Altered Sites of Tyrosine Phosphorylation in pp60^{c-src} Associated with Polyomavirus Middle Tumor Antigen

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We characterized the tyrosine phosphorylation sites of free pp60^{c-src} and of pp60^{c-src} associated with the polyomavirus middle tumor antigen (mT) in transformed avian and rodent cells. The sites of tyrosine phosphorylation in the two populations of pp60^{c-src} were different, both in vitro and in vivo. Free pp60^{c-src} was phosphorylated in vitro at a single site, tyrosine 416. pp60^{c-src} associated with mT was phosphorylated in vitro on tyrosine 416 and on one or more additional tyrosine residues located in the amino-terminal region of the molecule. Free pp60^{c-src} in polyomavirus mT-transformed cells was phosphorylated in vivo on tyrosine 527. In contrast, pp60^{c-src} associated with mT was phosphorylated in vivo on tyrosine 416 and not detectably on tyrosine 527. Thus, the in vivo phosphorylation sites of pp60^{c-src} associated with mT in transformed cells are identical to those of pp60^{v-src}, the Rous sarcoma virus transforming protein. The results suggest that altered phosphorylation of pp60^{c-src} associated with mT may play a role in the enhancement of the pp60^{c-src} protein kinase activity and in cell transformation by polyomavirus.

The Rous sarcoma virus transforming protein pp60^{v-src} and its cellular homolog pp60^{c-src} are membrane-associated phosphoproteins with protein-tyrosine kinase activity (9, 10, 14, 21, 23, 31, 33, 38). The specific activity of pp60^{c-src}, when assayed in vitro with peptide substrates, is 10% or less of the specific activity of pp60^{v-src} (17, 25). Phosphorylation may regulate the enzymatic activities of these two proteins: phosphorylation of amino-terminal tyrosine residues in pp60^{v-src} is accompanied by increased protein kinase activity (3, 7, 11, 37); phosphorylation of tyrosine in the carboxy-terminal region of pp60^{c-src} decreases its protein kinase activity (13). The major site of tyrosine phosphorylation in vivo is different in the viral and cellular protein; pp60^{v-src} is phosphorylated on tyrosine 416, whereas pp60^{c-src} is phosphorylated on a different tyrosine residue in the carboxy-terminal region of the molecule (8, 30, 36, 44).

The middle tumor antigen (mT) of polyomavirus plays a central role in transformation (50). mT is associated with a protein-tyrosine kinase activity (20, 39, 45) which correlates with the transforming ability of the protein (4, 20, 39, 45, 49). mT lacks intrinsic protein kinase activity (40, 41), but associates with a known cellular protein kinase, pp60^{c-src} (2, 9, 15, 16, 33, 38). pp60^{c-src} complexed to mT shows enhanced protein kinase activity and novel sites of tyrosine phosphorylation in vitro (2, 5, 13, 53). Because the protein kinase activity of pp60^{c-src} appears to be negatively regulated by carboxy-terminal tyrosine phosphorylation (13), we examined the possibility that binding to mT might prevent phosphorylation at this site on pp60^{c-src}, thereby increasing its protein kinase activity. We compared the phosphorylation sites of free pp60^{c-src} with those of pp60^{c-src} associated with mT. We find that pp60^{c-src} is phosphorylated in normal fibroblasts at tyrosine 527 (11a). Here we report that free pp60^{c-src} in polyomavirus-transformed cells is also phosphor-

ylated on tyrosine 527. In contrast, pp60^{c-src} associated with mT is not detectably phosphorylated on tyrosine 527, but is phosphorylated on tyrosine 416. The lack of phosphorylation of tyrosine 527 or the phosphorylation of tyrosine 416 in pp60^{c-src} associated with mT (or both) may be important for the increase in its protein kinase activity and for cell transformation by polyomavirus.

MATERIALS AND METHODS

Cell culture. Cells were explanted from 9- to 10-day chicken embryos (SPAFAS, Inc.) and plated at a density of 1.5×10^5 cells per cm^2 in 2:1:1 medium (2% tryptose phosphate [Difco Laboratories], 1% calf serum [GIBCO Diagnostics], 1% heat-inactivated [55°C, 1 h] chicken serum [GIBCO], 2 μg of polybrene [Sigma Chemical Co.] per ml in Dulbecco-Vogt modified Eagle medium). Cells were maintained at 41°C and passaged every third day, seeding 3×10^4 cells per cm^2 in 2:1:1 medium. Rat F2408, Py2408 (polyomavirus-transformed F2408 cells), and RC 3.7 (a rat cell line expressing high levels of chicken pp60^{c-src}) (35) cells were maintained at 37°C in Dulbecco-Vogt modified Eagle medium supplemented with 10% calf serum.

Virus infection. Freshly explanted chicken embryo cells were seeded at a density of 1.5×10^5 cells per cm^2 and infected 3 h later with an amphotropic pseudotype of a recombinant murine retrovirus, mT-murine leukemia virus (mT-MLV[ampho]) (19, 29). The cells were passaged for 2 to 3 weeks before use to allow the virus to spread throughout the culture. RC cells were seeded at a density of 1.5×10^4 cells per cm^2 and infected 24 h later with an ecotropic pseudotype of mT-MLV. An mT-transformed cell line, RC 1-18, was derived from these cells by continuous passage without selection.

Antisera. Mouse monoclonal antibody recognizing pp60^{c-src} (MAb 327) (32) was kindly provided by Joan Brugge (State University of New York at Stony Brook). Ascitic fluid
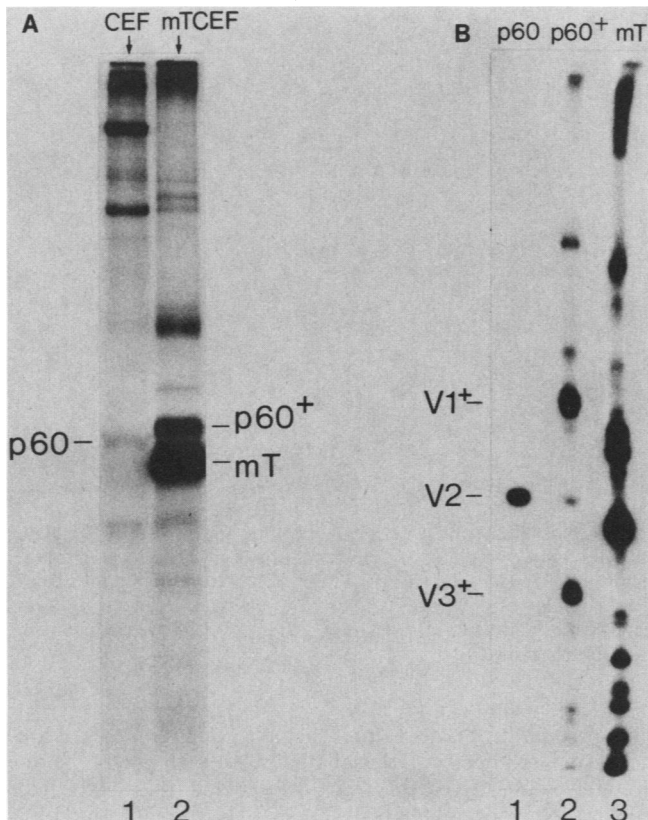
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FIG. 1. In vitro phosphorylation of pp60^{c-src} and mT from transformed chicken embryo cells. (A) Proteins were extracted from cells, immunoprecipitated, incubated with [γ-^{32}P]ATP, and resolved on a 7% SDS–polyacrylamide gel as described in Materials and Methods. Lanes: 1, pp60^{c-src} (p60) from chicken embryo cells (CEF), precipitated with MAb 327; 2, mT and associated pp60^{c-src} (p60$^+$) from mT-transformed chicken embryo cells (mTCEF), precipitated with anti-T ascites. Exposure time was 17 h. (B) V8 digestion of pp60^{c-src} and mT. Proteins were excised from the gel shown in A and digested with 50 ng of *S. aureus* V8 protease, and the peptides were separated on a 12% SDS–polyacrylamide gel. Lanes: 1, p60; 2, p60$^+$; 3, mT. Designation of V8 peptides of pp60^{c-src}: V1$^+$, 34-kilodalton amino-terminal peptide; V2, 26-kilodalton carboxy-terminal peptide; V3$^+$, 18-kilodalton amino-terminal peptide derived from further cleavage of V1$^+$. Exposure time, at −70°C with an intensifying screen, was 24 h.

containing polyclonal anti-tumor antibody (anti-T ascites) was obtained from Brown Norwegian rats inoculated with polyomavirus-transformed cells.

Radiolabeling of cells. Chicken embryo cells were seeded at a density of 4.5×10^4, and rat cells were seeded at 1.5×10^4 cells per cm^2, 24 h before labeling. Cells were rinsed three times in phosphate-free Dulbecco-Vogt modified Eagle medium and labeled for 18 h at 41°C (chicken cells) or 37°C (rat cells) in phosphate-free Dulbecco-Vogt modified Eagle medium with 5% dialyzed calf serum and 2.5 mCi of ^{32}P$_i$ (carrier free; ICN Pharmaceuticals, Inc.) per ml.

Extraction of proteins, immunoprecipitations, and protein kinase assays. Cells were washed three times with ice-cold Tris-buffered saline and lysed in RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 10 mM sodium phosphate [pH 7.0], 1% Trasylol, 50 µM leupeptin, 1 mM dithiothreitol). Lysates were clarified by centrifugation, and proteins were immunoprecipitated by a modification (5) of the method of

Hutchinson et al. (24). Briefly, clarified lysates were incubated at 4°C with anti-T ascites (10 µl per 2×10^5 cells) for 1 h or with MAb 327 (2 µl per 2×10^5 cells) for 1 h followed by rabbit anti-mouse immunoglobulin G (2 µl/µl of MAb 327) for 20 min. Immunoprecipitations of ^{32}P-labeled proteins were done in the presence of 75 µg of RNase A (Sigma) per ml. For the experiment shown in Fig. 7, lysates of ^{32}P-labeled RC 1-18 cells were incubated with anti-T ascites, and the mT–pp60^{c-src} immune complexes were removed by adsorption to Formalin-fixed *Staphylococcus aureus* bacteria. Supernatants were incubated with anti-T ascites, and the mT–pp60^{c-src} immune complexes were again removed. Supernatants were then aliquoted and incubated with either anti-T ascites or MAb 327. Methods then followed those described above. Protein kinase assays were performed by a modification (5) of the method of Eckhart et al. (20).

Polyacrylamide gel electrophoresis and autoradiography. Proteins were resolved on 7% SDS–polyacrylamide gels (20:1 acrylamide-bisacrylamide) and detected by autoradiography with Kodak XAR film. Exposure times and use of intensifying screens are indicated in each figure legend.

Peptide mapping and phosphoamino acid analysis. One-dimensional peptide mapping was done in 12% SDS–polyacrylamide gels (39:1 acrylamide-bisacrylamide) with 50 ng of *S. aureus* V8 protease (Miles Laboratories, Inc.), as previously described (6). For two-dimensional tryptic peptide mapping, proteins from 4×10^6 chicken cells or 2×10^6 rat cells were separated on SDS-polyacrylamide gels, extracted, oxidized, and digested with trypsin as described by Beemon and Hunter (1). Peptides were separated in two dimensions on 100-µm cellulose thin-layer plates by electrophoresis (pH 1.9, 1 kV, 25 min) and chromatography (23). Phosphoamino acid analyses were done by partially hydrolyzing peptides in 5.7 M HCl at 110°C for 1 h and separating the amino acids on 100-µm cellulose thin-layer plates by electrophoresis in two dimensions (12).

Preparation of the synthetic peptide. A peptide corresponding to amino acids 519–533 of pp60^{c-src} (NH$_2$-Tyr-Phe- Thr-Ser-Thr-Glu-Pro-Gln-Tyr-Gln-Pro-Gly-Glu-Asn-Leu-COOH), the synthesis of which is described elsewhere (11a), was phosphorylated by pp60^{c-src} immunoprecipitated from RC cells. Peptide (20 nmol), pp60^{c-src} precipitated from 2×10^5 RC cells, and 20 µCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp.) were incubated in 10 µl of kinase buffer [20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 7.0), 10 mM MnCl$_2$], for 20 min at 30°C. The phosphorylated peptide was purified from 1 µl of supernatant from the kinase reaction by electrophoresis at pH 3.5. The purified peptide or tryptic peptides of pp60^{c-src} were digested with 5 µg of chymotrypsin (Cooper Biomedical) in 20 µl of 0.05 M NH$_4$HCO$_3$ (pH 7.8) for 2 h at 37°C (modified from the method of Beemon and Hunter [1]). Chymotryptic peptides were separated in two dimensions by electrophoresis (pH 8.9, 1 kV, 10 min) and chromatography (23).

RESULTS

pp60^{c-src} associated with mT in transformed chicken cells has a novel site(s) of tyrosine phosphorylation in vitro. We previously showed that primary chicken embryo cells transformed by a recombinant murine retrovirus which encodes polyomavirus mT express an mT protein which associates with chicken pp60^{c-src} and enhances its protein kinase activity (29). To characterize the in vitro phosphorylation sites of pp60^{c-src} from chicken cells, proteins were immunoprecipitated from uninfected cell extracts with MAb 327 and from mT-transformed cell extracts wtih polyomavirus anti-tumor
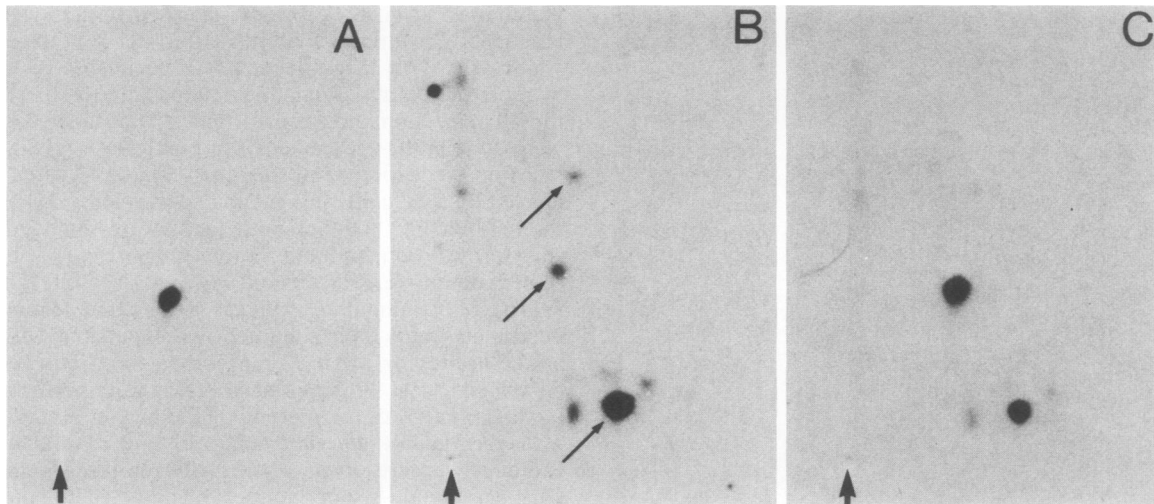
FIG. 2. Tryptic digestion of pp60$^{c\text{-}src}$ phosphorylated in vitro. Proteins were extracted from a gel similar to the one shown in Fig. 1A, oxidized, digested with trypsin, and separated in two dimensions on cellulose thin-layer plates by electrophoresis at pH 1.9 (horizontal direction, anode on the left) and chromatography (vertical direction). (A) p60 from CEF; (B) p60$^+$ from mTCEF; (C) mixture of peptides from p60 and p60$^+$. In each case the origin is marked (vertical arrow). The major peptides of p60$^+$ are indicated by diagonal arrows in B. The additional spots are partial digestion products and mT peptides. Cerenkov counts per minute loaded: (A) 170; (B) 800; (C) 170 of p60 and 170 of p60$^+$. Each autoradiogram was exposed for 3 days at $-70°C$ with an intensifying screen.

serum, incubated with [γ-^{32}P]ATP, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1A). The putative pp60$^{c\text{-}src}$ associated with mT (designated p60$^+$) in transformed cells (Fig. 1A, lane 2) migrated more slowly than the pp60$^{c\text{-}src}$ in untransformed cells (p60, lane 1), as shown previously (29). To confirm that p60$^+$ was related to p60, we analyzed the proteins by one-dimensional *S. aureus* V8 protease mapping (Fig. 1B). The patterns were similar to those observed previously with rodent cells (5, 53). Cleavage of p60 (Fig. 1B, lane 1) generated a single 26-kilodalton phosphopeptide, V2, derived from the carboxy-terminal portion of the molecule (7, 8), and phosphorylated on tyrosine 416 (44). Partial digestion of p60$^+$ yielded three major phosphopeptides (Fig. 1B, lane 2), one comigrating with V2 of p60 and the other two derived from the amino-terminal 34-kilodalton region of the molecule (5, 53). The p60$^+$ phosphopeptides were different from those generated by V8 cleavage of phosphorylated mT (Fig. 1B, lane 3). The additional phosphorylation(s) on p60$^+$ may explain why it migrated more slowly than p60 on an SDS gel.

We compared p60$^+$ and p60 further by two-dimensional tryptic mapping (Fig. 2). Tryptic cleavage of p60 generated a single phosphopeptide (Fig. 2A), known to be phosphorylated on tyrosine 416 (36, 44). Digestion of p60$^+$ yielded three major peptides (Fig. 2B), one of which was the tyrosine 416-containing peptide, as confirmed by a mixture of the tryptic phosphopeptides from p60 and p60$^+$ (Fig. 2C). Tyrosine was the only phosphorylated amino acid in each of the V8 and tryptic peptides of p60 and p60$^+$ (data not shown). Therefore pp60$^{c\text{-}src}$ not associated with mT is phosphorylated in vitro on tyrosine 416. pp60$^{c\text{-}src}$ associated with mT is phosphorylated in vitro both on tyrosine 416 and on one or more tyrosine residues, at least one of which is located in the amino-terminal region of the molecule.

pp60$^{c\text{-}src}$ associated with mT in transformed cells is phosphorylated on tyrosine 416 in vivo. To characterize the in vivo phosphorylation sites of pp60$^{c\text{-}src}$ bound to mT, we labeled mT-MLV-transformed chicken cells with ^{32}P$_i$ for 18 h, isolated mT and the associated pp60$^{c\text{-}src}$ by im-

munoprecipitating with anti-T ascites, and compared the mT-associated pp60$^{c\text{-}src}$ (designated p60a) with pp60$^{c\text{-}src}$ precipitated with MAb 327 from ^{32}P-labeled untransformed chicken cells (designated p60) (Fig. 3). The mobilities and V8 protease partial digestion patterns of the two proteins were similar. Phosphoamino acid analysis identified phosphotyrosine in the carboxy-terminal V2 peptides and phosphoserine in the amino-terminal V1, V3, and V4 peptides of each protein (data not shown). We compared the two proteins by two-dimensional tryptic mapping (Fig. 4). Cleavage of p60 generated six major phosphopeptides, which were numbered as shown (Fig. 4A). Peptides 1 and 2 contained phosphotyrosine; peptides 3 through 6 contained phosphoserine (data not shown). Digestion of p60a generated five tryptic phosphopeptides (Fig. 4B). Phosphopeptides 1 and 2 of p60 were not present in the p60a digest, and phosphopeptide 7 of p60a was not present in the p60 digest. Peptide 7 contained phosphotyrosine, and peptides 3 through 6 of p60a contained phosphoserine (data not shown). A mixture of the tryptic peptides from p60 and p60a (Fig. 4C) confirmed that the phosphoserine-containing peptides of the two proteins were identical, whereas the phosphotyrosine-containing peptides were different. From phosphoamino acid analysis of the V8 peptides we know that phosphotyrosine is present only in the carboxy-terminal regions of p60 and p60a (data not shown). Therefore, free pp60$^{c\text{-}src}$ in untransformed cells and pp60$^{c\text{-}src}$ associated with mT in transformed cells are phosphorylated on different carboxy-terminal tyrosine residues in vivo.

The phosphotyrosine-containing tryptic peptide of p60a, peptide 7, appeared to be similar to the tryptic peptide of pp60$^{c\text{-}src}$ phosphorylated in vitro on tyrosine 416. Analysis of the two peptides (Fig. 5) showed that they were identical. Since the tryptic peptide containing tyrosine 416 has no other tyrosine, we can conclude that pp60$^{c\text{-}src}$ associated with mT in transformed chicken cells is phosphorylated in vivo on tyrosine 416.

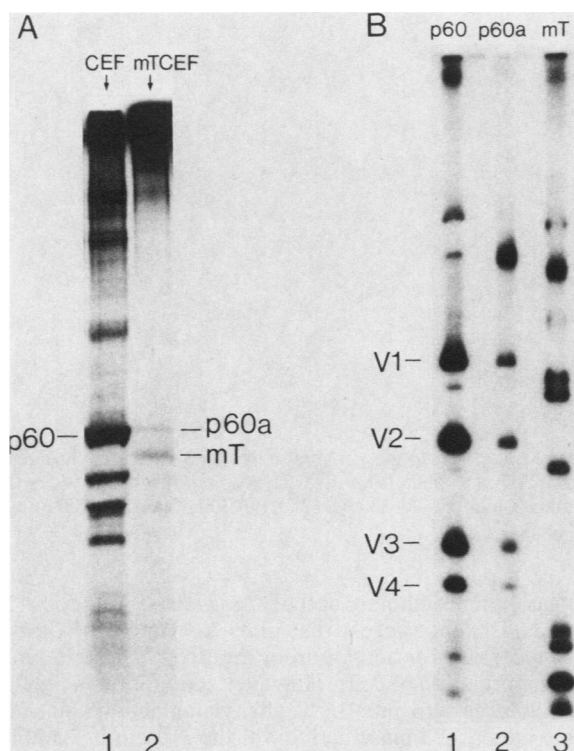We also studied the phosphorylation of pp60$^{c\text{-}src}$ associated with mT in an mT-MLV-transformed RC 3.7 rat cell

FIG. 3. In vivo phosphorylation of pp60$^{c\text{-}src}$ and mT in transformed chicken embryo cells. (A) Cells were radiolabeled with ^{32}P$_i$ for 18 h. The proteins were extracted, immunoprecipitated, and resolved on a 7% SDS–polyacrylamide gel. Lanes: 1, pp60$^{c\text{-}src}$ (p60) from chicken embryo fibroblasts (CEF), precipitated with MAb 327; 2, mT and associated pp60$^{c\text{-}src}$ (p60a) from mT-transformed chicken embryo fibroblasts (mTCEF), precipitated with anti-T ascites. Exposure time was 42 h. (B) V8 digestion of pp60$^{c\text{-}src}$ and mT. Proteins were excised from the gel shown in A and digested with 50 ng of *S. aureus* V8 protease, and the peptides were separated on a 12% SDS–polyacrylamide gel. Lanes: 1, p60; 2, p60a; 3, mT. Designation of V8 peptides of pp60$^{c\text{-}src}$: V1, 34-kilodalton amino-terminal peptide; V2, 26-kilodalton carboxy-terminal peptide; V3 and V4, 18- and 16-kilodalton amino-terminal peptides derived from further cleavage of V1. Exposure time, at −70°C with an intensifying screen, was 60 h.

line, RC 1-18. (RC 3.7 cells are an established line of rat cells which express high levels of chicken pp60$^{c\text{-}src}$ [35].) Figure 6 shows a comparison of tryptic digests of ^{32}P-labeled p60a from the transformed cells with p60 from the parental RC 3.7 cells. Cleavage of p60 generated 10 major phosphopeptides (Fig. 6A). Peptides 1 and 2, which were similar in mobility to the corresponding peptides from chicken cells, contained phosphotyrosine. Peptides 3 through 6 and 4r through 6r contained phosphoserine (data not shown). Digestion of p60a generated eight major phosphopeptides (Fig. 6B). The pattern was similar to that observed for p60a in mT-transformed chicken cells; phosphopeptides 1 and 2 of p60 were not present in p60a, and a new phosphotyrosine-containing peptide, peptide 7, similar to the peptide containing tyrosine 416 in chicken cells, was observed in p60a (Fig. 6C).

It appeared likely, based on their intensities, that peptides 4 through 6 were derived from chicken pp60$^{c\text{-}src}$ and peptides 4r through 6r were derived from rat pp60$^{c\text{-}src}$. A mixture of pp60$^{c\text{-}src}$ tryptic peptides from RC 3.7 cells (Fig. 6A) and

from rat F2408 cells (Fig. 6D) confirmed these assignments (Fig. 6E).

The experiments described above showed that the pp60$^{c\text{-}src}$ associated with mT in transformed cells was phosphorylated on a different tyrosine residue (tyrosine 416) than pp60$^{c\text{-}src}$ in untransformed cells. It was important to determine, however, whether phosphorylation of tyrosine 416 occurred in pp60$^{c\text{-}src}$ molecules not associated with mT in transformed cells, or whether it was unique to those pp60$^{c\text{-}src}$ molecules associated with mT. We removed all detectable mT and the pp60$^{c\text{-}src}$ complexed to it from ^{32}P-labeled lysates of RC 1-18 cells by immunoprecipitating twice with anti-T ascites (data not shown). The remaining, unassociated pp60$^{c\text{-}src}$ was then precipitated from the cleared lysates with MAb 327. Tryptic digests of the free pp60$^{c\text{-}src}$ from transformed cells were compared with those of pp60$^{c\text{-}src}$ from the untransformed parent RC 3.7 cells (Fig. 7). The phosphopeptides of free pp60$^{c\text{-}src}$ from transformed cells were identical to those of pp60$^{c\text{-}src}$ from untransformed cells. The phosphotyrosine-containing peptides 1 and 2 were present, and peptide 7 containing tyrosine 416 was absent, in both molecules. Therefore, only pp60$^{c\text{-}src}$ which is associated with mT in transformed cells is phosphorylated on tyrosine 416.

pp60$^{c\text{-}src}$ not associated with mT is phosphorylated on tyrosine 527. pp60$^{c\text{-}src}$ from chicken embryo cells or from mouse cells expressing high levels of chicken pp60$^{c\text{-}src}$ is phosphorylated on tyrosine 527 (11a). We confirmed this observation by using pp60$^{c\text{-}src}$ from RC 3.7 cells. A synthetic peptide corresponding to amino acids 519-533 of pp60$^{c\text{-}src}$ (Tyr-Phe-Thr-Ser-Thr-Glu-Pro-Gln-Tyr-Gln-Pro-Gly-Glu-Asn-Leu) was phosphorylated in vitro on the internal tyrosine (data not shown) by immunoprecipitated pp60$^{c\text{-}src}$ from RC cells. The phosphorylated peptide and tryptic peptide 1 of RC cell pp60$^{c\text{-}src}$ were digested with chymotrypsin and compared by two-dimensional peptide mapping (Fig. 8). Cleavage of tryptic peptide 1 generated a single chymotryptic phosphopeptide (Fig. 8A). (Other experiments showed that tryptic peptide 2 of pp60$^{c\text{-}src}$ contained the same chymotryptic peptide as tryptic peptide 1.) The digest of the synthetic peptide contained two major peptides (Fig. 8B). (Other experiments identified the upper of these peptides as undigested material.) Analysis of a mixture of the chymotryptic peptides of tryptic peptide 1 and the synthetic peptide showed that they were identical (Fig. 8C). This confirmed that tyrosine 527 is the major site of carboxy-terminal phosphorylation in vivo on pp60$^{c\text{-}src}$.

DISCUSSION

These data show that the major tyrosine phosphorylation site of free pp60$^{c\text{-}src}$ in polyomavirus mT-transformed cells is tyrosine 527. In contrast, pp60$^{c\text{-}src}$ complexed to mT is not detectably phosphorylated on tyrosine 527, but is phosphorylated on tyrosine 416. pp60$^{c\text{-}src}$ associated with mT is known to have enhanced protein kinase activity which correlates with the transforming ability of polyomavirus (2, 5, 13). Together, these data suggest that altered phosphorylation of pp60$^{c\text{-}src}$ associated with mT may play a role in activation of the pp60$^{c\text{-}src}$ kinase and possibly in cell transformation by polyomavirus.

Phosphorylation of tyrosine within the carboxy terminus of pp60$^{c\text{-}src}$ apparently decreases its protein kinase activity and may be used as a regulatory system (13). This suggests a mechanism by which mT-associated pp60$^{c\text{-}src}$ is activated; binding of mT to pp60$^{c\text{-}src}$ either prevents phosphorylation or induces dephosphorylation of tyrosine 527, resulting in acti-
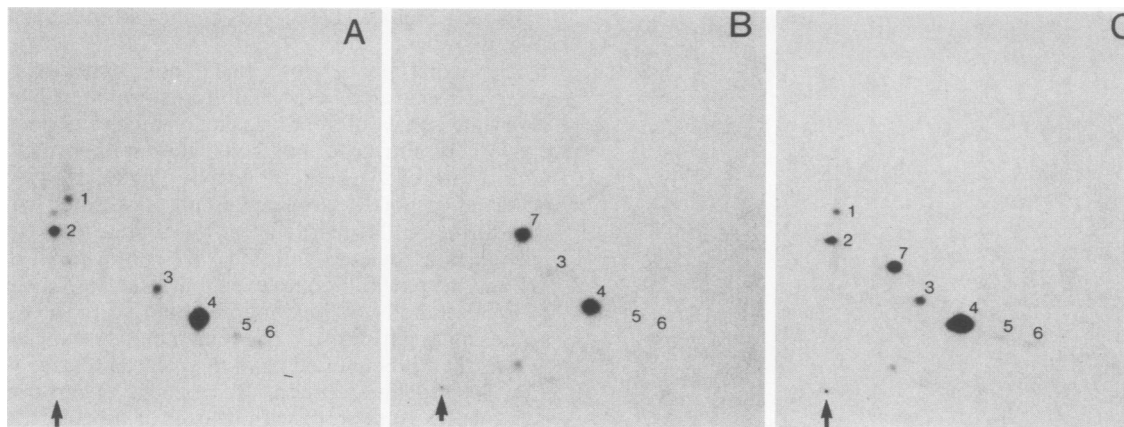
FIG. 4. Tryptic digestion of pp60$^{c\text{-}src}$ phosphorylated in vivo. Proteins were extracted from the gel shown in Fig. 3A, oxidized, digested with trypsin, and resolved as described in Materials and Methods. (A) p60 from CEF; (B) p60a from mTCEF; (C) mixture of peptides from p60 and p60a. In each case the origin is marked (arrow). Cerenkov counts per minute loaded: (A) 300; (B) 250; (C) 300 of p60 and 250 of p60a. Each autoradiogram was exposed for 6 days at −70°C with an intensifying screen.

vation of the pp60$^{c\text{-}src}$ kinase. It is, however, unlikely that lack of phosphorylation of tyrosine 527 is the only factor involved in activating the mT-associated pp60$^{c\text{-}src}$ kinase. The 2- to 3-fold difference in protein kinase activity observed between pp60$^{c\text{-}src}$ which is and is not phosphorylated on a carboxy-terminal tyrosine (13) (tyrosine 527) clearly cannot account for the greater than 20-fold difference in kinase activity observed between pp60$^{c\text{-}src}$ which is and is not bound to mT (2, 5, 13). Other factors, such as conformational changes in pp60$^{c\text{-}src}$ when it binds to mT, may also affect pp60$^{c\text{-}src}$ protein kinase activity.

It is interesting that mT-associated pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ are both phosphorylated in vivo on tyrosine 416. Similarly, pp60$^{c\text{-}src}$ produced after spontaneous mutations in a c-src-containing virus is phosphorylated on tyrosine 416 (and not

527), has enhanced kinase activity, and transforms cells (25). These data might suggest that phosphorylation of tyrosine 416 is necessary for activation of the pp60^{src} protein kinase and transformation of cells. However, a mutation in pp60$^{v\text{-}src}$ which changes tyrosine 416 to phenylalanine does not measurably affect the kinase activity of the protein or its ability to transform cells in culture (18, 46). Therefore, phosphorylation of tyrosine 416 may be a consequence rather than a cause of increased pp60^{src} protein kinase and may reflect the accessibility of the residue to kinases due to conformational changes in the molecule. Tyrosine 416 may also be the preferred phosphorylation site once tyrosine 527 is no longer available for phosphorylation.

There are a number of sequence differences between pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ which may be important for transform-
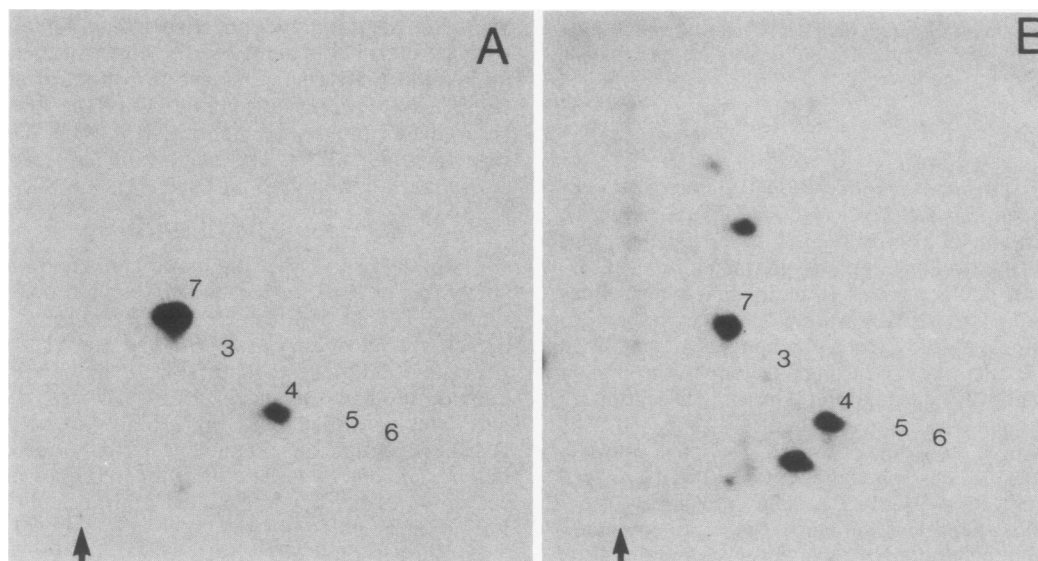

FIG. 5. Comparison of pp60$^{c\text{-}src}$ phosphorylated in vitro and in vivo. Proteins were extracted from gels similar to those shown in Fig. 1A and 3A, oxidized, digested with trypsin, and resolved as described in Materials and Methods. (A) Mixture of peptides from p60 phosphorylated in vitro and p60a phosphorylated in vivo; (B) Mixture of peptides from p60$^+$ phosphorylated in vitro and p60a phosphorylated in vivo. In each case the origin is marked (arrow). Cerenkov counts per minute loaded: (A) 270 of p60 and 250 of p60a; (B) 275 of p60$^+$ and 250 of p60a. Each autoradiogram was exposed for 6 days at −70°C with an intensifying screen.
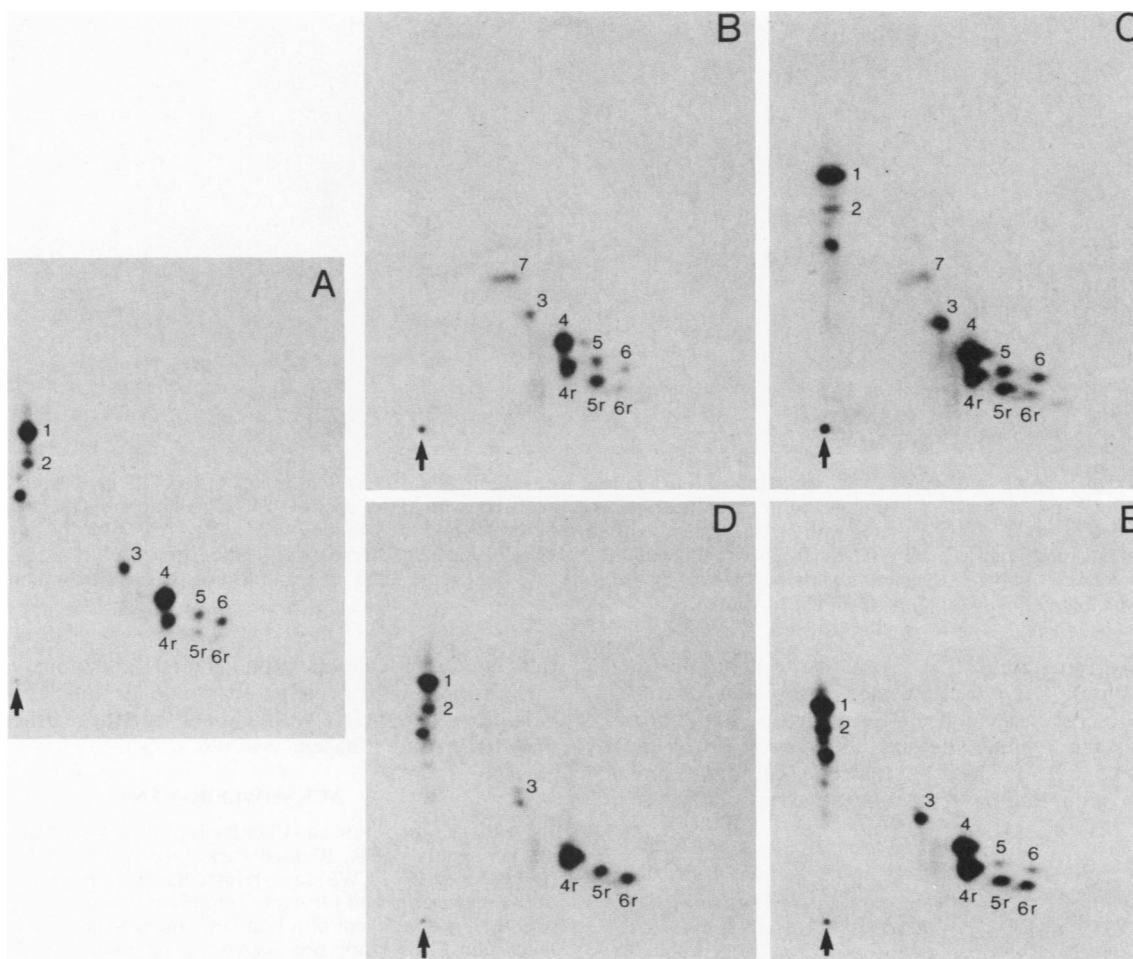
FIG. 6. Tryptic digestion of pp60$^{c\text{-}src}$ from rat cells phosphorylated in vivo. ^{32}P-labeled proteins were extracted from rat cells and digested with trypsin and the peptides were resolved as described in Materials and Methods. (A) p60 from RC 3.7 cells; (B) p60a from RC 1-18 cells; (C) mixture of peptides from A and B (D) p60 from F2408 cells; (E) mixture of peptides from A and D. In each case the origin is marked (arrow). Cerenkov counts per minute loaded: (A) 780; (B) 760; (C) 780 of A and 760 of B; (D) 780; (E) 1,560 of A and 1,560 of D. Each autoradiogram was exposed 67 h, except E which was exposed 24 h, at −70°C with an intensifying screen.

ing ability. It has been shown that differences in the amount of protein expressed in cells cannot account for the differences in transforming ability between the viral and cellular protein. Elevating the level of pp60$^{c\text{-}src}$ in cells to that achieved by p60$^{v\text{-}src}$ produces phenotypic changes, but is not sufficient to cause full transformation (26, 28, 35, 42). Furthermore, expression of pp60$^{v\text{-}src}$ at a level equivalent to endogenous pp60$^{c\text{-}src}$ induces transformation (27). Given that the level of pp60$^{v\text{-}src}$ is not responsible for transformation, there must be some other cause. One obvious difference is that the specific activity of the pp60$^{v\text{-}src}$ protein kinase is at least 10-fold greater than that of pp60$^{c\text{-}src}$ (25). Therefore, sequence differences which increase the activity of the protein kinase may be important for transforming ability.

The major sequence differences occur in the carboxy-terminal regions of the two proteins. In pp60$^{v\text{-}src}$ the 19 carboxy-terminal amino acids of pp60$^{c\text{-}src}$ have been replaced by 12 amino acids derived from a coding region 900 base pairs downstream from the translation termination codon of the pp60$^{c\text{-}src}$ gene (47, 48). Chimeric genes encoding a protein with the first 431 residues of pp60$^{c\text{-}src}$ and the 95 carboxy-terminal residues of pp60$^{v\text{-}src}$ can cause transformation (26, 42). Mutations in the carboxy terminus of pp60$^{v\text{-}src}$

which alter the last 11 or 23 amino acids abolish transforming ability in chicken cells (52). These observations suggest that the carboxy-terminal region of pp60$^{v\text{-}src}$ is important for transformation. However, replacement of the unique carboxy-terminal nine amino acids of pp60$^{v\text{-}src}$ with nine random amino acids does not eliminate transforming ability (43). Therefore, it may be that the unique amino acid sequence of pp60$^{v\text{-}src}$ downstream of residue 517 is not required for transformation and that what is important for transformation is the absence of the carboxy terminus of pp60$^{c\text{-}src}$ rather than the presence of the carboxy terminus of pp60$^{v\text{-}src}$. In view of the apparent involvement of tyrosine 527 phosphorylation in negative regulation of pp60$^{c\text{-}src}$ protein kinase activity, the loss of this region from pp60$^{v\text{-}src}$ could lead to constitutive activation of its protein kinase. In this sense pp60$^{v\text{-}src}$ and mT-associated pp60$^{c\text{-}src}$ may be similar. It will clearly be important to study the properties of pp60$^{c\text{-}src}$ molecules mutated specifically at the negative regulatory site which pp60$^{v\text{-}src}$ lacks.

Negative regulation by tyrosine phosphorylation may not be unique to pp60$^{c\text{-}src}$. Two other cellular protein-tyrosine kinase genes, c-fgr and c-yes, encode proteins with carboxy termini very similar to that of pp60$^{c\text{-}src}$ and have a tyrosine
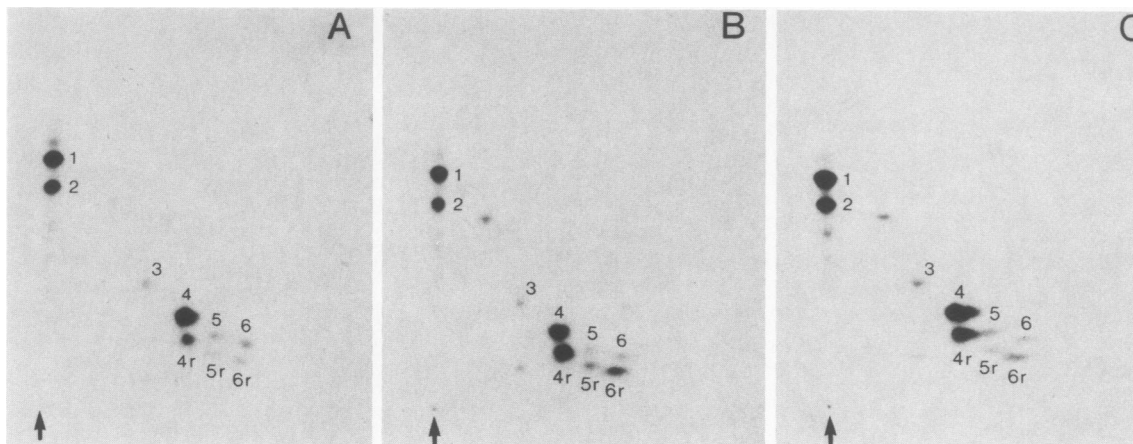
FIG. 7. Tryptic digestion of pp60^{c-src} unassociated with mT in transformed rat cells. ^{32}P-labeled lysates of RC 1-18 cells were cleared of all detectable mT and the pp60^{c-src} complexed to it by immunoprecipitating twice with anti-T ascites. The remaining, unassociated pp60^{c-src} was precipitated from the cleared lysates with MAb 327 and digested with trypsin and the peptides were resolved as described in Materials and Methods. (A) p60 from RC cell lysates; (B) p60 from cleared RC 1-18 cell lysates; (C) mixture of peptides from A and B. In each case the origin is marked (arrow). Cerenkov counts per minute loaded: (A) 720; (B) 810; (C) 720 of A and 810 of B. Each autoradiogram was exposed for 63 h at −70°C with an intensifying screen.

equivalent to tyrosine 527 (34, 51; K. Toyoshima, personal communication). The closely related viral oncogenes v-*fgr* and v-*yes* diverge in sequence from their respective proto-oncogenes a few residues before this tyrosine (for review, see reference 22). It may be that the carboxy-terminal tyrosine phosphorylation serves a negative regulatory role in

this family of cellular proteins and is eliminated in the transforming viral proteins. It would be interesting if mT were able to associate with and activate these other cellular protein-tyrosine kinases.
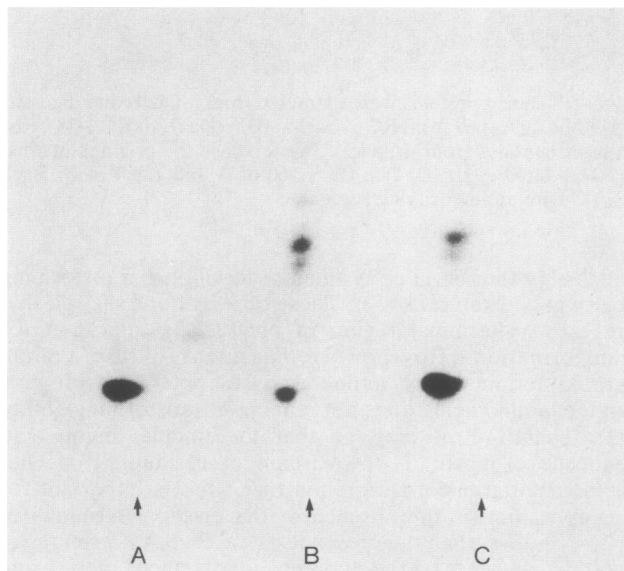
FIG. 8. Comparison of chymotryptic digests of a phosphopeptide of pp60^{c-src} and of a synthetic peptide corresponding to amino acids 519–533 of pp60^{c-src}. A tryptic phosphopeptide of pp60^{c-src} from RC cells and the synthetic phosphopeptide were digested with chymotrypsin and separated in two dimensions on cellulose thin-layer plates by electrophoresis at pH 8.9 and chromatography as described in Materials and Methods. (A) chymotryptic digest of tryptic phosphopeptide 1 of RC cell pp60^{c-src}; (B) chymotryptic digest of the synthetic phosphopeptide; (C) mixture of A and B. In each case the origin is marked (arrow). Cerenkov counts per minute loaded: (A) 200; (B) 230; (C) 200 of A and 230 of B. Exposure time was 2 days at −70°C with an intensifying screen.

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