Analysis of Middle Tumor Antigen and pp60^{c-src} Interactions in Polyomavirus-Transformed Rat Cells

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The relative abundance of pp60^{c-src} molecules associated with polyomavirus (Py) middle tumor antigen (MTAg) and the relative abundance of MTA g associated with pp60^{c-src} in a variety of Py-transformed rat cells was determined by quantitative immunoblot analyses which detect pp60^{c-src} or Py MTA g. The results demonstrate that approximately 5 to 10% of the total immunoprecipitable pp60^{c-src} molecules in Py-transformed rat cells are stably associated with MTA g and have elevated protein kinase activities. In these same cells, it was found that approximately 10 to 15% of the detectable MTA g molecules are stably associated with pp60^{c-src}. Other results presented in this report demonstrate that approximately 50 to 75% of the total MTA g-associated cellular tyrosine kinase activity potentially represents the enzymatic activity of pp60^{c-src}, while the remaining 25 to 50% represents the activity of other cellular tyrosine kinases. Our results also show that most pp60^{c-src} molecules associated with Py MTA g do not possess electrophoretic mobilities that are altered from those of pp60^{c-src} molecules not associated with MTA g or pp60^{c-src} molecules obtained from normal rodent cells.

Genetic and biochemical studies have shown that oncogenic transformation of rodent cells in culture by polyomavirus (Py) is dependent on the expression of the Py-encoded middle tumor antigen (MTAg) (for a review, see reference 20). The 56-kilodalton (kDa) MTA g is a phosphoprotein which is found in cellular membrane fractions and is capable of associating with a variety of cellular proteins to form high-molecular-weight complexes (15, 16, 22, 30, 41, 42). All known transformation-competent strains of Py encode MTA g molecules that can be found to possess associated tyrosine-specific protein kinase activity, which has been shown to minimally represent the functions of the c-src and c-yes gene products (8, 10, 15, 16, 19, 26, 34, 36).

Recent studies designed to analyze the role of the MTA g-associated c-src gene product in maintenance of the Py-transformed phenotype in rat cells by using inducible antisense or complementary c-src RNA have suggested that at least some of the characteristics of MTA g transformation are dependent on the abundance and activity of pp60^{c-src} (1). Interpretation of these experiments, however, is complicated by the fact that expression of complementary c-src RNA in normal rat cells was not evaluated. This fact raises the possibility that the alterations in the phenotype observed in the MTA g-transformed cells as a result of anti-sense c-src RNA synthesis represent consequences related to diminished pp60^{c-src} abundance that might be similar in normal rodent cells or rodent cells transformed by other oncogenes. The phenotype of the MTA g-transformed rat cells expressing complementary c-src RNA is unstable, and synthesis of high levels of complementary c-src RNA results in diminished cellular viability as a function of passage in culture (4). These findings support the idea that phenotypic alterations in these cells may be more closely associated with a cellular requirement for some minimal level of pp60^{c-src} expression than with an effect specific for functions of MTA g-associated pp60^{c-src} molecules. Additionally, the contribution of the

MTAg-associated cellular tyrosine kinases to the initiation of Py transformation is unclear since several transformation-defective mutants of Py encode MTAg capable of stably associating with and being phosphorylated by one or more cellular tyrosine kinases (10, 25, 29, 31, 39).

Stable association of MTA g in complexes with cellular pp60^{c-src} molecules results in an apparent increase in the specific activity of the c-src-encoded tyrosine phosphotransferase (8, 10, 14). The c-yes gene products associated with MTA g also possess increased tyrosine-specific protein kinase activity (26), although it is not currently known whether this increase represents an elevation in the specific activity of the p62^{c-yes} enzyme function. The high-molecular-weight MTA g-pp60^{c-src} complexes also contain an unidentified cellular phosphatidylinositol kinase whose activity is also apparently stimulated (25, 43).

In addition to the putative membrane-binding domain of MTA g which consists of a hydrophobic cluster of amino acids from residues 394 to 421 (9, 28, 37, 38), the region of MTA g which appears to be most critical for forming stable complexes containing pp60^{c-src} (and presumably p62^{c-yes}) has been localized to the amino-terminal portion of MTA g between amino acids 78 and 191 (5, 12, 29, 38). The association of MTA g with pp60^{c-src} in these complexes results in a shift of the carboxy-terminal phosphorylation site on pp60^{c-src} from tyrosine residue 527 to 416 (11, 14), a feature characteristic of c-src mutants that have oncogenic potential (23). The MTA g-associated pp60^{c-src} molecules also possess new sites of phosphorylation on one or more tyrosine residues in the amino-terminal portions of the c-src molecules, which are thought to result in diminishing the electrophoretic mobilities of these molecules on sodium dodecyl sulfate (SDS)-polyacrylamide gels (11, 44, 45).

In this report, we have attempted to calculate the relative percentage of cellular pp60^{c-src} molecules associated with MTA g and have estimated the amount of MTA g associated with pp60^{c-src} in Py-transformed rat cells. For these analyses, we used a quantitative immunoblot technique that uses

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FIG. 1. MAb 327/273 immunoblot analysis of pp60^{c-src} and PAb 815 immunoblot analysis of MTA g. (A) The proteins immunoprecipitated by MAb 327 from RIPA buffer lysates of FR 18-1 cells were eluted from the *Staphylococcus aureus* immunoadsorbent with SDS sample buffer and pooled. Different sample amounts were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose sheets, and MAb 327/273 immunoblot analysis was done. Lanes: 1, products of a single MAb 327 immunoprecipitation reaction from 500 μ g of FR 18-1 cell lysate; 2 to 10, different amounts of the pooled samples corresponding to 100, 80, 60, 40, 20, 10, 5, 1, and 0.1% of the sample analyzed in lane 1. (B) Different amounts of RIPA buffer lysates from uninfected MEC (lanes 3, 6, 9, and 12) and MEC infected with wt Py (lanes 1, 4, 7, and 10) or with dl45 Py (lanes 2, 5, 8, and 11) were immunoprecipitated with hamster T serum before the PAb 815 immunoblot analysis was performed. Protein concentrations for the immunoprecipitates were 5 (lanes 1 to 3), 10 (lanes 4 to 6), 25 (lanes 7 to 9), and 50 μ g/ml (lanes 10 to 12). (C) A total of 100 μ g of the RIPA buffer lysates described above was fractionated directly on 8% SDS gels before the PAb 815 immunoblot analysis. Positions of pp60^{c-src} (src), IgG, wt MTA g (WT MT), dl45 MTA g (dl45 MT), and the approximately 95-kDa protein recognized by PAb 815 are indicated at the left of the panels. Prestained molecular mass markers (in kilodaltons; Bethesda Research Laboratories) are indicated at the right of the panels.

monoclonal antibodies specific for pp60^{c-src} and MTA g. The results of these studies demonstrate that approximately 5 to 10% of the total pp60^{c-src} molecules present in Py-transformed rat cells are stably complexed with MTA g. In the cell lines examined, approximately 10 to 15% of the MTA g molecules present in the cells were found to be associated with pp60^{c-src}. Additional studies presented in this report demonstrate that approximately 50 to 75% of the MTA g-associated cellular tyrosine-specific protein kinase activity represents that of pp60^{c-src}. Our analyses further

show that most pp60^{c-src} molecules associated with MTA g do not possess altered mobilities on SDS-polyacrylamide gels.

Detection of pp60^{c-src} and MTA g by immunoblot analysis. The sensitivity and linearity of MAb 327/273 (mouse monoclonal antibodies that recognize pp60^{c-src} (27) immunoblot analysis of pp60^{c-src} in MAb 327 immune complexes from lysates of FR 18-1 cells is shown in Fig. 1A. FR 18-1 (FR10281/18-1) (1, 8) is a clonal cell line derived from a single focus after transfection of Fisher rat 3T3 (FR 3T3) cells with pPyMT-1, a recombinant plasmid capable of encoding only wild-type (wt) Py MTA g (40). For this experiment, the protein concentration of FR 18-1 RIPA buffer (8a, 33) lysates was adjusted to 500 μ g/ml, and equal amounts of lysate (1 ml) were immunoprecipitated with MAb 327 (6, 33). Different amounts of sample, ranging from 100 to 0.1% of the amount of pp60^{c-src} immunoprecipitated in a single MAb 327 immunoprecipitation reaction, were fractionated on an 8% SDS gel and electrophoretically transferred to nitrocellulose, and MAb 327/273 immunoblot analysis was conducted (8a, 33). The results of this experiment demonstrate that MAb 327/273 immunoblot analysis is capable of detecting, in a linear fashion, as little as 5% of the total pp60^{c-src} immunoprecipitated from 500 μ g of FR 18-1 RIPA buffer lysate with MAb 327. The approximately 55-kDa protein shown in the autoradiogram is the heavy chain of the MAb 327 immunoglobulin G (IgG) which reacts with the iodinated sheep anti-mouse IgG.

The sensitivity of PAb 815 (a rat monoclonal antibody that recognizes Py MTA g) (5) immunoblot analysis of MTA g in hamster T serum immune complexes and in lysates of wt Py- and dl45-infected primary mouse embryo cells (MEC) is shown in Fig. 1B and C. For these experiments, uninfected MEC or MEC infected with either wt Py or dl45 were harvested at 24 h postinfection by RIPA buffer lysis, and the protein concentration of the lysates was adjusted to 500 μ g/ml. Different amounts of the lysates, ranging from 5 to 50 μ g, were then immunoprecipitated with a constant amount of excess hamster T serum (8), and the immunoprecipitates were analyzed by the PAb 815 immunoblot technique. The PAb 815 immunoblot analysis was conducted in the same manner as that described for the MAb 327/273 immunoblot experiments (8a, 33) except that the PAb 815 IgG bound to MTA g was detected by the addition of ¹²⁵I-labeled sheep anti-rat IgG (Amersham Corp.). The results of this experiment demonstrate that the PAb 815 immunoblot procedure is capable of detecting MTA g in 10 μ g of these cellular lysates after immunoprecipitation with an overnight exposure of the blot. PAb 815 immunoblot analysis is also capable of detecting MTA g in cell lysates fractionated directly on SDS gels without prior immunoprecipitation (Fig. 1C). An additional protein with an approximate molecular mass of 95 kDa which reacted with PAb 815 can also be seen in cell lysates from wt Py-infected MEC. The origin of this protein is currently unknown and was not observed in either dl45-infected or uninfected MEC cell lysates. This 95 kDa protein is also not immunoprecipitated from RIPA buffer lysates of wt Py-infected MEC by either hamster T serum (Fig. 1B) or PAb 815 (data not shown).

An additional observation during the course of these studies was that some lots of ¹²⁵I-labeled sheep anti-rat IgG were capable of detecting MTA g in immunoblot assays without PAb 815. Unlabeled antibodies from the same commercial source (Amersham) were found to be incapable of immunoprecipitating MTA g from Py-infected or Py-transformed cell lysates. By using a series of Py deletion mutants, we were able to localize the apparent epitope recognized by

FIG. 2. Quantitation of MTA, $pp60^{c\text{-src}}$, and MTA-associated protein kinase activity in different Py-transformed rat cells. RIPA buffer lysates from FR 18-1 (lanes 1 and 2), FR *dl8* (lanes 3 and 4), FR *dl45* (lanes 5 and 6), and T1A1 (lanes 7 and 8) cells were adjusted to 500 $\mu\text{g/ml}$ per reaction, and immunoprecipitation assays of triplicate samples were conducted with either hamster T serum (lanes 2, 4, 6, and 8) or MAb 327 (lanes 1, 3, 5, and 7). (A) Immune complex-protein kinase assays. (B) PAb 815 immunoblot analysis. (C) MAb 327/273 immunoblot analysis. Positions of MTA (MT), *src*-related phosphoproteins, the approximately 85-kDa cellular tyrosine-containing phosphoprotein(s), $pp60^{c\text{-src}}$ (*src*), IgG, and prestained molecular mass markers (in kilodaltons) are indicated.

the iodinated anti-rat IgG to an area between MTA amino acids 301 and 335. The significance of these observations is currently unknown, but they point out that different lots of ^{125}I -labeled sheep anti-rat IgG should be assayed for this ability to react with MTA before PAb 815-dependent immunoblot detection of MTA can be conducted.

Quantitation of $pp60^{c\text{-src}}$ and MTA interactions. To analyze the level of MTA associated with $pp60^{c\text{-src}}$ in a variety of Py-transformed rat cells and to determine the proportion of $pp60^{c\text{-src}}$ associated with MTA in these same cells, we conducted PAb 815 and MAb 327/273 immunoblot analyses on immune complexes derived from parallel hamster T serum or MAb 327 immunoprecipitations of lysates from FR 18-1, FR *dl8*, FR *dl45*, and T1A1 cells. Additionally, we conducted immune complex-protein kinase assays on parallel immunoprecipitates (6–8). FR *dl8* clone 1 (FR *dl8*) and FR *dl45* clone 1 (FR *dl45*) are clonal cell lines derived from single foci after infection of FR 3T3 cells with Py deletion mutants *dl8* and *dl45* (3, 21). PyREWA5/T1A1 (T1A1) is a clonal cell line derived from infection of primary Wistar rat embryo cells with wt Py (24).

The results of the protein kinase assays (Fig. 2A) show that the amount of MTA phosphorylation in the hamster T serum and MAb 327 immune complexes was similar for a given cell line, although the level of MTA phosphorylation

and $pp60^{c\text{-src}}$ autophosphorylation differed between the various lines. The presence of various 60- to 64-kDa phosphoproteins potentially representing altered forms of $pp60^{c\text{-src}}$ or other cellular proteins can be observed in each of the hamster T serum immunoprecipitates. Also phosphorylated in the hamster T serum and MAb 327 immune complexes derived from the FR 18-1 and FR *dl45* cell lysates is a protein doublet with a molecular mass of approximately 85 kDa. We have determined in separate experiments that this pair of proteins is phosphorylated on tyrosine residues (data not shown) and may represent the 85-kDa protein previously described as a substrate in MTA- $pp60^{c\text{-src}}$ complexes for $pp60^{c\text{-src}}$ (29, 30), which potentially possesses phosphatidylinositol kinase activity (D. R. Kaplan and T. M. Roberts, personal communication). Interestingly, this protein or set of proteins was not found to be efficiently phosphorylated in hamster T serum or MAb 327 immune complexes derived from either FR *dl8* or T1A1 cell lysates.

The results of PAb 815 immunoblot analyses of hamster T serum and MAb 327 immune complexes formed in parallel with those used for the protein kinase assays described above are shown in Fig. 2B. The data show that the ratio of detectable MTA in the hamster T serum and MAb 327 immune complexes was approximately the same for each of these cell lines. In these immune complexes, we have

determined that the amount of MTA γ immunoprecipitated by MAb 327 represents approximately 10 to 15% of that immunoprecipitated by hamster T serum. This ratio appears to be constant even though the level of total detectable MTA γ differs by as much as eightfold (compare lanes 2 and 8).

The results of MAb 327/273 immunoblot analysis of parallel hamster T serum and MAb 327 immunoprecipitates are shown in Fig. 2C. The data show that the ratio of detectable pp60^{c-src} in the hamster T serum and MAb 327 immune complexes was not the same for each of the cell lines. No distinct correlation between the levels of total detectable pp60^{c-src} and pp60^{c-src} presumably associating with MTA γ was found. However, there is an apparent relationship between the level of MTA γ expression and the amount of associated pp60^{c-src} in these cell lines.

We did not detect by MAb 327/273 immunoblot analysis the p60⁺ form of pp60^{c-src}. In these studies, all detectable MTA γ -associated pp60^{c-src} comigrated on SDS gels with the bulk of cellular pp60^{c-src} which is not associated with MTA γ . The addition of unlabeled ATP (10 μ M) and Mg²⁺ to the MAb 327 and hamster T serum immunoprecipitates before fractionation of the proteins on SDS-polyacrylamide gels failed to generate altered migrating forms of pp60^{c-src} corresponding to p60⁺ which could be detected by MAb 327/273 immunoblot analysis (data not shown). Additionally, we found in other experiments that treatment of the nitrocellulose sheets with potato acid phosphatase under conditions that cleave this type of modification (13) did not reveal any additional MAb 327/273-reactive protein species (data not shown).

Evaluation of pp60^{c-src} specific activity in Py-transformed cells. During the course of analyzing pp60^{c-src} protein kinase activity in a variety of human tumor cells, we observed that the estimation of pp60^{c-src} abundance in lysates and immune complexes by metabolic labeling of cells with [³⁵S]methionine or [³²P]orthophosphate and immunoprecipitation with monoclonal antibodies, including MAb 327, can be inaccurate (J. O'Shaughnessy and J. B. Bolen, unpublished results). As with human neuroectodermal tumor cell lines, metabolic labeling and immunoprecipitation of pp60^{c-src} with a variety of antibodies suggested that the different tumor cells contain equivalent amounts of pp60^{c-src}. However, immunoblot analysis of these same immune complexes revealed that the amount of pp60^{c-src} actually present in the immunoprecipitates varied by as much as 12-fold. Since the concept that the specific activity of pp60^{c-src} molecules associated with MTA γ possesses elevated tyrosine phosphotransferase activity is also based on the determination of pp60^{c-src} abundance by metabolic labeling (8), we reevaluated the relative abundance of pp60^{c-src} in MAb 327 immune complexes from normal and MTA γ -transformed rat cells by MAb 327/273 immunoblot analysis.

For these experiments, FR 3T3 cells and FR 18-1 cells were lysed in RIPA buffer, the lysates were adjusted to the same protein concentrations, and different concentrations of FR 3T3 and FR 18-1 cell lysates were immunoprecipitated with a constant amount of MAb 327 before immune complex-protein kinase and immunoblot analyses were conducted. The results of these experiments show that pp60^{c-src} autophosphorylation (Fig. 3A) and phosphorylation of casein by pp60^{c-src} (Fig. 3C) was elevated approximately 5- to 10-fold in the MTA γ -transformed cells. The results of the MAb 327/273 immunoblot analysis of parallel immune complexes (Fig. 3B) show that over a wide range of protein concentrations, the abundance of pp60^{c-src} in the MAb 327

immunoprecipitates from the FR 3T3 cells is actually about twice that found in the MAb 327 immunoprecipitates from the FR 18-1 cells. These results demonstrate that the specific activity of pp60^{c-src} molecules associated with MTA γ is indeed apparently elevated.

Evaluation of MTA γ -associated pp60^{c-src} activity. To determine the amount of MTA γ kinase activity that is due to associated pp60^{c-src}, we performed serial immunoprecipitations of FR 18-1 RIPA buffer lysates using either MAb 327 or hamster T serum and conducted immune complex-protein kinase assays and MAb 327/273 immunoblot analyses on the immune complexes. The results of these experiments (Fig. 4) show that more than 97% of the detectable pp60^{c-src}, whether measured by tyrosine kinase activity (panel A) or by immunoblot analysis (panels C and E), was removed from the cell lysates after four serial immunoprecipitations with a constant amount of MAb 327 (panels A, C, and E, lanes 1 to 4). The ³²P-labeled casein bands observed in the reactions shown in lanes 4 of panels A and B were determined to result from background casein kinase activity present in this particular batch of casein. Phosphoamino acid analysis revealed that these casein molecules were phosphorylated principally on serine residues (data not shown). Immunoprecipitation of the supernatant after the final MAb 327 reaction with hamster T serum (panels A, C, and E, lanes 5) demonstrated that approximately 25 to 30% of the detectable MTA γ and casein-phosphorylating activity originally found in the cell lysates remained after pp60^{c-src} was removed from the lysates. In other similar experiments, the amount of detectable

FIG. 3. Analysis of pp60^{c-src} protein kinase activity and abundance in normal and MTA γ -transformed rat cells. RIPA buffer lysates of FR 3T3 and FR 18-1 cells were adjusted to the same protein concentrations (500 μ g/ml), and different amounts of triplicate samples were immunoprecipitated with MAb 327. Two sets of samples were used for protein kinase assays (final ATP concentration, 10 μ M) without (A) or with (C) the addition of α -casein, and one set of samples was used for MAb 327/273 immunoblot analysis (B). The total amount of protein in the samples was 500 (lanes 1 and 2), 250 (lanes 3 and 4), 150 (lanes 5 and 6, panels A and B only), and 75 μ g (lanes 7 and 8, panels A and B only). Each sample was also adjusted to the same total volume (1 ml). Positions of MTA γ (MT), pp60^{c-src} (src), α -casein, IgG, and prestained molecular mass markers (in kilodaltons) are indicated.

MTAg and casein-phosphorylating activity was occasionally found to be as high as 50% after all detectable pp60^{c-src} was removed (data not shown). For this determination, compare the kinase activity shown in panel A, lane 5, with that shown in panel B, lane 1. In parallel experiments, serial immunoprecipitations with hamster T serum (panels B, D, and F) were found to diminish the level of detectable pp60^{c-src} by less than 10% when assayed by MAb 327/273 immunoblot analysis (panels D and F). However, pp60^{c-src} kinase activity in the MAb 327 immune complexes was found to be decreased by approximately four- to eightfold (compare panel B, lane 5, with panel A, lane 1).

The results demonstrate that approximately 5 to 10% of the detectable cellular c-src gene product in the Py-transformed rat cells examined is stably associated with Py MTA. Our results further show that in these same cells, approximately 10 to 15% of the detectable MTA molecules are stably associated with pp60^{c-src}. These data support previous speculations that only a small fraction of the total pp60^{c-src} and MTA molecules within Py-transformed cells is present in a stable complex (8). Since we were unable to quantitate the actual amount of pp60^{c-src} detected by MAb 327/273 immunoblot analysis and the amount of MTA detected by PAb 815 immunoblot analysis, the stoichiometry of these two proteins in the pp60^{c-src}-MTA complexes in these rat cells is unclear. However, we believe that the percentages of the MTA and pp60^{c-src} molecules estimated to be in the complexes by immunoblot analysis are reasonable since similar percentages were obtained when we used a variety of Py-transformed rat cell lines (Fig. 2) and when we used other antibodies that recognize either pp60^{c-src} or MTA (data not shown).

The cellular tyrosine kinase activity associated with MTA has been shown to represent the functions of at least two members of the src subfamily of protein kinases, i.e., pp60^{c-src} (8, 10, 15, 16) and p62^{c-yes} (26). Our data suggest that pp60^{c-src} represents 50 to 75% of MTA-associated protein kinase activity that is detected in the Py-transformed rat cells analyzed (Fig. 4). Clearly, this ratio may be altered in Py-transformed cells from other species and may depend on the ratios of pp60^{c-src} and p62^{c-yes} in different cells. Whether the remaining MTA-associated protein kinase activity is solely a function of p62^{c-yes} is not known since we currently do not have antibodies that recognize this protein. The fact that serial immunoprecipitation of lysates from these cells with antibodies that recognize pp60^{c-src} did not remove all MTA-associated protein kinase activity suggests that MTA-pp60^{c-src} and MTA-p62^{c-yes} are present in separate complexes. Similar conclusions have been reached by Kornbluth et al. (26). Because the size of the MTA-cellular protein complex which possesses protein kinase activity is approximately 200 to 230 kDa (15, 16, 22, 30, 41, 42), it appears likely that the MTA-pp60^{c-src} and MTA-p62^{c-yes} complexes are approximately the same size and may contain the same group of cellular proteins, with either pp60^{c-src}, p62^{c-yes}, or possibly another tyrosine kinase substituting for one another.

Results reported from several laboratories have led to the idea that the pp60^{c-src} molecules associated with MTA possess elevated specific activities (1, 2, 6, 8, 10, 14, 44, 45). These specific activity determinations were based on comparisons between the levels of [³⁵S]methionine-labeled pp60^{c-src} in various immune complexes and the protein kinase activities detected in parallel immune complexes. The assumption in these experiments was that metabolic labeling of pp60^{c-src} in Py-transformed cells and in normal cells

FIG. 4. Correlation of MTA-associated protein kinase activity with presence of pp60^{c-src}. Duplicate samples of RIPA buffer lysates from FR 18-1 cells were subjected to serial immunoprecipitations (addition of antibody for 1 h and collection of the immune complexes with Formalin-fixed *S. aureus* followed by incubation of the supernatant with additional antibody, etc.) with either MAb 327 (panels A and C, lanes 1 to 4) or hamster T serum (panels B and D, lanes 1 to 4). One set of samples was used for protein kinase assays with alpha casein as an exogenous substrate (panels A and B), and the other set of samples was used for MAb 327/273 immunoblot analysis (panels C and D). Lanes 5 represent the supernatant from the fourth MAb 327 reaction immunoprecipitated with hamster T serum (panels A and C) or the fourth hamster T serum reaction immunoprecipitated with MAb 327 (panels B and D). (E) Longer exposure of the autoradiogram shown in panel C. (F) Longer exposure of experiments analogous to those shown in panel D. Lanes 1 and 2 in panel F are the same as lanes 5 and 1, respectively, in panel D. Positions of MTA (MT), pp60^{c-src} (src), alpha casein, IgG, and prestained molecular mass markers (in kilodaltons) are indicated. The positions of p60^{c-src} (+) and pp60^{c-src} are shown by the arrows adjacent to lanes 1 and 5 in panels A and B.

accurately reflected the relative abundance of pp60^{c-src} in both cell types. As mentioned previously, we have recently found that in at least some types of cells, metabolic labeling with [³⁵S]methionine followed by immunoprecipitation does not result in an accurate identification of the potential abundance of pp60^{c-src}. This observation prompted us to reevaluate whether the specific activity of pp60^{c-src} in Py-transformed cells was indeed elevated over the activity of pp60^{c-src} in corresponding normal cells. The results presented in Fig. 3 clearly demonstrate that the specific activity of pp60^{c-src} does appear to be enhanced in the Py-transformed cells we examined. As previously reported (8, 10, 14, 44, 45), pp60^{c-src} kinase activity was stimulated approximately 5- to 10-fold, a finding based on immunoprecipitating equal amounts of total cellular protein from the cellular lysates. However, since the normal FR 3T3 cells used for these evaluations were found to have approximately twice the detectable level of pp60^{c-src} when compared with that in the transformed FR 18-1 cells, the overall difference in specific activity of pp60^{c-src} in these immune complexes is more accurately 10- to 20-fold. Since roughly 10% of the pp60^{c-src} molecules immunoprecipitated are associated with MTag and since it has been shown that the pp60^{c-src} molecules within Py-transformed cells not associated with MTag have specific activities indistinguishable from those in normal c-src molecules (8, 44), our results suggest that the specific activities of the pp60^{c-src} molecules associated with MTag must be elevated 100-fold or more. Whether such a dramatic elevation in pp60^{c-src} specific activity is even possible is not clear since the difference between the specific activities of pp60^{v-src} and avian pp60^{c-src} is apparently no more than 10- to 20-fold when measured in immune complex-protein kinase assays under conditions very similar to those used in this study (17, 23). These observations raise the possibility that cellular tyrosine kinases distinct from pp60^{c-src} and p62^{c-yes} may be present in the MTag complexes. Alternatively, these observations may indicate that deregulated src molecules such as pp60^{v-src} and mutated pp60^{c-src} proteins possess the capacity for greater specific activity differences than are currently realized.

The level of MTag-associated pp60^{c-src} in the rat cell lines examined was found to be proportional to the amount of MTag expressed and apparently much less dependent on the level of pp60^{c-src} expression. Similarly, the amount of MTag phosphorylation in the immune complex-protein kinase assays was proportional to the amount of MTag in the T serum and MAb 327 immunoprecipitates. These results are consistent with previous observations (6, 18, 32) that the level of MTag expression dictates the apparent rate of stable MTag-pp60^{c-src} complex formation up to a given point. However, it has also been pointed out that gross overexpression of MTag in some cell types does not significantly increase the amount of complex (35, 46) since the appropriate species of pp60^{c-src} or other cellular factors are thought to become limiting. The level of the approximately 62- to 64-kDa protein(s) phosphorylated in the T serum immune complex-protein kinase assays did not appear to be as dependent on the level of MTag expression as did pp60^{c-src} (Fig. 2). The reasons for this are currently unclear, although, like pp60^{c-src}, the degree to which this protein or group of proteins is phosphorylated may not very accurately reflect the actual abundance of the protein(s). One unexpected finding of the immunoblot analysis of MTag-associated pp60^{c-src} molecules was the lack of detectable alterations in the migration of these molecules (Fig. 2). Previous analysis

of these phosphoproteins suggested that they represent altered migrating forms of pp60^{c-src} that possess at least one additional tyrosine phosphorylation site within the amino-terminal half of the protein (11, 45), diminished phosphorylation of carboxy-terminal tyrosine residues corresponding to the avian tyrosine 527 (11, 14), and elevated phosphorylation of tyrosine residues corresponding to the avian tyrosine 416 (11). Our current results suggest that most pp60^{c-src} molecules associated with MTag possess migrational properties indistinguishable from those of pp60^{c-src} not associated with MTag and those of pp60^{c-src} derived from normal rodent cells. Whether the p60⁺ protein does indeed represent an altered migrating form of pp60^{c-src} cannot be clearly demonstrated by our current data. However, it is possible that only a small fraction of the total pp60^{c-src} associated with MTag is phosphorylated in a manner that would generate this protein species, and the quantity of this subpopulation of pp60^{c-src} may simply be below the level of detection in the MAb 327/273 immunoblot assay. Although our results do not demonstrate that the previously noted analyses of MTag-associated pp60^{c-src} molecules are in any way inaccurate, our data do suggest that the identities and phosphorylation sites of the 60- to 64-kDa phosphoproteins associated with MTag molecules should continue to be evaluated.

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