In Vivo Effect of Sodium Orthovanadate on pp60c-src Kinase

JOHN W. RYDER AND JULIUS A. GORDON*

University of Colorado Health Sciences Center, Department of Pathology, Denver, Colorado 80262

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We have compared the tyrosine kinase activity of pp60c-src isolated from intact chicken embryo fibroblasts treated with micromolar sodium orthovanadate for 4 h and from untreated cells. We found an approximate 50% reduction in both autophosphorylation of pp60c-src and phosphorylation of casein when examined in the immune complex kinase assay. The reduction of in vitro enzymatic activity correlated with a vanadate-induced increase in in vivo phosphorylation of pp60c-src at the major site of tyrosine phosphorylation in the carboxyl-terminal half of the molecule and at serine in the amino-terminal half of the molecule. Our observations in vivo and those of Courtneidge in vitro (EMBO J. 4:1471–1477, 1985) suggest that vanadate may enhance a cellular regulatory mechanism that inhibits the activity of pp60c-src in normal cells. A likely candidate for this mechanism is phosphorylation at a tyrosine residue distinct from tyrosine 416, probably tyrosine 527 in the carboxyl-terminal sequence of amino acids unique to pp60c-src. The regulatory role, if any, of serine phosphorylation in pp60c-src remains unclear. The 36-kilodalton phosphoprotein, a substrate of pp60c-src, showed a significant phosphorylation at tyrosine after treatment of normal chicken embryo fibroblasts with vanadate. Assuming that pp60c-src is inhibited intracellularly by vanadate, either another tyrosine kinase is stimulated by vanadate (e.g., a growth factor receptor) or the 36-kilodalton phosphoprotein in normal cells is no longer rapidly dephosphorylated by a tyrosine phosphatase in the presence of vanadate.

Transformation of cells by Rous sarcoma virus (RSV) is correlated with an 8- to 10-fold increase in cellular phosphotyrosine (44) distributed among numerous cellular proteins (36). This increase is attributable to the expression of a functional (44), 60-kilodalton (kDa), virus-encoded (5, 38) cyclic AMP-independent (21), tyrosine-specific (12, 25, 33) protein kinase (pp60v-src). Several specific intracellular substrates for pp60v-src have been identified (for a review, see reference 24), although the role of tyrosine phosphorylation in transformation has not been elucidated. However, the consistent correlation between tyrosine phosphorylation and transformation indicates that expression of pp60v-src kinase activity is necessary for transformation (44).

The primary structure of the normal cellular homolog of pp60v-src, pp60c-src, is very similar but not identical to that of pp60v-src. The 19 carboxyl-terminal amino acids of chicken pp60c-src are replaced by a new set of 12 amino acids at the carboxyl-terminal end of pp60v-src encoded by the Schmidt-Ruppin A strain RSV, and 8 additional amino acid substitutions are found throughout the pp60v-src molecule (48). In the intact cell pp60v-src and pp60c-src are each phosphorylated at a serine residue in the amino-terminal half of the molecule, serine 17 (11, 19, 46), whereas pp60c-src is phosphorylated in the carboxyl-terminal half of the molecule at a tyrosine residue distinct from tyrosine 416, which is phosphorylated in pp60v-src (10, 29, 45). Recent studies have indicated that phosphorylation of pp60v-src in the intact cell occurs at tyrosine 527, located in the 19 carboxyl-terminal amino acids unique to pp60c-src (14).

Earlier experiments showed that although the range of exogenous substrates and other aspects of the in vitro tyrosine kinase activities of pp60v-src and pp60c-src are similar, the specific activities of pp60c-src for exogenous substrates and autophosphorylation are lower than those of pp60v-src (18), pp60c-src in normal cells occurs at a level 30 to 50 times lower than pp60v-src in cells infected and transformed by RSV (10). To test the hypothesis that cell transformation comes about as a result of a general overexpression of the src-related tyrosine kinase activity, pp60c-src was expressed in rodent fibroblasts at a level 7 to 10 times greater than the level of pp60v-src in RSV-transformed rodent fibroblasts. Only a limited focus-forming ability and minimal increases in total cellular phosphotyrosine and tyrosine phosphorylation of enolase and the 36-kDa phosphoprotein (intracellular substrates of pp60v-src [22, 24, 41]) were observed (18, 27). Additionally, these cells were not tumorigenic in vivo (27). One explanation for these results would be that the intracellular tyrosine kinase activity of pp60c-src is restricted relative to that of pp60v-src by an in vivo mechanism(s) (18, 27).

A possible regulatory mechanism of pp60v-src and pp60c-src tyrosine kinase activity is the phosphorylation state of the molecules. Incubation of partially purified preparations of pp60v-src with Mg2+ and ATP (13, 40), lysis of RSV-transformed cells in the presence of Mg2+ and ATP (9), incubation of intact RSV-transformed cells with vanadate (4, 9), and incubation of plasma membrane fractions from RSV-transformed cells with Mg2+, ATP, and vanadate (43) facilitate the identification of hyperphosphorylated forms of pp60v-src (at tyrosine residues in the amino-terminal half of the molecule) that are more enzymatically active. Recently, Courtneidge (17) showed that lysis of untransformed rodent cells in the presence of vanadate facilitates the recovery of a population of pp60v-src that is hyperphosphorylated at the major tyrosine residue in the carboxy-terminal half of the molecule (presumably tyrosine 527 [14]). In contrast to hyperphosphorylated pp60v-src, the in vitro kinase activity is depressed (17). Conversely pp60c-src bound to middle T antigen in polyomavirus-transformed cells exhibits enhanced tyrosine kinase activity in vitro (2, 6, 17) and is not detectably phosphorylated at tyrosine 527 (7). Studies correlating

* Corresponding author.
phosphorylation at tyrosine 527 with a reduction in in vitro tyrosine kinase activity suggest a mechanism by which the tyrosine kinase activity of pp60-src may be restricted. In our study, we incubated intact, untransformed chicken embryo fibroblasts (CEF) with micromolar concentrations of vanadate. We compared the in vitro kinase activity and phosphorylation state of immunoprecipitated pp60-src from vanadate-treated and untreated cells. Additionally, we determined the effect of vanadate on the phosphorylation level of several other phosphoproteins, including the 36-kDa phosphoprotein.

MATERIALS AND METHODS

**Materials.** Stock solutions of 1 mM sodium orthovanadate (Fisher Scientific Co.) were prepared by the method of Brown and Gordon (4). $^{32}$P was obtained as sodium orthophosphate in water from ICN Pharmaceuticals, Inc. (64014). $[^{35}S]$methionine was obtained from New England Nuclear Corp. (NEG 009H).

**Cell culture.** CEF were obtained by trypsinization of COFAL-negative, eviscerated 10-day chicken embryos (SPAFAS, Inc.). Cells were passaged on 100-mm plastic plates with Dulbecco modified minimal medium (Hazelton) supplemented with 5% calf serum, 1% heat-inactivated chicken serum, 10% tryptose phosphate broth (Difco Laboratories), penicillin (100 U/ml), and streptomycin (100 µg/ml). Third- to fifth-passage cells grown to confluency at 41°C over approximately 48 h were used for experiments.

**Endogenous labeling of intracellular proteins with sodium $[^{35}S]$methionine or $[^{32}P]$orthophosphate.** CEF plates (100 mm) of CEF were prepared for labeling with sodium $[^{32}P]$orthophosphate by rinsing cell monolayers once with 0.15 M NaCl. After a 30-min preincubation in phosphate-free Dulbecco minimal essential medium supplemented with 2% dialyzed calf serum, 0.75 to 1.3 µCi of sodium $[^{32}P]$orthophosphate per ml of medium was added with or without sodium orthovanadate, and the cultures were incubated for 4 h at 41°C. Cultures of CEF were prepared for labeling with $[^{35}S]$methionine by rinsing cell monolayers once with phosphate-buffered saline (pH 7.2) and preincubating for 0.5 h in methionine-free Dulbecco minimal essential medium (Flow Laboratories, Inc.) supplemented with 2% dialyzed calf serum. Then 100 µCi of $[^{35}S]$methionine per ml of medium was added with or without 50 µM sodium orthovanadate, and the cultures were incubated for 4 h at 41°C. For 18-h incubations with $[^{35}S]$methionine, the procedure was the same, except that subconfluent cultures of CEF plated for approximately 30 h were used, and incubation medium contained 10% of the complete complement of unlabeled methionine.

**Lysis and immunoprecipitation.** Cell lysates were prepared by washing cell monolayers three times with ice-cold phosphate-buffered saline (pH 7.2). Cells were harvested on ice into 1.0 ml RIPA buffer (150 mM NaCl-1% sodium deoxycholate-1% Triton X-100-0.1% sodium dodecyl sulfate [SDS]-10 mM Tris hydrochloride, pH 7.2) supplemented with 1 mM EDTA, aprotenin (60 IU), and soybean trypsin inhibitor (100 µg). In some instances, 100 µM vanadate was included in the RIPA buffer. Protein concentrations were determined by the method of Bradford (3). For immunoprecipitation of pp60-src, we used monoclonal antibody 327 (MAb 327) kindly provided by Joan Brugge (35). Samples of lysate containing 500 µg of protein were used to ensure antigen excess (data not shown). Samples were incubated for 45 min on ice with 10 µl of a 1:10 dilution (phosphate-buffered saline, pH 7.2) of MAb 327 followed by incubation with 1.0 µg of rabbit anti-mouse immunoglobulin G (Cappel-Coope Biomedical) diluted in 10 µl of phosphate-buffered saline (pH 7.2) for 20 min. Precipitation with protein A-bearing *Staphylococcus aureus* was by the method of Kessler (30). *S. aureus*-bound immune complexes were washed three times with ice-cold RIPA buffer and solubilized in SDS sample buffer (2% SDS-10% glycerol-5% 2-mercaptoethanol-2 mM EDTA-0.14 M Tris hydrochloride, pH 6.8) at 90°C for 3.5 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

For immunoprecipitation of the 36-kDa phosphoprotein a specific rabbit antisera kindly provided by S. Decker was employed (20). CEF incubated with sodium $[^{35}S]$methionine were harvested into RIPA buffer as described above. Samples containing 200 µg of protein were incubated for 30 min with 3 µl of the specific antiserum and precipitated with protein A-bearing *S. aureus*. Immune complexes were washed four times with RIPA buffer, solubilized in SDS sample buffer at 90°C for 3.5 min, and analyzed by SDS-PAGE.

**Kinase assay in the immune complex.** The medium was removed from confluent plates of CEF, and new medium supplemented with 5% calf serum was added. CEF were incubated with or without sodium orthovanadate for 4 h at 41°C. Cellular lysates were prepared, protein concentrations were determined, and pp60-src was immunoprecipitated with MAb 327 from samples containing 500 µg of protein as described above. *S. aureus*-bound immune complexes were washed three times with RIPA and once with Tris-buffered saline (0.15 M NaCl-50 mM Tris hydrochloride, pH 7.2).

In vitro phosphorylation of pp60-src was accomplished by suspending the *S. aureus*-bound immune complexes in 20 µl of 0.15 M NaCl-3 mM MgCl$_2$-1.5 µM ATP-50 mM Tris hydrochloride (pH 7.2) containing 75.0 to 150 µCi of $[^{32}P]$ATP prepared by the method of Johnson and Walseth (28) and incubating for 15 min at room temperature. The reaction was stopped by dilution into ice-cold RIPA and centrifugation. Immune complexes were solubilized in SDS sample buffer at 90°C for 3.5 min and analyzed by SDS-PAGE.

Immune complex kinase assays employing the exogenous substrate casein were performed by suspending the washed *S. aureus*-bound immune complexes in 20 µl of 5 mM MgCl$_2$-20 µM ATP-20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2) containing 5.0 µg of casein (Sigma Chemical Co.) and 12 to 25 µCi of $[^{32}P]$ATP. The mixture was incubated 10 min at room temperature, and the reaction was terminated by the addition of SDS sample buffer. Reaction products were analyzed by SDS-PAGE.

**Gel electrophoresis and autoradiography.** SDS-PAGE on 7.5 or 10% slab gels (as indicated below) was performed by the method of Laemmli (32). Gels were stained with Coomassie blue, destained, and dried. $[^{32}P]$-labeled proteins were detected by autoradiography carried out at −70°C with either Cronex (Du Pont Co.) or XAR-5 (Eastman Kodak Co.) film and Cronex Lightning-Plus intensifying screens (Du Pont) as indicated above. $[^{35}S]$methionine-labeled proteins were detected by fluorography after impregnation with 1 M sodium salicylate. Fluorography was performed at −70°C with XAR-5 film.

**Limited proteolysis of pp60-src.** $[^{32}P]$-labeled 60-kDa protein bands were excised from dried gels and hydrated with 20 to 40 µl of 0.1% SDS-5% 2-mercaptoethanol-10% glycerol-10 mM Tris hydrochloride (pH 6.8) (37). Limited proteolysis
was then performed by the method of Cleveland et al. (8) with 5.0 ng of *S. aureus* V8 protease. Peptide fragments were resolved on a 12.5% separating gel, which was stained, dried, and exposed for autoradiography.

**Tryptic peptide analysis.** The procedure for tryptic peptide analysis was that of Karess and Hanafusa (29), with the following exceptions. Tryptic peptides were dissolved in 10 μl of distilled water and spotted onto 20- by 20-cm Eastman plastic-backed cellulose sheets. Ascending chromatography was performed with *n*-butanol-acetic acid–water–pyridine (75:15:60:60). The plates were dried, and the second dimension was resolved by electrophoresis in pyridine-acetic acid–water (100:3:879), pH 6.5, at 600 V for 60 min on a Pharmacia flat bed. 32P-labeled tryptic peptides were visualized by autoradiography with XAR-5 film and intensifying screens.

**Phosphoamino acid analysis.** Phosphoamino acids were analyzed by the methods of Cooper et al. (16). Briefly, 32P-labeled protein bands of interest were excised from dried gels and hydrated with 20 to 40 μl of 0.1% SDS–5% 2-mercaptoethanol–10% glycerol–10 mM Tris hydrochloride (pH 6.8). Phosphoproteins were eluted with 0.1% SDS–5% 2-mercaptoethanol–50 mM NH₄HCO₃ (pH 8.0) and precipitated with 25% trichloroacetic acid; 50 μg of bovine serum albumin was used as a carrier. Precipitated protein was washed three times with a 1:1 mixture of ethanol and ether, dried, and suspended in 75 μl of 6.0 N HCl (Pierce Chemical Co.). Hydrolysis was carried out at 110°C for 75 min. The hydrolysates were dried, suspended in 4 μl of phosphoamino acid standard solution (2 mg each of phosphoserine, phosphothreonine, and phosphotyrosine per ml in 0.2% pyridine–2% acetic acid, pH 3.5) and spotted onto an Eastman plastic-backed cellulose sheet. The plastic-backed cellulose sheet was wetted with 0.2% pyridine–2% acetic acid (pH 3.5), and electrophoresis was carried out at 500 V on a Pharmacia flat bed. The run length was judged by the migration of Sanger dyes (0.7% xylene cyanol, 1.3% orange G, 0.7% acid fuchsins). The phosphoamino acid standards were visualized after spraying with ninhydrin. Autoradiography was carried out at -70°C with XAR-5 film and intensifying screens.

**RESULTS**

**Comparison of the in vitro kinase activity of pp60<sup>c-src</sup> from control and vanadate-treated cells.** Earlier work showed that incubation of RSV-transformed cells with micromolar concentrations of sodium orthovanadate for as little as 2.5 h and as long as 28 h facilitates the recovery of a hyperphosphorylated population of pp60<sup>c-src</sup> that exhibits enhanced in vitro tyrosine kinase activity (4, 9). To determine the effect of vanadate on pp60<sup>c-src</sup> from untransformed cells, confluent plates of CEF were incubated with or without micromolar vanadate for 2, 4, or 6 h, cells were lysed, pp60<sup>c-src</sup> was immunoprecipitated, and autophosphorylation activity was assayed in the immune complex. In contrast to the reported effect of vanadate on pp60<sup>c-src</sup>, we found an inhibition of pp60<sup>c-src</sup> specific activity assayed in vitro (Table 1). Consistent inhibition of in vitro autophosphorylation activity was obtained by incubating CEF with 50 μM vanadate for as little as 2 h. The autoradiogram in Fig. 1A shows a twofold increase in apparent in vitro autophosphorylation of pp60<sup>c-src</sup> from control CEF compared with pp60<sup>c-src</sup> from CEF incubated with 50 μM vanadate for 4 h.

![Image](Figure 1A.jpg)

**Table 1.** Sodium orthovanadate shows a concentration-dependent effect on pp60<sup>c-src</sup> activity<sup>a</sup>

<table>
<thead>
<tr>
<th>Period of incubation with vanadate (h)</th>
<th>Vanadate concn (μM)</th>
<th>% Autophosphorylated pp60&lt;sup&gt;c-src&lt;/sup&gt;</th>
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<sup>a</sup> Cells were incubated with 10, 50, or 100 μM sodium orthovanadate for 2, 4, or 6 h. The cells were extensively washed, lysed in RIPA buffer, and immunoprecipitated as outlined in the text. The activity of the pp60<sup>c-src</sup> was then assayed by its ability to autophosphorylate in the immune complex. Incorporation of 32P was quantitated by Cerenkov counting of pp60<sup>c-src</sup> in SDS-PAGE gel bands. The incorporation is expressed as the percent incorporation compared with untreated cells at each time period. The radiolabel was shown to reside in phosphotyrosine.
that phosphate was incorporated exclusively into the 26-kDa carboxyl-terminal peptide. Phosphoamino acid analysis of this peptide showed that phosphorylation occurred at tyrosine (data not shown).

pp60^src from control and vanadate-treated CEF was examined in immune complex kinase assays employing the exogenous substrate casein. The in vitro kinase activity of pp60^src from vanadate-treated cells was also inhibited when an exogenous substrate was used (Fig. 1B). In this study, the phosphorylation of casein by pp60^src from control CEF was 2.26-fold greater than that by pp60^src from vanadate-treated cells. Phosphoamino acid analysis of the casein bands indicated that phosphorylation occurred almost exclusively at tyrosine (data not shown).

Inhibition of pp60^src kinase activity after lysis of untransformed rodent cells in the presence of vanadate has recently been reported (17). We have repeatedly lysed CEF in the presence of 100 mM vanadate and assayed pp60^src tyrosine kinase activity in the immune complex (autophosphorylation and casein). We observed no difference in the in vitro tyrosine kinase activity of pp60^src from control CEF compared to pp60^src from CEF lysed in the presence of vanadate (data not shown). We conclude that the effect of vanadate is seen only with intact CEF. Similarly, lysis of RSV-transformed cells in the presence of vanadate has been reported to have no effect on the enzymatic activity of pp60^src (4, 9).

Comparison of the phosphorylation state of pp60^src from control and vanadate-treated CEF. Enhancement of the tyrosine kinase activity of pp60^src from vanadate-treated cells is correlated with the appearance of a structurally modified form of the enzyme containing phosphorylosine in the 34-kDa amino-terminal peptide (4, 9). To see whether inhibition of the kinase activity of pp60^src from vanadate-treated cells correlated with a change in the phosphorylation state of the enzyme, CEF were incubated for 4 h with sodium [32P]orthophosphate with or without 50 mM vanadate. Cells were lysed, and pp60^src was immunoprecipitated. Subsequent analysis of pp60^src from these experiments showed a modest but reproducible increase in the total phosphate content of pp60^src from vanadate-treated cells compared with that in pp60^src isolated from control cells (16 to 42% increase in nine independent experiments). Figure 2A is an autoradiogram of [32P]-labeled pp60^src from control and vanadate-treated cells. In this experiment, pp60^src from vanadate-treated cells contained 19% more radiolabel.

The protein bands depicted in Fig. 2A were subjected to limited proteolysis with S. aureus V8 protease. Phosphorylation of pp60^src from control and vanadate-treated cells occurred in the 26-kDa carboxyl-terminal peptide and in the 34-, 16-, and 18-kDa amino-terminal peptides (Fig. 2B). Densitometric analysis of the autoradiogram in Fig. 2B (Fig. 2C) showed that there was increased phosphorylation of pp60^src from vanadate-treated cells in both the amino- and carboxyl-terminal peptides. This finding was reproduced in four additional studies. Only phosphoserine was found in the amino-terminal peptides, and only phosphothreonine was present in the carboxyl-terminal peptides, of pp60^src from both control and vanadate-treated cells (Fig. 3).

We examined the effect of just lysing untransformed CEF in the presence of vanadate. CEF incubated with sodium [32P]orthophosphate were lysed with or without 100 mM vanadate, and pp60^src was immunoprecipitated. We found that lysis of CEF in the presence of vanadate had no detectable effect on the phosphorylation level of pp60^src.

FIG. 2. (A) Incubation of intact CEF with vanadate leads to increased in vivo phosphorylation of pp60^src. Sister cultures of CEF were incubated with or without 50 µM vanadate and sodium [32P]orthophosphate and lysed in RIPA, and samples containing 500 µg of protein were immunoprecipitated with MAb 327 as described in Materials and Methods. Proteins were analyzed by SDS-PAGE (7.5% polyacrylamide separation gel), and [32P]-labeled proteins were visualized by autoradiography with Cronex film exposed for 48 h with an intensifying screen. Lanes: 1 and 2, control cells; 3 and 4, vanadate-treated cells. (B) Intracellular phosphorylation of pp60^src occurs at amino acid residues in the 34-, 16-, and 18-kDa amino-terminal peptides and at amino acid residues in the 26-kDa carboxyl-terminal peptides. [32P]-labeled 60-kDa bands depicted in (A) were excised from dried gels, hydrated, and subjected to limited proteolysis with 5.0 ng of S. aureus V8 protease as described in Materials and Methods. Peptide fragments were resolved on a 12.5% polyacrylamide gel. Autoradiography was performed with Cronex film exposed for 4 days with an intensifying screen. Lanes: 1, proteolysis of the control 60-kDa bands depicted in lanes 1 and 2 in (A); 2, proteolysis of the vanadate 60-kDa bands depicted in lanes 3 and 4 in (A). (C) Incubation of intact CEF with vanadate leads to increased phosphate incorporation at amino acid residues in both carboxyl- and amino-terminal peptides. The autoradiogram in (B) was subjected to densitometric analysis with a Joyce-Loebl scanning densitometer. Peaks corresponding to the 34-, 26-, 16-, and 18-kDa peptides are indicated.
Tryptic peptide analysis of pp60<sup>src</sup> from control and vanadate-treated CEF. To determine whether increased phosphorylation of pp60<sup>src</sup> induced by vanadate involved phosphorylation of novel amino acid residues, tryptic peptide analyses were performed. Figure 4 shows autoradiograms of 32P-labeled tryptic peptides of pp60<sup>src</sup> from control and vanadate-treated cells incubated with sodium [32P]orthophosphate. The major phosphorylated tryptic peptides of pp60<sup>src</sup> from control and vanadate-treated cells are identical (in repetitions of this experiment, the small peptide between peptides 3 and 4 was variably present in both control and vanadate conditions); a mixing experiment confirmed this observation (data not shown). Phosphoamino acid analysis revealed phosphoserine in peptide 1, phosphotyrosine in peptides 2 and 3, and phosphoserine and phosphotyrosine in peptide 4 (data not shown). Presumably peptide 4 is partially trypsinized or untrypsinized pp60<sup>src</sup>.

Two sites of tyrosine phosphorylation have been detected in the carboxyl-terminal 26 kDa of pp60<sup>src</sup>: tyrosine 416 (45), the site that is autophosphorylated in in vitro kinase reactions, and tyrosine 527, the site that is phosphorylated in the intact cell (14). pp60<sup>src</sup> labeled with 32P in the intact cell and autophosphorylated pp60<sup>src</sup> were mixed and subjected to tryptic peptide analysis. Another 32P-labeled peptide in addition to those depicted in Fig. 4 was present on autoradiograms (data not shown). Since this additional 32P-labeled peptide presumably contained tyrosine 416, then pp60<sup>src</sup> was only phosphorylated in vivo at tyrosine 527 in control and vanadate-treated cells.

Avian pp60<sup>src</sup> from untransformed cells treated with various tumor promoters is phosphorylated at serine 12 and serine 48 in addition to serine 17 and yields several phosphoserine-containing tryptic peptides in addition to those shown in Fig. 4 (23). In the absence of any new 32P-labeled tryptic peptides in the vanadate condition, we conclude that the vanadate-induced increase in phosphorylation of pp60<sup>src</sup> occurred at the two major sites of phosphorylation in untransformed CEF (serine 17 and tyrosine 527).

Vanadate treatment of CEF does not alter the amount of pp60<sup>src</sup> immunoprecipitated by MAb 327. Immunoprecipitation with MAb 327 was performed with samples of lysate containing the same amount of protein and also in antigen excess. Thus, we expected the same amount of pp60<sup>src</sup> to be immunoprecipitated from control and vanadate-treated CEF. However, to ensure that the apparent changes in the in vitro kinase activity and phosphate content of pp60<sup>src</sup> induced by vanadate were not a function of differences in the amount of immunoprecipitated pp60<sup>src</sup>, control and vanadate-treated cultures of CEF were incubated with [35S]methionine and lysed, and samples containing 500 μg of protein were immunoprecipitated with MAb 327. There was little difference in the amount of [35S]methionine-labeled pp60<sup>src</sup> immunoprecipitated from lysates of control and vanadate-treated CEF (Table 2).

Vanadate treatment of CEF increases phosphorylation of a 30-kDa protein at serine and the 36-kDa phosphoprotein at tyrosine. We were somewhat surprised to find a vanadate-induced increase in phosphorylation at serine in pp60<sup>src</sup>, since vanadate does not have this effect on pp60<sup>src</sup> (4, 9). Vanadate is reported to be an in vitro inhibitor of phosphotyrosyl phosphatases, causing an increase in the amount of recoverable phosphotyrosine (47). However, we identified a 30-kDa protein that was precipitated from lysates of CEF...
with MAb 327 and a secondary antibody (Fig. 2A), with the secondary antibody alone (data not shown), with a specific rabbit antiserum against the 36-kDa phosphoprotein, or with nonimmune rabbit serum (Fig. 5A). The phosphorylation state of this 30-kDa molecule was enhanced, quite reproducibly, by vanadate treatment of CEF. Phosphoamino acid analysis of this protein from both control and vanadate-treated cells revealed only phosphoserine (data not shown).

A 36-kDa phosphoprotein has been identified as an intracellular substrate of pp60-src in RSV-transformed cells (22, 24, 41). Increased tyrosine phosphorylation of this phosphoprotein also occurs when untransformed cells are treated with epidermal growth factor or platelet-derived growth factor (15, 42). The 36-kDa phosphoprotein is only slightly phosphorylated at serine in untransformed CEF (26). Since vanadate stimulated tyrosine phosphorylation of pp60-src and since vanadate treatment of untransformed rodent cells has been reported to stimulate tyrosine phosphorylation of the 36-kDa phosphoprotein (31), we undertook studies to determine whether vanadate has a similar effect on the 36-kDa phosphoprotein in untransformed CEF. CEF were incubated with sodium [32P]orthophosphate with or without 50 μM sodium orthovanadate. Cells were lysed, and the 36-kDa phosphoprotein was immunoprecipitated. Vanadate treatment of CEF did enhance phosphorylation of this protein (74% in the study depicted in Fig. 5A), and the increased phosphorylation occurred predominantly at tyrosine (Fig. 5B).

**DISCUSSION**

Recent experiments have shown that very high-level overexpression of pp60-src in untransformed cells does not cause full cellular transformation or in vivo tumorigenicity. The cells can form small foci and show a modest increase in cellular protein phosphotyrosine. These studies suggest that the tyrosine kinase activity of pp60-src may be limited in the intact cell (18, 27).

In this paper we have presented studies showing that short-term incubation of intact CEF with added sodium orthovanadate results in the recovery of a population of pp60-src that has decreased in vitro kinase activity. This decreased in vitro kinase activity correlates with an increase in in vivo tyrosine phosphorylation in the carboxyl-terminal half of the molecule and serine in the amino half of the molecule. Our findings concerning the effect of vanadate on pp60-src in intact cells are similar to those of Courtneidge, who reported that lysis of untransformed rodent cells in the presence of sodium orthovanadate facilitates recovery of pp60-src that has decreased in vitro tyrosine kinase activity and is more heavily phosphorylated at tyrosine in the carboxyl-terminal half of the molecule (17). Increased tyrosine phosphorylation induced by incubation of intact cells or lysis of cells in the presence of vanadate does not involve novel sites of phosphorylation and presumably occurs at tyrosine 527. Further evidence supporting the notion that phosphorylation-dephosphorylation events at tyrosine 527 regulate enzymatic activity stems from an observation that pp60-src from polyomavirus-transformed cells bound to mIgD T antigen exhibits enhanced in vitro tyrosine kinase activity (2, 6, 17) and is not detectably phosphorylated at tyrosine 527 (7). Increased phosphorylation at tyrosine in the carboxyl-terminal half of the molecule in vanadate-treated cells could result from inhibition of a tyrosine-specific phosphatase (47), stimulation of a tyrosine-specific kinase, or alteration in the structure or location (or both) of pp60-src. Phosphorylation at tyrosine 527 is peculiar to pp60-src, since this amino acid residue is located in the 19 carboxyl-terminal amino acids unique to pp60-src (14, 48). Since there is not a homologous site in pp60-vsrc, phosphorylation at this residue apparently constitutes a regulatory mechanism that is unique.
to pp60\textsuperscript{src}. Therefore, if the immune complex pp60\textsuperscript{src} kinase assay is an accurate reflection of in vivo enzymatic activity, then phosphorylation of pp60\textsuperscript{src} at this tyrosine residue may serve to limit its intracellular tyrosine kinase activity.

pp60\textsuperscript{src} from intact RSV-transformed cells (4, 9) or from plasma membrane fractions (43) incubated with vanadate has hyperphosphorylated tyrosine in the amino-terminal half of the molecule. This hyperphosphorylated form of the molecule has increased tyrosine kinase activity both in vitro (4, 9) and in vivo (4), in contrast to our findings with pp60\textsuperscript{src}. Tyrosine residues in the amino-terminal half of the molecule are apparently autophosphorylated (43), suggesting that pp60\textsuperscript{src} can enhance its own enzymatic activity. It is not known whether tyrosine 527 of pp60\textsuperscript{src} is autophosphorylated or phosphorylated by another tyrosine kinase. If phosphorylation at this residue inhibits the in vivo kinase activity of pp60\textsuperscript{src}, and if this residue is autophosphorylated, then pp60\textsuperscript{src} has the capacity to inhibit its own kinase activity. Alternatively, it has been suggested that regulation may relate to compartmentalization of pp60\textsuperscript{src} (18).

Recent studies have shown that pp60\textsuperscript{src} is capable of autophosphorylating tyrosine in the amino-terminal half of the molecule in vitro kinase assays when bound to middle T antigen from polyomavirus-transformed cells (6, 49) or when extracted from neuroblastoma cell lines (1). These hyperphosphorylated forms of pp60\textsuperscript{src} may be found in vivo (1, 49) and are more active in in vitro kinase assays when exogenous substrates are employed (1, 2, 6). pp60\textsuperscript{src} from quiescent murine fibroblasts that have been treated with platelet-derived growth factor is also phosphorylated at tyrosine in the amino-terminal half of the molecule and demonstrates enhanced in vitro kinase activity (42). Although the phosphorylation state of tyrosine 527 in pp60\textsuperscript{src} from neuroblastoma cell lines and from platelet-derived growth factor-stimulated fibroblasts has not been investigated, tyrosine 527 in pp60\textsuperscript{src} bound to middle T antigen is not detectably phosphorylated (7). If there is decreased phosphorylation of tyrosine 527 in the former two cases, then lack of phosphorylation at this site may enable pp60\textsuperscript{src} to autophosphorylate tyrosine residues in the amino-terminal half of the molecule. Such autophosphorylation may simply be a reflection of enhanced tyrosine kinase activity, or it may serve to further stimulate tyrosine kinase activity as is apparently the case with pp60\textsuperscript{src} (4, 9). It would be of interest to know whether two recently identified single-amino-acid substitutions that enhance the tyrosine kinase activity and transforming potential of pp60\textsuperscript{src} (34) somehow inhibit phosphorylation at tyrosine 527.

We also found increased phosphorylation at serine, probably serine 17, in the amino-terminal half of pp60\textsuperscript{src} (42). Interestingly, vanadate treatment of intact RSV-transformed cells does not increase phosphorylation at serine in pp60\textsuperscript{src} (4, 9), and Courtneidge did not observe increased phosphorylation at serine when untransformed cells were lysed in the presence of vanadate (17). Since phosphorylation of the homologous serine 17 site of pp60\textsuperscript{src} appears not to greatly influence the in vitro tyrosine kinase activity of the molecule (19), increased phosphorylation at tyrosine 527 in the carboxyl-terminal half of pp60\textsuperscript{src} is most likely responsible for the inhibition of in vitro kinase activity. The serine 17 residue of pp60\textsuperscript{src} is apparently phosphorylated in vivo by a cAMP-dependent protein kinase (39). Increased phosphorylation at serine 17 of pp60\textsuperscript{src} in intact, vanadate-treated cells could come about as a result of inhibition of a phosphatase, stimulation of cAMP-dependent kinase, or alteration in the structure or location (or both) of pp60\textsuperscript{src}.

Incubation of CEF with micromolar vanadate alters neither the transport of P, nor the levels of cAMP, ADP, or ATP (Brown and Gordon, unpublished data).

We found that lysis of normal CEF in the presence of vanadate has no effect on the tyrosine kinase activity or phosphorylation state of pp60\textsuperscript{src}. Additionally, lysis of RSV-transformed CEF and RSV-transformed vole cells has no effect on the phosphorylation state or tyrosine kinase activity of pp60\textsuperscript{src} (4, 9). Thus, it is not clear why lysis of the untransformed rodent cells used by Courtneidge in the presence of vanadate facilitates identification of a more highly phosphorylated, less active form of pp60\textsuperscript{src} (17). Perhaps these cells contain a phosphatase or kinase that is active and subject to modulation by vanadate in lysates.

Tyrosine phosphorylation of the 36-kDa phosphoprotein substrate of pp60\textsuperscript{src} is less than expected in rodent cells containing very high levels of overexpressed pp60\textsuperscript{src} (18, 27) and negligible in CEF overexpressing pp60\textsuperscript{src} (26). However, we found increased phosphorylation of this protein in untransformed CEF treated with vanadate; a similar observation has been made with untransformed rodent cells (31). Intracellular substrates for pp60\textsuperscript{src} have not been identified, although in rodent fibroblasts treated with platelet-derived growth factor the appearance of pp60\textsuperscript{src} phosphorylated at tyrosine in the amino-terminal half of the molecule is paralleled by an increase in tyrosine phosphorylation of the 36-kDa phosphoprotein, suggesting a possible relationship (42). However, epidermal growth factor treatment of cells stimulates increased tyrosine phosphorylation of the 36-kDa phosphoprotein that is not paralleled by the appearance of structurally modified or more active form of pp60\textsuperscript{src} (15). These studies suggest that although pp60\textsuperscript{src} may phosphorylate the 36-kDa phosphoprotein in normal cells, it may not be the only tyrosine kinase activity that is capable of doing so. Regardless of which tyrosine kinase phosphorylates the 36-kDa phosphoprotein, such phosphorylation may be transient and kept at low and even undetectable levels presumably by phosphotyrosyl phosphatases.

Inhibition of these phosphatases may well lead to a net (and observable) increase in tyrosine phosphorylation of the 36-kDa phosphoprotein even if the responsible kinase is inhibited.

Finally, a recent report suggested that increased intracellular tyrosine phosphorylation after vanadate treatment of untransformed rodent cells leads to transformation as evidenced by cell rounding, modest increase in 2-deoxyglucose transport, and some growth in methocel; a remarkable increase in total cell phosphotyrosine was reported (31). Alternatively, long-term incubation of cells with sodium orthovanadate, acting as a mitogen or leading to cell injury, may mimic changes suggestive of cell transformation. We observed no changes in CEF that were suggestive of transformation, although this may be due to the fact that our experiments involved short-term incubation of CEF with vanadate. However, our studies and those of Courtneidge (17) indicate that any involvement of pp60\textsuperscript{src} in transformation of vanadate-treated rodent cells may involve mechanisms other than stimulation of pp60\textsuperscript{src} tyrosine kinase activity.

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LITERATURE CITED


40. Puchico, A. F., A. F. Wells, and M. S. Collett. 1983. Increase in the phosphotransferase specific activity of purified Rous sar-


