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In Vitro Mutagenesis of the Putative Membrane-Binding Domain of Polyomavirus Middle-T Antigen

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Polyomavirus middle-T antigen contains a contiguous sequence of 22 hydrophobic amino acids near the carboxyl terminus, which is the putative membrane-binding domain of the protein. The DNA encoding this region was mutated to form a series of deletions, insertions, and substitutions called RX mutants. The phenotypes of these mutants fall into three groups based on the transforming and biochemical properties of their encoded proteins. The first group, with deletions outside but proximal to the hydrophobic domain, displayed an essentially wild-type phenotype. A second group, with extensive deletions within the region encoding the hydrophobic domain, expressed middle-T species which did not fractionate with cellular membranes or associate with pp60$^{c\text{-}src}$ and which were defective in their ability to transform. A third group of mutants with more subtle predicted alterations in the hydrophobic domain were wild type for the biochemical parameters investigated but were unable to transform cultured rodent cells. These observations are consistent with previous findings that membrane association plays an important role in transformation by middle-T and that, whereas association between middle-T and pp60$^{c\text{-}src}$ is a necessary correlate of transformation, it is not sufficient. A comparison of murine polyomavirus middle-T and a newly described hamster papovavirus putative middle-T revealed a strong homology between their respective hydrophobic-domain amino acid sequences. This homology is not observed in the anchorage domains of other model proteins, and this may imply that the middle-T hydrophobic domain is important in transformation for reasons other than simple membrane association.

Polyomavirus codes for three tumor-associated antigens: large-T, middle-T, and small-t. The three proteins share a common amino terminus, but each has unique carboxyl-terminal sequences of differing length resulting from the differential splicing of the parental early mRNA transcript (26). Middle-T alone is able to transform the growth properties of established cultured rodent cells (27). A middle-T-associated tyrosine-specific kinase activity has been demonstrated in vitro (13, 20, 22), the presence of which correlates with the transforming potential of different polyomavirus variants (10). It is probable that middle-T itself is not a tyrosine kinase but that this activity is due to its association with pp60$^{c\text{-}src}$, a known cellular tyrosine kinase and the product of a proto-oncogene (9). The interaction between transformation-competent middle-T species and pp60$^{c\text{-}src}$ leads to an increase in the specific activity of this tyrosine kinase (4) and an alteration in the phosphorylated state of pp60$^{c\text{-}src}$ (7, 29). The kinase-active fraction of middle-T appears to be located near the plasma membrane fraction of cells (3, 19, 21).

Middle-T is a protein of 421 amino acids with a distinct, predominantly hydrophobic, 22-amino-acid domain near the carboxyl terminus (23). The protein is found associated with membranes in infected and transformed cells, although its precise location remains unclear (30). The lipid-spanning segments of other viral and cellular proteins that bridge the plasma membrane or internal membranes contain hydrophobic sequences; however, these proteins, unlike middle-T, also contain an amino-terminal signal sequence which is responsible for insertion into the membrane by the classical route exemplified by vesicular stomatitis virus glycoprotein (VSV-G) (for a review, see reference 28). Middle-T is assumed to be positioned with its amino terminus facing into the cytoplasm since it cannot be labeled from the outside of cells. Presumably, it spontaneously associates with membrane fractions after synthesis in a manner similar to the process used by cytochrome b_5 (2). Whether the hydrophobic sequence of middle-T spans the lipid bilayer with the terminal six amino acids outside the cell remains unknown.

Mutations and deletions have been previously introduced into the DNA encoding the hydrophobic domain and adjacent regions. Deletions were introduced into a plasmid encoding middle-T (18), resulting in a series of middle-T antigens lacking the extreme carboxyl-terminal amino acids and in increasingly greater segments extending into the hydrophobic domain. Analysis of the mutant plasmids showed that any mutations causing changes in the carboxyl-terminal sequences of middle-T reduced drastically their transforming ability. In another study, a termination codon was introduced into the middle-T coding sequence, yielding a truncated 28,000-molecular-weight (28K) (261-amino-acid) protein (MOP) that was unable to transform and that lacked associated in vitro kinase activity (24). Another mutant (Py1387T), containing a termination codon engineered within the DNA encoding middle-T (5), expressed a truncated protein (51K) lacking only the final 37 amino acids. The middle-T encoded by this transformation-defective mutant was located in the cytoplasm and lacked in vitro kinase activity.

VSV-G is typical of one class of membrane-spanning proteins. It has an amino-terminal signal sequence which is cleaved soon after synthesis, it is glycosylated, and it has a

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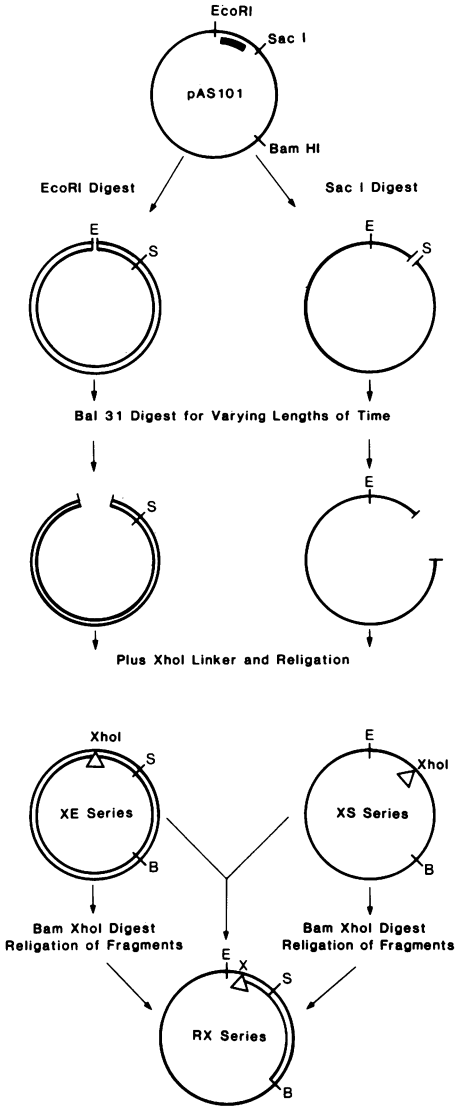

FIG. 1. Procedure for the generation of mutations within the DNA encoding the putative membrane-binding domain of middle-T (indicated by a solid bar in the parent plasmid pAS101). Two series of deletion-mutant plasmids (the XE and XS series) were created by cleaving pAS101 at either the *Eco*RI or the *Sac*I site, followed by exonuclease digestion and the insertion of an *Xho*I linker. The extent of the deletion was determined by DNA sequencing. After digestion of the XE and XS plasmids with *Bam*HI and *Xho*I, appropriate DNA fragments were ligated to create a new series of in-phase mutations within the DNA encoding the hydrophobic domain called the RX series of plasmids.

hydrophobic membrane anchorage domain that spans the lipid bilayer. A hybrid plasmid was constructed (25) which expressed a protein composed of the amino-terminal 379 amino acids of middle-T and the carboxyl-terminal 60 amino acids of VSV-G. This chimeric protein (MT-G1) was expressed in COS-1 cells, where it was shown to be located in the cellular membrane fraction and to exhibit associated kinase activity. However, the mutant was unable to transform established rodent cells in vitro.

Many studies to date are consistent with the view that transformation by polyomavirus requires an active association between middle-T and pp60$^{c\text{-}src}$. Since middle-T and pp60$^{c\text{-}src}$ are both associated with membranes, and since changes in the hydrophobic putative membrane-binding domain of middle-T invariably affect transformation, it would appear that membrane binding must play an important role in the transformation process. Here, we report studies in which further mutations have been generated within the DNA encoding the putative membrane-binding domain. The effects of such lesions on middle-T-induced transformation were investigated.

MATERIALS AND METHODS

Construction of the hydrophobic domain mutants. The parent wild-type plasmid pAS101 (containing the polyomavirus DNA *Bam*HI-*Eco*RI fragment cloned within the vector, pAT153) was separately digested at the *Eco*RI and *Sac*I sites (Fig. 1). The linearized DNA molecules were double-strand-exonuclease digested with BAL 31 for various lengths of time to generate deletions of differing extent. The cut ends were filled in with the Klenow fragment of DNA polymerase in the presence of deoxynucleotide triphosphates and then blunt end ligated to *Xho*I linkers (5′ CCTCGAGG 3′). This treatment generated two series of deletion mutants around either the *Eco*RI site (the XE series) or the *Sac*I site (the XS series), the endpoints of which mapped within the genomic region coding for the hydrophobic domain of the middle-T antigen. In-phase deletions, insertions, repeats, and replacements were generated by religating the *Bam*HI-*Xho*I fragments of the appropriate XE and XS deletions to generate restructured plasmids called the RX series (Fig. 1 and 2). The termination mutant (RXT) was generated by digestion of RX38 (Fig. 2) with *Xho*I, followed by filling in of the cohesive ends with the Klenow fragment of DNA polymerase. To the blunt ends were ligated a synthetic oligonucleotide (5′ CTAGTTAACTAG 3′) which contains termination signals in all three reading frames. The RX mutant sequences were verified by DNA sequencing.

Cells and assays. Transformation assays were carried out by introducing purified plasmid DNA onto a monolayer of Rat-1 cells by the calcium phosphate transfection method (14).

The RX series of plasmid DNAs was cotransfected with the Tn*5*-encoded phosphotransferase (*neo*) gene (pSV$_2$Neo), in the ratio 10:1, by the calcium phosphate technique onto NIH 3T3 cells, followed by growth in the presence of the antibiotic G418. G418-resistant clones were selected after 10 to 14 days, followed by growth in the selective medium. The individual clones were assayed for the presence of middle-T, associated kinase activity, pp60$^{c\text{-}src}$ association, and subcellular locations as described below.

Growth conditions for labeling with [^{35}S]methionine, preparation of cell lysates, immunoprecipitation, and the in vitro kinase assay have all been previously described (9, 22). Cellular fractionation was carried out as described previously (25).

RESULTS

Hydrophobic domain mutants of middle-T. The DNA encoding the hydrophobic domain of middle-T (nucleotides 1,417 to 1,479) was extensively mutated to create in-phase deletions, insertions, repeats, and replacements. The predicted alterations in amino acid sequence within and around the 22-amino-acid putative membrane-binding domain

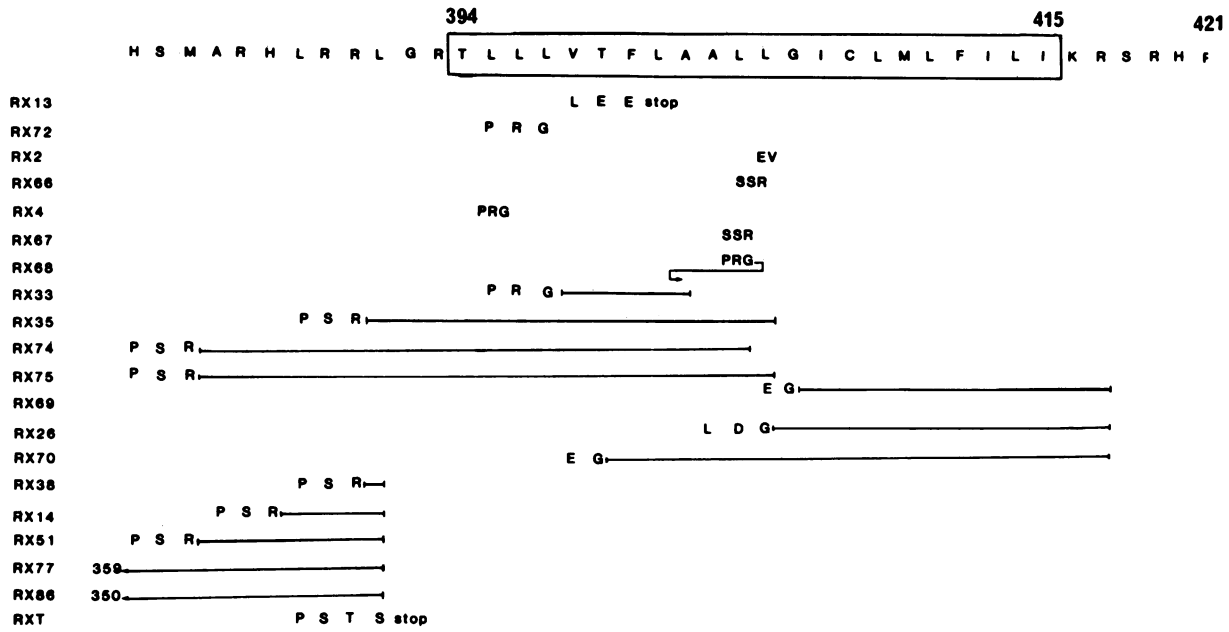

FIG. 2. Predicted lesions in the RX-mutant middle-T antigens. The carboxyl-terminal amino acid sequence of middle-T is shown with the hydrophobic domain boxed. The individual lesions of the RX mutants are indicated. The amino acid changes indicated are due to the *Xho*I linker. Deletions are indicated by straight lines, except in RX68, where this indicates a repeated amino acid sequence. Stop indicates the introduction of a translational termination codon. Abbreviations: A, alanine; C, cysteine; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; R, arginine; S, serine; T, threonine; V, valine.

(amino acids 394 to 415) can be grouped into several types: mutations in which an uncharged amino acid was replaced by a charged amino acid, deletion mutations in which portions of the hydrophobic domain were removed, mutations in which termination codons were introduced at various places, and deletion mutations in which sequences outside but proximal to the hydrophobic domain were removed.

TABLE 1. Properties of selected RX-mutant-encoded middle-T antigens[a]

Middle-T mutant or wild type	Focus formation (%)	Predicted size (kDa)[b]	Phosphate acceptor activity[c]	pp60$^{c\text{-}src}$ Association[c]	Protein location[d]
Wild type	100	56.0	+	+	Membrane
RX38	54	56.0	+	ND	Membrane
RX51	126	55.1	+	ND	Membrane
RX77	10	51.7	+	ND	Membrane
RX13	1	53.2	−	−	Cytosol
RX26	1	54.4	−	−	Cytosol
RX69	1	54.5	−	−	Cytosol
RXT	1	52.0	−	−	Cytosol
RX2	1	56.0	+	+	Membrane
RX67	1	56.0	+	+	Membrane
RX68	1	56.8	+	+	Membrane

[a] Focus formation assays were carried out by transfecting 5 μg of plasmid DNA onto a monolayer (5×10^5) of Rat-1 cells. Focus formation was compared with wild-type middle-T (which typically forms 150 to 200 foci after 14 days).

[b] kDa, Kilodaltons.

[c] Phosphate acceptor activity and pp60$^{c\text{-}src}$ association were determined for each of the mutant middle-T antigens in an in vitro kinase assay after immunoprecipitation with rodent tumor cell sera or peptide antibodies raised against the carboxyl terminus of pp60$^{c\text{-}src}$, respectively.

[d] Protein location was determined after metabolic labeling and differential centrifugation. +, Presence, and −, absence of the biochemical property. ND, Not determined.

Mutant middle-T antigens which contain small alterations (Fig. 2) include the RX72-encoded protein, in which three leucine residues (amino acids 395 to 397) near the amino extreme of the hydrophobic domain are replaced by a charged amino acid (arginine) and two uncharged amino acids (proline and glycine) coded for by the inserted linker sequence. In RX4-encoded middle-T, the same combination of amino acids replaces the first leucine (amino acid 395). Similar small mutations were generated at the center of the hydrophobic domain. RX2-encoded middle-T has a single leucine (amino acid 405) replaced by a nonpolar valine and a charged glutamic acid, whereas in the RX66-encoded protein, a pair of leucine residues (amino acids 404 and 405) are replaced by two serine resides and a charged arginine. In the RX68-encoded protein, proline, arginine, and glycine residues replace a leucine moiety (amino acid 404), followed by a repeat of amino acids 402 to 405.

Mutant plasmids RX69, RX26, and RX70 encode middle-T species in which increasing portions of the carboxyl-terminal half of the hydrophobic domain are deleted and replaced by a charged amino acid (glutamic, aspartic, and glutamic acids, respectively). Deletions within the region encoding the amino-terminal half of the hydrophobic domain include plasmid RX33, encoding a middle-T in which amino acids 398 to 401 are deleted and a charged arginine moiety is introduced, and plasmids RX35, RX74, and RX75, encoding proteins in which increasing portions of the middle-T hydrophobic domain are deleted from the center towards the amino-terminal end of the hydrophobic domain and beyond. A series of deletion mutants which was generated outside the

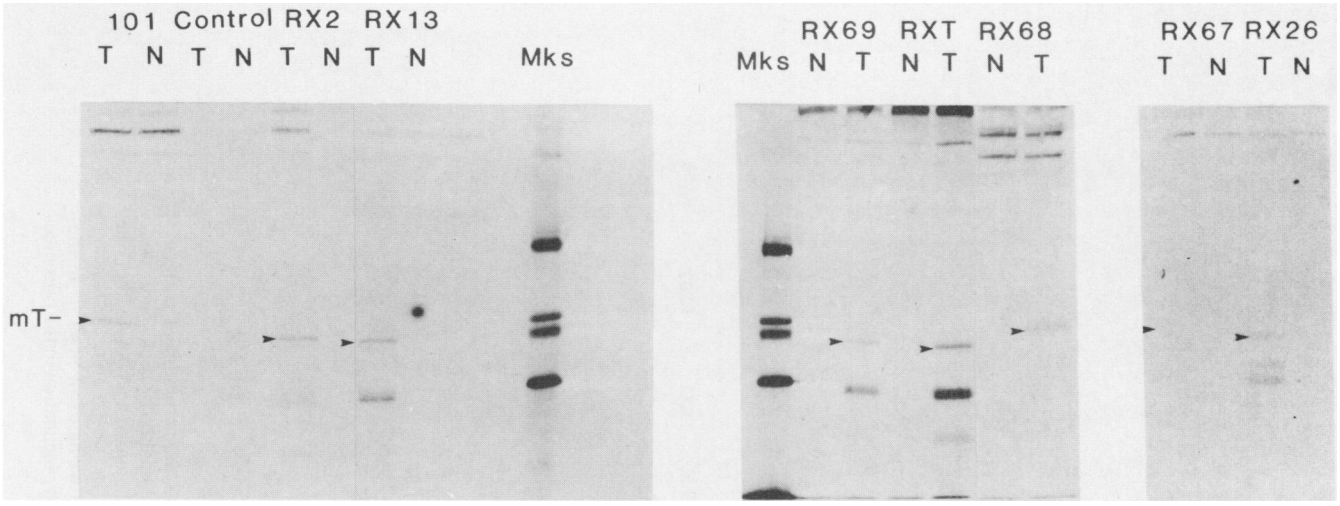

FIG. 3. Metabolic labeling of neomycin-resistant NIH 3T3 cell lines expressing selected RX-mutant middle-T antigens. The cell lines were incubated in the presence of [^{35}S]methionine, lysed, and immunoprecipitated with either normal rat serum (N) or rat polyomavirus anti-tumor cell serum (T), followed by analysis by polyacrylamide gel electrophoresis and fluorography. Abbreviations: mT, middle-T; Mks, molecular weight markers of 81, 60, 50, and 40 kilodaltons.

region encoding the hydrophobic domain (RX38, RX14, RX51, RX77, and RX86) contains lesions from amino acid 391 towards the amino terminus, with endpoints indicated in Fig. 2.

Finally, two mutants which contained termination codons upstream of the natural TAG were generated. RX13 encodes a truncated middle-T molecule of 400 residues generated by a frame-shift mutation, whereas RXT was generated by the insertion of a synthetic oligonucleotide linker which contains termination signals in all three reading frames. Compared with termination mutants previously published, the RXT termination codon is closer to the region encoding the hydrophobic domain, being only two residues upstream.

Hydrophobic domain mutants of polyomavirus middle-T are transformation defective. The transforming abilities of the RX mutants were determined by focus-formation assays on monolayers of Rat-1 and NIH 3T3 cells. Every mutant with a change within the region encoding the hydrophobic domain of middle-T or which contained a termination codon upstream of this region was totally defective in the focus-formation assay (selected examples are shown in Table 1). This assay was repeated extensively to confirm this finding.

Middle-T species with lesions outside the hydrophobic domain, encoded by RX14, RX38, and RX51, induced focus formation in Rat-1 cells at a level comparable to that of wild-type middle-T (Table 1); however, in the case of the largest deletions (RX86 and RX77), the number of foci formed was reduced, and the time for focus appearance was somewhat delayed. When possible, foci of transformed cells were picked and expanded into cell lines. Of the transformed Rat-1 cells tested (RX14, RX38, and RX51), all demonstrated anchorage-independent growth in soft agar and induced tumors when injected into Fischer rats at levels comparable to Rat-1 cells transformed by wild-type middle-T. Rat-1 cells transformed by RX77 showed delay in the time of appearance of soft agar foci and tumors when injected into rats (data not shown).

Expression of the mutant middle-T proteins. To study the proteins encoded by the transformation-defective RX mutants, cell lines expressing certain mutant proteins were picked after cotransfection of the RX plasmid DNA into NIH 3T3 cells with a plasmid encoding the marker for neomycin resistance (pSV_2Neo). Cells containing the gene for neomycin resistance were selected in a medium containing the antibiotic G418. Resistant clones were analyzed for the presence of a middle-T species by metabolic labeling, followed by immunoprecipitation with polyomavirus anti-tumor cell serum and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Selected [^{35}S]methionine-labeled RX-mutant middle-T species are shown in Fig. 3. The middle-T molecules with small mutations (encoded by RX2, RX67, and RX68) comigrated with wild-type middle-T. The middle-T species encoded by the hydrophobic domain deletion mutants (RX13, RX26, RX69), the termination mutant (RXT), and proteins with lesions outside the hydrophobic region all had mobilities consistent with their predicted lesions (Table 1). The lower-molecular-weight proteins seen in Fig. 3 are probably a truncated form of large-T encoded by the wild-type and mutant plasmids and proteolytic fragments of the middle-T species.

In vitro kinase activities of the hydrophobic-domain mutant middle-T proteins. The standard in vitro kinase assay for middle-T involves immunoprecipitation with either rat or hamster anti-polyomavirus tumor cell sera followed by extensive washing and incubation in the presence of [^{32}P]ATP. Under these conditions, middle-T (hamster tumor serum) or middle-T and the serum immunoglobulin fraction (rat tumor serum) are phosphorylated, indicating the presence or relative activity of the associated kinase activity, respectively (10). This assay was carried out with lysates from the NIH 3T3 neomycin-resistant cell lines expressing the transformation-defective RX-encoded middle-T species and with lysates from Rat-1 cells transformed by the transformation-competent RX-encoded middle-T antigens.

Transformation-competent mutants with lesions outside the hydrophobic domain (encoded by RX14, RX51, RX76, and RX77) demonstrated an associated kinase activity comparable to wild-type middle-T (Fig. 4a). In this instance, peptide antibodies raised against the carboxyl terminus of middle-T were used for the immunoprecipitation. Middle-T antigens encoded by nontransforming mutants with deletions

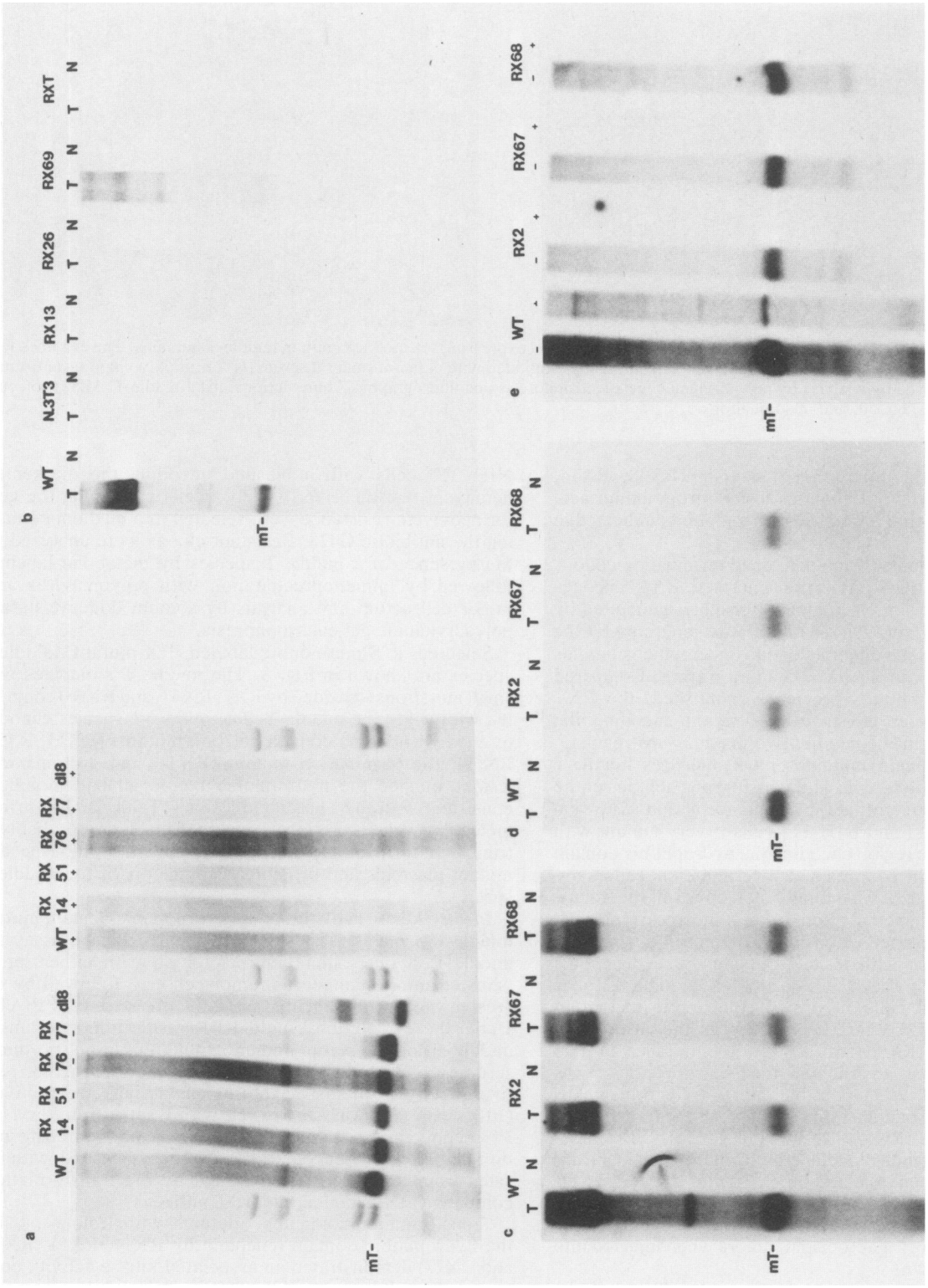

FIG. 4. In vitro kinase assays with lysates from rodent cell lines expressing wild-type (WT) and selected RX-mutant middle-T antigens. Lysates were made from transformed Rat-1 cells (a) or neomycin-resistant NIH 3T3 cells (b to e) expressing the middle-T species indicated. Immunoprecipitations were carried out with (a) a peptide antibody (mTc) raised against the carboxyl-terminal six amino acids of middle-T in the presence (+) or absence (−) of competing peptide, (b and c) rat anti-tumor cell serum (T) or normal rat serum (N), (d) hamster anti-tumor-cell serum (T) or normal hamster serum (N), or (e) a peptide antibody raised againt the carboxyl terminus of $pp60^{c\text{-}src}$ in the presence (+) or absence (−) of competing peptide. The immunoprecipitates were washed extensively, followed by incubation in the presence of [^{32}P]ATP, polyacrylamide gel electrophoresis, and autoradiography.

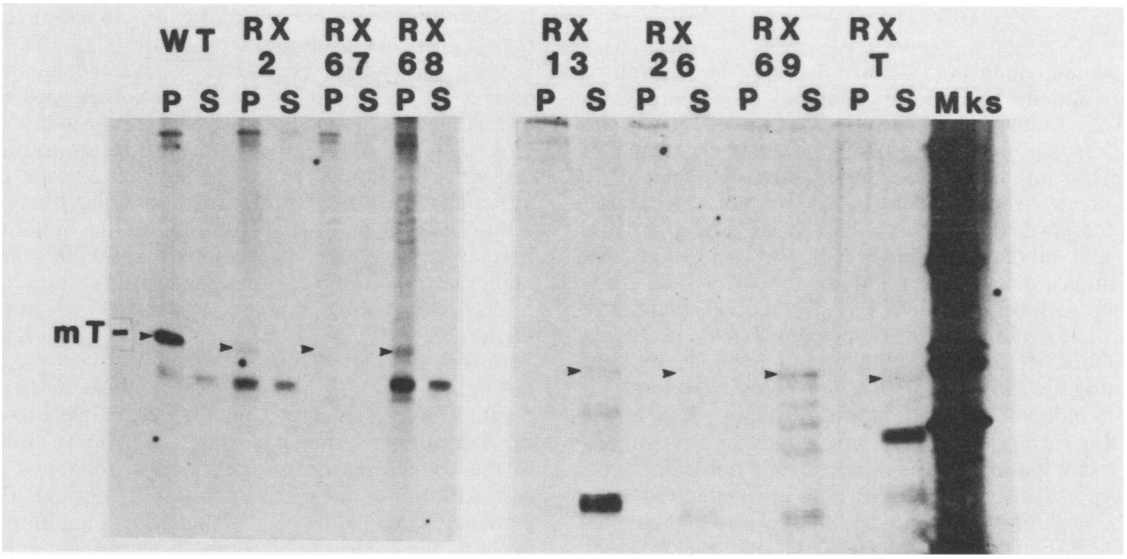

FIG. 5. Cellular membrane association of the middle-T antigens. Neomycin-resistant cell lines expressing selected RX-mutant-encoded middle-T antigens were incubated in the presence of [^{35}S]methionine, followed by lysis in a hypotonic buffer. The lysate was fractionated into a crude membrane fraction (P) and cytosol (S) by differential centrifugation, followed by immunoprecipitation with rat polyomavirus anti-tumor cell serum and analysis by polyacrylamide gel electrophoresis and fluorography. mT, Middle-T; Mks, markers.

inside the hydrophobic domain (encoded by RX13, RX26, RX69, and RXT) did not show associated kinase activity (Fig. 4b). Middle-T species with only a single predicted charged amino acid within the hydrophobic region had phosphate acceptor activity, although the level of phosphorylation was reduced when compared with wild-type middle-T (Fig. 4c). These RX2-, RX67-, and RX68-encoded middle-T antigens also induced the phosphorylation of the immunoglobulin fraction when rat anti-tumor cell serum was used, but to a lesser extent than wild-type middle-T (Fig. 4d).

To establish whether the RX-mutant-encoded middle-T species were able to associate with pp60$^{c\text{-}src}$, a similar kinase assay was carried out after immunoprecipitation with an antibody specific for pp60$^{c\text{-}src}$ in place of an antibody to middle-T. Wild-type middle-T, as well as RX2-, RX67-, and RX68-encoded middle-T antigens, demonstrated an association with pp60$^{c\text{-}src}$, since they were phosphorylated in the kinase reaction (Fig. 4e). The reduced level of phosphorylation for the mutant middle-T species was similar to that observed when an antibody to middle-T was used. Middle-T species encoded by RX13, RX26, RX69, and RXT did not associate with pp60$^{c\text{-}src}$ when the same assay was performed (data not shown).

Membrane association of the hydrophobic-domain mutant middle-T species. The subcellular distribution of the RX-mutant-encoded middle-T species was determined by the methodology of Templeton et al. (25). Neomycin-resistant NIH 3T3 cell lines expressing the RX-mutant middle-T antigens were metabolically labeled with [^{35}S]methionine and then lysed in a hypotonic buffer and fractionated by differential centrifugation. The cytosol and membrane fractions were analyzed by polyacrylamide gel electrophoresis, followed by fluorography. The results are shown in Fig. 5 and summarized in Table 1.

Wild-type middle-T, as well as the proteins with lesions outside the hydrophobic domain, were found to be present in the fraction sedimenting at 100,000 × g, which contains the bulk of cellular membranes. The RX-mutant-encoded proteins with deletions within the hydrophobic domain (RX13, RX26, RX69, and RXT) were present in the supernatant fraction, whereas the middle-T species with small lesions within the hydrophobic domain (RX2, RX67, and RX68) were present in the fraction containing cellular membranes.

	389		421
MuPY m-T	RRLGR	TLLLVTFLAALLGICLMLFILI	KRSRHF STOP
	369		401
HapV m-T	RNRLRR	LVLMIFLLSLGGFFLTLFFLI	KRKMHL STOP
	391		424
pIII	INLFR	GVFAFLLYVATTMYVFSFTANIL	RNKES STOP
	457		489
VSV-G	WFSSWK	SSIASFFFIIGLIIGLFLVL	RNGIHLC
	528		560
Inf-HA	YKD	WILWISFAISCFLLCVVLLGFIMW	ACQKGN

FIG. 6. Hydrophobic domains and surrounding amino acids of murine polyomavirus middle-T (MuPY m-T), hamster papovavirus middle-T (HapV m-T), bacteriophage f1 protein III (pIII), vesicular stomatitis virus glycoprotein (VSV-G), and influenza virus hemagglutinin (Inf-HA). The amino acids are represented by a single-letter code, the limits of the hydrophobic regions are represented by gaps, and those residues showing homology to polyomavirus middle-T are underlined. The numbers refer to the location of the sequences in the parent protein, and stop indicates the carboxyl terminus of the protein. The single-letter amino acid code is given in the legend to Fig. 2.

DISCUSSION

Hydrophobic-domain mutants of middle-T. The region of DNA encoding the hydrophobic domain of polyomavirus middle-T was mutated to determine both the effects on the membrane association of the protein and the consequence of changes in the putative membrane-binding domain on the biological properties of middle-T. The mutants generated could be categorized into three groups as judged by the biological and biochemical properties investigated in this study. Examples of each grouping are shown in Table 1.

One group of mutants displayed an essentially wild-type phenotype. The proteins they encode contain lesions outside the hydrophobic domain (affecting amino acids 392 to 350), demonstrating that this section of amino acids is not required in middle-T-induced transformation. Proteins with larger lesions in this region produced fewer foci in the transformation assay, with a delay in appearance when compared with the wild type. However, we attribute this effect to conformational problems associated with the size of the predicted lesion rather than to the deletion of essential residues from this particular region of middle-T.

The second group of proteins did not associate with cellular membrane fractions or with pp60$^{c\text{-}src}$ and were unable to transform established rodent cells in vitro. They either have extensive lesions within the hydrophobic domain or are truncated, lacking all or most of the putative membrane-binding domain, similar to the published middle-T termination mutants MOPS (24) and Py1387T (5).

A third group of RX mutant-encoded proteins retained all of the biochemical properties associated with wild-type middle-T investigated in this study yet were unable to transform the growth properties of Rat-1 cells or NIH 3T3 cells. These mutants contain small substitution mutations within the region encoding the putative membrane-binding domain, which includes the introduction of a single charged amino acid. This phenotype is similar to that reported for the MT-G1 mutant middle-T (25), in which the hydrophobic domain of middle-T had been replaced by the VSV-G membrane anchorage domain.

Mechanisms of transformation. A current model for the transformation of cultured rodent cells by polyomavirus middle-T suggests that an interaction between middle-T and pp60$^{c\text{-}src}$ increases the activity and possibly the specificity of this tyrosine kinase (4, 8). The first and second group of RX mutants can be understood within the framework of this model. The first group was essentially wild type, whereas the second group encoded proteins which were unable to associate with pp60$^{c\text{-}src}$ and hence unable to induce the processes involved in cellular transformation. The third group of RX mutants can be included in a class together with *dl*23 (15), *dl*1015 (16), and *dl*2208 (17), whose protein products associate with pp60$^{c\text{-}src}$ but nevertheless fail to induce transformation. This observation implies that although the association of middle-T with pp60$^{c\text{-}src}$ is necessary for transformation, it is not sufficient (10). A reason for the transformation defect in the RX-encoded middle-T proteins could potentially be their instability. The half-lives of the mutant-encoded proteins are being investigated.

A comparison of hydrophobic domains. Recently published data regarding the importance of putative membrane-binding domains within model proteins have involved mutagenic techniques similar to that detailed in this study. Deletion mutagenesis of the DNA encoding the 23-uncharged-amino-acid carboxyl-terminal domain of bacteriophage f1 gene III demonstrated that this domain is necessary for the correct localization and processing of the protein within the bacterial membrane (11). A mutational analysis of the membrane-binding domain of VSV-G (1) converted an internal isoleucine into either glutamine (a polar uncharged amino acid) or arginine (a charged amino acid). The former lesion had no effect on the protein, whereas the latter interrupted the transport of the glycoprotein to the plasma membrane, although it did not prevent it from spanning the lipid bilayer. Subtle effects on the interaction of the mutant middle-T species with cellular membranes may explain, in part, the defect in transformation observed in this study.

The hydrophobic domain of middle-T is important for membrane association; however, it has been demonstrated here that the mere presence of middle-T in the membrane fraction of cellular homogenates and an association with pp60$^{c\text{-}src}$ are insufficient to induce transformation. Whether the hydrophobic domain of middle-T plays an additional role in the transformation process, other than just membrane association, requires consideration. Although there is no evidence that middle-T is an intrinsic membrane-spanning protein, the hydrophobic domain shares common features with the anchorage domains of VSV-G, the pIII protein of the f1 bacteriophage, and influenza virus hemagglutinin. They have a minimum of 20 contiguous uncharged and predominantly hydrophobic amino acids bounded by a group of basic charged residues (Fig. 6). The nature of this sequence is thought to reflect the need for an alpha helical structure which both spans the lipid bilayer and maximizes hydrophobic interactions between protein and lipid (11). The hydrophobic domains of polyomavirus middle-T, f1 pIII, VSV-G, and influenza hemagglutinin (Fig. 5) show little similarity in amino acid sequence other than the general considerations described above. In contrast, comparison of murine polyomavirus middle-T with that of a recently sequenced, closely related papovavirus found in hamsters (12) demonstrates a strong conservation in amino acid sequence within the respective hydrophobic domains. This homology is all the more striking in that, within the carboxyl-terminal half of the middle-T species, the only other conserved region is an eight-amino-acid sequence around tyrosine 315 of murine polyomavirus middle-T. There is a strong possibility that the middle-T antigens of the hamster and murine viruses play a similar role. The conserved nature of the hydrophobic domain implies that this region is more than a simple tract of hydrophobic residues and possibly suggests that the amino acid sequence has some other significance, for example, in the interaction with another membrane-associated molecule.

It is apparent from this and several earlier studies that the hydrophobic domain of middle-T is required for membrane association and that membrane association is required for both pp60$^{c\text{-}src}$ association and the induction of transformation. However, the reasons for the transformation defect in the RX-mutant-encoded middle-T species, which are able to associate with cellular membranes and pp60$^{c\text{-}src}$, are unknown and are being investigated. One implication of this study is that the interaction between middle-T and pp60$^{c\text{-}src}$ is easily perturbed by mutations, even though in some instances it is not abolished. The perturbations may stem from effects upon the interaction between the two molecules (6) or the cellular localization of the complex. Their result may be to prevent the complex from interacting with key elements in the cascade that eventually triggers transformation. The identification of those key elements, whether they are proteins, lipids, or other molecules, is essential for our further understanding of polyomavirus-induced transformation.

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