Analysis of pp60^{c-src} Protein Kinase Activity in Hamster Embryo Cells Transformed by Simian Virus 40, Human Adenoviruses, and Bovine Papillomavirus 1

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We have examined the effect of DNA tumor virus transformation of primary hamster embryo cells on the tyrosyl kinase activity of pp60^{c-src}. Our present study demonstrates that some clones of hamster embryo cells transformed by simian virus 40, adenovirus type 2, adenovirus type 12, or bovine papillomavirus 1 can possess elevated pp60^{c-src} kinase activity when compared with normal hamster embryo cells. However, other clones of hamster embryo cells transformed by these same viruses were found to have normal levels of pp60^{c-src} kinase activity. In those clones of transformed cells where pp60^{c-src} kinase activity was elevated, the increased levels of kinase activity were the result of an apparent increase in the specific activity of the pp60^{c-src} phosphotransferase rather than an increase in the amount of the *src* gene product. Additionally, pp60^{c-src} was not found to be physically associated with tumor antigens known to be encoded by these viruses. These results indicate that elevated levels of pp60^{c-src} kinase activity can be found in hamster embryo cells transformed by several different DNA tumor viruses and suggest that the molecular mechanism by which pp60^{c-src} kinase activity is elevated may differ from that previously observed in polyomavirus-transformed cells. These results also imply that elevation of pp60^{c-src} kinase activity is not required for the transformation of hamster cells by these viruses.

Oncogenic transformation of cells by DNA tumor viruses is thought to require the continued expression of one or more of the virus-encoded early gene products (19). Since many of these early gene products do not share significant amino acid homology, it appears that the mechanism by which they initiate alterations in cellular growth properties may vary. It is also possible that any given virus may have the potential to initiate or maintain the phenotype of the transformed cell by altering more than one cellular biochemical pathway.

Oncogenic transformation of rodent cells by polyomavirus (Py) requires the continued expression of the 56-kilodalton middle tumor antigen (MTAg) (reviewed in reference 15). The MTA_g possesses an associated tyrosyl kinase activity (10, 16, 18) which has been shown to be a property of a tightly bound cellular enzyme, pp60^{c-src} (2, 7, 8, 13). The significance of the MTA_g-associated tyrosyl kinase in Py-mediated transformation is suggested by the finding that all transformation-competent strains of Py are capable of encoding MTA_g molecules which possess this associated protein kinase activity. Recent studies have demonstrated that the association of MTA_g with pp60^{c-src} results in an increase in the specific activity of the MTA_g-associated pp60^{c-src} tyrosyl kinase function (4, 5) and an increase in the pp60^{c-src}-associated phosphoinositide kinase activity (22). Py is the only known DNA tumor virus which has been shown to encode a transforming protein which possesses an associated tyrosyl kinase activity. It therefore seemed possible that cells transformed by other DNA tumor viruses might not possess elevated levels of pp60^{c-src} kinase activity, a feature characteristic of Py-transformed cells. To determine whether the activation of pp60^{c-src} enzyme activity was

specific for Py-transformed cells, we screened clones of primary hamster embryo cells (HEC) transformed by other DNA tumor viruses for elevated pp60^{c-src} tyrosyl kinase activity. We report here that some clones of HEC transformed by other DNA tumor viruses also exhibited increased levels of pp60^{c-src} protein kinase activity. In contrast to transformation by Py, however, pp60^{c-src} was not found to be associated with proteins encoded by these other DNA viruses.

In our initial experiments, we compared the level of pp60^{c-src} kinase activity in HEC transformed by a variety of DNA tumor viruses, including Py, with primary cultures of HEC. The level of pp60^{c-src} kinase activity was determined by either *src* autophosphorylation (Fig. 1A through C) or casein phosphorylation (Fig. 1D through F) in immune complex kinase assays. The level of pp60^{c-src} tyrosyl kinase activity was found to be significantly elevated in two of four simian virus 40 (SV40)-transformed hamster cell lines (Fig. 1A), two of two adenovirus type 2 (Ad2)-transformed hamster cell lines, two of two Ad12-transformed hamster cell lines (Fig. 1B), and three of three bovine papillomavirus 1 (BPV-1)-transformed hamster cell lines (Fig. 1C). In the cell lines described in Fig. 1, the amount of pp60^{c-src} kinase activity was found to be generally equal to or greater than that observed in Py-transformed HEC. However, in contrast to these results, examination of an additional four SV40-transformed, two Ad2-transformed, and two Ad12-transformed hamster cell lines revealed that they contained normal levels of pp60^{c-src} kinase activity (data not shown). These results demonstrate that elevated pp60^{c-src} kinase activity can be observed in hamster cells transformed by DNA viruses other than Py and suggest that elevated pp60^{c-src} kinase activity is not required for SV40, Ad2, or

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FIG. 1. Immune complex protein kinase assays of HEC transformed by different DNA tumor viruses. A, Four independent SV40-transformed HEC cell lines (SVHE-1 through -4) derived from foci after SV40 infection of primary HEC were lysed in buffer (100 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1% Nonidet P-40), adjusted to the same protein concentration (400 μ g/ml), and immunoprecipitated with MAb 327, a monoclonal antibody directed against pp60^{c-src} (12). Protein kinase assays were performed, and the phosphorylated reaction products were analyzed by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels and autoradiography as previously described (1, 3). Parallel assays were conducted with a previously characterized Py-transformed HEC line (5) and primary HEC. B, Same experiment conducted with two Ad2- and two Ad12-transformed HEC lines. C, Same experiment conducted with three BPV-1-transformed HEC cell lines. The positions of pp60^{c-src} and Py MTag are indicated in the left margin, and the positions of prestained molecular weight markers (Bethesda Research Laboratories) are indicated in the right margin. D, Casein kinase assays (5) run in parallel to the experiment shown in A. E, Casein kinase assays run in parallel to the experiment shown in B. F, Casein kinase assays run in parallel to the experiment shown in C. For this figure, the phosphorylated casein bands were excised from sodium dodecyl sulfate gels, and the number of ³²P counts per gel slice was determined by liquid scintillation spectroscopy as previously described (2, 5).

Ad12 transformation of HEC. The situation is less clear with regard to BPV-1 transformation of HEC, since all BPV-1 HEC transformants tested showed enhanced pp60^{c-src} kinase activity.

The data presented in Fig. 1 established that some SV40-, Ad2-, Ad12-, and BPV-1-transformed HEC lines contained elevated pp60^{c-src} protein kinase activity. It was of interest to characterize several of these HEC transformants to determine whether the mechanism of pp60^{c-src} activation was similar to that proposed after Py transformation (3, 23).

The pp60^{c-src} molecules physically associated with Py MTag in Py-transformed cells possess both quantitative and

qualitative differences compared with pp60^{c-src} molecules not complexed with MTag (3, 5, 23). The most apparent quantitative difference is the increased in vitro protein kinase activity of the MTag-associated src molecules (4, 5). Qualitative changes in MTag-associated src molecules include one or more probable phosphotyrosine modifications within the amino-terminal half of this protein (23) in addition to phosphoserine modifications normally observed in the amino-terminal portion of src molecules (6, 17). Recently, V8 peptide analysis of MTag-associated pp60^{c-src} has demonstrated that pp60^{c-src} can be phosphorylated on as yet undefined amino-terminal tyrosine residues in addition to

tyrosine moieties (presumably tyrosine 416) in the carboxy-terminal portion of the molecule (3, 23). We, therefore, analyzed the in vitro-phosphorylated pp60^{c-src} proteins from several of the cell lines shown in Fig. 1 in addition to in vitro-phosphorylated pp60^{c-src} from three other independent Py-transformed HEC lines by partial proteolytic digestion with V8 protease. Significant levels of amino-terminal in vitro phosphorylation of c-src molecules occurred only in Py-transformed HEC (Fig. 2). These data indicate that the src molecules associated with MTag in the Py-transformed HEC can be qualitatively distinguished from those in the other virus-transformed HEC lines, even though the pp60^{c-src} kinase activities in these transformed HEC are quantitatively indistinguishable. These results can also be interpreted to suggest that the amino-terminal tyrosine phosphorylation of src molecules associated with MTag may be a consequence of src activation rather than a causal event.

We have previously demonstrated that the level of c-src RNA is not elevated in Py-transformed rodent cells (4, 5). Similarly, the overall level of c-src protein in Py-transformed rodent cells is indistinguishable from that found in the corresponding normal rodent cell (4, 5). We analyzed the level of c-src RNA and protein in the various virus-transformed cells to determine whether the increased levels of pp60^{c-src} kinase activity might reflect concomitant increases in the level of the src protein.

To estimate the amount of pp60^{c-src} synthesis in these cells, we labeled representative cell cultures with either [³⁵S]methionine (250 μ Ci/ml, ca. 1,300 Ci/mmol; Amersham Corp.) or ³²P_i (1 mCi/ml, carrier free; Amersham) for 4 h and

immunoprecipitated the radiolabeled pp60^{c-src} molecules with MAb 327, a monoclonal antibody that recognizes mammalian c-src molecules (12). No significant differences in the level of pp60^{c-src} synthesis (Fig. 3A) or in vivo phosphorylation (Fig. 3B) were observed among the various transformed and normal hamster cells. Similarly, the level of c-src transcripts as determined by dot-blot hybridization (Fig. 3C) or Northern analysis of poly(A)-selected cytoplasmic RNA (Fig. 3D) demonstrates that the steady-state level of c-src mRNA is indistinguishable among the various cell cultures tested. These results demonstrate that, as in Py-transformed cells, the level of pp60^{c-src} protein and c-src RNA is not significantly altered in HEC transformed by SV40, Ad2, or BPV-1. Partial proteolytic peptide mapping of the [³⁵S]methionine- and ³²P-labeled src protein (Fig. 3A and B) by V8 digestion also did not reveal detectable differences (data not shown).

The results presented above suggest that, as in Py-transformed HEC, the increased level of pp60^{c-src} kinase activity observed in HEC transformed by the other viruses represents an apparent enhancement of the pp60^{c-src}-specific activity. Unlike with Py transformants, however, we have not been able to coimmunoprecipitate pp60^{c-src} with any of the known early gene products encoded by either SV40, Ad2, Ad12, or BPV-1 (data not shown). These experiments have been conducted with several different polyclonal antisera from hamsters bearing SV40- and adenovirus-induced tumors and with several different monoclonal antibodies directed against SV40 large tumor antigen and cellular p53. Antisera or monoclonal antibodies to putative proteins encoded by the early open reading frames of BPV-1 are not available. We also have been unable to identify any probable early gene products from these DNA viruses in MAb 327 immunoprecipitates from metabolically radiolabeled transformed cells (Fig. 3A and B) (data not shown) or as phosphorylated species after immune-complex kinase assays (Fig. 1; unpublished results). Similar results were obtained with three additional monoclonal antibodies directed against pp60^{c-src} (data not shown). Also, in contrast to our previous findings with Py (4, 5), we have found no evidence that the stimulation of pp60^{c-src} kinase activity is directly related to the expression of the early gene products from either SV40, Ad2, or Ad12. Acute infection of HEC by these viruses, using multiplicities of infection which resulted in the majority of cells becoming fluorescence positive for the appropriate early gene products, did not result in the stimulation of pp60^{c-src} kinase activity in subsequent MAb 327 immune complex kinase assays (our unpublished results). This observation suggests that the elevated pp60^{c-src} kinase activity found in the cells examined in these experiments may have occurred secondary to the initial cellular events associated with viral transformation. Since we have not analyzed clones of normal HEC which have been passaged the number of times that the transformed cells used in these experiments have been passaged, it is also possible that the elevated levels of c-src kinase activity observed in the various cell lines may represent either a property preexisting in a small population of the cells before transformation or a property gained by some cellular clones as a result of serial passage during selection for transformants.

The results presented in this report demonstrate that pp60^{c-src} tyrosyl kinase activity can be significantly elevated in hamster embryo cells oncogenically transformed by several different DNA tumor viruses. The level of pp60^{c-src} kinase activity observed in several of the transformed HEC lines, particularly those transformed by Ad2, was found to

FIG. 2. Partial proteolytic mapping of in vitro-phosphorylated pp60^{c-src} from transformed HEC. In vitro-phosphorylated 60-kilodalton bands were excised from gels, placed on parallel 12.5% sodium dodecyl sulfate-polyacrylamide gels, and digested with 50 ng of V8 protease per lane; the resulting peptides were resolved by electrophoresis and detected by autoradiography as previously described (2-5). The peptide maps from the pp60^{c-src} band derived from normal HEC were exposed to X-ray film twice as long as the other samples in this figure. The position of the amino-terminal src phosphopeptide from Py-transformed HEC is indicated by the arrow. The position of the 26-kilodalton carboxy-terminal V8 pp60^{c-src} peptide (17) is indicated in the left margin, and the positions of prestained molecular weight markers are shown in the right margin.

FIG. 3. Analysis of $\text{pp60}^{\text{c-src}}$ synthesis and c-src transcripts in DNA virus-transformed HEC. (A) Py HE-1, SV HE-1, Ad2 HE-2, BPV HE-3, and HE cells were labeled for 3 h in the presence of [^{35}S]methionine, lysed in buffer (100 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 1% Nonidet P-40), adjusted to the same protein concentration (400 $\mu\text{g}/\text{ml}$), and immunoprecipitated with either MAb 327 (M) or normal mouse serum (N); the radiolabeled products were analyzed by electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gels and detected by autoradiography. (B) Same experiment as in A, except the cells were labeled with ^{32}P . (C) Dot-blot analysis of total cellular RNA (14, 21) from transformed or normal HE cells. Dilutions of the RNA (1 μg in the first lane) were spotted on nitrocellulose and reacted as indicated with either Py early region (32-P Py) (11) or v-src (32-P src) probes (9). (D) Northern analysis of c-src transcripts from transformed and normal HE cells. Poly(A)-selected RNA (5 $\mu\text{g}/\text{lane}$) was electrophoresed on a formaldehyde-agarose gel, transferred to nitrocellulose, and reacted with a ^{32}P -labeled v-src DNA probe (14). The position of the approximately 4.0-kilobase c-src RNA band is indicated in the left margin along with the positions of 28S and 18S rRNA detected by ethidium bromide staining on a parallel gel.

be higher than that observed in Py-transformed cells. The level of c-src transcripts and $\text{pp60}^{\text{c-src}}$ protein did not change from that found in normal cells after transformation with these viruses. Thus, similar to Py transformation, the increased levels of $\text{pp60}^{\text{c-src}}$ kinase activity in SV40-, Ad2-, Ad12-, and BPV-transformed cells apparently reflect an alteration in the specific activity of the $\text{pp60}^{\text{c-src}}$ phosphotransferase.

In contrast to that from Py-transformed cells, $\text{pp60}^{\text{c-src}}$ from the other DNA virus HEC transformants did not possess a significant ability to autophosphorylate tyrosine residues within the amino-terminal half of the src molecules. These results could be interpreted to indicate that amino-terminal phosphorylation of $\text{pp60}^{\text{c-src}}$ in Py-transformed cells reflects an effect of activated src molecules associated with Py MTag rather than the biochemical cause of the enhanced enzyme activity. The association of MTag with $\text{pp60}^{\text{c-src}}$ might lead to a conformational alteration in the activated c-src molecules which favors autophosphorylation at additional sites within the amino-terminal portion of the protein. Alternatively, since the associated MTag and $\text{pp60}^{\text{c-src}}$ proteins have been reported to exist as a high-molecular-weight

complex (>200,000) (20), it is possible that src molecules associated with one MTag molecule could transphosphorylate other MTag-associated src molecules by intermolecular mechanisms similar to those of other exogenous substrates such as casein.

The majority of the evidence presented here argues that increased levels of $\text{pp60}^{\text{c-src}}$ kinase activity are not required for transformation of hamster embryo cells by SV40, Ad2, and Ad12. The situation is less clear for BPV-1 transformation and will require additional study. We have found no obvious phenotypic differences (e.g., growth rate, growth in soft agar, and tumor induction) between HEC transformed by the adenoviruses or SV40 which correlate with the level of src kinase activity (unpublished results). These results raise the possibility that activation of $\text{pp60}^{\text{c-src}}$ kinase activity is not required for Py transformation. Our current model suggests that as a result of binding to MTag, $\text{pp60}^{\text{c-src}}$ kinase activity becomes elevated, and through increased phosphorylation of src substrates cellular growth properties become altered. In this view, the main role of MTag is to mediate some change in $\text{pp60}^{\text{c-src}}$ which leads to increased levels of src enzymatic activity. It is possible, however, that the

MTAg molecules associated with pp60^{c-src} could represent the effector molecules within the MTA_g-pp60^{c-src} complex by either interacting directly with other cellular proteins or simply altering the substrate specificity of pp60^{c-src}, thereby eliminating the requirement for increased levels of *src* kinase activity. Alternatively, MTA_g might associate with a small population of *src* molecules which already possess elevated kinase activity and alter the interaction of these activated *src* proteins with other cellular proteins or phospholipids. Clearly, these potential mechanisms are not mutually exclusive.

In summary, elevation of pp60^{c-src} kinase activity may occur in HEC transformed by several different DNA tumor viruses. However, in contrast to previous observations with Py-transformed cells, the elevation in pp60^{c-src} protein kinase activity is not invariable, and the transforming proteins encoded by SV40, Ad2, Ad12, and BPV-1 are not found in association with pp60^{c-src}. These results suggest that the interaction between pp60^{c-src} and virus-encoded proteins is probably not a general mechanism responsible for oncogenic transformation. However, viruses other than Py may not encode an effector protein such as MTA_g which, by association with pp60^{c-src}, results in the alteration of some cellular growth control processes.

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