Structurally and Functionally Modified Forms of pp60^{v-src} in Rous Sarcoma Virus-Transformed Cell Lysates

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When analyzed from transformed cell lysates, pp60^{v-src}, the product of the Rous sarcoma virus *src* gene, typically appears as a single polypeptide of 60,000 molecular weight, phosphorylated at two major sites, an amino-terminal region serine residue and carboxy-terminal region tyrosine residue. We describe here the identification of variant forms of pp60^{v-src} present in transformed cell lysates that exhibited an altered electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels. This change in migration appeared to be the result of some alteration in the amino-terminal portion of the molecule and paralleled the appearance of extensive amino-terminal region tyrosine phosphorylation on the pp60^{v-src} molecule. These structural modifications were further correlated with a dramatic increase in the protein kinase-specific activity of pp60^{v-src}. The detection of these variant forms of pp60^{v-src} depended on the prior treatment of the transformed cell cultures with vanadium ions or the inclusion in the cell disruption buffer of Mg²⁺ or ATP-Mg²⁺. The implication is that modified, highly active forms of the pp60^{v-src} protein exist in transformed cells, but are transient and rapidly converted to stable forms, possibly by specific dephosphorylation. We suggest that amino-terminal region tyrosine phosphorylation of pp60^{v-src}, presumably the result of autophosphorylation, serves to greatly enhance *src* protein enzymatic activity, but that much of the regulation of this transforming protein's function may involve a phosphotyrosyl protein phosphatase.

The protein responsible for the oncogenic capabilities of Rous sarcoma virus (RSV) is pp60^{v-src}, a phosphoprotein encoded by the viral *src* gene (2, 20, 35, 46). pp60^{v-src} appears to possess at least one enzymatic activity, that of a tyrosine-specific protein kinase (6, 12, 13, 18, 21, 28, 29, 31, 41). pp60^{v-src} as isolated from transformed cell lysates itself contains two major phosphorylated residues: a phosphoserine located 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 (7, 8), and a phosphotyrosine residue, initially mapped to the carboxy-terminal one-third of the polypeptide (7, 8) and subsequently identified as the tyrosine residue at amino acid position 416 in the pp60^{v-src} molecule (33, 42). Phosphorylation of tyrosine 416 may be the result of pp60^{v-src} autophosphorylation since this same tyrosine residue was also found to be phosphorylated in vitro by purified pp60^{v-src} enzyme preparations (9, 34, 42). Phosphorylation and dephosphorylation of proteins and, in particular, enzymes have been shown to often regulate protein function (19, 24). Thus, pp60^{v-src} function could potentially be regulated by such a mechanism at at least two sites in the molecule by two different protein kinase activities, without considering protein phosphatase involvement (22).

Recently, Synder et al. reported the construction of an RSV mutant in which the tyrosine residue at position 416 in the pp60^{v-src} polypeptide was replaced with phenylalanine (43). They demonstrated that the resultant mutant protein still possessed protein kinase activity and was capable of inducing transformation of cultured cells. These results suggest that phosphorylation of tyrosine 416 is not required for pp60^{v-src} function. A similar conclusion was reached by Cross and Hanafusa (10). They constructed RSV mutants expressing pp60^{v-src} proteins that lacked either the major phosphorylated tyrosine (*tyr-416*) or the major phosphorylated serine (*ser-17*) residue, and they showed that cells infect-

ed with these mutants were morphologically transformed and possessed pp60^{v-src} tyrosine kinase activities similar to wild-type levels. These two studies therefore seem to indicate that phosphorylation of pp60^{v-src}, at either *tyr-416* or *ser-17*, plays little, if any, role in the function of *src*.

We too have been interested in evaluating the functional consequences of phosphorylation, specifically tyrosine phosphorylation, on the pp60^{v-src} protein. However, our approach to this problem differs from those of the above-described studies. Using partially purified preparations of the wild-type pp60^{v-src} enzyme, we have defined protein kinase reaction conditions whereby pp60^{v-src} can be extensively phosphorylated on tyrosine residues (9, 36). This more extensive in vitro tyrosine phosphorylation was not only reflected in an overall increase in pp60^{v-src} phosphate incorporation, but involved, in addition to phosphorylation of tyrosine 416, phosphorylation of previously undescribed sites on the pp60^{v-src} molecule. The most prominent new phosphorylated site appeared in a heterodisperse amino-terminal region tryptic peptide. Phosphorylation at this amino-terminal site appeared to slow the migration of the intact pp60^{v-src} polypeptide in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Thus, in protein kinase reactions containing purified pp60^{v-src}, performed under conditions allowing for extensive tyrosine phosphorylation, the normally observed single polypeptide band of pp60^{v-src} was converted to a closely spaced doublet band (9). This increased amino-terminal tyrosine phosphorylation and the appearance of the pp60^{v-src} polypeptide of altered electrophoretic mobility were correlated with a dramatic increase in the protein kinase specific activity of pp60^{v-src} (36). These results suggested that amino-terminal tyrosine phosphorylation, a previously unrecognized secondary modification on pp60^{v-src}, serves to regulate the phosphotransferase activity of this transforming protein (9, 36).

When isolated from cells in culture, pp60^{v-src} typically appears as a single polypeptide band of molecular weight 60,000 (2, 7), although on occasion proteolytic breakdown products of pp60^{v-src} have been observed (13, 47). The

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inability to detect any variant forms of pp60^{V-src} in transformed cells raises the question of the physiological significance of the extensive amino-terminal tyrosine phosphorylation and associated increase in protein kinase specific activity of pp60^{V-src} observed in vitro (9, 36). We report here the identification and initial characterization of pp60^{V-src} molecules isolated from transformed cells that have all the structural and functional features of the invitro-modified *src* protein we have previously described. The detection of these new forms of pp60^{V-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.

MATERIALS AND METHODS

Cells and virus. The cells used in these experiments were European field vole (*Microtus agrestis*) cells transformed by subgroup D Schmidt-Ruppin strain of RSV (clone 1-T) and were propagated as described previously (9, 36).

Radiolabeling of cell cultures and immunoprecipitation. Cell cultures were incubated for various periods of time as indicated in the legends to the figures in culture medium containing 20% of the normal level of methionine supplemented with 100 μ Ci of [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml or culture medium containing 20% of the normal level of phosphate supplemented with 1 mCi of ³²P_i (ICN Pharmaceuticals Inc., Irvine, Calif.) per ml. After the labeling period, cells were scraped from the culture dishes in the labeling medium, pelleted, and suspended in buffer H (100 mM NaCl, 10 mM Tris-hydrochloride [pH 7.2], 2 mM EDTA, 0.5% deoxycholate, 1% Nonidet 40) or buffer H containing various supplements as described in the legends to the figures. After 10 min on ice, cellular debris was removed by centrifugation at 25,000 $\times$ *g* for 20 min at 4°C. The cleared lysates were then aliquoted and immunoprecipitated with 2 to 5 μ l of tumor-bearing rabbit serum as previously described (2). Samples of the radiolabeled immunoprecipitated polypeptides were fractionated on SDS-10% polyacrylamide gels (25) and were either fluorographed (3) and exposed to X-ray film (for [³⁵S]methionine label), or autoradiographed (for ³²P_i label) with Cronex 4 X-ray film and Lightning-Plus intensifying screens (Du Pont Co., Wilmington, Del.).

Peptide mapping and phosphoamino acid analysis. Radiolabeled polypeptides resolved by SDS-polyacrylamide gel electrophoresis were localized by autoradiography and excised, and the individual protein-containing gel pieces were subjected to a second cycle of gel electrophoresis, localization, and excision. Proteins in gel slices were analyzed by one-dimensional partial proteolysis mapping with *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) as previously described (5, 7). Proteins were eluted from gel slices and subjected to two-dimensional tryptic peptide mapping exactly as described elsewhere (7). Eluted proteins and peptides were subjected to acid hydrolysis and electrophoresis at pH 3.5 for analysis of phosphoamino acids as described previously (8).

Purification of pp60^{V-src} and protein kinase reactions. pp60^{V-src} was prepared from cell lysates by immunoaffinity chromatography essentially as described previously (12, 13). Cultures (routinely 24 100-mm dishes) were radiolabeled with [³⁵S]methionine (20 μ Ci/ml) as described above for 24 to 30 h (in the absence or presence of 50 μ M Na₃VO₄) before a cleared lysate was prepared. For the preparation of enzyme from vanadium-treated cells, cells were disrupted in buffer H containing 200 μ M Na₃VO₄ and loaded onto the

immunoaffinity column equilibrated in the same buffer. After loading, the column was washed with 1 column volume of Na₃VO₄-containing buffer and then washed with buffers lacking Na₃VO₄. For the preparation of ATP-Mg⁺2 enzyme (untreated), cell cultures were disrupted in buffer H containing 1 mM ATP-10 mM MgCl₂ and loaded onto the column equilibrated with buffer H. All enzymes were eluted from the column with 1 M KSCN and then dialyzed against 50% glycerol buffer. For every enzyme purification, the molar amount of pp60^{V-src} protein present in the final preparation was estimated as described elsewhere (36). Standard analytic protein kinase reactions were carried out in a total volume of 30 μ l containing 5 mM MgCl₂, 0.01 M Tris-hydrochloride (pH 7.2), 1 μ M [γ -³²P]ATP (600 Ci/mmol), and 0.17 to 0.35 pmol of pp60^{V-src} protein. Protein substrates added to reactions included casein (Sigma Chemical Co., St. Louis, Mo.) at 300 μ g/ml, vinculin (purified from chicken gizzards [14]) at 70 μ g/ml, 34K protein-containing preparations (purified as described in reference 36) at 70 μ g/ml, and TBR immunoglobulin G (IgG, ammonium sulfate fractionated) at 500 μ g/ml. Reactions containing vinculin were supplemented with 10 μ g of phosphatidylinositol per reaction (23). Reactions proceeded for 30 min at 22°C and were terminated by the addition of 30 μ l of 2 $\times$ SDS sample buffer followed by boiling for 1 min and then analyzed by electrophoresis in SDS-10% polyacrylamide gels.

RESULTS

Identification of variant *src*-specific polypeptides in transformed cell lysates. Our in vitro studies with purified pp60^{V-src} indicated that extensive tyrosine phosphorylation of the *src* protein could be correlated with a dramatic increase in the *src* kinase specific activity (36). This extensive phosphorylation was characterized by the appearance of a new, more slowly migrating (in SDS-polyacrylamide gels) form of pp60^{V-src} that possessed previously unobserved amino-terminal region phosphorylated tyrosine residues (9). However, an analogous form of pp60^{V-src} has never been observed in lysates of transformed cells, thus raising a question of the real physiological significance of the more highly active, modified pp60^{V-src} polypeptide observed in vitro. Going on the premise that this in vitro variant form of pp60^{V-src} had some in vivo correlate, we reasoned that our inability to detect a pp60^{V-src} protein of altered electrophoretic mobility and increased enzymatic specific activity in transformed cells was due to the highly unstable or metabolically transient nature of the putative form. Such a highly active species of pp60^{V-src} might be short lived and rapidly modified to the normally observed pp60^{V-src} form by cellular enzymes in intact cells and, in particular, upon cell disruption. If this were the case, the most likely cellular enzymatic activity to effect this modification would be a phosphotyrosyl protein phosphatase. Such phosphatase activities have been described (1, 4, 15-17, 26, 44, 45) and, furthermore, have been shown to be inhibited by orthovanadate (26, 45) and Zn²⁺ (1, 17). We have therefore explored the use of these phosphatase inhibitors in our search for modified species of pp60^{V-src} in transformed cells.

Figure 1 shows the results of the *src*-specific immunoprecipitation of proteins from lysates of RSV-transformed cells that had been cultured for various periods of time in the presence of several concentrations of vanadyl ions (in this case, VOSO₄). Figure 1A shows an autoradiogram of a polyacrylamide gel separation of ³²P_i-labeled proteins immunoprecipitated with anti-*src* antiserum. From untreated cultures, pp60^{V-src} appeared as a single polypeptide band

(Fig. 1A, track 1). However, the inclusion of VOSO₄ in the culture medium at a concentration above 20 μ M and for a period as brief as 2.5 h allowed for the detection of a new *src*-specific polypeptide that migrated just above the normally observed pp60^{v-src} protein band (Fig. 1A, tracks 2 through 5). Incubation of cultured cells with low concentrations of vanadyl, even for extended periods of time, did not allow for the detection of the new *src* species (Fig. 1A, track 6). Concentrations of vanadyl ion above 50 μ M did not alter the results shown in Fig. 1A, but did appear to be slightly toxic to the cell cultures. Figure 1B shows the results of the *src*-specific immunoprecipitation of [³⁵S]methionine-labeled lysates from cells cultured in the absence (Fig. 1B, track 1) or the presence (Fig. 1B, track 2) of vanadium (in this case, Na₃VO₄). From the treated cells, a new [³⁵S]methionine-labeled polypeptide was observed migrating just above the normally observed pp60^{v-src} band. We have explored the use of a variety of inorganic vanadium compounds, representing several different oxidation states, and have found no significant differences from the results shown in Fig. 1.

Vanadium ions have been previously described as potent inhibitors of alkaline phosphatase activities (30, 40, 44), and

FIG. 1. Identification variant *src*-specific polypeptides by immunoprecipitation of lysates from vanadium ion-treated transformed cell cultures. (A) Transformed cells grown on 60-mm culture dishes were incubated with culture medium containing 20% of the normal level of phosphate for a total period of 20 or 66 h and supplemented at various times with several concentrations of VOSO₄. All cultures were radiolabeled with ³²P_i (in appropriately supplemented medium) for 2 h before cell harvest. Cleared cell lysates were prepared and immunoprecipitated with TBR serum, and immune-specific radiolabeled proteins were analyzed on SDS-10% polyacrylamide gels as described in the text. Tracks: 1, untreated culture (20 h); 2, culture medium supplemented with 25 μ M VOSO₄ for 20 h; 3, culture medium supplemented with 50 μ M VOSO₄ for 20 h; 4, culture medium supplemented with 50 μ M VOSO₄ for 5 h before cell harvest; 5, culture medium supplemented with 50 μ M VOSO₄ for 2.5 h before cell harvest; 6, culture medium supplemented with 10 μ M VOSO₄ over a period of 66 h (with one medium change). The numbers at the left represent the positions of molecular mass standards (in kilodaltons) included in all gels. (B) Transformed cells were grown in either the absence or presence of 50 μ M Na₃VO₄ in culture medium containing 20% of the normal level of methionine for a period of 18 h, at which time each medium was supplemented with 100 μ Ci of [³⁵S]methionine per ml. After a 2-h labeling period, cells were harvested, and cleared lysates were prepared. Immunoprecipitation and gel analysis proceeded as outlined in the text. Tracks: 1, untreated culture; 2, Na₃VO₄-treated culture. The higher-molecular-weight [³⁵S]methionine-labeled protein seen in this panel, and also in Fig. 2, was unrelated to pp60^{v-src} as demonstrated by peptide mapping procedures (data not shown). Its nature is unclear at this time.

FIG. 2. Effect of various supplements in the cell disruption buffer on the immunoprecipitation of *src*-specific polypeptides from untreated and vanadium ion-treated transformed cells. Transformed cell cultures were radiolabeled with either ³²P_i or [³⁵S]methionine for 28 h as described in the text in the absence (A) or presence (B) of 50 μ M Na₃VO₄. Cells were then suspended in buffer H (—, tracks 1 and 5), buffer H supplemented with 10 mM MgCl₂ (Mg, tracks 2 and 6), buffer H with 1 mM ATP–10 mM MgCl₂ (ATP Mg, tracks 3 and 7), or buffer H with 100 μ M Na₃VO₄–1 mM ATP–10 mM MgCl₂ (VO₄ ATP Mg, tracks 4 and 8). After 10 min at 4°C, the lysates were cleared, immunoprecipitated, and analyzed on SDS-10% polyacrylamide gels as described in the text.

phosphotyrosyl protein phosphatase activities (26, 45). Zn²⁺ has also been described as specifically inhibiting phosphotyrosyl protein phosphatase activities (1, 17). However, in our hands, we have not observed the variant *src* protein in immunoprecipitates from cell cultures treated with Zn²⁺ under a variety of conditions (data not shown).

The results in Fig. 1A suggested that cell cultures must be treated with vanadium ions at a sufficient concentration for some period of time before the variant *src*-specific polypeptide is detectable. However, in these experiments, no precautions were taken to prevent rapid modifications of the *src* protein during the preparation of the cell lysate for immunoprecipitation. The mere inclusion of vanadium ions in the cell disruption buffer, without prior treatment of the cell culture, failed to allow for the detection of the altered *src*-specific polypeptide (data not shown). We have found, however, that the inclusion of either Mg²⁺ alone or ATP–Mg²⁺ in the cell lysis buffer did aid in the detection of the new polypeptide, whether or not the cell cultures had been treated with vanadium (Fig. 2).

Structural analyses of the variant forms of pp60^{v-src}. These new *src*-specific polypeptides found in transformed cells were further characterized by comparative peptide mapping procedures and phosphoamino acid analyses. The upper and lower pp60^{v-src}-specific polypeptides immunoprecipitated from vanadium-treated cells were resolved by SDS-polyacrylamide gel electrophoresis, localized by autoradiography, excised, and individually reelectrophoresed. These two twice-gel-purified polypeptides were then compared with similarly prepared pp60^{v-src} from untreated cells by the partial proteolysis procedure of Cleveland et al. (5). The use of an appropriate amount of *Staphylococcus aureus* V8 protease in this analysis results in the cleavage of pp60^{v-src} to an NH₂-terminal 34,000-dalton fragment and a COOH-terminal 26,000-dalton fragment (7). Figure 3A shows such an

FIG. 3. One-dimensional partial V8 proteolysis of the variant $pp60^{v-src}$ polypeptides. Transformed cell cultures, radiolabeled with either $^{32}P_i$ or [^{35}S]methionine for 28 h in either the absence or presence of 50 μM Na_3VO_4 , were disrupted in buffer H and immunoprecipitated with TBR serum. The immunoprecipitated, radiolabeled polypeptides were resolved by electrophoresis in SDS-10% polyacrylamide gels. After localization by autoradiography, the $pp60^{v-src}$ polypeptides were excised and individually subjected to a second cycle of gel electrophoresis, localization, and excision. These twice-gel-purified polypeptides were then electrophoresed in an SDS-10% polyacrylamide gel in the presence of 20 ng of *S. aureus* V8 protease as previously described (7). (A) ^{32}P -labeled $pp60^{v-src}$ polypeptides. Tracks: 1, mixture of the $pp60^{v-src}$ polypeptide immunoprecipitated from untreated cells (–) and the upper, more slowly migrating $pp60^{v-src}$ protein immunoprecipitated from Na_3VO_4 -treated cells (U); 2, $pp60^{v-src}$ from untreated cells; 3, upper $pp60^{v-src}$ polypeptide immunoprecipitated from Na_3VO_4 -treated cells (U); 4, the lower, more rapidly migrating $pp60^{v-src}$ polypeptide immunoprecipitated from Na_3VO_4 -treated cells (L); 5, mixture of the Na_3VO_4 upper (U) and lower (L) $pp60^{v-src}$ polypeptides. (B) [^{35}S]methionine-labeled $pp60^{v-src}$ polypeptides. Tracks: 1, $pp60^{v-src}$ from untreated cells (–); 2, the upper (U) $pp60^{v-src}$ polypeptide from Na_3VO_4 -treated cells; 3, the lower (L) $pp60^{v-src}$ polypeptide from Na_3VO_4 -treated cells. The 34,000-dalton fragment (34) and the 26,000-dalton fragment (26) represent the NH_2 -terminal and $COOH$ -terminal portions of the $pp60^{v-src}$ molecule (7), respectively.

analysis of ^{32}P -labeled *src* polypeptides. The $COOH$ -terminal fragments of all three polypeptides ($pp60^{v-src}$ from untreated cells and the upper and lower $pp60^{v-src}$ polypeptides from vanadium-treated cells) appeared to migrate identically. However, the NH_2 -terminal fragments from both the upper and lower vanadium $pp60^{v-src}$ protein bands appeared to migrate more slowly than the corresponding fragment of $pp60^{v-src}$ from untreated cells, with the fragment from the upper polypeptide migrating the slowest (Fig. 3A, tracks 2 through 4). A similar analysis of the corresponding [^{35}S]methionine-labeled polypeptides is shown in Fig. 3B. The altered migration of the NH_2 -terminal fragment from the upper $pp60^{v-src}$ polypeptide was clearly visible, although there did not appear to be a discernable change in the migration of the lower $pp60^{v-src}$ NH_2 -terminal fragment.

We have gone on to compare, by a two-dimensional separation technique, the tryptic phosphopeptides of these

three species of $pp60^{v-src}$. Figure 4A shows the pattern of ^{32}P -labeled tryptic phosphopeptides generated from $pp60^{v-src}$ obtained from untreated transformed cells, revealing the major amino-terminal phosphoserine-containing and $COOH$ -terminal phosphotyrosine-containing peptides (7, 21). Similar analysis of the lower and upper $pp60^{v-src}$ protein bands from vanadium-treated cells is shown in Fig. 4B and C, respectively. The lower $pp60^{v-src}$ had a pattern of tryptic phosphopeptides similar to that of $pp60^{v-src}$ from untreated cells, except for the appearance of a considerable amount of phosphorylated material spread out in the vertical (chromatography) dimension. This material represents the amino-terminal tyrosine phosphorylation previously described on $pp60^{v-src}$ in vitro (9). The phosphopeptide pattern of the upper $pp60^{v-src}$ protein was very much like that of the lower $pp60^{v-src}$; however, the proportion of the phosphoserine-containing peptides appeared to be reduced.

The phosphoamino acid composition of all of the tryptic phosphopeptides was established by scraping the appropriate region from the two-dimensional maps and subjecting the

FIG. 4. Two-dimensional tryptic phosphopeptide fractionation of the $pp60^{v-src}$ polypeptides isolated from untreated and vanadium ion-treated transformed cells. ^{32}P -labeled $pp60^{v-src}$ polypeptides were immunoprecipitated and purified by two cycles of polyacrylamide gel electrophoresis as described in the legend to Fig. 3. After autoradiographic localization in the second gel, the individual polypeptides were excised, eluted, and processed for two-dimensional tryptic peptide mapping (7). (A) $pp60^{v-src}$ from untreated cells; (B) lower, more rapidly migrating $pp60^{v-src}$ polypeptide from Na_3VO_4 -treated cells; (C) upper, more slowly migrating $pp60^{v-src}$ polypeptide from Na_3VO_4 -treated cells; (D) schematic representation of the phosphopeptides and their phosphoamino acid composition. Phosphoamino acid composition was determined by scraping and collecting the respective regions of cellulose from the plastic-backed sheets and eluting the ^{32}P -labeled phosphopeptides with several changes of water. After lyophilization, samples were hydrolyzed in 6 N HCl at 110°C for 1 h and analyzed by paper electrophoresis at pH 3.5 (8).

eluted peptides to acid hydrolysis and electrophoresis at pH 3.5 (8). A schematic representation of the tryptic phosphopeptides and their phosphoamino acid composition is provided in Fig. 4D.

The results from the two-dimensional tryptic peptide analyses suggested that both the vanadium upper and lower pp60^{v-src} polypeptides had a considerably higher phosphotyrosine content than did pp60^{v-src} isolated from untreated cells. This was dramatically demonstrated when we compared the ratios of phosphoamino acid composition of three intact polypeptides (Fig. 5).

Functional analyses of partially purified enzyme preparations containing the variant pp60^{v-src} polypeptides. All of the above-described structural features of both the upper and lower pp60^{v-src} polypeptides immunoprecipitated from vanadium-treated cells appeared analogous to those described for pp60^{v-src} purified from (untreated) cells and subsequently phosphorylated (autophosphorylated) extensively in vitro (9).

We next wanted to compare the functional capabilities of pp60^{v-src} from vanadium-treated cells and cells disrupted in the presence of ATP-Mg²⁺ with those of pp60^{v-src} from untreated cells. The simplest and most rapid means of doing this is to compare the ability of *src*-specific immunoprecipitates from each of these cell lysates to phosphorylate the heavy chain of anti-*src* IgG in a standard immune complex protein kinase assay (6, 28). This procedure is routinely used by workers in the field to compare and quantitate pp60^{v-src} protein kinase activity. We have performed this assay with immunoprecipitates from untreated cells, vanadium-treated cells, and cells lysed in the presence of ATP-Mg²⁺. Our

FIG. 5. Phosphoamino acid composition of pp60^{v-src} polypeptides immunoprecipitated from untreated and vanadium ion-treated cells. ³²P-labeled pp60^{v-src} polypeptides immunoprecipitated from untreated and Na₃VO₄-treated transformed cells were purified as described in the legend to Fig. 3 by two cycles of gel electrophoresis, eluted from the gel slices, and subjected to phosphoamino acid analyses (7). Tracks: 1, pp60^{v-src} from untreated cells; 2, upper, more slowly migrating pp60^{v-src} polypeptide from Na₃VO₄-treated cells; 3, lower, more rapidly migrating pp60^{v-src} polypeptide from Na₃VO₄-treated cells. The radioactivity present in the various phosphoamino acids was quantitated by scintillation spectrometry, and the ratio of phosphotyrosine to phosphoserine was determined. P-ser, P-threo, and P-tyro represent the positions of migration of authentic phosphoserine, phosphothreonine, and phosphotyrosine, respectively, included in all samples.

FIG. 6. Protein kinase activity in partially purified pp60^{v-src} enzyme preparations obtained from various transformed cell lysates. pp60^{v-src} enzyme preparations were obtained by immunoaffinity chromatography of lysates from untreated cells (-), Na₃VO₄-treated cells (VO₄), and untreated cells disrupted in the presence of ATP and MgCl₂ (ATP Mg) as described in the text. Standard analytic protein kinase reactions were carried out with the three enzyme preparations in the absence of exogenously added proteins (tracks 1 through 3), and in the presence of casein (tracks 4 through 6), vinculin (tracks 7 through 9), 34K protein-containing fraction (tracks 10 through 12), and TBR IgG (tracks 13 through 15) as described in the text. C, Casein; V, vinculin; HC, Heavy chain of IgG; 50, 34, and 30, polypeptides of 50,000, 34,000, and 30,000 daltons, respectively, readily phosphorylated in the 34K protein-containing fraction. All phosphorylations occurred exclusively on tyrosine residues. The 34,000-dalton protein in this preparation represents a previously described putative target protein of pp60^{v-src} (11, 36, 37). The nature of the 50,000- and 30,000-dalton polypeptides remains unclear at this time.

results, however, revealed no significant activity differences among the three reactions (data not shown). We have previously indicated that this immune complex protein kinase assay may not be appropriate for the quantitative evaluation of pp60^{v-src} protein kinase activity (47). The misleading nature of this assay was further illustrated in the functional characterization of the variant pp60^{v-src} forms generated in vitro (36). Therefore, to more rigorously evaluate the protein kinase activity of the modified pp60^{v-src} species found in transformed cells, we have partially purified the *src*-specific polypeptides from untreated cells, vanadium-treated cells, and cells disrupted in the presence of ATP-Mg²⁺ by immunoaffinity chromatography, and we have compared their ability to phosphorylate a variety of protein substrates in soluble protein kinase reactions. Figure 6 (tracks 1 through 3) shows the results of autophosphorylation by the three enzyme preparations. The standard pp60^{v-src} enzyme purified from untreated cells showed the characteristic phosphorylation of a single polypeptide band at 60,000 molecular weight. However, the enzyme preparations obtained from the vanadium-treated cells (VO₄) and the cells disrupted in the presence of ATP-Mg²⁺ revealed the phosphorylation of two closely spaced polypeptides at 60,000 molecular weight, corresponding to the upper and lower pp60^{v-src} proteins found in immunoprecipitates from metabolically radiolabeled cells (Fig. 1 and 2). When exogenous phosphate-acceptor proteins such as casein, vinculin, and chicken cell protein fraction containing 34K protein (11, 37) were added to these protein kinase reactions, they were phosphorylated to significantly higher levels by the enzyme preparations containing the variant forms of pp60^{v-src} (VO₄

TABLE 1. Relative protein kinase specific activities of *src* enzyme preparations containing the variant forms of pp60^{v-src}

Protein substrate	Kinase sp act ratio ^a	
	VO ₄ /standard enzyme	ATP-Mg/standard enzyme
Casein	7.3 (4.7–11.7)	5.0 (4.4–5.5)
Vinculin	9.7 (7.5–18.6)	5.4 (3.8–9.6)
34K protein	16.7 (9.3–20.4)	9.4 (6.3–13.4)
50K protein	21.9 (15.8–35.0)	14.3 (8.6–18.7)
30K protein	19.2 (15.2–22.1)	11.3 (9.8–13.0)
IgG	4.1 (2.7–7.6)	4.3 (2.9–5.4)

^a Analytic protein kinase reactions containing the various pp60^{v-src} enzyme preparations and protein substrates were carried out as described in the text and analyzed as shown in Fig. 6. After autoradiographic localization of the various phosphorylated protein bands, the appropriate regions of the gels were excised, and the amount of radioactivity in the gel slices was determined by liquid scintillation counting. Taking into account the estimated amount of pp60^{v-src} polypeptide in the respective kinase reactions (36), we were able to determine the relative protein kinase specific activities of the preparation obtained from untreated cells (standard), cells treated with Na₂VO₄ (VO₄), and untreated cells disrupted in the presence of ATP and MgCl₂ (ATP-Mg). The data are presented as a ratio of activity between two enzymes and represent the average values obtained from several independent reactions employing several different preparations of the enzymes. The numbers within parentheses indicate the ranges of values obtained.

enzyme and ATP-Mg enzyme) compared with the standard enzyme (Fig. 6). Even the phosphorylation of purified anti-*src* IgG was higher with the variant enzyme preparations (Fig. 6, tracks 13 through 15), in contrast to the results from the immune complex protein kinase assay (data not shown). Taking into account the exact amount of pp60^{v-src} polypeptide present in the three enzyme preparations and the degree to which the various protein substrates were phosphorylated, we have compared the relative protein kinase specific activities of the VO₄ enzyme and the ATP-Mg enzyme to that of the standard enzyme (Table 1). Both enzyme preparations containing the modified pp60^{v-src} forms had dramatically higher kinase specific activities compared with that of the standard pp60^{v-src} enzyme preparation.

DISCUSSION

We report here the identification of previously unobserved variant forms of the pp60^{v-src} protein in transformed cell lysates. These new species of pp60^{v-src} exhibited a slightly reduced electrophoretic mobility in SDS-polyacrylamide gels compared with the normal form of pp60^{v-src}. This change in migration appeared to be the result of some alteration in the amino-terminal portion of the molecule and could be correlated with the appearance of extensive NH₂-terminal region tyrosine phosphorylation on the pp60^{v-src} polypeptide. These structural features of the variant pp60^{v-src} molecules found in the transformed cell lysates were very similar to those found when partially purified standard pp60^{v-src} was extensively autophosphorylated in vitro (9). The detection of these modified pp60^{v-src} polypeptides depended on the prior treatment of the transformed cell cultures with vanadium ions. Orthovanadate has previously been shown to inhibit alkaline phosphatase activities (30, 40, 44) and, specifically, phosphotyrosyl-protein phosphatase activities (26, 45). Vanadate may, however, exert other effects on cells (38). Thus, it may be the case that our previous inability to detect the high phosphotyrosine-containing species of pp60^{v-src} in transformed cells was a consequence of the rapid modification of these forms to the normally observed pp60^{v-src} by putative phosphotyrosyl protein phosphatases. Only when we inhibited these phosphatase activities with vanadium ions were we

able to observe the hyper-phosphorylated pp60^{v-src} forms. It has been reported that Zn²⁺ also inhibits phosphotyrosyl protein phosphatase activities (1, 17). We have not observed the variant forms of pp60^{v-src} in transformed cells treated under a variety of conditions with Zn²⁺. We found, however, that the inclusion of Mg²⁺ alone or ATP-Mg²⁺ in the cell disruption buffer did allow for the detection of the variant pp60^{v-src} polypeptides. Clearly the provision of high levels of ATP in the presence of Mg²⁺ would promote pp60^{v-src} autophosphorylation in the cell lysate, similar to what has been observed with the purified pp60^{v-src} enzyme (9, 36). The fact that the addition of Mg²⁺ alone to the lysis buffer was able to preserve some of the modified pp60^{v-src} molecules may imply that there is a sufficiently high level of ATP present in the cell lysate to allow for the maintenance of pp60^{v-src} autophosphorylation, or that Mg²⁺ itself inhibits the putative phosphatase activities.

Under all of the conditions we have examined, we have observed a discrepancy in the ratio of the new, upper polypeptide band to the normal pp60^{v-src} band between ³²P-labeled protein and [³⁵S]methionine-labeled protein. Under any given set of conditions in which the new *src*-specific polypeptides were detectable, there always appeared to be a higher ratio of the new upper forms to the normal form in the ³²P-labeled material than in the corresponding ³⁵S-labeled material. This might suggest that the new, highly phosphorylated variant forms represent only minor species of the total *src* protein present in cell lysate.

Recently, several reports have attempted to address the functional role of the phosphorylation of pp60^{v-src}. Through elegant genetic engineering approaches, Synder et al. (43) and Cross and Hanafusa (10) have concluded that the phosphorylation of tyrosine 416 on pp60^{v-src} is not essential for *src*-specific protein kinase activity or cellular transformation. Cross and Hanafusa have further suggested that phosphorylation of serine 17 is also nonessential for these functions of pp60^{v-src} (10). This latter conclusion is substantiated by earlier results (13, 27, 32, 47). However, these studies did not address the issue of the quantitative control of *src* enzyme activity by phosphorylation-dephosphorylation of these sites. On the other hand, Roth et al. (39) concluded from a quite different approach that cyclic AMP-stimulated phosphorylation of serine 17 served to increase the activity of pp60^{v-src} in transformed cells. Finally, from studies with the purified pp60^{v-src} enzyme in vitro, we have reported that extensive tyrosine phosphorylation of pp60^{v-src} could be correlated with a significant increase in its kinase specific activity (36). However, this extensive tyrosine phosphorylation, in addition to involving the *tyr-416* site, involved new residues located in the NH₂-terminal region of the pp60^{v-src} molecule (9). Thus we suggested that NH₂-terminal region tyrosine phosphorylation of pp60^{v-src} served to control (activate) *src* function (9, 36). The possible involvement of tyrosine 416 in the regulation of *src* activity, however, still remains an unresolved issue. Localization of the NH₂-terminal sites of tyrosine phosphorylation on the pp60^{v-src} molecule would allow for the molecular engineering of pp60^{v-src} mutant proteins with deleted or altered residues, as has been done at other phosphorylation sites on pp60^{v-src} (10, 43). This would further elucidate the role of NH₂-terminal region tyrosine phosphorylation in the regulation of pp60^{v-src} function.

In this report we have characterized variant pp60^{v-src} forms isolated from transformed cells that appear to be very closely analogous to the modified pp60^{v-src} we have generated in vitro (9, 36). Our functional evaluation of the variant

forms from transformed cells demonstrated that enzyme preparations containing these pp60^{v-src} species of high phosphotyrosine content were significantly more active than the commonly studied *src* protein kinase (Table 1). A more detailed comparison of the enzymatic characteristics of these kinase preparations will be presented elsewhere (submitted for publication).

All of our functional assays of pp60^{v-src} protein kinase activity have involved the analysis of the partially purified enzyme and have taken into account the absolute amount of pp60^{v-src} polypeptide present in the actual enzymatic reactions. This has allowed us to compare the relative specific activities of the various enzyme preparations. The above-cited studies from other laboratories have all employed the immune complex protein kinase assay to quantitate pp60^{v-src} activity. We have found in this work and have indicated in previous communications (36, 47) that this assay can be misleading. We feel that this assay is inappropriate for the quantitative estimation of pp60^{v-src} enzymatic activity, and that studies employing this assay must be reevaluated. For example, it would be interesting to analyze the kinase activities of the purified, genetically engineered mutant *src* polypeptides.

The results presented here and elsewhere (36) demonstrate that extensive phosphorylation of pp60^{v-src} at tyrosine residues can be correlated with a significant increase in the activity of the enzyme. Considering the results of others (10, 43), it appears that NH₂-terminal region tyrosine phosphorylation on pp60^{v-src} is most important in this correlation. Since this tyrosine phosphorylation of pp60^{v-src} appears to be the result of autophosphorylation (9, 36), the real controlling factor of the *src* kinase activity may lie in the dephosphorylation of these NH₂-terminal phosphotyrosine residues. The identification and characterization of such a putative pp60^{v-src}-specific phosphotyrosyl phosphatase would greatly advance our understanding of the functional regulation of this and other transforming proteins.

The procedures we have employed here (cell culture in the presence of vanadium ions, cell lysis in the presence of ATP-Mg²⁺) have allowed us to detect structurally altered and functionally more active forms of the RSV *src* protein in transformed cells. This suggests that within transformed cells there may be transient, rapidly modified molecules of pp60^{v-src} that are highly active on cellular proteins, possibly in appropriate subcellular environments. There may still be other forms of pp60^{v-src} that have escaped our detection with the methodologies used here. Furthermore, studies involving the phosphorylation of cellular proteins in RSV-transformed cells have not taken into account the possibility of rapid modifications (dephosphorylation) of putative substrates or targets of the pp60^{v-src} kinase activity. The methods employed here may be helpful in future studies aimed at identifying transient phosphorylations that may be the result of transforming proteins and also other cell growth regulators.

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