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Polyomavirus Middle T-Antigen NPTY Mutants

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A polyomavirus middle T-antigen (MTAg) mutant containing a substitution of Leu for Pro at amino acid 248 has previously been described as completely transformation defective (B. J. Druker, L. Ling, B. Cohen, T. M. Roberts, and B. S. Schaffhausen, J. Virol. 64:4454–4461, 1990). This mutant had no alterations in associated proteins or associated kinase activities compared with wild-type MTAg. Pro-248 lies in a tetrameric sequence, NPTY, which is reminiscent of the so-called NPXY sequence in the low-density-lipoprotein receptor. In the low-density-lipoprotein receptor, mutations in the NPXY motif but not in the surrounding amino acids abolish receptor function, apparently by decreasing receptor internalization (W. Chen, J. L. Goldstein, and M. S. Brown, J. Biol. Chem. 265:3116–3123, 1990). To determine whether this sequence represents a functional motif in MTAg as well, a series of single amino acid substitutions was constructed in this region of MTAg. All of the mutations of N, P, T, or Y, including the relatively conservative substitution of Ser for Thr at amino acid 249, resulted in a transformation-defective MTAg, whereas mutations outside of this sequence allowed mutants to retain near-wild-type transformation capabilities. Transformation-defective mutants with mutations in the NPTY region behaved similarly to the mutant with the original Pro-248–to–Leu-248 mutation when assayed for associated proteins and activities in vitro; that is, they retained a full complement of wild-type activities and associated proteins. Further, insertion of the tetrameric sequence NPTY downstream of the mutated motif restored transforming abilities to these mutants. Thus, the tetrameric sequence NPTY in MTAg appears to represent a well-defined functional motif of MTAg.

Polyomavirus induces a variety of tumors in rodents and is capable of transforming primary cell lines. The early region of polyomavirus, which encodes three tumor (T) antigens, large, middle, and small T antigens, is responsible for the transforming properties of the virus (reviewed in reference 42). Although the three T antigens cooperate to produce a fully transformed phenotype, middle T antigen (MTAg) is both necessary and sufficient for transformation of established cell lines (2, 9, 14, 19, 23, 27, 36, 43).

MTAg transforms cells at least in part through its association with intracellular signal-transducing proteins. The list of MTAg-associated proteins includes *src* family members ($pp60^{c\text{-}src}$, $pp59^{c\text{-}fyn}$, and $pp62^{c\text{-}yes}$ [4, 8, 10, 12, 13, 24]), the 85-kDa subunit of a phosphatidylinositol (PI)-3 kinase (11, 20, 21, 32), and the catalytic and regulatory subunits of protein phosphatase 2a (35, 44). In the case of *src* family members, association with MTAg results in an increase in specific activity. The specific activity of $pp60^{c\text{-}src}$ associated with MTAg is increased as much as 50-fold (3). Mutational analysis of MTAg has demonstrated that each of these activities appears necessary for transformation (reviewed in reference 31). However, mutants such as *dl*1015 also associate with and activate the *src* family members and PI-3 kinase, as measured in vitro, yet are transformation defective (11, 32). This leads to the conclusion that not even the combination of all three activities as measured in vitro is sufficient for transformation by MTAg.

Another mutant, with properties similar to those of *dl*1015, is 248m, which encodes a MTAg with a single amino acid substitution of Leu for Pro at amino acid 248 (15, 16). This mutant retains all known MTAg-associated proteins and activities as measured in vitro (15). The mutant 248m lies in a tetrameric amino acid sequence (Asn, Pro, Thr, Tyr [NPXY]) spanning amino acids 247 to 250. A similar sequence in the low-density-lipoprotein (LDL) receptor has a role in receptor-mediated internalization into clathrin-coated pits. Thus, mutations in N, P, or Y but not X resulted in decreased receptor internalization following ligand binding (7). In addition to the Pro-to-Leu substitution at 248, a mutant containing Tyr-250–to–Phe-250 (pT250) is known to be transformation defective (30). Although pT250 is known to associate with and activate $pp60^{c\text{-}src}$, its association with or activation of the PI-3 kinase has not been evaluated. Further, mutation of Ser-251 to Phe-251 (30) or deletion of amino acids 252 to 283 (*dl*8) (18) has no deleterious effect on transformation by MTAg. In fact, *dl*8 appears to be a more-potent transforming protein than wild-type MTAg as measured by focus formation and growth in soft agar (15a). These data suggested that the NPXY motif of MTAg could be a functional motif. To test this hypothesis, a variety of point mutations were introduced into MTAg from amino acids 246 to 250. These mutants demonstrate that all substitutions in amino acids 247 to 250, the NPXY sequence, including a relatively conservative substitution of Ser for Thr at amino acid 249 (X), result in a transformation-defective MTAg. All of these mutants behave similarly to the mutant containing the original Pro-to-Leu substitution at amino acid 248 in that they associate with and fully activate all of the known MTAg-associated proteins as measured in vitro. Transforming capabilities can be restored to these mutants by the insertion of a wild-type NPTY sequence downstream of the mutated version of this motif.

MATERIALS AND METHODS

Construction of MTAg point mutants. All plasmid constructions were carried out by using standard molecular cloning techniques (37). Restriction enzymes were purchased from New England BioLabs. The *Sph*I-*Nco*I frag-

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ment of pUC8-MT (32) (nucleotides 701 to 1274) (40) was cloned into pBD5 as previously described (16). Single-stranded DNA for mutagenesis was obtained by standard methods (37). Mutagenesis was performed with synthetic oligonucleotides used according to the manufacturer's instructions (Amersham) (41) with minor modifications. As the plasmid used for mutagenesis contained five *Nci*I sites, the time of exposure to exonuclease III was reduced to 20 min. Further, in case the second strand was digested completely with exonuclease III, a reverse primer corresponding to the 5′ end of the ampicillin resistance gene was annealed to the digested plasmid prior to incubation with DNA polymerase I. Oligonucleotides used for mutagenesis were as follows: GTCTTCTG(A/G)(C/T)CACAAAGAC for substitutions at Ser-246, CTGAGCG(G/A/T/C)CCCGACC for substitutions at Asn-247, GAGCAAC(G/A/T/C)(G/A/T)(T/C)ACCTATTC for substitutions at Pro-248, GCAACCCG(T/G)(T/C)CTAT TCTG for substitutions at Thr-249, and CCCGACC(G/T)(C/T)TTCTGTTATG for substitutions at Tyr-250. Nucleotides in parentheses represent degenerate sequences. Following mutagenesis, individual colonies were screened for desired mutations by sequencing of single-stranded DNA by dideoxy sequencing (Sequenase; USB). The entire *Sph*I-*Nco*I fragments of plasmids containing mutations were sequenced to ensure that no second-site mutations had occurred. The *Sph*I-*Nco*I fragment containing mutations was then cloned into a similarly digested full-length MTAg cDNA pBD15-MT (16). Single-stranded DNA obtained from this plasmid was sequenced to ensure that the wild-type sequence had been replaced with the desired mutation. The MTAg cDNAs containing the desired mutations were then cloned into the retrovirus pLJ (22).

Construction of NPTY insertion mutants. Oligonucleotides were synthesized such that they would encode the amino acids NPTY or controls in the proper reading frame and could be cloned into the unique *Esp*I site of pBD15-MT. By the nature of the oligonucleotide constructs, an additional leucine would be encoded at the end of each of the various constructs. Single-stranded DNAs from all insertion mutants were sequenced to ensure that the proper insertion had been obtained. The MTAg cDNAs containing the desired mutations were then cloned into the retrovirus pLJ.

Cells and tissue culture. BALB/3T3 (clone A31) cells (from C. D. Scher) and Psi-2 cells (gift from C. Cepko) (6) were maintained in Dulbecco modified Eagle medium supplemented with 10% donor calf serum (GIBCO) at 37°C in 10% CO_2.

Retrovirus infections and establishment of BALB/3T3 clones. Transfection of Psi-2 cells, infections with virus supernatants, drug selection, and isolation of G418-resistant clones were carried out as previously described (9).

Transformation assays. Focus formation assays were performed as previously described (32). At the end of the assay period (3 to 5 weeks), cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and stained with methylene blue as previously described (9).

Radiolabeling of cells and immunoprecipitations. The procedures for radiolabeling of cells and immunoprecipitations have been published in detail elsewhere (38, 39). Immunoprecipitates were boiled for 5 min in a sodium dodecyl sulfate (SDS) dissociation buffer (0.0625 M Tris [pH 6.8], 5% [wt/vol] SDS, 5% [wt/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol, 0.015% [wt/vol] bromophenol blue) and analyzed on discontinuous buffer SDS-polyacrylamide gels (26). Washed immunoprecipitates from [^{35}S]methionine-labeled cultures were subjected to a second round of immunoprecipitation as previously described (15).

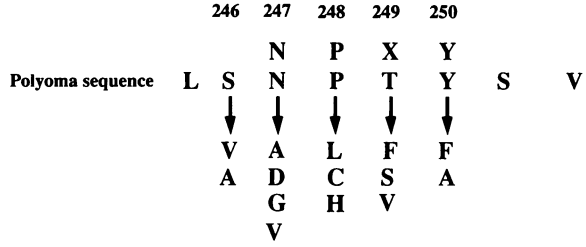

FIG. 1. Schematic of mutations introduced into MTAg. The amino acid sequence of MTAg from amino acid 245 through 252 (numbering per reference 40) is depicted with single-letter amino acid abbreviations. Single amino acid substitutions introduced into MTAg are indicated with arrows below the corresponding wild-type sequence. NPXY above the wild-type sequence indicates the position of this tetrameric sequence in MTAg.

Kinase reactions in vitro. Confluent cultures of MTAg-expressing cells were lysed, immunoprecipitated, and washed as previously described (46) except that lysis buffers contained 1 mM sodium orthovanadate. Protein amounts were determined by using the Bradford assay (Bio-Rad), and immunoprecipitations were performed on extracts normalized for MTAg as determined by immunoblotting or by immunoprecipitation of metabolically labeled cultures. In vitro protein kinase assays were performed as described elsewhere (20) except that PI kinase assays were performed in the presence of 200 μM adenosine, as this has been shown to inhibit contaminating PI-4 kinase activity (45).

Immunoblotting. Immunoblotting was performed as previously described (33) except that 3% bovine serum albumin in phosphate-buffered saline was used to block excess binding sites on the nitrocellulose. The rabbit anti-polyomavirus T-antigen serum used was provided by D. Pallas (34) and was diluted 1:2,200 for incubation with blots.

RESULTS

Construction of mutants. Mutations in the tetrameric sequence NPXY are known to affect receptor-mediated internalization of the LDL receptor (7). In MTAg, two mutations in this sequence, Pro to Leu at amino acid 248 and Tyr to Phe at amino acid 250, result in a transformation-defective MTAg (15, 29), while mutations outside of this region, Ser to Phe at amino acid 251 or deletion of amino acids 252 to 283 (*dl*8) (18, 29), do not result in any decrease in the transforming ability of MTAg. In order to evaluate more thoroughly whether this tetrameric sequence is a functional motif of MTAg, a variety of single amino acid substitutions were introduced into this region of MTAg by using site-directed mutagenesis (Fig. 1). The fragment used for mutagenesis was sequenced in its entirety and replaced into its proper sequence context in an MTAg cDNA (16). The resulting MTAg was again sequenced through the mutated site to confirm that a substitution had been created. Each mutant will be referred to by the amino acid which was mutated and by the substitution induced (e.g., Ala-247 and Cys-248, etc.).

Transformation assays. Each mutant was placed into the retroviral vector pLJ (22). Mutants, wild-type MTAg, and control retrovirus were transfected into a packaging cell line, Psi-2, and the transiently expressed virus was used to infect BALB/3T3 cells. BALB/3T3 cells were split 2 days following infection, and half were selected for geneticin (G418) resis-

TABLE 1. Focus-forming assay of MTAg mutants

Construct	No. of:		%[a]
	G418-resistant colonies	Foci	
pLJ	618	3	<1
WT-MT	460	337	73
Ala-246	114	63	55
Val-246	93	49	53
Ala-247	212	2	1
Asp-247	221	4[b]	2
Gly-247	124	1	1
Val-247	111	1	1
Cys-248	136	1	<1
His-248	102	0	<1
Phe-249	119	6[b]	5
Ser-249	238	9[b]	4
Val-249	102	2[b]	2
Ala-250	91	1[b]	1
Phe-250	104	5[b]	5

[a] Percentage of G418-resistant colonies forming foci. Values are combinations of values from at least three separate experiments.

[b] Foci formed late in the course of the experiments and were smaller than wild-type foci.

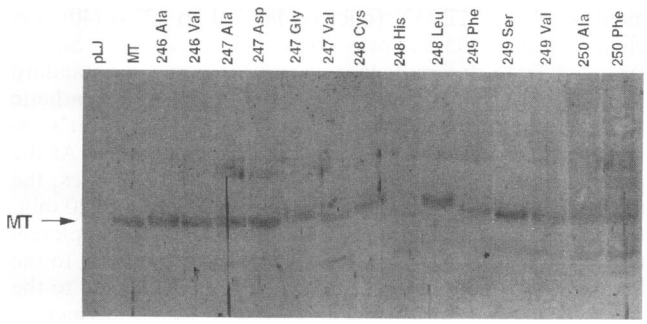

FIG. 2. Immunoblot analysis of MTAgs expressed in BALB/3T3 fibroblasts. Nonidet P-40 lysates of individual G418-resistant colonies expressing control retrovirus (pLJ), wild-type (MT), or the various MTAg mutants were separated on a 9% SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. Blots were probed with rabbit polyclonal antipolyomavirus T-antigen serum as described in Materials and Methods. MTAg mutants are referred to by the amino acid which was mutated and by the substitution induced, e.g., 247 Ala. The migration of MTAg is indicated with an arrow.

tance, while the other half were allowed to grow for 3 to 5 weeks as a monolayer. The numbers of G418-resistant colonies versus the numbers of foci formed on monolayers are compiled in Table 1. As can be seen, mutations at amino acid residue 246 resulted in a slight decrease in the efficiency of focus formation. However, morphologically, cells expressing Ala-246 or Val-246 did not differ from wild-type-MTAg-expressing cells. In contrast, all mutations in amino acids 247 to 250 (NPTY) resulted in a severely transformation-defective MTAg. Foci were observed at a low frequency in cells expressing Ser-249, Phe-249, and Phe-250; however, these foci appeared late in the experiments and were never as large as foci obtained with wild-type MTAg. Thus, all mutations in the NPTY sequence of MTAg, including the relatively conservative substitution of Ser for Thr at amino acid 249, resulted in a transformation-defective MTAg, while changes outside of this region had little effect on the transforming ability of MTAg.

MTAg protein production. In order to determine whether the transformation defect in any of these mutants was simply a lack of MTAg protein production, pooled G418-resistant colonies and 6 to 10 individual G418-resistant colonies of each mutant were selected for further analysis. By immunoblot analysis with a polyclonal rabbit anti-T-antigen serum, a protein of 56 to 58 kDa was seen in all MTAg-expressing clones; this protein is presumed to represent MTAg (data not shown). An average expressing clone of each mutant was selected for further comparative analysis. An anti-T-antigen immunoblot of these clones is shown in Fig. 2. With the exception of Ala-250 and Phe-250, similar amounts of protein were required to obtain similar intensities of MTAg staining by this immunoblot analysis. The Ala-250 and Phe-250 mutants appear to express lower steady-state levels of MTAg protein, and this difference may contribute to their transformation-defective phenotype. However, this is not the case for the rest of the mutants. Similar results were obtained by immunoprecipitation of MTAg from [^{35}S]methionine-labeled cultures (Fig. 3). The relative amounts of MTAg observed by immunoblotting (Fig. 2) were similar to those observed in this assay, suggesting that there is no apparent difference in labeling of the various MTAg mutants. As assessed by one-dimensional analysis, each of the mutants appears to have a similar complement of associated proteins in comparison to wild-type MTAg (data not shown). In particular, associated proteins of 85, 63, and 36 kDa can be identified in one-dimensional gels of immunoprecipitations of these mutants.

MTAg-associated kinase activities. Colonies selected as described above for relatively similar levels of MTAg expression were analyzed for the abilities of MTAg immunoprecipitates to phosphorylate an exogenous substrate in vitro. Normalization for MTAg was performed by densitometric scanning of the [^{35}S]methionine-labeled immunoprecipitated MTAg. All of the MTAg mutants demonstrated increased levels of tyrosine kinase activity in vitro compared with that of the control (Fig. 4). Although there was some variation in the level of kinase activity from experiment to experiment, the only mutant which consistently demonstrated lower levels of kinase activity than wild-type MTAg was the mutant containing Cys-248. In experiments in which

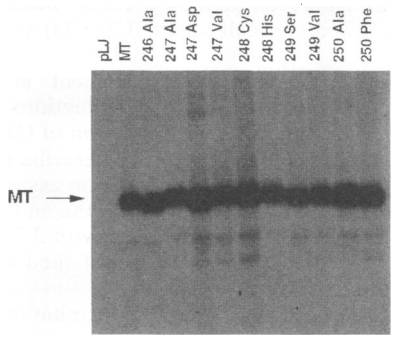

FIG. 3. [^{35}S]methionine labeling and immunoprecipitation of MTAg expressed in BALB/3T3 fibroblasts. BALB/3T3 cells expressing control retrovirus, wild-type MTAg, or selected mutants were labeled with [^{35}S]methionine (1 mCi/ml) for 4 h prior to lysis. Extracts were double immunoprecipitated by using anti-T-antigen serum as described in reference 15. Samples were resolved on a 9% SDS–polyacrylamide gel and visualized by fluorography. The figure is labeled as described for Fig. 2.

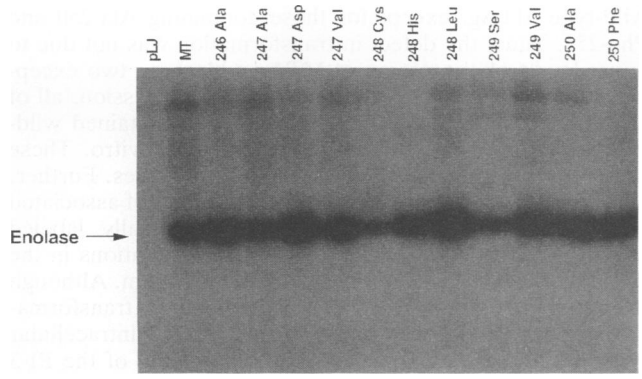

FIG. 4. Tyrosine kinase activity associated with mutant MTAgs. Anti-T-antigen immunoprecipitates were assayed for their abilities to phosphorylate enolase as an exogenous substrate. Immunoprecipitates were from extracts normalized for MTAg expression by densitometric scanning of the [^{35}S]methionine-labeled extracts (Fig. 3). Samples were resolved on a 9% SDS–polyacrylamide gel and visualized by autoradiography. The migration of enolase is indicated with an arrow. The figure is labeled as described for Fig. 2.

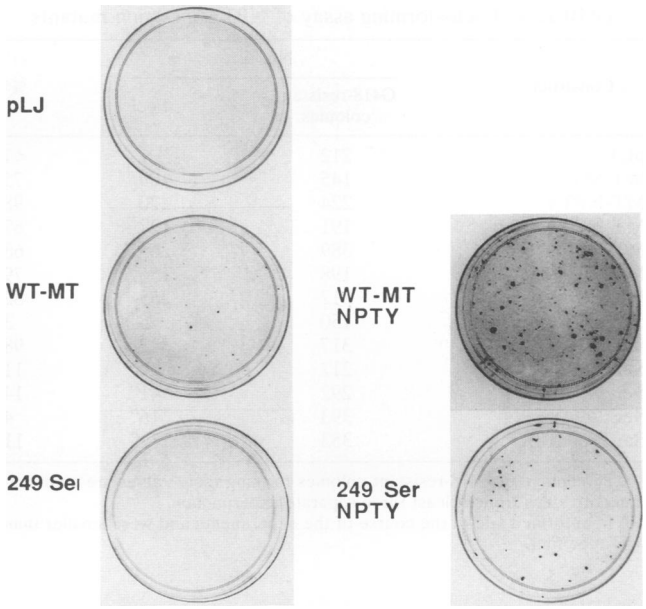

FIG. 6. Focus-forming assay of NPTY insertion mutants. Focus formation on monolayers of BALB/3T3 cells after infection with control retrovirus (pLJ), wild-type MTAg, or the indicated mutant. Cultures were incubated in Dulbecco modified Eagle medium plus 10% donor calf serum for 2 weeks, fixed, and stained with methylene blue. At 2 weeks, the MTAg foci typically are relatively small, as seen here.

no exogenous substrate was added to the kinase reaction mixture, a protein of relative molecular size 85 kDa was phosphorylated in this immune complex kinase assay by all of the mutants, including the one containing Cys-248 (data not shown). This 85-kDa protein is presumed to be a subunit of the MTAg-associated PI-3 kinase. To address the levels of lipid kinase associated with each MTAg mutant, PI-3 kinase assays were performed on these mutants (Fig. 5). Again, all of the mutants had increased levels of associated PI-3 kinase activity compared with that of the control, and with the exception of the mutant containing Cys-248, all of the mutants had levels of associated PI-3 kinase activity similar to that of wild-type MTAg.

NPTY restores transforming ability to mutants with mutations in the NPTY motif. In order to further investigate whether NPTY represents a functional motif of MTAg, an attempt was made to complement the mutations in NPTY by inserting a second wild-type copy of the motif at another site in the mutant protein. Insertions encoding NPTY were placed at an *Esp*I site of MTAg corresponding to amino acid 283. This site was selected because it is deleted in the mutant *dl*8 without any adverse effect on function. When placed into wild-type MTAg, this insertion surprisingly enhanced MTAg transforming function as measured in a focus-forming assay (Fig. 6). Thus, foci are formed earlier, by 5 to 7 days as opposed to 7 to 10 days for wild-type MTAg, and foci are larger and form at a higher percentage of G418-resistant colonies than of wild-type MTAg colonies (Table 2 and Fig. 6). When NPTY was inserted downstream of mutated versions of NPTY, transforming ability was restored to each of the mutants tested (Fig. 6 and Tables 1 and 2). In order to make sure that this was not a consequence of any insertion at this *Esp*I site, various controls for the NPTY sequence were inserted at this site in wild-type MTAg and in the mutant containing Ser-249. These controls included NPTA, based on changing Tyr-250 to Phe; NLTY, based on changing Pro-248 to Leu; YTPN, which is NPTY backwards; and IRSG, which is four random amino acids. None of the insertions restored transforming ability to Ser-249, and none

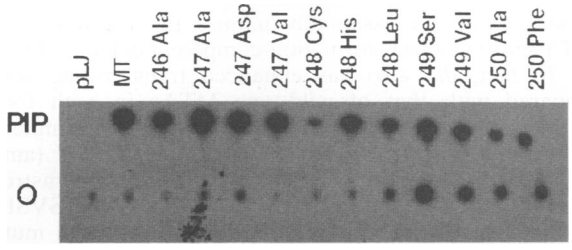

FIG. 5. PI kinase activity associated with MTAg mutants. Anti-T-antigen immunoprecipitates, normalized as described for Fig. 4, were analyzed for their abilities to phosphorylate PI, as described in Materials and Methods. Products of the reaction in vitro were analyzed by thin-layer chromatography and autoradiography as described elsewhere (20). O, origin; PIP, PI phosphate. The figure is labeled as described for Fig. 2.

TABLE 2. Focus formation by NPTY insertion mutants

Construct	No. of:		%[a]
	G418-resistant colonies	Foci	
pLJ	332	1	<1
WT-MT	140	104	74
MT-NPTY	341	310	91
Ala-247–NPTY	216	202	94
His-248–NPTY	51	41	80
Ala-249–NPTY	240	228	95
Ser-249–NPTY	208	204	98
Phe-250–NPTY	163	153	94
Val-250–NPTY	90	83	92

[a] Percentage of G418-resistant colonies forming foci. Values are combinations of values from at least three separate experiments.

TABLE 3. Focus-forming assay of NPTY insertion mutants

Construct	No. of:		%[a]
	G418-resistant colonies	Foci	
pLJ	212	1	<1
WT-MT	145	109	75
MT-NPTY	224	220	98
MT-NPTA	191	127	67
MT-NLTY	389	263	68
MT-YTPN	198	156	79
MT-IRSG	312	255	82
Ser-249	150	5[b]	3
Ser-249–NPTY	317	312	98
Ser-249–NPTA	212	24[b]	11
Ser-249–NLTY	292	41[b]	14
Ser-249–YTPN	393	16[b]	4
Ser-249–IRSG	353	39[b]	11

[a] Percentage of G418-resistant colonies forming foci. Values are combinations of values from at least three separate experiments.

[b] Foci formed late in the course of the experiments and were smaller than wild-type foci.

seemed to alter significantly the focus-forming ability of wild-type MTAg (Table 3). Although a slight increase in the number of foci was observed for these insertions into Ser-249, the increase occurred at a very low percentage of G418-resistant colonies, and the foci were smaller than wild-type foci and formed late in the course of the experiments. Thus, only the sequence NPTY was capable of restoring full transforming capabilities. All of these insertion mutants encode a stable MTAg at levels similar to that of the wild-type MTAg, as assessed by immunoblotting, and retain a full complement of associated activities when assayed in vitro (data not shown).

DISCUSSION

A series of mutations was created in the NPTY sequence of MTAg. All of the mutations in the NPTY sequence of MTAg, including the relatively conservative substitution of Ser for Thr at amino acid 249, resulted in a transformation-defective MTAg. Changes outside of this region had little effect on the transforming ability of MTAg. Further, transforming ability could be restored to these mutants by the insertion of an NPTY sequence, but not control sequences, at a site downstream of the mutant site. These data suggest that the tetrameric sequence NPTY is a functional motif of MTAg.

The data obtained for MTAg are slightly different from those obtained for the LDL receptor in that mutations in the Thr residue of MTAg decreased function. Mutations at this position of the LDL receptor had little effect on function, and thus the position was termed X, representing any amino acid (7). In our assays, mutations at the Thr residue retained a small amount of residual transforming ability, suggesting that this residue may be less important than surrounding residues to the transforming ability of MTAg. Further, it has been suggested that in other systems, a polar or positively charged amino acid residue just preceding the Tyr is relatively important to this functional motif (25). Although this might explain the transformation defect of several of the amino acid substitutions at amino acid 249, it could not explain the transformation-defective phenotype of Ser-249.

All of the mutants containing mutations in the NPTY sequence encode a stable MTAg at levels similar to those of wild-type MTAg, except for those containing Ala-250 and Phe-250. Thus, the defect in transformation was not due to lower levels of expression of MTAg with these two exceptions. When matched for levels of MTAg expression, all of the mutants except that containing Cys-248 retained wild-type levels of kinase activity as measured in vitro. These included tyrosine kinase and PI-3 kinase activities. Further, all of the mutants had a wild-type complement of associated proteins in immunoprecipitates of metabolically labeled MTAg. Therefore, all of the mutants with mutations in the NPTY motif behave similarly to the original 248m. Although the mutant containing Phe-250 was known to be transformation defective and to associate with and activate intracellular tyrosine kinases, its association and activation of the PI-3 kinase had not previously been evaluated.

The abnormality responsible for the transformation-defective phenotypes of these mutants remains unclear. The NPTY motif has a role in receptor-mediated internalization of the LDL receptor (7). Certainly, if MTAg and its associated proteins and activities were required in a particular subcellular compartment for transformation to occur, it would not be unreasonable to think that a transport protein might be required for this function. However, using immunofluorescence and subcellular fractionation, we have thus far been unable to document a difference between wild-type MTAg and NPTY mutants. Although an alteration in subcellular localization of MTAg or its associated activities remains a formal possibility, another possibility is that the NPTY sequence is a binding motif for an as-yet-unknown protein which is not involved in internalization. Nuclear magnetic resonance spectroscopy has shown that the NPXY motif adopts a reverse-turn conformation (1, 17). These data suggest that this motif could either participate in a binding interaction or be a structural motif for internalization signals.

Recently, it has been demonstrated that cells expressing 248m and *dl*1015 do not contain elevated levels of the products of the PI-3 kinase, particularly PI-3,4,5-trisphosphate, in vivo compared with wild-type-MTAg-expressing cells (28). This finding could represent an explanation for the transformation-defective phenotypes of these mutants. Since these mutants do associate with the PI-3 kinase as measured in vitro, it remains possible that the defect in these mutants is an inability to transport the activated PI-3 kinase to the correct subcellular compartment. This defect might then result in the inability to produce the necessary mitogenic PI-3 kinase products.

A singular result of these studies is that an MTAg which contains two wild-type NPTY sequences has enhanced transforming capabilities compared with wild-type MTAg as measured in a focus-forming assay. The site of insertion was chosen because it essentially mimics the location of the NPTY motif in a transformation-competent deletion mutant, *dl*8. In fact, *dl*8 also has enhanced transforming ability compared with that of wild-type MTAg in both focus-forming assays and soft-agar growth (15a). This mutation deletes amino acids 252 to 283. Thus, the NPTY (amino acids 247 to 250) sequence is moved to a downstream location, creating the amino acid sequence NPTYSVSPM. This sequence is nearly recreated in the insertion mutant NPTYLSPM. These sequences are similar to the proposed binding site for the PI-3 kinase of MTAg and the platelet-derived growth factor receptor (5). Thus, it is possible that a binding site for a new protein or activity is created or that a better binding site for a previously associated activity is created. Transport to a particular subcellular compartment could also be enhanced. Finally, it is of interest to consider

why MTAg can be engineered into a better transforming protein. Normally, MTAg is encoded by DNA sequences which include large and small T antigens through alternative splicing. Thus, MTAg is normally under sequence constraints from all three T-antigen functions. When removed from these constraints, insertions such as the NPTY sequence, which might interfere with large T-antigen function in the viral situation, become possible.

ACKNOWLEDGMENTS

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