Journal of Cellular Biochemistry 26:95–106 (1984)
Membrane Receptors and Cellular Regulation 31–42

Enzymatic Characteristics of pp60$^{v\text{-}src}$ Isolated From Vanadium-Treated Transformed Cells

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The transforming protein of Rous sarcoma virus (RSV) typically appears as a single phosphorylated polypeptide designated pp60$^{v\text{-}src}$. pp60$^{v\text{-}src}$ possesses a protein kinase activity specific for tyrosine residues on select protein substrates. Treatment of RSV-transformed cells with vanadium ions resulted in the appearance of an electrophoretic variant of pp60$^{v\text{-}src}$ and was paralleled by a significant increase in the src kinase specific activity in purified enzyme preparations. Both the normal (standard) src kinase and the src kinase preparations obtained from vanadium-treated cells exhibited similar optimal activity profiles for $MgCl_2$, KCl, and pH. Furthermore, their site specificities of phosphorylation of the substrates casein and vinculin were the same. The reaction kinetic profile of the standard src kinase showed a nonlinear pattern, while the vanadium enzyme exhibited conventional linear Michaelis-Menten kinetics. These results are discussed with respect to the possible functional regulation of pp60$^{v\text{-}src}$ activity by a vanadium-sensitive protein phosphatase activity.

Key words: RSV src protein, retrovirus transformation, tyrosine phosphorylation, protein kinase, vanadium ion, oncoprotein

Oncogenic transformation by Rous sarcoma virus (RSV) is the result of the expression of the viral src gene product pp60$^{v\text{-}src}$. This protein possesses at least one enzymatic activity: that of a protein kinase capable of phosphorylating suitable protein substrates on tyrosine residues [1]. Thus at the present time, it is generally thought that the mechanism by which pp60$^{v\text{-}src}$ causes neoplastic conversion involves the aberrant, or otherwise inappropriate, phosphorylation of cellular proteins at tyrosine residues. Many laboratories are presently concerned with identifying phosphotyrosine-containing proteins that may be physiologically important targets of the src kinase. However, it should still be kept in mind that pp60$^{v\text{-}src}$ may yet have other functional activities that contribute to the diverse phenotypic and biologic manifestations that characterize the transformed state.

pp60$^{v\text{-}src}$ is itself a phosphoprotein, and when isolated from transformed cell lysates typically contains two major phosphorylated residues: a phosphoserine located

Received March 25, 1984; revised and accepted June 6, 1984.

near the amino-terminal end of the protein (ser-17), which appears to be the result of phosphorylation by a cellular cyclic AMP-dependent protein kinase, and a phosphotyrosine residue in the carboxy-terminal region of the molecule (tyr-416), which may be the result of $pp60^{v\text{-}src}$ "autophosphorylation" [2–8]. The potential regulation of $pp60^{v\text{-}src}$ function by phosphorylation-dephosphorylation modifications of the $pp60^{v\text{-}src}$ polypeptide has been the concern of our laboratory, as well as others. The two major phosphorylated residues on $pp60^{v\text{-}src}$ (ser-17 and tyr-416) do not appear to be required for the transforming capabilities and kinase activity of this transforming protein [7,8]. However, we have found that extensive autophosphorylation of purified $pp60^{v\text{-}src}$ in vitro served to dramatically increase the specific activity of the src protein kinase [9]. This extensive tyrosine phosphorylation of $pp60^{v\text{-}src}$ correlated with the appearance of an electrophoretic variant of $pp60^{v\text{-}src}$ possessing previously unrecognized amino-terminal phosphotyrosine [10]. This suggested that tyrosine phosphorylation of $pp60^{v\text{-}src}$ at sites other than tyr-416 may be involved in regulating the functional activity of this transforming protein [9,10]. However, such variant forms of $pp60^{v\text{-}src}$ had not been observed in transformed cells, casting doubt as to the physiologic significance of the in vitro variant $pp60^{v\text{-}src}$ forms. Very recently we reported the identification of $pp60^{v\text{-}src}$ molecules isolated from transformed cells that have all the structural and functional features of the in vitro modified src protein we had previously described [11]. Detection of this new form of $pp60^{v\text{-}src}$ depended on culturing transformed cells in the presence of vanadium ions, or disrupting cells under conditions that promote kinase activities and inhibit phosphatase activities. Here we extend our studies of the variant $pp60^{v\text{-}src}$ molecules identified in vanadium-treated cells by comparing various parameters of the protein kinase activity of partially purified $pp60^{v\text{-}src}$ enzyme preparations obtained from untreated and vanadium-treated transformed cells.

MATERIALS AND METHODS

Cells and Virus

The cells used in these experiments were European field vole cells transformed by subgroup D Schmidt-Ruppin strain of RSV and were propagated as described [9,10].

Radiolabeling of Cultures and Immunoprecipitation

Cell cultures were radiolabeled with culture medium containing 20% of the normal level of methionine supplemented with 50 μCi/ml [^{35}S]-methionine, or culture medium containing 20% of the normal level of phosphate supplemented with 1 mCi/ml [^{32}P]-orthophosphate for 28 hr prior to being harvested. Cleared cell lysates were prepared, immunoprecipitated, and analyzed on SDS-polyacrylamide gels, all as previously described [9,10].

Purification of $pp60^{v\text{-}src}$ and Protein Kinase Reactions

$pp60^{v\text{-}src}$ was prepared from untreated or vanadium-treated (50 μM Na_3VO_4, 28 hr) cells by immunoaffinity chromatography as previously described [9–11]. The molar amount of $pp60^{v\text{-}src}$ polypeptide present in the reactions was estimated by calculating the specific activity of steady-state [^{35}S]-methionine-labeled total cell protein and the amount of [^{35}S]-methionine radioactivity in the $pp60^{v\text{-}src}$ polypeptide band (as resolved by gel electrophoresis) of a known volume of enzyme preparation, as previously described [9]. Except where noted in the legends to the figures, protein

kinase reactions were carried out at 22°C for 30 min in a total volume of 30 μl containing 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 μM [γ-^{32}P]-ATP (600 Ci/mmol), and 0.12–0.32 pmol pp60$^{v\text{-}src}$ protein. When included, casein (Sigma, St. Louis, MO) was present at 300 μg/ml, vinculin (purified from chicken gizzards [12]) at 70 μg/ml, and phosphatidylinositol (Sigma) at 10 μg per reaction [11,13].

Peptide Mapping

Radiolabeled polypeptides resolved by gel electrophoresis were localized by autoradiography of unfixed gels and processed for two-dimensional tryptic peptide analysis as previously described [2]. Two-dimensional fractionation of [^{32}P]-labeled pp60$^{v\text{-}src}$ peptides proceeded exactly as described [2,10]. Casein tryptic phosphopeptides were subjected to ascending chromatography in butanol-acetic acid-water (5:1:4) prior to second dimension electrophoresis at pH 3.5 (pyridine-acetic acid-water: 1:10:189). The tryptic phosphopeptides of vinculin were chromatographed in sec-butanol-*n*-propanol-isoamyl alcohol-pyridine-water (1:1:1:3:3) in the first dimension, followed by electrophoresis at pH 3.5 in the second.

RESULTS

Figure 1 shows an autoradiographic representation of radiolabeled polypeptides immunoprecipitated from RSV-transformed vole cells with tumor-bearing rabbit (TBR) serum, as resolved by SDS-polyacrylamide gel electrophoresis. The cell cultures were labeled with either [^{35}S]-methionine or [^{32}P]-orthophosphate under steady-state conditions in either the absence (std) or presence (VO_4) of 50 μM Na_3VO_4. When standard [^{35}S]-labeled or [^{32}P]-labeled lysates were immunoprecipitated with TBR serum, the typical single pp60$^{v\text{-}src}$ polypeptide band was observed (Fig. 1, tracks 1 and 3). However, similar immunoprecipitation of cells cultured in the presence of vanadium ions resulted in the appearance of a new, additional src-specific polypeptide migrating slightly more slowly than the "standard" pp60$^{v\text{-}src}$ protein (Fig. 1, tracks 2 and 4). Our ability to detect this novel pp60$^{v\text{-}src}$ polypeptide in vanadium-treated cells radiolabeled with [^{35}S]-methionine was variable: in some cases the relative amount of the new polypeptide and standard pp60$^{v\text{-}src}$ protein were equivalent; on other occasions we were barely able to detect the variant molecule. On the other hand, vanadium-treated cells radiolabeled with [^{32}P]-orthophosphate routinely revealed the new, variant pp60$^{v\text{-}src}$ molecule, and in some cases represented the major component of the closely-spaced *src* protein doublet. One possible interpretation of these results suggests that the new variant form of pp60$^{v\text{-}src}$ represents a minor, transiently modified (highly phosphorylated) species within the total *src* protein present in the cell lysate [11].

The src-specific proteins from untreated and vanadium-treated cells have been partially purified by immunoaffinity chromatography, and the resultant enzyme preparations used in protein kinase reactions [11]. Figure 2 shows a sampling of phosphorylations by these two enzyme preparations. When the standard enzyme preparation (std), obtained from untreated cells, was incubated in analytic kinase reactions containing [γ-^{32}P]-ATP, the pp60$^{v\text{-}src}$ polypeptide itself was radiolabeled (Fig. 2, track 1), presumably the result of autophosphorylation [3,5,6,10]. When src enzyme preparations obtained from vanadium-treated cells (VO_4) were similarly analyzed, both the new, variant polypeptide and the standard pp60$^{v\text{-}src}$ protein were labeled (Fig. 2, track 2). The phosphorylation of casein by these respective src enzyme

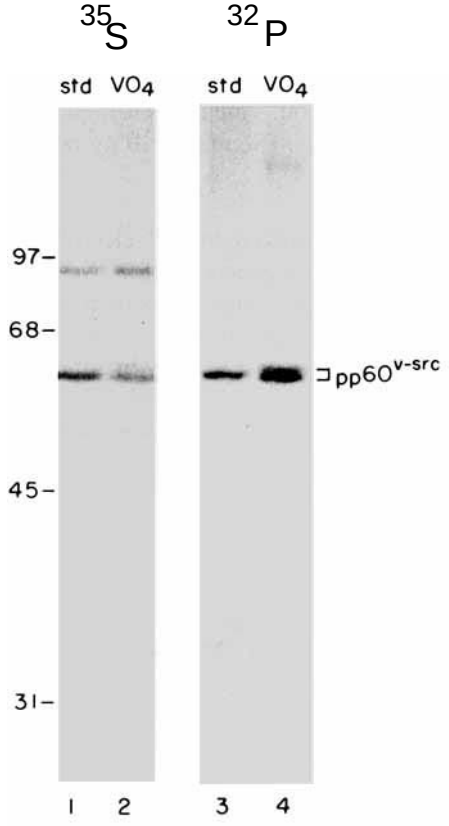

Fig. 1. Variant form of $pp60^{v\text{-}src}$ immunoprecipitated from lysates of vanadium ion–treated transformed cell cultures. RSV-transformed vole cell cultures were radiolabeled with either [^{35}S]-methionine or [^{32}P]-orthophosphate in either the absence (std) or presence (VO_4) of 50 μM Na_3VO_4 for a period of 28 hr as described in the Materials and Methods. Cleared cell lysates were prepared and immunoprecipitated with TBR serum as described [10]. Portions of the immunoprecipitated polypeptides were subjected to electrophoresis in SDS-10% polyacrylamide gels, and the gels were then processed for fluorography or autoradiography as described [10]. The number at the left represent the positions of molecular weight standards (in kilodaltons) included in all gels.

preparations is shown in Figure 2, tracks 3 and 4, demonstrating the greatly increased (seven-fold) phosphorylating ability of the vanadium enzyme preparation.

Vinculin has been implicated as a possible target of the src kinase activity [14]. Purified vinculin may be phosphorylated in vitro by the src kinase, and furthermore, this phosphorylation can be greatly enhanced by the inclusion of anionic phospholipids in the kinase reaction mixtures [13]. We have been able to confirm these results. We find that the phosphorylation of vinculin by both our standard $pp60^{v\text{-}src}$ enzyme preparations and our src enzyme preparations obtained from vanadium-treated cells was very considerably stimulated (some 20-fold) by the presence of phosphatidylinositol (Fig. 2, tracks 5–8). Again, the VO_4 enzyme preparation exhibited a substantially greater capacity (ten-fold) to phosphorylate vinculin than did the standard $pp60^{v\text{-}src}$ enzyme.

Since the src enzyme preparations obtained from vanadium-treated transformed cells exhibited a dramatically increased protein kinase specific activity when compared with standard preparations obtained from untreated cells (Fig. 2) [11], we have investigated in detail various parameters of the kinase activities of these two enzyme preparations in the hope of further understanding this difference. We have compared

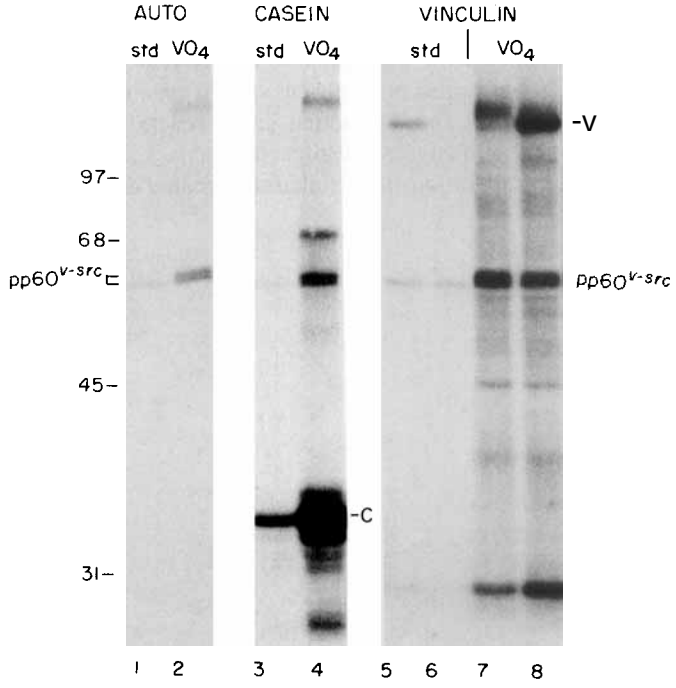

Fig. 2. Protein phosphorylation by partially purified pp60$^{v\text{-}src}$ enzyme preparations containing the variant *src* protein. pp60$^{v\text{-}src}$ enzyme preparations were obtained by immunoaffinity chromatography of lysates from untreated (std) or Na_3VO_4-treated (VO_4) cells [9,11]. Standard analytic protein kinase reactions (Materials and Methods) were carried out with the two enzyme preparations in the absence of exogenously added protein (AUTO, tracks 1 and 2), in the presence of casein (C, tracks 3 and 4), and in the presence of vinculin (V, tracks 4–8). Vinculin phosphorylation reactions were performed in either the absence (tracks 6 and 7) or presence (tracks 5 and 8) of 300 μg/ml phosphatidylinositol [13]. Phosphorylated polypeptides other than pp60$^{v\text{-}src}$, casein, and vinculin were also observed. In particular, a high molecular weight, diffuse polypeptide in the VO_4 enzyme preparation, a 70-kilodalton polypeptide in the casein reactions, and many minor bands, as well as one major (28-kilodalton) polypeptide in the vinculin reactions were observed. These phosphorylations are attributable to the presence of contaminating polypeptides in the respective protein preparations.

the ability of the two enzyme preparations to phosphorylate themselves ("autophosphorylation"), casein, and vinculin (in the presence of phosphatidylinositol), over a range of $MgCl_2$ concentrations, KCl concentrations, and pH. The results of these analyses are summarized in Figure 3. Although the vanadium *src* enzyme preparation showed a greatly increased activity for all three substrates measured, the optimal range of $MgCl_2$ concentrations, KCl concentrations, and pH. The results of these analyses are summarized in Figure 3. Although the vanadium src enzyme preparation showed a greatly increased activity for all three substrates measured, the optimal $MgCl_2$ concentration, KCl concentration, and pH were very similar to those of the standard src kinase. Under the assay conditions employed, a reaction buffered with Tris-HCl at pH 8.0, containing 5 mM $MgCl_2$, and devoid of KCl, was optimal for both the standard and vanadium src kinase preparations. In our hands with these enzyme preparations, we have found that the substitution of $MgCl_2$ with $MnCl_2$, at any concentration, resulted in at least an order of magnitude reduction in kinase activity (data not shown).

To compare further the kinase activities of the standard and vanadium src enzyme preparations, we have analyzed their kinetics of phosphorylation of casein. In these analyses, considerable care was taken to ensure that equivalent amounts of total $pp60^{v\text{-}src}$ polypeptide were present in all enzymatic reactions, that the substrate casein was in substantial excess, and that actual initial reaction velocities (V_0) were estimated. The results of these analyses are presented as a Lineweaver-Burk plot in Figure 4. The src enzyme preparation from vanadium-treated cells exhibited standard

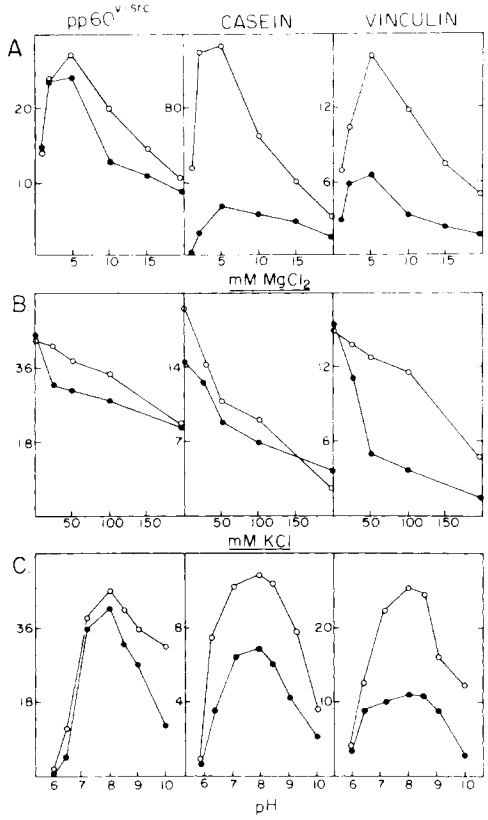

Fig. 3. Optimum reaction conditions for the src protein kinase activity isolated from untreated and vanadium ion-treated transformed cells. Analytic protein kinase reactions containing either the standard src enzyme preparation or the src enzyme preparation obtained from vanadium-treated cells were set up to establish optimal $MgCl_2$ and KCl concentrations, and reaction pH, for the phosphorylation of $pp60^{v\text{-}src}$ itself (auto), casein, and vinculin. All phosphorylation reactions containing vinculin were supplemented with phosphatidylinositol (Materials and Methods). Reactions were terminated after 30 min at 22°C and subjected to SDS-polyacrylamide gel electrophoresis. The various phosphorylated polypeptides were localized by autoradiography of the dried gels, excised, and the radioactivity quantitated by scintillation spectrometry. A) Reaction mixtures were composed of 10 mM Tris-HCl, pH 7.2, and varying concentrations of $MgCl_2$. B) Reaction mixtures were composed of 10 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, and varying concentrations of KCl. C) Reaction mixtures were composed of 5 mM $MgCl_2$ and were supplemented with 50 mM Tris-HCl, adjusted to various pH values (at 22°). In all cases [γ-^{32}P]-ATP was present at 1 μM. Standard src kinase, (●—●); VO_4 src kinase, (○—○). Values for autophosphorylation (auto) are presented as cpm × 10^{-2} (●), or cpm × 10^{-3} (○). Values for casein phosphorylation are presented as cpm × 10^{-3} (●, ○) for $MgCl_2$, and cpm × 10^{-3} (●), or cpm × 10^{-4} (○) for KCl and pH. Values for vinculin phosphorylation are presented as cpm × 10^{-2} (●), or cpm × 10^{-3} (○).

linear Michaelis-Menten kinetics. However, the standard enzyme preparation obtained from untreated cells presented nonlinear and, in fact, biphasic reaction kinetics. The K_m value for ATP of both enzyme preparations was nearly identical, approximately 7 μM, while the relative V_{max} value for casein phosphorylation by the vanadium enzyme was 5–6-fold higher than that of the standard enzyme.

In order to compare and assess further the ability of these two enzyme preparations to phosphorylate protein substrates, we have attempted to estimate the molar extent of phosphorylation of various phosphate acceptor proteins—namely, pp60$^{v\text{-}src}$ itself, casein, and vinculin. We have performed analytic protein kinase reactions under optimal reaction conditions (Fig. 3), with a high concentration of ATP (100 μM), for an extended period of time (2 hr). Under these conditions, all phosphorylation reactions had reached plateau levels (as determined by time course studies; data not shown). We then quantitated the amount of phosphate incorporated into a known amount of acceptor protein. The results of these analyses are presented in Table I. With all three substrates, the vanadium enzyme was capable of transferring, on a molar basis, 3–5 times more phosphate onto a given acceptor protein. Since at high ATP concentrations in the kinase reactions, the standard enzyme appeared to alter its phosphorylation kinetics (Fig. 4), the values presented in Table I probably represent underestimates of the differences in the actual phosphorylating capabilities of the two src enzyme preparations.

The src enzyme preparations from vanadium-treated cells clearly exhibited an increased ability to phosphorylate various protein substrates when compared with standard enzyme preparations. This increase appeared to be related to enzyme activation as reflected in an increased V_{max} value (Fig. 4). However, it was important to establish whether or not this apparent activation of src enzymatic activity involved any alteration in the site-specificity of phosphorylation by the modified src kinase. Therefore we have compared the sites of protein phosphorylation, by the standard and vanadium src enzyme preparations, on three substrates: pp60$^{v\text{-}src}$, casein, and

TABLE I. Extent of Substrate Phosphorylation by src Protein Kinase Preparations*

	molPO$_4$/mol polypeptide	
Substrate	std enzyme	VO$_4$ enzyme
pp60$^{v\text{-}src}$	0.43	1.92
Casein	0.21	0.70
Vinculin	0.04	0.14

*Standard analytic protein kinase reactions were performed as described in the Materials and Methods except that [γ-^{32}P]-ATP was present at 100 μM (21 Ci/mmol). After 2 hr at 22°C, reactions were terminated, and samples were subjected to gel electrophoresis. The molar incorporation of phosphate was determined as previously described [9,10]. The amount of casein and vinculin polypeptide comigrating with the quantitated radioactivity was estimated by Coomassie blue staining, using bovine serum albumin as a standard. The values represent the average of two independent determinations.

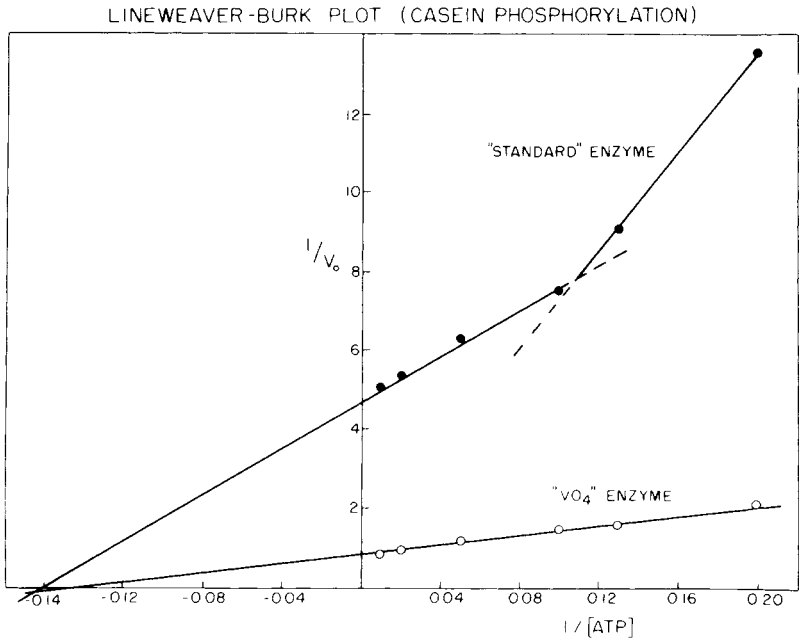

Fig. 4. Lineweaver-Burk plot of the phosphorylation of casein by the standard src enzyme and by the src enzyme isolated from vanadium-treated cells. The standard and VO_4 enzyme preparations (0.24 pmol $pp60^{v\text{-}src}$ polypeptide) were incubated in 120 μl kinase reaction mixtures containing 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mg/ml casein, and various concentrations of [γ-^{32}P]-ATP (5–100 μM; 30 Ci/mmol). At each concentration of ATP, equal portions (30 μl) were removed to SDS-sample buffer after 2, 5, and 10 min of reaction at 22°C. The phosphorylation of casein was quantified by scintillation counting of the phosphorylated protein bands resolved by SDS-polyacrylamide gel electrophoresis. A plot of the phosphorylation at the three time points for each ATP concentration was used to estimate initial reaction velocities (V_0) by extrapolation to time zero. The inverse of V_0 was then plotted against the inverse of the ATP concentration. (●), standard enzyme; (○), VO_4 enzyme.

vinculin. The various proteins were phosphorylated under standard kinase reaction conditions (see Methods) by the two enzyme preparations. Under these reaction conditions, phosphorylation of the various acceptor proteins was in the linear range of phosphate incorporation and well below the plateau levels of phosphorylation described in Table I. The phosphorylated protein bands were isolated from polyacrylamide gels and digested with trypsin. In the case of $pp60^{v\text{-}src}$ "autophosphorylated" in the vanadium enzyme preparation, no effort was made to separate and analyze individually the two phosphorylated polypeptides. Rather the closely spaced $pp60^{v\text{-}src}$ doublet was excised and processed as one. Phosphorylation of all proteins, by either enzyme preparation, occurred exclusively on tyrosine residues (data not shown). The resultant tryptic phosphopeptides were fractionated in two dimensions, and their autoradiograms are presented in Figure 5. The phosphopeptide pattern of the autophosphorylated $pp60^{v\text{-}src}$ protein from the standard enzyme preparation revealed one predominant phosphopeptide, the major COOH-terminal tryptic phosphopeptide of $pp60^{v\text{-}src}$ previously described [3,10]. The pattern of tryptic phosphopeptides from the autophosphorylated vanadium $pp60^{v\text{-}src}$ doublet revealed this same phosphopeptide, as well as several additional phosphopeptides. This phosphopeptide pattern of

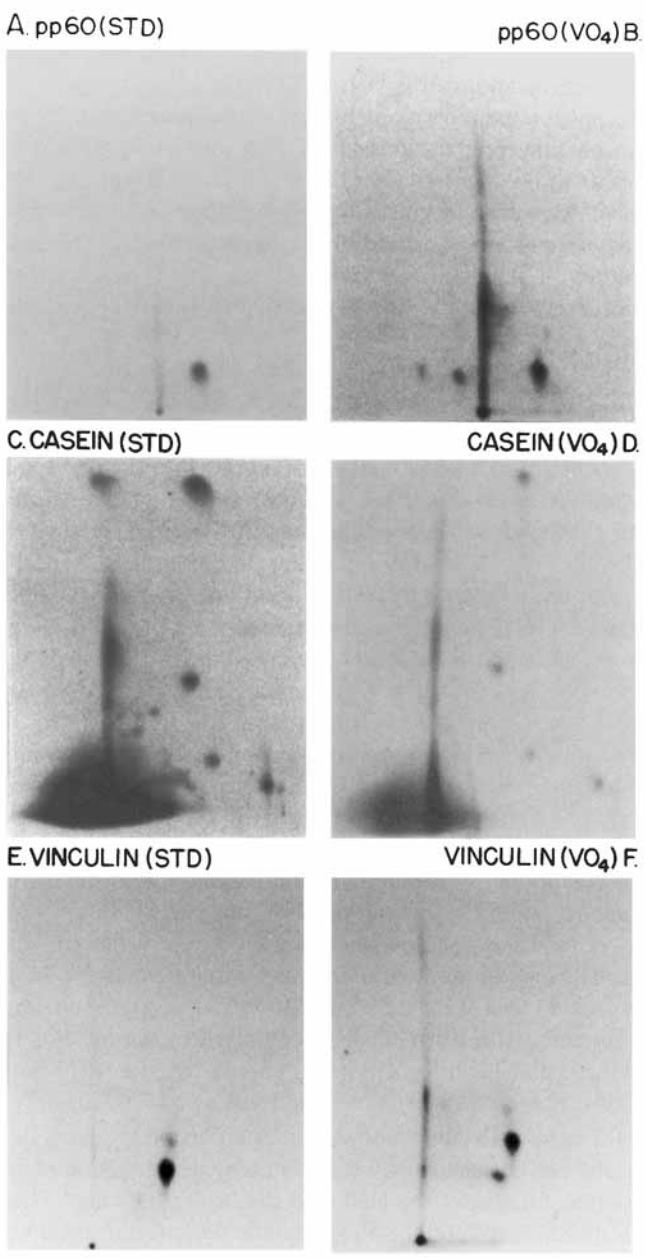

Fig. 5. Two-dimensional tryptic phosphopeptide fractionation of proteins phosphorylated by either the standard src kinase or the VO_4 src enzyme preparation. Phosphorylation reactions, as described in the legend to Figure 2, were carried out preparatively by the two enzyme preparations, and the various phosphorylated polypeptides excised from polyacrylamide gels, eluted, and processed for two-dimensional tryptic peptide mapping as previously described [2]. Two-dimensional fractionation was as described in the Materials and Methods. A) pp60$^{v\text{-}src}$ autophosphorylated in the standard enzyme reaction. B) pp60$^{v\text{-}src}$ (doublet) autophosphorylated in the VO_4 enzyme reactions. C) Casein phosphorylated by the standard src kinase. D) Casein phosphorylated by the VO_4 src kinase. E) Vinculin phosphorylated by the standard src kinase. F) Vinculin phosphorylated by the VO_4 src kinase. Vinculin phosphorylation reactions were conducted in the presence of phosphatidylinositol (see Materials and Methods).

the autophosphorylated vanadium pp60$^{v\text{-}src}$ polypeptide doublet was identical to that previously observed for purified standard pp60$^{v\text{-}src}$ that had been incubated with MgCl2 and high concentrations of [γ-^{32}P]-ATP [10].

The tryptic phosphopeptide maps of casein phosphorylated by the standard and by the vanadium enzyme preparations are shown in Figure 5C and C, respectively. The patterns appear to be qualitatively very much alike. Similarly, no major changes in the phosphopeptide pattern of vinculin phosphorylated by either the standard or the vanadium src enzyme preparations was observed (Fig. 5E,F). These data suggest that no major alteration in the site specificity of phosphorylation of either casein or vinculin was occurring with the more enzymatically active vanadium src kinase preparation.

DISCUSSION

When analyzed from transformed cells, pp60$^{v\text{-}src}$ typically appears as a single 60,000-dalton protein band. We have recently shown that a variant form of the pp60$^{v\text{-}src}$ protein can be found in transformed cell lysates [11]. Identification of this new species of pp60$^{v\text{-}src}$ depended on either the treatment of cell cultures with vanadium ions prior to cell disruption or disruption of (untreated) cells in the presence of $MgCl_2$ or $MgCl_2$/ATP. The structural characteristics of this new species of pp60$^{v\text{-}src}$ isolated from transformed cells were identical to those previously described for the novel form of purified pp60$^{v\text{-}src}$ that was generated after extensive autophosphorylation in vitro [10]. That is, the altered electrophoretic mobility of the variant form was the result of some modification in the amino-terminal region of the molecule and was paralleled by the appearance of amino-terminal region tyrosine phosphorylation on the pp60$^{v\text{-}src}$ polypeptide. Furthermore, purified enzyme preparations containing either the variant form generated in vitro or the modified pp60$^{v\text{-}src}$ isolated from transformed cells exhibited a substantially increased protein kinase specific activity compared to standard pp60$^{v\text{-}src}$ enzyme preparations [9,11].

In this report we have gone on to compare certain of the enzymatic features of src protein kinase preparations containing the variant pp60$^{v\text{-}src}$ molecules isolated from vanadium-treated transformed cells (VO_4 enzyme) to enzyme preparations obtained from untreated transformed cells (standard enzyme). For this comparison we have studied three phosphate-acceptor proteins as substrates for the two src protein kinase preparations: pp60$^{v\text{-}src}$ itself, casein and vinculin (Fig. 2).

Ito et al [13] have previously shown that the phosphorylation of vinculin by src kinase preparations can be specifically stimulated by the inclusion of anionic phospholipids in the reaction mixtures. We also find this to be the case. Phosphorylation of vinculin by both the standard and the VO_4 enzyme preparations was very dramatically enhanced (some 20-fold) by the inclusion of phosphatidylinositol (Fig. 2), whereas the presence of phospholipid had no effect (positive or negative) on the phosphorylation of casein or pp60$^{v\text{-}src}$ by either enzyme preparation (data not shown).

Although the VO_4 src enzyme preparation was routinely 7–12-fold more active on all protein substrates tested [11], the optima for $MgCl_2$, KCl, and pH were all very similar to those of the standard enzyme (Fig. 3). The standard and VO_4 enzyme preparations did differ however in the kinetics of casein phosphorylation (Fig. 4). While the VO_4 kinase exhibited standard linear Michaelis-Menten saturation of enzyme by ATP, the standard src enzyme preparation showed non-linear reaction

kinetics. This pattern suggests that during the course of the casein phosphorylation reaction, the standard enzyme was being functionally altered. This alteration most likely is related to the more extensive phosphorylation (autophosphorylation) of pp60$^{v\text{-}src}$ itself with increasing ATP concentrations. These results are very similar to those obtained when the kinetics of casein phosphorylation by purified standard src kinase were compared to those of purified, extensively autophosphorylated pp60$^{v\text{-}src}$ [9], suggesting that the VO_4 enzyme preparation obtained from transformed cells and pp60$^{v\text{-}src}$ extensively autophosphorylated in vitro may be similarly modified.

To determine if the apparent increased kinase activity exhibited by the VO_4 enzyme caused any alteration in its site specificity of phosphorylation of various acceptor proteins, we compared the tryptic phosphopeptide patterns of pp60$^{v\text{-}src}$, casein, and vinculin that resulted from their phosphorylation by either standard kinase preparation or the VO_4 enzyme. Both enzyme preparations were still capable of adding radiolabeled phosphate to the tryptic peptide containing tyrosine residue 416 (Fig. 5A,B). However, the VO_4 enzyme was further able to phosphorylate additional sites (Fig. 5B); sites previously shown to reside on both the amino-terminal and carboxy-terminal regions of the pp60$^{v\text{-}src}$ molecule [10]. The continued ability of these enzyme preparations to autophosphorylate, an observation previously made for purified pp60$^{v\text{-}src}$ that had been incubated with $MgCl_2$ and high concentrations of ATP for an extended period of time prior to its inclusion in a kinase reaction containing [γ-^{32}P]-ATP [9], suggests that phosphate accepting sites are still available on the molecules, or, alternatively, that some form of phosphate exchange is occurring. We have been unable to obtain any evidence for phosphate exchange.

The patterns of tryptic phosphopeptides resulting from the phosphorylation of either casein or vinculin by the two enzyme preparations appeared in both cases to be very similar (Fig. 5C–F), indicating that there was no major difference in the site specificity of phosphorylation of these proteins by the two enzyme preparations.

Vanadium ions have been previously shown to be potent inhibitors of alkaline phosphatase, and specifically, phosphotyrosyl protein phosphatase activities [15–18]. Thus the implication is that treatment of RSV-transformed cells with vanadium serves to inhibit the tyrosine dephosphorylation of pp60$^{v\text{-}src}$ by a putative phosphotyrosyl protein phosphatase. The result is the appearance of a physically modified species of pp60$^{v\text{-}src}$, and this can be correlated with an increased specific activity of the src protein kinase [11]. This increase in kinase activity appears to be a straightforward activation of the enzyme (increased V_{max}), since the optimal conditions for kinase activity and enzyme specificity appear to be unaltered. Moreover, our results suggest that phosphorylation, specifically, tyrosine phosphorylation, of pp60$^{v\text{-}src}$ may be involved in this activation. The direct demonstration of the involvement of specific protein dephosphorylation by a vanadium-sensitive (cellular) phosphotyrosyl protein phosphatase in the ultimate regulation of this transforming protein's function may provide insight into means for the control of oncogenesis by pp60$^{v\text{-}src}$.

ACKNOWLEDGMENTS

These studies were supported by a grant from the Leukemia Research Fund, University of Minnesota Foundation, and American Cancer Society grant IN-13-W-4. We thank Y. Guptill for her invaluable assistance in the preparation of this manuscript.

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