

Association of p62^{c-yes} with Polyomavirus Middle T-Antigen Mutants Correlates with Transforming Ability

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A number of mutants of polyomavirus middle T antigen (MTag) were constructed into replication-competent avian retroviruses. To assess the ability of these MTag variants to transform and to associate with the avian p60^{c-src} and p62^{c-yes} proto-oncogene products, we used these viruses to infect chicken embryo fibroblasts. We found that the ability of individual mutant MTags to associate with p62^{c-yes} correlated well with the ability of these mutants to transform, as has been previously shown for the association of MTag with p60^{c-src}. All transformation-competent mutant MTags retained the ability to complex with p62^{c-yes}. Two transformation-defective mutants, RX67 and RX68, which could weakly associate with p60^{c-src}, were unable to associate with p62^{c-yes}. dl1015, a transformation-defective mutant which could associate with p60^{c-src} and with a phosphatidylinositol kinase activity, was also able to associate with p62^{c-yes}. Therefore, some as yet unmeasured biochemical property is defective in this mutant.

Transformation by polyomavirus is mediated primarily by the middle T antigen (MTag), a 58-kilodalton membrane-associated phosphoprotein encoded by the early region of the virus (9, 15, 26, 29, 32). Transfection of a plasmid encoding only the MTag can transform established rat cells (33). Similarly, MTag expressed from a murine leukemia virus vector can transform NIH 3T3 cells and MTag expressed from an avian retroviral vector or an amphotrophic murine leukemia virus vector can transform primary chicken embryo fibroblasts (CEF) (9, 17, 18). In addition, it is clear that MTag can induce a specific tumor type, hemangioma, upon expression in a variety of hosts, including chickens, transgenic mice, and chimeric mice (2, 18, 36).

The ability of MTag to transform cells in culture and induce tumors in animals may be due to its association with a variety of cellular proteins (4, 7, 11, 12, 19, 20). MTag can associate with and modulate the tyrosine kinase activity of several proto-oncogene products (p60^{c-src}, p62^{c-yes}, and p59^{c-fyn}). It has also been reported to form a complex with a phosphatidylinositol kinase (PI kinase) activity, which leads to the production of phosphatidylinositol-3-phosphate (PI-3-P) (7, 16, 34). Association with other, as yet unidentified proteins has also been reported (11, 12, 28).

The association of MTag with p60^{c-src} appears to be necessary but not sufficient for transformation by the MTag (3, 8, 22). Specifically, all mutants of MTag which can transform can associate with p60^{c-src}. However, there are mutants of MTag which do not transform but still associate with p60^{c-src}. Similar analyses of several mutants indicate that the association of the MTag-p60^{c-src} complex with PI kinase activity may also be necessary but not sufficient for transformation (7, 16). Examination of the transforming MTag mutant *dl8* and the nontransforming mutants *dl23*, *dl1015*, and *NG59* has shown *c-fyn* protein to behave like p60^{c-src} in terms of its ability to associate with these mutants (4, 20).

To assess the possible role of the MTag-p62^{c-yes} complex in transformation by the MTag, we have examined the association between p62^{c-yes} and a number of transformation-competent and transformation-defective MTag mutants. Since our anti-*yes* antibody recognizes avian p62^{c-yes}, we have cloned the battery of MTag mutants into avian retroviral vectors and examined the ability of the mutants to transform CEF, to induce hemangiomas in chickens, and to associate with avian *c-yes* protein.

MATERIALS AND METHODS

Cells and virus. Primary and secondary CEF were prepared as described by Hanafusa (13). Cell culture conditions were as described previously (13). pSR-XDMT1, the MTag-carrying plasmid used as the starting material for the construction of mutants into the avian retroviral vector, was previously described (18). Viral infections of cultured cells and injection into chickens were performed with 0.2 ml containing 2×10^5 focus-forming units of virus (after harvesting from transfected cultures). For focus formation, infected CEF were overlaid with 0.37% soft agar. Anchorage-independent growth was measured by plating fully infected CEF in 0.4% agar.

SRMT1 virus and its derivatives, carrying the MTag in the place of *v-src* of Rous sarcoma virus was injected into the wing webs of 1-week-old chickens. Beginning 1.5 weeks after injection, chickens were examined for the blood-filled hemangiomas characteristically formed at the site of wing web injection with the retrovirus carrying wild-type MTag (18).

Plasmid constructions. To facilitate insertion of the various MTag mutants into the avian retroviral vector pNR200 (18), the constructions were done in two steps. pBR322 was digested with *Aat*II and *Hind*III, and the DNA ends were made flush with T4 DNA polymerase. *Bgl*II linkers (CAG ATCTG) were ligated to these blunt ends with T4 DNA ligase, the plasmid was digested with *Bgl*II, and the linear

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fragment was isolated by gel electrophoresis. The plasmid was recircularized and transformed into *Escherichia coli* DH5. With this construction, the *EcoRI* site was eliminated from pBR322 and a *BglII* site was inserted.

The wild-type MTag-encoding sequences had been flanked with *BglII* linkers prior to cloning into pNR200 (to form pSR-XDMT1) (18). The wild-type sequences were excised with *BglII*, and the 1.5-kilobase MTag-containing fragment was ligated into the *BglII* site of the pBR322 derivative described above. The resulting construction, pBRMT, was digested with *BstXI* and *EcoRI*, which cut before the start codon of MTag and after the stop codon, respectively. The plasmids containing the mutant MTag-encoding sequences were digested with *BstXI* and *EcoRI*, and the individual mutant DNAs were cloned into the digested pBRMT vector. The resultant clones, containing mutant DNAs flanked by *BglII* linkers, were digested with *BglII*. The MTag-containing fragments were isolated by gel electrophoresis and ligated to *BglII*-digested, dephosphorylated pNR200. The mutant plasmids have been named pSR-XDmutant (i.e., specific mutant name).

Transfection. pSR-XDMT1 and pSR-XDmutant derivatives were digested with *SalI*, ligated to *SalI*-digested pREP-B (18) or BH-REP (14), and transfected onto CEF by the calcium phosphate method (35).

Protein biochemistry. To detect metabolically labeled MTag, fully infected CEF were starved for 2 h in minimal essential medium lacking methionine but containing 5% dialyzed calf serum. Plates (diameter, 60 mm) were labeled with 300 μ Ci of [³⁵S]methionine per ml for 4 to 6 h in 1 ml of methionine-free medium. Cells were lysed in RIPA buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units of aprotinin per ml), and lysates were clarified by centrifugation. Lysates were precleared with preimmune serum and then immunoprecipitated with anti-T antibody by using urea reversal to reduce the background as follows. Following addition of polyclonal anti-T serum and incubation for 2 h, immunoprecipitates were harvested with protein A-Sepharose. Urea (10 M) was used to resuspend the Sepharose, and then the Sepharose was spun down. The supernatant was diluted into lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 150 mM NaCl, 1 mM EDTA, 100 μ M sodium orthovanadate, 1% Nonidet P-40, 1% aprotinin) and incubated overnight at 4°C. Immune complexes were re-collected on protein A-Sepharose, washed with RIPA buffer, suspended in loading buffer, and electrophoresed on SDS-8.5% polyacrylamide gels. Gels were fluorographed in Amplify for 30 min.

Kinase assays were performed as described previously (18), with monoclonal antibody (MAb) 327, a gift from J. Brugge (21), or anti-*yes* antibody, a gift from M. Sudol (31), except that the kinase buffer contained 10 mM MnCl₂ and 50 mM HEPES (pH 7.0). For gradient sedimentation analyses, cells were lysed in RIPA buffer without SDS and loaded on a 10 to 30% continuous glycerol gradient. Centrifugation was carried out for 16 h at 44,000 rpm in an SW50.1 rotor. Each gradient fraction was immunoprecipitated with either MAb 327 or anti-*yes* antibody and assayed for kinase activity. Proteins were separated on SDS-8.5% polyacrylamide gels.

PI kinase assays and product analyses were performed as described previously (10).

TABLE 1. Published properties of MTag mutants when expressed in rodent cells

Mutant	Transforming ability ^a	p60 ^{c-src} association
SRMT1	+++	+
RX67	—	+
RX68	—	+
NG59	—	—
MG13	±	+ ^b
MG3	+++	+
pAS131	++	+
pTH	±	±
d11015	— ^c	+

^a As measured by focus formation or colony formation.

^b Association was not seen in chicken cells.

^c Can form some small foci but no colonies or tumors.

RESULTS

MTag mutants and retroviral constructs. The existing mutants of polyomavirus MTag fall broadly into three categories: (i) those which can transform and associate with p60^{c-src}, (ii) those which cannot transform but still associate with p60^{c-src}, and (iii) those which fail to associate with p60^{c-src} and cannot transform (5, 7, 8, 22, 23). We have selected a number of mutants from these three classes to characterize their ability to transform CEF and to associate with p62^{c-src}.

The previously reported properties of the mutants used in this study are described in Table 1. Briefly, the NG59, MG13, and MG3 proteins contain lesions in the N terminus of MTag, in the region of the molecule thought to be directly involved in p60^{c-src} binding (5). pTH and pAS131 contain mutations at the in vitro tyrosine phosphoacceptor sites of the MTag, and RX67 and RX68 carry mutations in and around the putative membrane-binding domain of the MTag, located at the C terminus of the molecule (22, 23). d11015 is a deletion mutant of MTag, with deletions from amino acids 338 to 347. The ability of these mutants to associate with p60^{c-src} when expressed in rodent cells is described in Table 1.

We constructed a series of avian retroviruses encoding the various mutants of MTag. We previously reported the construction of pSR-XDMT1, a plasmid encoding the wild-type MTag in the position of *v-src* in the Rous sarcoma virus genome (18). The sequences encoding each MTag mutant were exchanged with the wild-type MTag gene in pSR-XDMT1. Since pSR-XDMT1 and its derivatives do not contain all the genes necessary for retroviral replication, we ligated these MTag-carrying plasmids (digested with *SalI* to release the retroviral sequences from the pBR322 vector) to *SalI*-digested pREP-B (subgroup B *env*) (18), containing the remainder of the Rous sarcoma virus replicative genes, thereby forming complete Rous sarcoma virus proviral structures with MTag mutants in place of *v-src*.

Analysis of transforming ability. CEF were transfected with pSR-XDmutants ligated to pREP-B, and at 2 days posttransfection, cells were split into two culture dishes. Within 7 days, foci of transformed cells appeared in the dishes transfected with wild-type MTag. Cultures were again split, and virus was harvested from all transfected plates on days 10, 11, and 12 following transfection. These virus stocks were used for reinfection of CEF in all of the following experiments. To assess the ability of the various MTag mutants to promote anchorage-independent growth of CEF, we trypsinized cultures fully infected with mutant-carrying viruses and plated them in soft agar. These assays

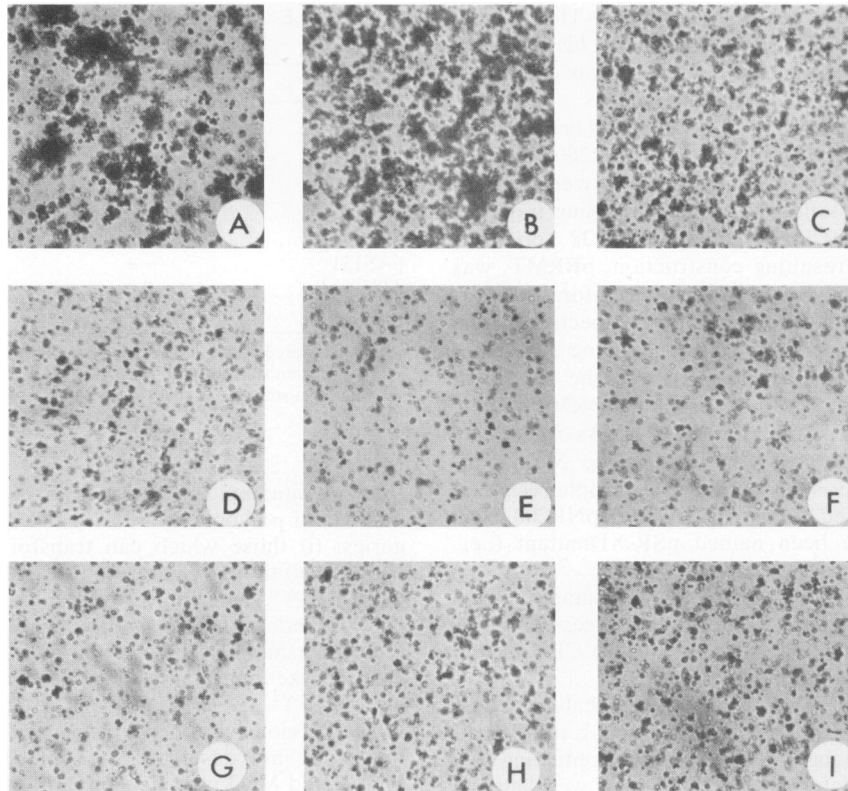


FIG. 1. Soft-agar colony formation by MTag mutants. Cells were infected with the following MTag mutant-carrying retroviruses: SRMT1 (A); MG3 (B); pAS131 (C); *dl1015* (D), mock infection (E); MG13 (F); pTH (G); RX67 (H); and RX68 (I). Each 60-mm dish of infected cells was trypsinized, and infected cells were plated in soft agar to assay for anchorage-independent growth.

were qualitative in nature (either positive or negative for growth) rather than quantitative. Wild-type MTag and the mutant MG3 induced the rapid (within 2 weeks) formation of colonies in soft agar (Fig. 1). Cells infected with mutants previously characterized as transformation defective (NG59 [data not shown], RX67, and RX68) formed no colonies in soft agar. Although mutant MG13 had 22% of the colony-forming activity of wild-type MTag in rodent cells, we detected no colony-forming activity in CEF infected with this mutant. The MTag mutant pAS131, carrying a tyrosine-to-phenylalanine mutation at the major *in vitro* site of tyrosine phosphorylation, tyrosine 315, exhibited a slight delay in transformation, forming smaller, compact colonies in soft agar relative to the wild type. *dl1015*, a mutant which can associate with $p60^{c\text{-}src}$ but cannot transform rodent cells, did not induce the formation of colonies in soft agar but was able to promote some focus formation in monolayer cultures of CEF, as has been recently reported for mouse cells carrying this mutant (25).

We injected the various mutant viruses into 1-week-old chickens. The mutants capable of inducing growth of CEF in soft agar were also able to promote the formation of hemangiomas, the main tumor type seen in chickens injected with wild-type SRMT1 virus (18). The mutants unable to transform cells in culture were incapable of inducing tumor formation.

Association of MTag mutants with $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$. To assay for association of the MTag mutants with $p60^{c\text{-}src}$ and $p62^{c\text{-}yes}$, RIPA lysates from CEF infected with the SRMT1 mutant derivatives were split in half and immunoprecipitated with either MAb 327 (directed against *src* protein) or anti-*yes*

antibody. *In vitro* kinase assays were performed on the immunoprecipitates, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). With the exception of MG13, association of $p60^{c\text{-}src}$ was as reported for the battery of mutants expressed in mammalian cells (Table 1). The prototypic transformation-defective mutant, NG59, did not associate with either $p60^{c\text{-}src}$ or $p62^{c\text{-}yes}$. In addition, the association of the MTag mutant RX67 with $p60^{c\text{-}src}$ was weak and variable. $p62^{c\text{-}yes}$ was able to associate with all of the MTag mutants which could associate with $p60^{c\text{-}src}$, except RX67 and RX68, two transformation-defective mutants (Fig. 2).

CEF infected with RX68 were examined further by fractionating cell lysates on glycerol gradients. Following centrifugation and fractionation, each fraction was divided into two aliquots and assayed for kinase activity with anti-*yes* antibody or with MAb 327. $p60^{c\text{-}src}$ kinase activity was found in the same regions of the gradient as the *in vitro*-phosphorylated MTag, suggesting that these two proteins were complexed (Fig. 3). This distribution of MTag and $p60^{c\text{-}src}$ kinase activity across the gradient was the same as has been previously reported for wild-type MTag (8). Conversely, $p62^{c\text{-}yes}$ kinase activity precipitable by anti-*yes* antibody peaked in fraction 4, corresponding to the uncomplexed, monomer weight of the protein (Fig. 3); this supports the finding that this mutant cannot bind to $p62^{c\text{-}yes}$. With wild-type MTag, the profile of $p62^{c\text{-}yes}$ kinase activity across the gradient was very similar to that of $p60^{c\text{-}src}$ complexed to MTag (data not shown).

dl1015, a transformation-defective mutant with the ability to associate with $p60^{c\text{-}src}$ and with PI kinase activity, was

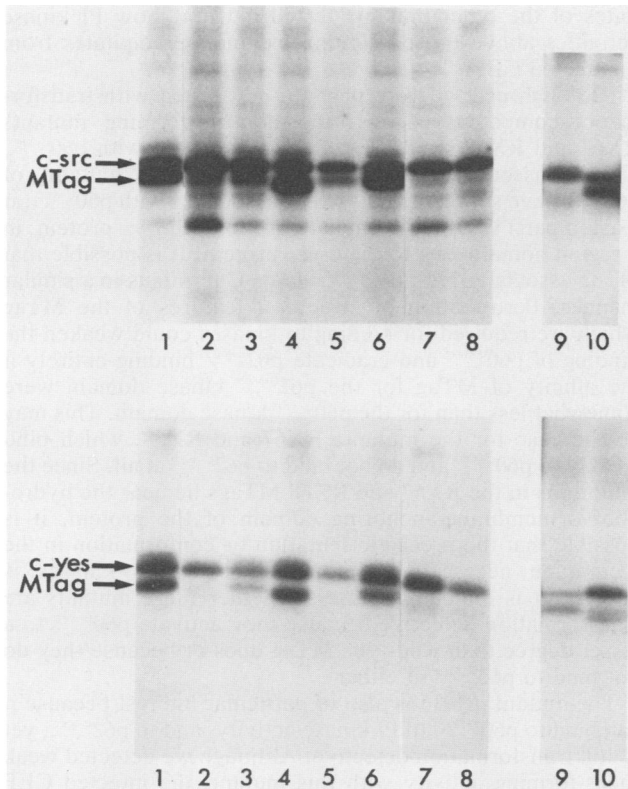


FIG. 2. MTag mutant-associated kinase activity. Lysates of CEF infected with various MTag mutants were immunoprecipitated with either MAb 327 or anti-yes antibody, and *in vitro* kinase assays were performed. Proteins were resolved by SDS-polyacrylamide gel electrophoresis. Lanes: 1, SRMT1; 2, RX67; 3, pTH; 4, *d/1015*; 5, RX68; 6, MG3; 7, MG13; 8, NG59; 9 and 10 (top panel), RX68; 9 and 10 (bottom panel), pAS131. MAb 327 was used in lanes 1 to 8 and 10 in the top panel and lane 9 in the bottom panel. All other samples were immunoprecipitated with anti-yes sera. Equal amounts of total cell protein were used for all immunoprecipitates.

also able to bind to and be phosphorylated by p62^{c-yes}. In addition, the mutant pAS131, able to associate with both p60^{c-src} and p62^{c-yes}, was only slightly impaired in its ability to transform, supporting results obtained with this mutant expressed in rodent cells (23, 24, 27).

Metabolic labeling of MTag mutant-infected cultures with [³⁵S]methionine, followed by immunoprecipitation of cell lysates with a polyclonal anti-T serum, allowed detection of the mutants NG59 and MG13, which could not be seen in the *in vitro* kinase assay. These proteins were produced at the same levels as MTag in wild-type-infected cultures (Fig. 4). In addition, RX67, RX68, and pTH, which were quite weak phosphoacceptors, were produced at levels comparable to the wild-type MTag in SRMT1-infected CEF.

To further assess the possible role of the MTag-p62^{c-yes} complex in transformation, we have assayed immunoprecipitates of the mutant MTags for PI kinase activity. Immunoprecipitates of the transforming MG3 mutant and of wild-type MTag with either MAb 327 or anti-yes antibody contained high levels of PI kinase activity, converting PI to PI-3-P (Fig. 5). The nontransforming mutant *d/1015* protein associated with PI kinase activity in both anti-yes and anti-*src* immunoprecipitates. In the immunoprecipitates of wild-type MTag and MG3 and *d/1015* MTags, a phosphorylated 85-kilodalton protein was detectable in *in vitro* kinase

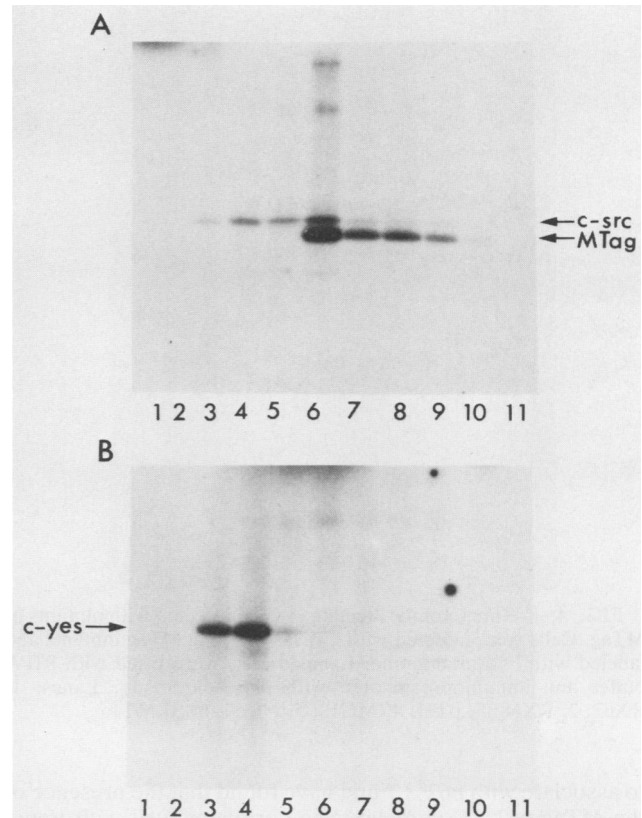


FIG. 3. Gradient sedimentation analysis of the RX68 mutant. RX68-infected CEF were lysed in RIPA buffer without SDS and were loaded onto 10 to 30% continuous glycerol gradients. Each gradient fraction was immunoprecipitated with MAb 327 (A) or anti-yes antibody (B) and assayed for kinase activity. Proteins were separated on SDS-8.5% polyacrylamide gels.

assays when samples were lysed in a mild buffer, containing 1% Nonidet P-40 as the only detergent (data not shown). All of the nontransforming mutants failed to associate with both PI kinase activity and p85.

DISCUSSION

Although MTag can bind to a number of *src*-family tyrosine kinases (*src*, *yes*, and *fyn*) and can activate the kinase activities of p60^{c-src} and p62^{c-yes} (4, 6, 19, 20), it is still an open question whether protein kinase activation is the mechanism of transformation by MTag. That MTag is associated with tyrosine kinases before detergent extraction is suggested by experiments in which MTag becomes labeled on tyrosine when [³²P]ATP is added to isolated plasma membranes from polyomavirus-transformed cells (1, 30). Moreover, although novel tyrosine-phosphorylated proteins are difficult to detect in MTag or polyomavirus-transformed cells, these proteins can be seen if cells are treated with sodium orthovanadate, a phosphatase inhibitor, presumably because the novel phosphorylations are stabilized (37).

Previous analyses of MTag mutants have indicated that p60^{c-src} probably does play an important role in polyomavirus transformation, since there is a good correlation between transforming ability and the ability to activate the kinase activity of the *c-src* protein (3, 5, 8, 22, 23). We have analyzed a series of MTag mutants to investigate their ability

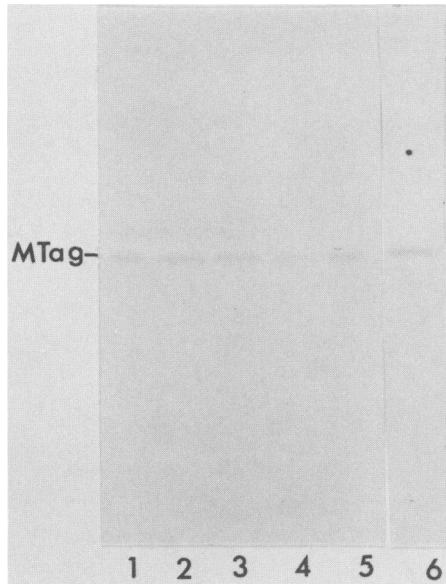


FIG. 4. [^{35}S]methionine labeling of CEF infected with mutants of MTag. Cells were infected with viruses carrying MTag mutants and labeled with [^{35}S]methionine. Labeled cells were lysed with RIPA buffer and immunoprecipitated with anti-T antibody. Lanes: 1, RX67; 2, RX68; 3, pTH; 4, MG13; 5, NG59; 6, SRMT1.

to associate with $\text{p62}^{\text{c-yes}}$ and have found that the presence of the MTag- $\text{p62}^{\text{c-yes}}$ complex also correlates well with transforming ability. In addition, PI kinase activity was found in both anti-*yes* and anti-*src* immunoprecipitates of wild-type MTag and of the mutants MG3 and *d/1015*. Immunoprecip-

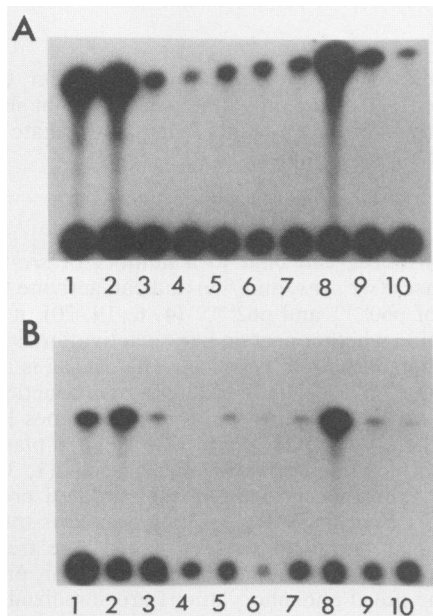


FIG. 5. Following immunoprecipitation with either MAb 327 (A) or anti-*yes* sera (B), PI kinase assays were performed. (A) Lanes: 1, SRMT1; 2, MG3; 3, MG13; 4, NG59; 5, RX67; 6, RX68; 7, pTH; 8, *d/1015*; 9, pAS131; 10, uninfected CEF. (B) Lanes: 1, SRMT1; 2, MG3; 3, MG13; 4, NG59; 5, RX67; 6, RX68; 7, pAS131; 8, *d/1015*; 9, pTH; 10, uninfected CEF.

itates of the other mutants tested did not show PI kinase activities above those of control immunoprecipitates from uninfected CEF.

Although $\text{p62}^{\text{c-yes}}$, like $\text{p60}^{\text{c-src}}$, associated with transformation-competent MTags, the nontransforming mutants RX67 and RX68, which are able to associate with $\text{p60}^{\text{c-src}}$, did not associate with $\text{p62}^{\text{c-yes}}$. Studies with mutants of $\text{p60}^{\text{c-src}}$ have suggested that MTag associates with $\text{p60}^{\text{c-src}}$ (at least in part) via the C-terminal portion of the *src* protein, in a region homologous to the *c-yes* protein. It is possible that MTag associates with $\text{p62}^{\text{c-yes}}$ via its C terminus in a similar manner. Perturbation of structural features of the MTag which are required for binding to kinases could weaken the binding of $\text{p60}^{\text{c-src}}$ and eradicate $\text{p62}^{\text{c-yes}}$ binding entirely if the affinity of MTag for the $\text{p62}^{\text{c-yes}}$ kinase domain were somewhat less than for the $\text{p60}^{\text{c-src}}$ kinase domain. This may be the case for the mutants RX67 and RX68, which bind weakly to $\text{p60}^{\text{c-src}}$ and do not bind to $\text{p62}^{\text{c-yes}}$ at all. Since the mutations in the RX67 and RX68 MTags lie near the hydrophobic membrane-anchoring domain of the protein, it is possible that the precise orientation or conformation in the membrane, necessary for proper binding of the kinases, is altered. It is difficult to assess whether these mutants are transformation defective because they activate $\text{p60}^{\text{c-src}}$ to a lesser degree than wild-type MTag does or because they do not bind to $\text{p62}^{\text{c-yes}}$ (if either).

The mutant *d/1015* is also of particular interest because it can bind to $\text{p60}^{\text{c-src}}$, to PI kinase activity, and to $\text{p62}^{\text{c-yes}}$, yet is still transformation defective. Although we detected weak focus-forming activity with this mutant, the infected CEF could not grow in an anchorage-independent manner and the mutant virus did not induce the formation of hemangiomas upon injection into chickens. Further studies are required to determine the specific biochemical defect of this mutant.

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