Phosphorylation of the Transforming Protein of Rous Sarcoma Virus: Direct Demonstration of Phosphorylation of Serine 17 and Identification of an Additional Site of Tyrosine Phosphorylation in p60^{v-src} of Prague Rous Sarcoma Virus

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We provide direct evidence that serine 17 is the major site of serine phosphorylation in p60^{v-src}, the transforming protein of Rous sarcoma virus, and in its cellular homolog, p60^{c-src}. The amino acid composition of the tryptic peptide containing the major site of serine phosphorylation in p60^{v-src} was deduced by peptide map analysis of the protein labeled biosynthetically with a variety of radioactive amino acids. Manual Edman degradation revealed that the phosphorylated serine in this peptide was the amino terminal residue. These data are consistent only with the phosphorylation of serine 17. The major site of serine phosphorylation in chicken p60^{c-src}, the cellular homolog of p60^{v-src}, is contained in a tryptic peptide identical to that containing serine 17 in p60^{v-src} of Schmidt Rupp Rous sarcoma virus of subgroup A. Serine 17 is therefore also phosphorylated in p60^{c-src}. The p60^{v-src} protein encoded by Prague Rous sarcoma virus was found to contain two sites of tyrosine phosphorylation. The previously unrecognized site of tyrosine phosphorylation may be tyrosine 205 or possibly tyrosine 208. Treatment of Prague Rous sarcoma virus-infected cells with vanadyl ions stimulated the protein kinase activity of p60^{v-src} and increased the phosphorylation of tyrosine 416 but not the phosphorylation of the additional site of tyrosine phosphorylation.

The transforming protein of Rous sarcoma virus (RSV), p60^{v-src}, is a protein kinase which phosphorylates substrates on tyrosine (8, 16, 18). This enzymatic activity of p60^{v-src} appears to be necessary, and may in fact be sufficient, for both the initiation and maintenance of cellular transformation by this virus (29). p60^{v-src} is itself a phosphoprotein (7, 18) and is the substrate of at least three different protein kinases in transformed cells. The cyclic AMP-dependent protein kinase phosphorylates p60^{v-src} at a serine located somewhere near the amino terminus (7): protein kinase C will phosphorylate the protein at serine 12 and serine 48 (15, 23), and the protein undergoes apparent autophosphorylation at tyrosine 416 (16, 20, 22, 32).

It is presumed that the phosphorylation of cellular proteins by p60^{v-src} affects their activity or function. An important question is whether the activity of p60^{v-src} is also regulated by phosphorylation. The most persuasive evidence that this might occur are the recent observations of Collett et al. (6) and Brown and Gordon (4), who found that p60^{v-src} isolated from cells treated with vanadyl ions is both phosphorylated to an increased extent on tyrosine at an unknown site(s) in its amino-terminal domain and is measurably more active as a protein kinase when assayed in vitro.

The phosphorylation of tyrosine 416 is also of apparent importance. Site-directed mutations which either delete or replace tyrosine 416 have no effect on the activity of p60^{v-src} as a protein kinase in vitro or on the ability of the virus to transform cells in culture (13, 34). They do however reduce tumorigenicity. A virus encoding p60^{v-src} lacking residues 415, 416, and 417 induces tumors in birds more slowly than

does the wild-type virus (13). Additionally, mouse cells transformed in vitro with viral DNA encoding a p60^{v-src} with phenylalanine at position 416 induce tumors only in immunodeficient mice (33).

There is conflicting data as to whether the phosphorylation of the protein by the cyclic AMP-dependent protein kinase can increase its activity. Small deletions in the 5' end of the *src* gene prevent phosphorylation of the serine which is the substrate of the cyclic AMP-dependent protein kinase. These have, however, no measurable effect on the activity of p60^{v-src} as a protein kinase in vitro or as a transforming protein in cultured cells (13). Roth et al. (25) have, however, reported that treatment of RSV-transformed CHO cells with agents which should stimulate the cyclic AMP-dependent protein kinase and hence the phosphorylation of p60^{v-src} on serine increase the activity of p60^{v-src} as a protein kinase when assayed in vitro. This suggests that in some cells the phosphorylation of p60^{v-src} on serine by the cyclic AMP-dependent protein kinase may stimulate its activity.

Although protein kinase C will phosphorylate both p60^{v-src} and its cellular homolog p60^{c-src} at serine 12 and serine 48, no change in the protein kinase activity of the proteins assayed in vitro has been detected (15, 23). The importance of this modification therefore remains unclear.

Finally, there is recent evidence that the activity of p60^{c-src} is inhibited by phosphorylation of the protein (12), probably at tyrosine 527 (11). Although p60^{v-src} does not contain tyrosine 527, the fact that the activity of p60^{c-src} may be regulated by phosphorylation strengthens the notion that p60^{v-src} could be subject to similar regulation.

There is indirect evidence that serine 17 is the major site of serine phosphorylation in p60^{v-src} and is phosphorylated by the cyclic AMP-dependent protein kinase (13). To demonstrate this directly, we took the same approach we used to identify tyrosine 416 as a site of phosphorylation in p60^{v-src}

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(20). We determined a partial amino acid composition of the tryptic peptide containing the phosphorylated serine and compared it with the predicted amino acid composition of the potential serine-containing tryptic peptides in the amino-terminal portion of the protein. We have been able to show conclusively that serine 17 is phosphorylated in the p60^{src} protein of Schmidt-Ruppin (SR) RSV of subgroup A and have obtained indirect evidence that this is also true of the p60^{src} proteins encoded by the other strains of RSV and of p60^{c-src}.

An analysis of the p60^{v-src} proteins encoded by several strains of RSV and by B77 virus by two-dimensional analysis of methionine-containing tryptic peptides revealed considerable differences (2). These apparent differences among the several viral p60^{v-src} proteins were in fact much more pronounced than are the differences between the p60^{c-src} proteins of chickens and humans (28). We wished to know whether this somewhat unexpected polymorphism of these viral proteins was also apparent when phosphorylated tryptic peptides were examined. We therefore extended our earlier studies to an analysis of the phosphorylation sites of the p60^{v-src} proteins of seven different strains of RSV and of cellular p60^{c-src} by two-dimensional tryptic peptide mapping. We found that the p60^{v-src} proteins of the several strains of Prague (PR) RSV contained a novel site of tyrosine phosphorylation. Because of the recent suggestions that tyrosine phosphorylation can stimulate the protein kinase activity of p60^{v-src}, we characterized this site in some detail.

MATERIALS AND METHODS

Cells and viruses. Primary chicken embryo fibroblasts were prepared from embryonated eggs (SPAFAS, Inc., Norwich, Conn.) as described before (24). The SR strain of RSV, subgroup D (SR-D), and the PR strain of RSV, subgroup B (PR-B), were obtained originally from P.K. Vogt (University of Southern California). The SR strain of RSV, subgroup A (SR-A), originated in the laboratory of H. Hanafusa (The Rockefeller University). Three stocks of the PR strain of RSV, subgroup C (PR-C), were used. PR-C (stock 1) was obtained from M. Stone (Duke University). PR-C (stock 2) was obtained through the Viral Oncology Program of the National Cancer Institute. PR-C (clone 4) was a gift from R. Guntaka (University of Missouri). This virus stock is identical to that used for the preparation of the recombinant plasmid, pATV-8, from which the nucleotide sequence of the PR-C genome was determined (26). The B77 virus-transformed rat cell line NB77 was obtained from E. Nigg (University of California, San Diego). Primary or secondary cultures of chicken fibroblasts were infected with these viruses as described (27, 30) and maintained at 41°C.

Radioactive labeling of cells. Transformed chicken cells in 100-mm petri dishes were labeled with ³²P_i for 18 h at 41°C as described previously (16, 21). B77 virus-transformed NRK cells were labeled with ³²P_i for 18 h at 37°C. Cells were labeled with [³H]histidine (1 mCi/ml; 51.3 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 5 ml of Dulbecco minimal essential medium containing 5% of the normal concentration of histidine and 4% dialyzed calf serum for 18 h at 41°C. Labeling with [³H]proline (1 mCi/ml; 28 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was carried out in 5 ml of complete Dulbecco minimal essential medium supplemented with 4% dialyzed calf serum for 18 h at 41°C. Labeling of the cells with other radioactive amino acids has been described previously (20).

To prepare ³²P-labeled p60^{c-src}, secondary chicken cells were seeded at 6 × 10⁶ cells per 100-mm dish and labeled 24

h later with ³²P_i at a concentration of 2 mCi/ml for 18 h at 38°C. The labeling medium was identical to that used for the transformed cells.

Cell lysis and immunoprecipitation. Except in the case of cells treated with vanadyl ions, labeled cells were lysed with phosphate-buffered RIPA buffer supplemented with 2 mM EDTA, and immunoprecipitation was carried out with serum from rabbits bearing SR-D-induced tumors as described previously (21, 27).

Treatment of cells with vanadyl ions. To prepare a stock solution of sodium orthovanadate, Na₃VO₄ (Fisher Scientific Co., Pittsburgh, Pa.) was dissolved in H₂O at a concentration of 10 mM and sterilized by filtration. The pH of the solution was greater than 10 and was not adjusted. The solution was stored at room temperature in a plastic vial. Chicken cells transformed with PR-C (stock 1) were incubated with 50 μM Na₃VO₄ for 24 h at 41°C. To measure the effect of this treatment on the protein kinase activity of p60^{v-src}, unlabeled cells were lysed in 1% Nonidet P-40–0.5% sodium deoxycholate–50 mM Tris hydrochloride (pH 7.2 at 4°C)–2 mM EDTA (6), and p60^{v-src} was isolated by immunoprecipitation with an anti-peptide antiserum specific for the carboxy terminus of p60^{v-src} (31). Immunoprecipitation was with antigen in excess so that the specific activity of the protein could be assessed. Protein kinase activity was assayed with [Val⁵]angiotensin II as an exogenous substrate (38). The immunoprecipitated protein was incubated for 5 min at 30°C in 8 μl of 10 mM sodium phosphate (pH 7.2) which contained 2 mM [Val⁵]angiotensin II, 1 μM [γ-³²P]ATP (625 Ci/mmol; Amersham), and 5 mM MgCl₂. Phosphorylated angiotensin was identified by electrophoresis on cellulose thin-layer plates at pH 3.5 for 27 min at 1,500 V. The cellulose containing the phosphorylated angiotensin was scraped off the plate, and incorporation was quantified by scintillation counting using diphenyloxazole dissolved in toluene as the scintillation fluid.

Sister cultures were labeled for 15 h at 41°C with ³²P_i at a concentration of 1 mCi/ml in the presence or absence of 50 μM Na₃VO₄. The cells were lysed in the same buffer used for the protein kinase assays, and the protein was isolated by immunoprecipitation with rabbit anti-RSV tumor serum.

Gel electrophoresis, polypeptide elution, and tryptic digestion. Immunoprecipitated proteins were purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described before (21, 27). The proteins were located by autoradiography and eluted either electrophoretically (21, 37), or from mechanically homogenized gels (1).

After addition of 5 to 10 μg of carrier protein, the proteins were precipitated by trichloroacetic acid, oxidized with performic acid, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Diagnostics, Freehold, N.J.) as described in detail elsewhere (1). In some experiments, the time of performic acid oxidation was reduced to 1 h, and the proteins were digested with 30 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin for 4 h at 37°C and then for an additional 4 h at 37°C with 20 μg of fresh trypsin. This procedure yielded results indistinguishable from those obtained with the previously described protocol. To ensure that the additional peptides in the maps of the p60^{v-src} proteins of the PR-RSV viruses did not arise from partial digestion, the proteins were redigested with 4 μg of trypsin for 2 h at 37°C.

Two-dimensional peptide analysis, *Staphylococcus aureus* V8 protease digestion, and phosphoamino acid analysis. Two-dimensional analysis of phosphorylated tryptic peptides was performed by electrophoresis at pH 8.9 and by ascending

FIG. 1. Amino acid composition of the phosphoserine-containing tryptic peptide α of SR-A p60^{v-src}. Tryptic digests of p60^{v-src} labeled biosynthetically either with $^{32}\text{P}_i$ or individual radioactive amino acids were prepared as described in the text and analyzed by electrophoresis at pH 8.9 in the first dimension followed by ascending chromatography. The origin in the maps shown here and in all figures was approximately in the center, at the bottom of the plate. The anode was at the left. The radioactive label, the amount of radioactivity loaded, and the exposure time were as follows: (A) $^{32}\text{P}_i$, 260 cpm, 4 days; (B) [^3H]tryptophan, 8,100 cpm, 46 days; (C) $^{32}\text{P}_i$, 260 cpm, and [^3H]proline, 9,800 cpm, 21 days; (D) [^3H]proline, 9,800 cpm, 25 days; (E) $^{32}\text{P}_i$, 170 cpm, and [^3H]histidine, 1,700 cpm, 86 days; (F) [^3H]histidine, 1,700 cpm, 62 days. Except for A, only fluorographs of the maps are shown. In panels A, C, and E, the $^{32}\text{P}_i$ -labeled tryptic peptides are each labeled with both α and β . The $^{32}\text{P}_i$ -labeled peptides can still be detected in the maps containing mixtures of $^{32}\text{P}_i$ - and ^3H -amino acid-labeled peptides (C and E). The positions of peptides α and β in maps containing only ^3H -amino acid-labeled peptides (B, D, and F) are indicated by arrowheads and arrows, respectively.

chromatography in modified chromatography buffer as described before (16). Digestion of whole tryptic digests or purified peptides with *S. aureus* V8 protease under conditions where only glutamoyl bonds are cleaved have been described (20, 21). Partial acid hydrolysis of purified peptides and two-dimensional analysis of phosphoamino acids have been described (16, 21). For phosphoamino acid anal-

ysis, the phosphotyrosine-containing peptide of p60^{c-src} was eluted from the cellulose with a buffer consisting of *n*-butanol-pyridine-glacial acetic acid-water (75:50:15:60 [vol/vol]) so as to improve yields. ^{32}P -labeled peptides and phosphoamino acids were detected by preflashed film and an intensifying screen. ^3H -amino acid-labeled peptides were visualized by fluorography (3, 20).

FIG. 2. Phosphate is released in the first cycle of Edman degradation of phosphoserine-containing peptide α . The phosphorylated tryptic peptides of p60^{V-src} of SR-A were separated by electrophoresis at pH 8.9 on cellulose thin-layer plates. Peptide α was eluted and subjected to three cycles of manual Edman degradation as described in the text. A portion of the total sample remaining after steps 1 and 2 was then analyzed by electrophoresis at pH 8.9 on a cellulose thin layer plate. Exposure was for 21 h with a fluorescent screen. The origin is indicated with arrowheads. The anode was at the left. Numbers: 0, starting material; 1, peptide and its cleavage products after one cycle; 2, after two cycles.

Edman degradation. The eluted peptide was dissolved in 20 μ l of H₂O, mixed with 20 μ l of 5% phenylisothiocyanate in pyridine, and incubated for 30 min at 45°C. The reaction mixture was then extracted twice with 200 μ l of heptane-ethyl acetate (10:1 [vol/vol]), the organic phase being discarded each time. Trifluoroacetic acid (50 μ l) was then added to the aqueous phase, and the mixture was incubated for 10 min at 45°C. The sample was dried and suspended in 15 μ l of H₂O, and 5 μ l was removed for analysis of the products of step 1 by thin-layer electrophoresis. The remainder of the sample was diluted to 20 μ l with H₂O, and two more cycles of degradation were performed as described above. The samples were analyzed by electrophoresis at pH 8.9 on cellulose thin-layer plates.

RESULTS

Identification of the major phosphorylated serine in p60^{V-src} of SR-A. Tryptic digestion produces two major phosphorylated peptides from p60^{V-src}. The peptide that we have termed α contains only phosphoserine, and peptide β contains only phosphotyrosine (16). Peptide α corresponds to the peptide designated 1 by Gould et al. (15), and β corresponds to the peptide they designated 2. To identify rigorously the site of serine phosphorylation giving rise to peptide α , a partial amino acid composition of the peptide was determined and compared with the compositions of the predicted tryptic peptides of the protein.

To do this, we prepared two-dimensional tryptic peptide maps of p60^{V-src} of SR-A labeled biosynthetically with ³²P_i or with one of the following amino acids: [³⁵S]cysteine, [³H]glycine, [³H]histidine, [³H]isoleucine, [³⁵S]methionine, [³H]proline, [³H]tryptophan, or [³H]tyrosine. The amino acid composition of the phosphoserine-containing tryptic peptide α was then determined by identification of peptides labeled with the specific amino acids that comigrated with the ³²P_i-labeled peptide α by analysis of appropriate mixtures. This approach was facilitated by the fact that this site is phosphorylated to an extent of at least 0.6 mol of phosphate per mol of p60^{V-src} (30). Some of these results were published previously (20). Peptide α was labeled with [³H]proline (Fig. 1C and D), [³H]histidine (Fig. 1E and F), and with [³H]glycine (20). It was not labeled detectably with

[³H]tryptophan (Fig. 1B), [³⁵S]cysteine, [³H]isoleucine, or [³⁵S]methionine (20), or [³H]tyrosine (not shown).

p60^{V-src} of SR-A should yield 22 serine-containing tryptic peptides (14, 35). An inspection of the amino acid sequences of these tryptic peptides revealed with only one peptide would contain serine, histidine, and proline, and lack cysteine, isoleucine, methionine, tryptophan, and tyrosine. This peptide is composed of amino acids 17 to 37.

There are three serine residues present in this peptide, at positions 17, 23, and 32. Manual amino terminal sequence analysis of ³²P-labeled peptide α demonstrated that all of the label was lost from the peptide and released as ³²P_i in the first cycle of degradation (Fig. 2). Since phosphoserine in a peptide is stable under these conditions (39), this demonstrated that serine 17 was the single site of phosphorylation in this peptide.

Comparison of phosphorylation sites in the different viral p60^{V-src} proteins. We have analyzed here two-dimensional maps of phosphorylated tryptic peptides of p60^{V-src} encoded by seven different RSVs. Tryptic digests were prepared from proteins isolated from ³²P_i-labeled cells by immunoprecipitation. In most cases the nature of the phosphorylated amino acid in a given peptide was determined by phosphoamino acid analysis (data not shown). (In each of the figures, the major phosphoserine-containing peptide is labeled α and the tryptic peptide containing tyrosine 416 is labeled β .)

The p60^{V-src} proteins encoded by SR-D, B77, and SR-A were found to yield only two major phosphorylated tryptic peptides (Fig. 3). Analysis of mixtures showed that the phosphotyrosine-containing β peptides were identical (Fig. 3B and E). In contrast, the phosphoserine-containing α_2 peptide of p60^{V-src} of SR-A was different from the α_1 peptide of the p60^{V-src} of both SR-D and B77 (Fig. 3B and E). Inspection of the maps of p60^{V-src} of SR-D (Fig. 3A) and B77 (Fig. 3D) suggested that the α_1 peptides in these two proteins were the same.

PR-B p60^{V-src} (Fig. 4A) yielded two major phosphorylated tryptic peptides, α_3 and β , and a third minor peptide, termed here γ , which contained only phosphotyrosine. This novel peptide was not a partial digestion product containing peptide β , since it, unlike β , was resistant to digestion with *S. aureus* protease (data not shown). The β peptide of the p60^{V-src} protein of PR-B was identical to that of the p60^{V-src} protein of SR-A (Fig. 4B). The phosphoserine-containing α peptides were different.

Maps of phosphorylated tryptic peptides of p60^{V-src} proteins encoded by three different stocks of PR-C (Fig. 4C through F) were also more complex than those of SR-RSV. Redigestion with trypsin did not simplify the maps. p60^{V-src} of PR-C (stock 1) contained three major phosphorylated peptides, designated α_3 , β , and δ (Fig. 4C). Peptide δ , which was more abundant than peptide β , contained only phosphotyrosine. This peptide was different from the novel phosphotyrosine-containing γ peptide of p60^{V-src} of PR-B (Fig. 4A). Like peptide γ , peptide δ was insensitive to secondary digestion with *S. aureus* protease (data not shown).

The maps of the p60^{V-src} proteins of PR-C (stock 2) (Fig. 4D) and PR-C clone 4 (Fig. 4E) both contained minor peptides which appeared to be identical to the phosphotyrosine-containing γ peptides of the p60^{V-src} proteins of PR-B (Fig. 4A) and PR-C (stock 2) (Fig. 4D). The presence of three phosphotyrosine-containing tryptic peptides in the map of PR-C (stock 2) probably reflects sequence heterogeneity in the p60^{V-src} proteins encoded by this uncloned stock of virus. Virus recovered from cloned viral DNA obtained from

FIG. 3. Phosphorylated tryptic peptides of $p60^{v\text{-src}}$ encoded by SR-D, SR-A and B77. Tryptic digests of the $p60^{v\text{-src}}$ proteins labeled biosynthetically with $^{32}\text{P}_i$ were prepared and analyzed as described in the text and the legend to Fig. 1. In B and E, α_1 is the peptide derived from the $p60^{v\text{-src}}$ of SR-D and B77, and α_2 is the peptide derived from the $p60^{v\text{-src}}$ of SR-A. The source of the digest, the amount of radioactivity loaded, and the exposure time were as follows: (A) SR-D, 570 cpm, 48 h; (B) SR-D, 300 cpm, and SR-A, 280 cpm, 48 h; (C) SR-A, 560 cpm, 48 h; (D) B77, 720 cpm, 18h; (E) B77 800 cpm, and SR-A, 740 cpm, 18 h.

this stock of virus encodes a $p60^{src}$ containing only peptide δ (5). None of the PR-RSV $p60^{v\text{-src}}$ proteins contained the same phosphoserine-containing α tryptic peptide as the SR-A protein. Analysis of mixtures of peptides (Figure 4B and F) suggested that the PR-RSV $p60^{v\text{-src}}$ proteins yielded three different phosphoserine-containing peptides, α_3 , α_4 , and α_5 . In contrast, the phosphotyrosine-containing β peptide was ubiquitous.

Analysis of the phosphorylation sites in $p60^{c\text{-src}}$. $p60^{c\text{-src}}$ is also phosphorylated on both serine and tyrosine (9). We wished to determine whether the phosphoserine-containing

tryptic peptide of $p60^{c\text{-src}}$ was similar to any of the five different phosphoserine-containing peptides found in the viral $p60^{v\text{-src}}$ proteins. Analysis of a mixture of phosphorylated tryptic peptides of $p60^{c\text{-src}}$ from chicken cells and the $p60^{v\text{-src}}$ protein of SR-A showed that the major phosphoserine-containing peptides were identical, and the major phosphotyrosine-containing peptides, labeled β_v and β_c , were different (Fig. 5). $p60^{c\text{-src}}$ yielded a low level of a peptide apparently identical to the peptide containing phosphorylated tyrosine 416 from $p60^{v\text{-src}}$.

Effect of treatment of infected cells with vanadyl ions on the

FIG. 4. Phosphorylated tryptic peptides of p60^{v-src} encoded by different stocks of PR-RSV. Tryptic digests of the p60^{v-src} proteins were prepared and analyzed as described in the legend to Fig. 1. In B and F, α₂ is the peptide derived from the p60^{v-src} of SR-A, α₃ is the peptide derived from the p60^{v-src} of PR-B, α₄ is the peptide derived from the p60^{v-src} of PR-C clone 4, and α₅ is the peptide derived from the p60^{v-src} of PR-C (stock 1). The source of the digest, the amount of radioactivity loaded, and the exposure time were as follows: (A) PR-B, 1,150 cpm, 18 h; (B) PR-B, 1,150 cpm, and SR-A, 1,100 cpm, 18 h; (C) PR-C (stock 1), 500 cpm, 3 days; (D) PR-C (stock 2), 400 cpm, 3 days; (E) PR-C (clone 4), 400 cpm, 3 days; (F) PR-C (stock 1), 500 cpm, and PR-C (clone 4), 400 cpm, 3 days.

phosphorylation of p60^{v-src}. Collett et al. (6) and Brown and Gordon (4) have shown that treatment of RSV-transformed cells with vanadyl ions leads to greatly increased phosphorylation of one or more tyrosine residues within the amino-terminal 16 to 20 kilodaltons (kDa) of the polypeptide. To examine whether the site which is phosphorylated to an increased extent in the presence of vanadyl ions corresponds to the additional site of tyrosine phosphorylation apparent in the p60^{v-src} protein of PR-C, we examined the effect of vanadyl ions on the phosphorylation of p60^{v-src} of PR-C (stock 1).

Treatment of PR-C-transformed chicken cells for 18 h with

50 μM Na₃VO₄ increased the kinase activity of p60^{v-src} 2.6-fold, as assayed *in vitro* with [Val⁵]angiotensin II as a substrate (data not shown). In addition, it increased the ratio of phosphotyrosine to phosphoserine in the protein 1.7-fold (data not shown). Peptide mapping revealed significantly increased labeling of peptide β, containing tyrosine 416, relative to the labeling of peptide α (Fig. 6). Although partial proteolysis and phosphoamino acid analysis detected increased tyrosine phosphate in the amino-terminal half of the protein (data not shown), no increased labeling of peptide δ relative to the labeling of peptide α was apparent (Fig. 6).

Localization of the additional site of tyrosine phosphoryla-

tion in PR-RSV p60^{v-src}. To localize the site of tyrosine phosphorylation giving rise to peptide δ in p60^{v-src} of PR-C (stock 1), the protein was digested partially with *S. aureus* V8 protease, and the fragments produced were subjected to two-dimensional tryptic peptide mapping. Peptide δ was present in the 34-kDaV1 fragment, which contains the amino terminus (7) of the protein (Fig. 6). It was absent, however, from the 18-kDaV3 fragment, which also contains the amino

terminus of the protein (Fig. 6). This localizes this site of phosphorylation to the region of the protein between the two sites of proteolysis which give rise to the 18- and 34-kDa fragments.

DISCUSSION

Sites of serine phosphorylation. We have demonstrated directly that serine 17 is the major site of serine phosphorylation in p60^{v-src} of SR-A. Collett et al. (7) have shown that the phosphorylation of the tryptic peptide containing serine 17 is stimulated by treatment of cells with cyclic AMP. This suggests that the cyclic AMP-dependent protein kinase phosphorylates serine 17. Like many amino acid substrates of the cyclic AMP-dependent protein kinase (17), this residue is flanked on its amino-terminal side by multiple arginine residues (NH₂-Arg-Arg-Arg-Ser-Leu-Glu-COOH). The phosphoserine-containing tryptic peptide from chicken p60^{c-src} is identical to that from the p60^{v-src} protein of SR-A. In addition, the two proteins are identical in sequence in the region flanking serine 17 (36). It is almost certain therefore that serine 17 is also the major site of serine phosphorylation in this cellular tyrosine protein kinase.

There is noticeable polymorphism in the phosphoserine-containing tryptic peptides of p60^{v-src}. The p60^{v-src} proteins of seven stocks of RSV yielded five different major phosphoserine-containing tryptic peptides. We suspect that each of these peptides contains serine 17, and that the apparent polymorphism arises from differences in the residues flanking this site, rather than from the phosphorylation of fundamentally different serine residues in the several p60^{v-src} proteins. Because of the minor differences in the sequences of the two proteins (14, 26, 35), the sites of cleavage giving rise to the tryptic peptide containing serine 17 in the p60^{v-src} protein of PR-C (clone 4) yield a peptide containing nine more amino acids than that from p60^{v-src} of SR-A. Similarly, a single asparagine-to-aspartic-acid substitution in the p60^{v-src} protein of B77 makes the tryptic peptide containing serine 17 noticeably more acidic than that derived from the p60^{v-src} of SR-A (19). The variability in the properties of the tryptic peptides containing serine 17 suggests that the sequences of the other p60^{v-src} proteins will also be found to differ in this region.

Sites of tyrosine phosphorylation. A phosphorylated peptide known to contain tyrosine 416 was present in the maps of all the viral proteins. It is clear, therefore, that this site is phosphorylated in all the viral p60^{v-src} proteins, and that none of the viral proteins differ in sequence within the 10 residues (410 to 419) constituting this tryptic peptide. This sequence is present in p60^{c-src} but is phosphorylated only to a very limited extent.

Polymorphism was also observed in the sites of tyrosine phosphorylation. The p60^{v-src} proteins encoded by PR-C contained one other major site of tyrosine phosphorylation not apparent in the maps of p60^{v-src} of SR-RSV or B77 virus, or p60^{c-src} of chicken cells. In some experiments, the extent of phosphorylation of this site was greater than that of tyrosine 416 (Fig. 4 and 6). Tryptic peptide mapping of fragments of p60^{v-src} showed the additional site of tyrosine phosphorylation to be located approximately between residues 160 and 310. Additionally, secondary digestion with *S. aureus* protease of the peptide containing this site showed it lacked a glutamic acid residue. p60^{v-src} of PR-C contains two tyrosines at positions 205 and 208, which are both absent from the p60^{v-src} of SR-A and chicken p60^{c-src} and present in tryptic peptides lacking glutamic acid. We suggest that

FIG. 5. Comparison of the phosphorylated tryptic peptides of p60^{c-src} from chicken cells and p60^{v-src} of SR-A. Tryptic digests of the biosynthetically labeled proteins were prepared and analyzed as described in the text and the legend to Fig. 1. β_c is the peptide derived from p60^{c-src}, and β_v is the peptide derived from p60^{v-src}. The source of the digest, the amount of radioactivity loaded, and the exposure time were as follows: (A) p60^{c-src}, 440 cpm, 12 days; (B) p60^{v-src} (SR-A), 350 cpm, and p60^{c-src}, 440 cpm, 5 days; (C) p60^{v-src} (SR-A), 350 cpm, 5 days.

FIG. 6. Analysis of the effect of vanadate on the phosphorylation of peptide δ and localization of peptide δ in $p60^{v\text{-src}}$. $p60^{v\text{-src}}$ of PR-C (stock 1) was isolated from chicken cells which had been simultaneously treated with vanadyl ions and labeled biosynthetically with $^{32}\text{P}_i$. The preparation and analysis of the phosphorylated tryptic peptides was as described in the text and the legend to Fig. 1. To localize the site of phosphorylation in peptide δ , the $p60^{v\text{-src}}$ protein of PR-C (stock 1), labeled biosynthetically with $^{32}\text{P}_i$, was digested partially with *S. aureus* protease, and the resulting 34 and 18 kDa fragments were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Elution of the fragments, digestion with trypsin, and analysis of the peptides were as described in the text and the legend to Fig. 1. Exposure was for 24 to 48 h with a fluorescent screen. Abbreviations: CON, total $p60^{v\text{-src}}$ from untreated cells; VAN, total $p60^{v\text{-src}}$ from cells treated with vanadate; N34, 34-kDa amino-terminal fragment of $p60^{v\text{-src}}$; N18, 18-kDa amino-terminal fragment of $p60^{v\text{-src}}$.

tyrosine 205, or possibly tyrosine 208, may be the additional site of tyrosine phosphorylation in the $p60^{v\text{-src}}$ of PR-C. It is noteworthy that neither of these sites is located in the vicinity of acidic residues. Acidic residues are often, but not always, found to flank sites of tyrosine phosphorylation (10, 20, 32).

The fact that treatment of RSV-transformed cells with vanadyl ions leads to both an increase in the tyrosine protein kinase activity of $p60^{v\text{-src}}$ and an increase in the amount of the phosphotyrosine in the amino-terminal domain of the protein has led to the idea that the phosphorylation of the protein at one or more tyrosine residues in this domain stimulates its activity. We therefore asked whether treatment of cells with sodium orthovanadate led to increased labeling of the phosphorylated tyrosine located in the amino-terminal half of $p60^{v\text{-src}}$ of PR-C. It did not. We did observe both increased tyrosine protein kinase activity in the protein and increased tyrosine phosphorylation of $p60^{v\text{-src}}$, both at tyrosine 416 and in the amino-terminal half of the molecule, in the presence of orthovanadate. We found however no

increase in the phosphorylation of the PR-RSV-specific site of tyrosine phosphorylation. Additionally, no new phosphorylated tryptic peptide was found. The sites of tyrosine phosphorylation which potentially activate the enzyme therefore remain elusive.

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