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Stimulation of pp60$^{c\text{-}src}$ Tyrosyl Kinase Activity in Polyoma Virus–Infected Mouse Cells Is Closely Associated With Polyoma Middle Tumor Antigen Synthesis

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We have examined the effect of polyoma virus infection of primary mouse embryo cells on the tyrosyl kinase activity associated with the cellular src gene product, pp60$^{c\text{-}src}$. The results of our studies demonstrate that infection of mouse cells with wild-type polyoma virus or viral mutants capable of transforming rodent cells in culture and inducing tumors in animals results in the stimulation of pp60$^{c\text{-}src}$ tyrosyl kinase activity. The level of pp60$^{c\text{-}src}$ kinase stimulation in infected cells was found to be proportional to both the oncogenic potential of the virus strain used for infection and the characteristic phenotype of rodent cells transformed by the various strains of polyoma virus. Stimulation of pp60$^{c\text{-}src}$ kinase activity was not observed in mouse cells infected with transformation-defective strains of polyoma virus. In examining the kinetics of pp60$^{c\text{-}src}$ kinase stimulation in mouse cells at various times following wild-type polyoma virus infection, we found that the level of pp60$^{c\text{-}src}$ kinase activity correlated directly with the synthesis of polyoma virus-encoded tumor antigens. By comparing wild-type polyoma virus with other viral mutants in these experiments, we conclude that the stimulation of pp60$^{c\text{-}src}$ kinase activity in mouse cells following polyoma virus infection is associated with the synthesis of middle tumor antigen.

Key words: polyoma virus, pp60$^{c\text{-}src}$, polyoma middle T antigen, tyrosyl kinase, viral infection

The region of the polyoma virus (Py) genome required for both Py-mediated tumorigenesis in vivo and oncogenic transformation in vitro encodes three known nonstructural proteins: the large, middle, and small tumor antigens (TAgs) [1]. Of these three proteins, the Py-encoded middle tumor antigen (MTAg) appears to be critical for the maintenance of transformation [2,3]. The MTAg has an associated protein kinase activity that transfers a phosphoryl group from ATP or GTP to tyrosine

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residues on MTAg in immunoprecipitates formed between Py-infected or Py-transformed cell lysates and sera from animals bearing Py-induced tumors (T sera) [4–6]. The importance of this enzyme activity for Py-mediated oncogenesis is suggested by the observation that Py mutants that lack the ability to induce morphologic transformation of rodent cells in culture or tumors in animals also lack detectable MTAg-associated tyrosyl kinase activity [6,7].

Currently, there is no direct evidence to demonstrate that MTAg possesses intrinsic protein kinase activity [8,9] and recent evidence has been presented indicating that MTAg is associated with the cellular src gene product $pp60^{c\text{-}src}$ [10]. Since both the viral and cellular forms of the src gene product possess intrinsic tyrosyl kinase activity [11–13], it has been proposed that the MTAg-associated kinase is a property of the MTAg-associated $pp60^{c\text{-}src}$ [10].

We have previously analyzed the association between MTAg and $pp60^{c\text{-}src}$ in Py-transformed and Py-infected rodent cells by using a series of monoclonal antibodies (MAb) that recognize mammalian $pp60^{c\text{-}src}$ [14,15]. The results of those studies demonstrated that infection of mouse cells with Py results in the stimulation of $pp60^{c\text{-}src}$ kinase activity several times that observed in uninfected mouse cells or mouse cells infected with transformation-deficient mutants of Py. Similarly, significant enhancement of $pp60^{c\text{-}src}$ kinase activity was found in Py-transformed hamster, rat, and mouse cells when compared to the activity found in the corresponding normal rodent cells. Since no differences were detected in the level of c-src RNA or $pp60^{c\text{-}src}$ synthesis in Py-infected and uninfected mouse cells or Py-transformed and normal rodent cells, we proposed that the association between MTAg and $pp60^{c\text{-}src}$ could result in an increase in the specific activity of the $pp60^{c\text{-}src}$ tyrosyl kinase [15].

In the present study, we have examined the effect of Py-infection on the kinetics of $pp60^{c\text{-}src}$ tyrosyl kinase enhancement in mouse cells. The results of these studies demonstrate that the degree of stimulation of $pp60^{c\text{-}src}$ kinase activity is directly related to the level of Py-encoded TAg synthesis at early times following Py infection of mouse cells.

MATERIALS AND METHODS

Virus Strains and Cell Culture

The Pasadena large plaque strain of Py was the wild-type strain (Py wt) used in these experiments [16]. Other virus strains included d18 [17], a viable deletion strain that encodes an MTAg with a 30-amino-acid deletion between residues 252 to 282 [18]; d123 [17], a viable deletion strain that encodes an MTAg with a 34-amino-acid deletion between residues 301 to 335 [18]; NG59 [19], a host range-transformation defective (hr-t) mutant that has an in-phase insertion-substitution (Asp to Ile-Asn) at amino acid 179 [20]; and NG18 [19], an hr-t mutant that does not produce detectable MTAg or small TAg [21]. The hr-t mutants NG59 and NG18 are both defective for transformation and MTAg-associated kinase activity [6,22]. The d123 has severely diminished transforming activity and little detectable MTAg-associated kinase activity [4,17]. The Py wt and d18 transform rodent cells in culture and have easily detectable MTAg-associated kinase activity [4–7].

Primary mouse embryo cells (MEC) isolated from 12- to 14-day-old embryos were infected with 50 plaque-forming units per cell of Py and were maintained in serum-free Dulbecco modified Eagle's medium until harvested.

Antisera and Monoclonal Antibodies

Hamster T serum was obtained from Syrian golden hamsters bearing tumors induced by inoculation with the Py-induced tumor cell line PyT54 [23]. Mouse monoclonal antibodies directed against pp60$^{c\text{-}src}$ were prepared as previously described [24].

Immune-Complex Protein Kinase Assay

Py-infected cells were washed three times with cold phosphate-buffered saline, scraped into the same buffer, and pelleted by centrifugation. The cell pellets were resuspended on ice in modified RIPA buffer (0.15 M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet-P40, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.01 M Tris-hydrochloride (pH 7.2), 0.001 M EDTA) containing 600 Kallikrein inhibitor units per ml aprotinin (Boehringer, Mannheim, FRG) and 1×10^{-4} M sodium vanadate (Fisher, Pittsburgh, PA). Following a 20 min incubation, the lysates were clarified by centrifugation in an Eppendorf microcentrifuge for 5 min at 4°C. The cleared extracts were adjusted to the same protein concentration (generally 1–2 mg/ml) and incubated with the appropriate antibody for approximately 12 hr on ice. The antigen-antibody complexes were collected by adsorption onto formalin-fixed Staphylococcus aureus Cowan I (Staph A) [25] either directly or to Staph A that had been prereacted with affinity-purified rabbit antimouse IgG (Cappel, Malvern, PA). The immune complexes immobilized on Staph A were washed three times with modified RIPA buffer, once with modified RIPA buffer containing 1.0 M NaCl, and once with kinase buffer (.02 M morpholinopropane-sulfonic acid (pH 7.0), 0.005 M $MgCl_2$). The washed immunoprecipitates were suspended in kinase buffer containing 20 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear, Boston, MA) and the reaction was allowed to proceed for 15 min at 23°C. The reactions were terminated by the addition of modified RIPA buffer. The immunoprecipitates were washed twice in modified RIPA buffer and once in TNN buffer (.05 M Tris (pH 8.0), .15 M NaCl, .05% (v/v) Nonidet P-40). The bound proteins were eluted by addition of SDS sample buffer and analyzed on SDS-polyacrylamide gels as described previously [26].

Quantitation of the phosphorylated reaction products from the immune complex kinase assays was conducted by localization of the band of interest by autoradiography, excision of the corresponding gel region, and counting in a liquid scintillation spectrometer. The gels, in each case, were reexposed to X-ray film to insure that only the bands of interest had been excised.

Radiolabeling of Cells and Immunoprecipitation

Py-infected or mock-infected MEC were labeled between 15 and 30 hr postinfection for 15 hr with 400 μCi of [^{35}S]methionine (ca 1,400 Ci/mmol, Amersham, Arlington Heights, IL) or 1 mCi of [^{32}P]orthophosphate (carrier free, New England Nuclear) per ml in methionine or phosphate-free Eagle's minimal essential medium supplemented with 5% complete Eagle's minimal essential medium plus 2% dialyzed fetal calf serum. All cells were lysed in modified RIPA buffer as described above and immunoprecipitations were conducted as previously described [14].

Peptide Analysis of Phosphoamino Acid Analysis

Peptide mapping by limited proteolysis in SDS-polyacrylamide gels was conducted as described by Cleveland et al [27] using S aureus V8 protease (Pierce,

Rockford, IL). Phosphoamino acid analysis was conducted by a modification of the method of Hunter and Sefton [28] as described by Blithe et al [29].

RESULTS

Stimulation of pp60$^{c\text{-}src}$ Kinase Activity in Py-Infected Mouse Cells

Primary MEC were infected with an equal number of plaque-forming units of Py wt or mutant Py and harvested 24 hr postinfection as described in Materials and Methods. When immune-complex kinase assays were conducted with T sera immunoprecipitates from these lysates, MTAg was found to be the major phosphorylated protein (Fig. 1, lanes 1). The extent of MTAg in vitro phosphorylation in these assays was consistent with the previous reports that transformation competent strains of Py possess MTAg-associated kinase activity proportional to their oncogenic potential [4–6,17]. When immune-complex kinase assays were conducted with parallel immunoprecipitates formed between the same lysates and a MAb (MAb 327) that recognizes mammalian pp60$^{c\text{-}src}$ [24], both MTAg and pp60$^{c\text{-}src}$ were found to be phosphorylated in each case where the viral-encoded MTAg was also found to possess associated kinase activity (Fig. 1, lanes 3). The level of pp60$^{c\text{-}src}$ in vitro phosphorylation in lysates of MEC infected with transformation-competent strains of Py was increased three- (d123), eight- (wt), or tenfold (d18) over the phosphorylation of pp60$^{c\text{-}src}$ in lysates of uninfected MEC or MEC infected with transformation-deficient strains (NG59 and NG18) of Py (Fig. 1).

The in vitro phosphorylated MTAg and pp60$^{c\text{-}src}$ proteins shown in Figure 1 were excised and analyzed by partial proteolytic digestion with V8 protease. Repre-

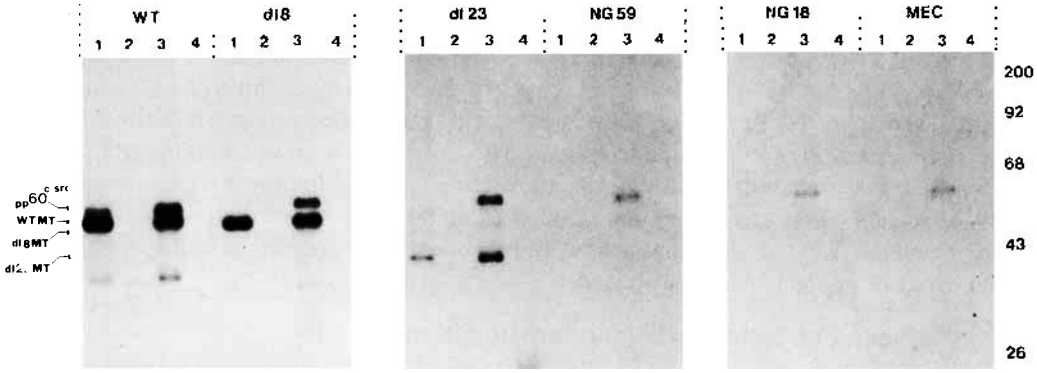

Fig. 1. Protein kinase activity in T sera and MAb 327 immunoprecipitates of MEC infected with different strains of Py. The Py-infected MEC lysates were made 24 hr postinfection and immunoprecipitated with the appropriate antibodies and the phosphorylated reaction products of immune-complex kinase assays were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. Lane 1, T sera; lane 2, normal hamster sera (NHS); lane 3, MAb 327; lane 4, normal mouse sera (NMS). The positions of various prestained molecular weight markers (BRL) (200 K, myosin heavy chain; 92 K, phosphorylase b; 68 K, bovine serum albumin; 43 K, ovalbumin; 26 K, α-chymotrypsinogen; 18 K, β-lactoglobulin) are indicated in the margin as are the positions of the various MTAgs and pp60$^{c\text{-}src}$.

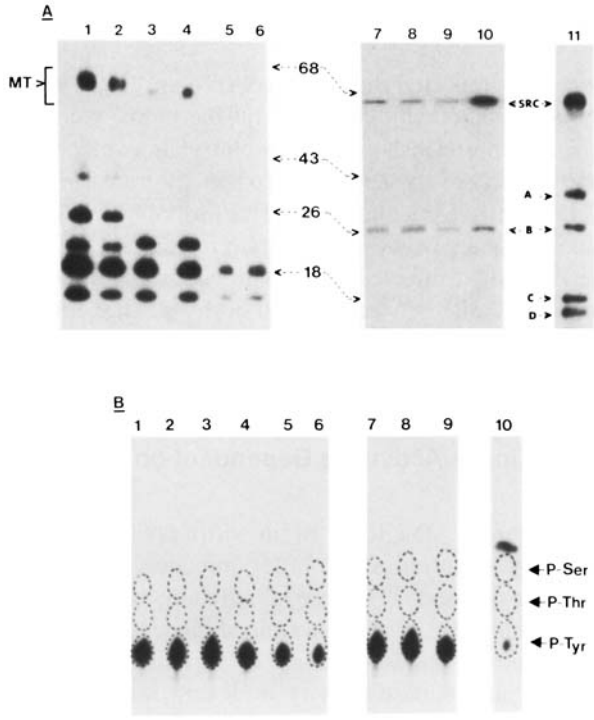

Fig. 2. A) Peptide mapping of immune-complex kinase reaction products by limited V8 proteolysis. The in vitro phosphorylated MTAg bands (lanes 1–6) and pp60$^{c\text{-}src}$ bands (lanes 7–9) were excised from the gels shown in Figure 1A, placed on parallel 10% SDS-polyacrylamide gels, overlaid with 200 ng/lane V8 protease, and digestion performed as described in Materials and Methods. Lanes 1 (wt MTAg), 3 (d18 MTAg), and 5 (d123 MTAg) immunoprecipitated with T sera; lanes 2 (wt MTAg), 4 (d18 MTAg), and 6 (d123 MTAg) immunoprecipitated with MAb 327; lanes 7–9, pp60$^{c\text{-}src}$ immunoprecipitated with MAb 327 from wt, d18, or d123-infected MEC, respectively. Lane 10, in vitro phosphorylated pp60$^{v\text{-}src}$ immunoprecipitated with MAb 327 from mouse cells (Balb/c 3T3) transformed with Rous sarcoma virus. Lane 11, in vivo phosphorylated pp60$^{c\text{-}src}$ from uninfected MEC immunoprecipitated with MAb 327. B) phosphoamino acid analysis of immune-complex kinase reaction products. Lanes 1 (wt MTAg), 2 (d18 MTAg), and 3 (d123 MTAg) immunoprecipitated with T sera; lanes 4 (wt MTAg), 5 (d18 MTAg), and 6 (d123 MTAg) immunoprecipitated with MAb 327; lanes 7–10, pp60$^{c\text{-}src}$ immunoprecipitated with MAb 327 from wt, d18, or d123-infected MEC, or uninfected MEC, respectively. The positions of Ninhydrin-stained phosphoamino acid standards are indicated in the right margin.

sentative results from this experiment are shown in Figure 2A. The phosphorylated V8-derived peptides of MTAg from either T sera (lanes 1, 3, 5) or MAb 327 (lanes 2, 4, 6) immunoprecipitates were found to be indistinguishable from one another and similar to those previously reported for in vitro phosphorylated MTAg [7]. The phosphorylated V8-derived peptides from pp60$^{c\text{-}src}$ in this experiment (lanes 7–10) demonstrated that the 26 Kd V8 fragment representing the carboxy-terminal portion of this protein was the principal region of in vitro phosphorylation. The results of phosphoamino acid analysis of the in vitro phosphorylated pp60$^{c\text{-}src}$ (lanes 7–10) and MTAg proteins (lanes 1–6) from parallel experiments are shown in Figure 2B and demonstrate that phosphotyrosine was the only phosphorylated amino acid detected.

Synthesis and Phosphorylation of pp60$^{c\text{-}src}$ Is Not Increased in Py-Infected Mouse Cells

We have previously reported that the level of c-src RNA is not elevated in Py-transformed or Py-infected rodent cells [15]. Furthermore, we have shown that the synthesis and degree of posttranslational phosphorylation of pp60$^{c\text{-}src}$ in such cells was not significantly increased when measured in Py-transformed and Py-infected cells labeled for 3 hr with [^{35}S]methionine or [^{32}P]orthophosphate [15]. To determine whether longer labeling times would demonstrate increased levels of pp60$^{c\text{-}src}$ in Py-infected MEC, we labeled wt-infected MEC with either [^{35}S]methionine (Fig. 3A) or [^{32}P]orthophosphate (Fig. 3B) between 15 and 30 hr postinfection and estimated the level of pp60$^{c\text{-}src}$ by immunoprecipitation of cellular lysates with MAb 327. As shown, no significant differences in the level of pp60$^{c\text{-}src}$ synthesis or in vivo phosphorylation between Py-infected and -uninfected MEC was observed.

Increased pp60$^{c\text{-}src}$ Kinase Activity Is Dependent on Py Early Gene Product Synthesis

As shown in Figure 1, the level of in vitro pp60$^{c\text{-}src}$ phosphorylation was increased approximately eightfold 24 hr after infection of MEC with Py wt. To examine the time course of pp60$^{c\text{-}src}$ kinase stimulation, we infected parallel cultures of MEC with 50 PFU/cell of Py wt and determined the level of both MTAg-associated kinase activity and pp60$^{c\text{-}src}$ kinase activity at various times following infection. Detectable MTAg-associated kinase activity in T sera immunoprecipitates was first found at 8 hr postinfection (Fig. 4A and B, lane e). This activity then increased dramatically over the next several hours and was maximal at about 22 to 24 hr postinfection (Fig 4A and B, lanes l and m). As shown in Figure 4A and C (lanes b and c) small increases in pp60$^{c\text{-}src}$ kinase activity (1.5 to twofold) were observed as

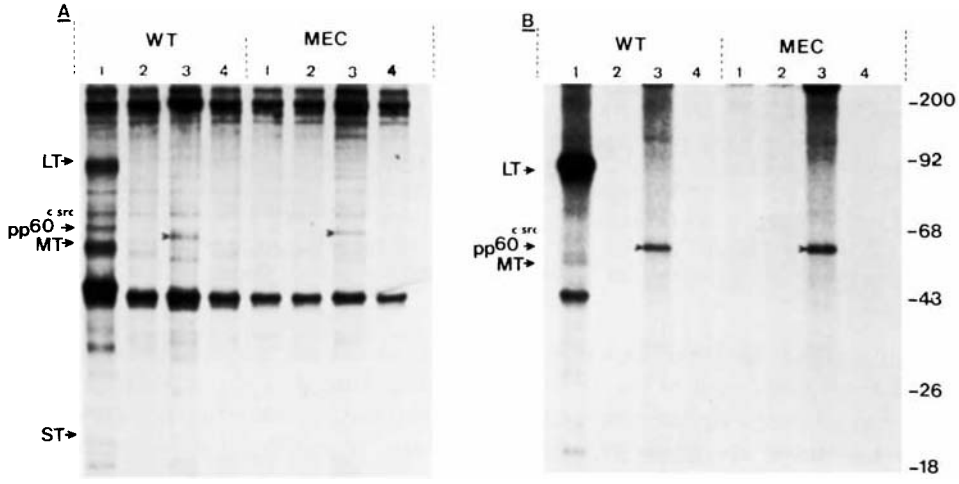

Fig. 3. Immunoprecipitation of pp60$^{c\text{-}src}$ from Py-infected MEC lysates. Primary MEC were infected with Py wt and were labeled between 15 and 30 hr postinfection with either [^{35}S]methionine (A) or [^{32}P]orthophosphate (B) as described in Materials and Methods. The cells were lysed and immunoprecipitated with T sera (lane 1), NHS (lane 2), MAb 327 (lane 3), or NMS (lane 4) and the radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The positions of prestained molecular weight markers are indicated in the right margin and the positions of Py TAgs and pp60$^{c\text{-}src}$ are shown in the left margin.

early as 2 to 4 hr postinfection. The level of pp60$^{c\text{-}src}$ kinase was found to increase gradually thereafter until 22 hr postinfection. Interestingly, detectable MTAg was found to coimmunoprecipitate with pp60$^{c\text{-}src}$ and be phosphorylated in MAb 327 immune-complex kinase assays at 6 hr postinfection (Fig. 4C, lane d) 2 hr before the time MTAg-associated kinase activity was detected in T sera immunoprecipitates. In parallel experiments, detectable [^{35}S]methionine-labeled MTAg in T sera immunoprecipitates was not found until approximately 10 hr postinfection. The level of metabolically labeled MTAg was found to increase until approximately 24 hr postinfection (data not shown).

Since pp60$^{c\text{-}src}$ kinase activity was found to be increased at very early times following infection of MEC with Py wt (Fig. 4), we next examined whether or not

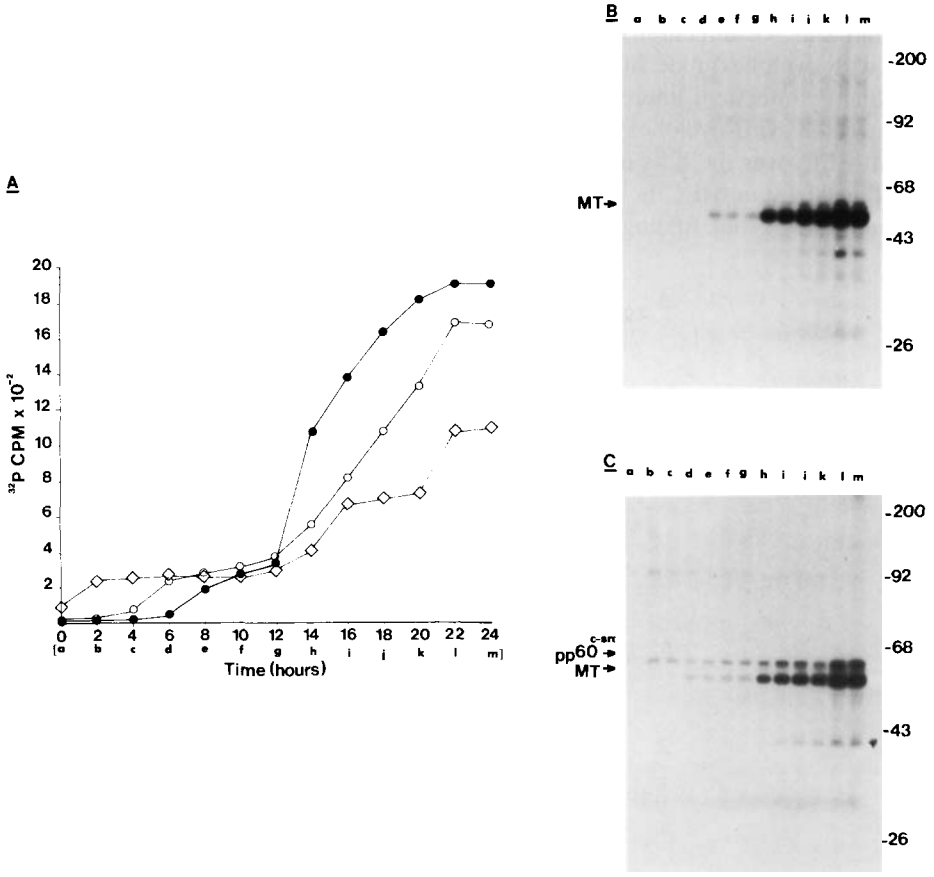

Fig. 4. Stimulation of pp60$^{c\text{-}src}$ kinase activity following infection of MEC with Py wt. Parallel plates of MEC were infected with Py wt and the cells were harvested at 2 hr intervals. Following the last time point (24 hr postinfection), the cells were lysed and the lysates divided into two equal aliquots and immunoprecipitated with either T sera (B) or MAb 327 (C) and the phosphorylated products of immune-complex kinase assays were separated on SDS-polyacrylamide gels and detected by autoradiography. The positions of prestained molecular weight markers are indicated in the right margin and the positions of MTAg and pp60$^{c\text{-}src}$ are shown in the left margin. The MTAg and pp60$^{c\text{-}src}$ bands were excised from the gels represented in B and C and quantitated by counting in a liquid scintillation spectrometer and the results are depicted graphically (A). (●) MTAg immunoprecipitated with T sera, (○) MTAg immunoprecipitated with MAb 327, (□) pp60$^{c\text{-}src}$ immunoprecipitated with MAb 327.

this increase in kinase activity was dependent on TAg synthesis. For these experiments, we infected MEC with 50 PFU/cell of either Py wt or NG18, which does not encode detectable MTAg or small TAg [21], and quantitated the amount of $pp60^{c\text{-}src}$ phosphorylated in MAb 327 immune-complex kinase assays. The data shown in Figure 5A demonstrate that infection of MEC with NG18 did not result in the early stimulation of $pp60^{c\text{-}src}$ kinase activity. These results suggested that the synthesis of either MTAg and/or small TAg might be responsible for the increased $pp60^{c\text{-}src}$ kinase activity in wt-infected MEC.

To determine whether or not protein synthesis was required for the early stimulation of $pp60^{c\text{-}src}$ kinase activity in Py-infected cells, MEC were infected with 50 PFU/cell of Py wt in the presence or absence of 50 μg/ml cycloheximide and the amount of $pp60^{c\text{-}src}$ phosphorylated in MAb 327 immune-complex kinase assays at 2, 4, 6, and 8 hr postinfection was determined. As is shown in Figure 5B, treatment of MEC with cycloheximide inhibited the early stimulation of $pp60^{c\text{-}src}$ kinase activity following Py infection. Interestingly, the level of $pp60^{c\text{-}src}$ kinase activity in uninfected MEC treated with cycloheximide was indistinguishable from that observed in untreated MEC over the 8 hr time course of this experiment. Since the adsorption and uncoating of Py in MEC is independent of protein synthesis (RA Consigli, personal communication), our findings suggest that the lack of $pp60^{c\text{-}src}$ kinase stimulation in

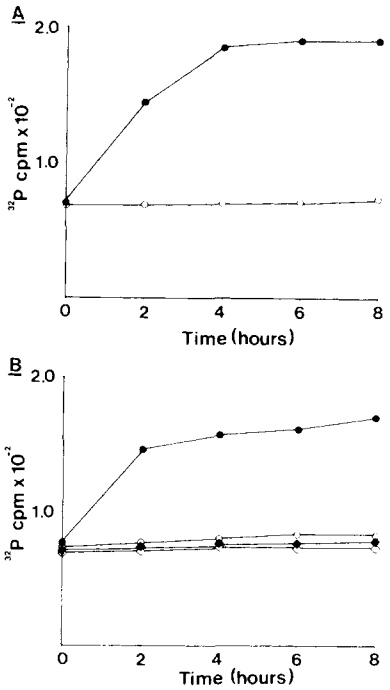

Fig. 5. Quantitation of in vitro phosphorylated $pp60^{c\text{-}src}$ at early times following infection of MEC with Py. A) primary MEC were infected with an equal amount of either wt (●) or NG18 (○), the cells were harvested at the various times shown postinfection, and the amount of in vitro phosphorylated $pp60^{c\text{-}src}$ determined by performing immune-complex kinase reactions on MAb 327 immunoprecipitates as described in Figure 3. B) primary MEC were infected with Py wt in the presence (○) or absence (●) of 50 μg/ml cycloheximide and the amount of in vitro phosphorylated $pp60^{c\text{-}src}$ determined as described above. The effect of cycloheximide treatment on $pp60^{c\text{-}src}$ in vitro phosphorylation in uninfected MEC was also determined, (■) untreated, (□) cycloheximide-treated (50 μg/ml).

cycloheximide-treated Py-infected MEC is not the result of inhibiting Py entry into the cells.

DISCUSSION

Oncogenic transformation of rodent cells by Py is a multistep phenomenon dependent on the interaction of both Py-encoded proteins and cellular factors that vary between different rodent cells [1]. Several mutants of Py that affect the coding region for MTAg have been reported [17–20] and, among the mutants that are transformation-competent, the phenotype of cells transformed by these Py mutants varies dramatically [30]. The selection of Py-transformed cells by the appearance of dense foci on plastic surfaces in liquid culture or the growth of anchorage-independent colonies in soft agar undoubtedly reflects numerous biochemical alterations within the transformants that result from a series of interactions between viral and cellular gene products. To analyze the effects of the Py-encoded early gene products (the TAgs) on cellular events, we have studied MEC cells infected with Py to minimize some problems likely to be encountered by the examination of cell lines selected for transformation.

In this report, we have examined the effect of Py infection on pp60$^{c\text{-}src}$ tyrosyl kinase activity. Our results demonstrate that, though small increases in pp60$^{c\text{-}src}$ kinase activity can be observed prior to the detection of TAg synthesis or MTAg-associated kinase activity in Py wt-infected MEC (Figs. 4 and 5), the stimulated pp60$^{c\text{-}src}$ kinase activity generally parallels the level of Py early gene product synthesis (Fig. 4). Since increased pp60$^{c\text{-}src}$ kinase activity was not observed in NG18- and NG59-infected MEC (Fig. 1) and was increased only to a small degree in d123-infected MEC (Fig. 1), we interpret our results to suggest that the increased level of pp60$^{c\text{-}src}$ kinase activity in Py-infected MEC is related to the synthesis of Py-encoded MTAg and its association with pp60$^{c\text{-}src}$. Furthermore, the results presented in this report demonstrate that the maximum level of pp60$^{c\text{-}src}$ kinase activity detected in MEC infected with various strains of Py is proportional to the oncogenic potential of the Py strain used for infection and to the amount of MTAg-associated kinase activity present in the infected cells (Fig. 1). This concept is supported by our finding that, although wt, d123, and NG59-infected MEC contain approximately the same amount of detectable MTAg, the d18-infected MEC used in these experiments were found to contain less than 20% of the amount of MTAg found in wt-infected MEC (data not shown). This discrepancy is presumably due to the diminished level of Py DNA synthesis in d18-infected cells [17].

The results shown in Figure 1 provide additional evidence that MTAg and pp60$^{c\text{-}src}$ can be coimmunoprecipitated from lysates of Py-infected MEC by MAb 327 directed against pp60$^{c\text{-}src}$ [24]. As previously observed [14, 15], the coimmunoprecipitation of MTAg and pp60$^{c\text{-}src}$ was evidenced by our finding that both proteins were phosphorylated in immune-complex kinase assays. The identities of the phosphorylated proteins were confirmed by peptide mapping (Fig. 2A) and the phosphorylated amino acid product of these reactions was found to be phosphotyrosine (Fig. 2B). Infection of MEC with transformation-competent strains of Py resulted in significantly increased pp60$^{c\text{-}src}$ phosphorylation when compared with the level of pp60$^{c\text{-}src}$ phosphorylation in parallel assays conducted with MAb 327 immunoprecipitates from either uninfected MEC or MEC infected with transformation-defective strains of Py (NG59 and NG18). Interestingly, the level of increased pp60$^{c\text{-}src}$ phosphorylation (presumably autophosphorylation) in these immune-complex kinase assays was pro-

portional to both the potential of these Py strains to induce oncogenic transformation in rodent cells and the phenotype of the subsequent cellular transformants [17,19,30]. The clearest example of this phenomenon in observed with the Py-viable deletion strains d18 and d123. Rodent cells transformed by d18 grow faster as colonies in soft agar or dense foci on plastic dishes when compared to parallel rodent cells transformed by Py wt [30]. Rodent cells transformed by d123 grow in either soft agar or as dense foci on plastic surfaces at rates significantly below those of wt transformants [30].

As is shown in Figure 3, the increased levels of $pp60^{c\text{-}src}$ kinase activity were not the result of increased synthesis of $pp60^{c\text{-}src}$. When wt-infected MEC and uninfected MEC were labeled in parallel for 15 hr in the presence of [^{35}S]methionine or [^{32}P]orthophosphate, no significant differences were noted in amount of radiolabeled $pp60^{c\text{-}src}$. These results are in agreement with our previous finding that the level of c-src RNA in wt-infected MEC was indistinguishable from that found in uninfected MEC and that similar levels of metabolically labeled $pp60^{c\text{-}src}$ were observed in both wt-infected and uninfected MEC following shorter (3 hr) labeling periods [15]. The results shown in Figure 3 further suggest that the turnover of $pp60^{c\text{-}src}$ is not significantly altered in Py-infected MEC.

The results shown in Figure 4 demonstrate that the level of $pp60^{c\text{-}src}$ kinase activity, as measured by in vitro phosphorylation of $pp60^{c\text{-}src}$ in MAb 327 immune-complex kinase assays, parallels the increase in MTAg-associated kinase activity and TAg synthesis (data not shown) following Py wt infection of MEC. We have also observed a similar pattern of increased $pp60^{c\text{-}src}$ kinase activity following Py infection by measuring α-casein phosphorylation in MAb 327 immunoprecipitates (unpublished results). During the first several hours following wt infection of MEC, $pp60^{c\text{-}src}$ kinase activity was found to increased approximately twofold (Fig. 4). We therefore took advantage of this early event to examine whether TAg synthesis at levels below our limits of detection was also responsible for the observed increase in $pp60^{c\text{-}src}$ kinase activity. As is shown in Figure 5A, infection of MEC with NG18 did not stimulate $pp60^{c\text{-}src}$ kinase activity, whereas infection of parallel MEC cultures with an equal amount of wt resulted in a significant increase in $pp60^{c\text{-}src}$ kinase activity by 4 to 5 hr postinfection. These results suggest that either Py-encoded MTAg or small TAg might be responsible for the observed increase in $pp60^{c\text{-}src}$ kinase activity. Since the d123 mutant encodes a normal small TAg [17,18], which is synthesized at approximately the same level as wt-encoded small TAg in infected cells, the low level of $pp60^{c\text{-}src}$ kinase stimulation in d123-infected MEC suggests that MTAg synthesis is responsible for the increased $pp60^{c\text{-}src}$ kinase activity. The lack of early $pp60^{c\text{-}src}$ kinase activity stimulation of NG18-infected cells further argues that this increased activity is not a nonspecific response to virus infection or the conditions utilized for virus adsorption in our experiments. The inability of wt infection to stimulate this early increase of $pp60^{c\text{-}src}$ kinase activity in cycloheximide-treated MEC suggests that TAg synthesis is required (Fig. 5B). The results of these studies, therefore, suggest that the early stimulation of $pp60^{c\text{-}src}$ kinase activity is dependent on the synthesis of Py-encoded MTAg. However, because the level of MTAg at these early times following infection is below our limits of detection, we cannot conclude that the early stimulation of $pp60^{c\text{-}src}$ kinase activity is dependent on the direct association of MTAg and $pp60^{c\text{-}src}$.

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