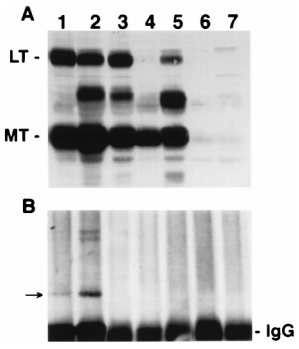

FIG. 6. Mutant middle T-induced tumors do not contain middle T-Shc complexes. Protein extracts from tumors and normal tissues were immunoblotted with anti-T antigen antibodies (A) to show the expression of the T antigens. Migration of large T (LT) and middle T (MT) are indicated. In panel B, extracts were first immunoprecipitated with anti-middle T antibody Pab762, and immune complexes were immunoblotted with anti-Shc antibodies. Lane 1, PTA-induced thymic tumor; lane 2, PTA-induced mammary tumor; lanes 3 and 5, PTA-mt250YS-induced mammary tumors; lane 4, PTA-mt250YS-induced salivary gland tumor; lanes 6 and 7, normal tissue from mammary gland and salivary gland. The arrow in panel B shows migration of the Shc isoform. IgG, immunoglobulin G.

The similarities and differences in the tumor profiles generated by PTA-1178T and PTA-mt250YS suggest that both pathways have some common downstream components and some unique ones. Some tumor types, such as kidney tumors, require the deregulation of both the PI 3-kinase pathway and the *ras* pathway, while in others, such as salivary gland epithelium tumors, deregulation of the PI 3-kinase pathway is sufficient. Other cell types, such as thymic epithelium, need both pathways deregulated to become fully transformed, while deregulation of either pathway alone results in partial transformation. In a majority of cases, this partial transformation results in proliferation of the normal lymphocyte population in the thymus but not in the neoplastic epithelial component. The mo-

lecular mechanism of this is not clear, but the expansion of the lymphocytes in the thymus suggests that the neoplastic epithelium secretes a substance that is mitogenic for lymphocytes. In addition, the result suggests that both signaling pathways must be activated for deregulated cell growth of the thymic epithelium. In the case of the mammary tumors, the association of both PI 3-kinase and Shc with middle T results in predominantly intraductal carcinomas of the comedo type, while deregulation of each pathway by itself induces a large proportion of the neoplasms with altered morphologies. One possible explanation of these altered histotypes is that each is the result of transformation of a unique cell type within the mammary epithelium. Another explanation is that full transformation requires a certain threshold level of a common downstream element that is easily reached when both pathways are deregulated, while other morphological histotypes require lower levels of activation that are reached when either one of the two pathways is deregulated. However, this is unlikely since the histotype patterns of the two mutants are different. Alternatively, full transformation may require the activation of multiple unique downstream signaling pathways, and activation of a subset of these pathway could result in altered morphologies.

This report supports recent biochemical studies showing that the two pathways have both unique and common downstream elements. Studies have shown that the association of both PI 3-kinase and Shc with middle T is needed to fully activate the mitogen-activated protein kinase pathway (57), while only the association of Pl 3-kinase with middle T is necessary to activate the pp70 S6 kinase (10). Furthermore, it has been shown that there is a direct link between the activation of *ras* and the PI 3-kinase pathway (12, 46). The catalytic subunit of PI 3-kinase, p110, has been reported to be directly associated with *ras* (46). In addition, the association of inositol polyphosphate 5-phosphatases with Shc and GRB2 further suggests a link between the *ras* activation and PI 3-kinase signaling pathways (12).

In summary, middle T antigen associates with a number of different signaling molecules through its phosphorylated tyrosines. Previous studies have indicated that the association of PI 3-kinase with middle T is critical for the induction of some tumor types. This study shows that activation of *ras*, through the middle T-Shc interaction, is necessary for the induction of a subset of the tumors induced by the wild-type virus. It has recently been shown that middle T antigen also associates with PLC-γ. Our preliminary results indicate that disruption of this association does not result in the abrogation of any tumor types. By using viruses disrupting these various interactions, we have shown that activation of different signaling pathways by polyomavirus middle T antigen results in different tumor profiles, and we can begin to assess the role of each of the different signaling pathways in tumor formation. The correlation of these interactions, their downstream signal transduction pathways, and their effects on the induction of specific tumors will begin to elucidate the molecular mechanism of tissue specific neoplasia.

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ADDENDUM IN PROOF

We have recently learned of a similar report of the tumor capabilities of PTA-mt250YS (R. Bronson, C. Dawe, J. Caroll, and T. Benjamin, Proc. Natl. Acad. Sci. USA, in press).

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