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Platelet-Derived Growth Factor Induces Multisite Phosphorylation of pp60$^{c\text{-}src}$ and Increases Its Protein-Tyrosine Kinase Activity

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We have shown previously that pp60$^{c\text{-}src}$ is a substrate for protein kinase C in vitro and in vivo and that the target of protein kinase C phosphorylation in mammalian pp60$^{c\text{-}src}$ is serine 12. We now demonstrate that in addition to tumor promoters, all activators of phosphatidylinositol turnover that we have tested in fibroblasts (platelet-derived growth factor, fibroblast growth factor, serum, vasopressin, sodium orthovanadate, and prostaglandin $F_{2\alpha}$) lead to the phosphorylation of pp60$^{c\text{-}src}$ at serine 12. In addition to stimulating serine 12 phosphorylation in pp60$^{c\text{-}src}$, platelet-derived growth factor treatment of quiescent fibroblasts induces phosphorylation of one or two additional serine residues and one tyrosine residue within the N-terminal 16 kilodaltons of the enzyme and activates its immune complex protein-tyrosine kinase activity.

pp60$^{c\text{-}src}$ is a membrane-associated phosphoprotein with intrinsic protein-tyrosine kinase activity (for a review, see reference 32). It can be activated to transform cells by C-terminal truncation or various point mutations (6, 35, 36, 40, 49, 56). pp60$^{c\text{-}src}$ is expressed ubiquitously in animal cells, in which its transforming potential is normally suppressed and its specific tyrosine protein kinase activity is lower than that of its transforming counterparts (24, 34, 45). The highest levels of pp60$^{c\text{-}src}$ have been found in the brain (21), platelets (28), peripheral blood lymphocytes (28), and adrenal medullary chromaffin cells (46). Although this distribution has suggested that pp60$^{c\text{-}src}$ functions in development, maintenance of a differentiated state, or secretion, its role in normal cellular physiology and its mode of regulation are not understood.

We are interested in the role of phosphorylation in the regulation of pp60$^{c\text{-}src}$ function. Under most conditions, serine 17 (S17) is the major site of serine phosphorylation in pp60$^{c\text{-}src}$ (48, 61). pp60$^{c\text{-}src}$ is also phosphorylated in cells to near stoichiometry on a tyrosine near its C terminus, tyrosine 527 (Y527) in chicken pp60$^{c\text{-}src}$ (16). Phosphorylation of Y527 constrains the protein kinase activity of the molecule (18, 22), and deletion or mutation of this residue results in a protein with transforming ability and higher protein kinase activity (6, 36, 49, 56). Another tyrosine residue within pp60$^{c\text{-}src}$, tyrosine 416 (Y416), is the major site of autophosphorylation in vitro (47, 61). In vivo, Y416 is phosphorylated when Y527 has been mutated (as in Y527 to phenylalanine 527) or deleted (as in pp60$^{v\text{-}src}$) (6, 36, 49, 56). If it is assumed that pp60$^{c\text{-}src}$ is active as a protein kinase under certain circumstances in normal cells, a mechanism(s) must exist for activating and subsequently inhibiting its activity. Such regulation could be achieved by the transient dephosphorylation of Y527, but to date, there have been no reports that this occurs in nontransformed cells.

We (29) and others (26, 62) have shown that pp60$^{c\text{-}src}$ is phosphorylated rapidly and stoichiometrically at a normally unphosphorylated serine(s) when cells are treated with tumor promoters that bind and activate protein kinase C. The same sites, serine 12 (S12) in mammalian pp60$^{c\text{-}src}$ and both S12 and serine 48 in chicken pp60$^{c\text{-}src}$, can be phosphorylated by purified protein kinase C in vitro (29). In this report, we demonstrate that all agonists of phosphatidylinositol turnover, which we have tested in fibroblasts, lead to the near stoichiometric phosphorylation at S12. With one exception, however, these agents do not alter the immune-complex protein kinase activity of the protein. The exception we have found is platelet-derived growth factor (PDGF). In addition to inducing protein kinase C phosphorylation of pp60$^{c\text{-}src}$, PDGF stimulates other phosphorylation events within the N-terminal region of pp60$^{c\text{-}src}$ on both serine and, as previously reported by Ralson and Bishop (53), tyrosine residues. The multiple phosphorylation events induced by PDGF are accompanied by a two- to threefold increase in pp60$^{c\text{-}src}$ immune-complex protein kinase activity, which suggests that (i) pp60$^{c\text{-}src}$ might be regulated by N-terminal phosphorylation and (ii) pp60$^{c\text{-}src}$ might be involved in mediating the cellular response of fibroblasts to PDGF.

MATERIALS AND METHODS

Cells. NIH 3T3 cells, NR6 3T3 cells (50), and A431 cells were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% calf serum. Swiss 3T3 cells were maintained in DMEM containing 10% fetal calf serum. For experiments, cells were plated at low density and were grown for 3 to 4 days before they reached confluence. At confluence, their media were removed and replaced with DMEM containing 0.5% serum. Biosynthetic labeling was performed 48 h later.

Mitogens and hormones. Partially purified human PDGF and human PDGF purified to homogeneity were generous gifts from Russell Ross (University of Washington) and Rusty Williams (University of California at San Francisco). Mark Murray (Zymogenetics) kindly supplied purified human PDGF produced in yeast. This preparation is a B-chain homodimer. Epidermal growth factor (EGF) was generously provided by Bob Holley (Salk Institute). Vasopressin and bradykinin were kindly given to us by Jean Rivier (Salk Institute), and basic fibroblast growth factor (FGF) was from Dave Schubert (Salk Institute). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), insulin, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were from Sigma Chemical Co. Growth factors were used at the following final concentrations: PDGF, 10 to 50 ng/ml; EGF, 50 ng/ml; FGF, 60 ng/ml; insulin, 50 ng/ml; TPA, 50 ng/ml; vasopressin, 50 nM; bradykinin, 200 nM; Na_3VO_4, 50 μM; and $PGF_{2\alpha}$, 500 ng/ml. Growth factors were dissolved

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in 1 mM acetic acid–0.1% bovine serum albumin (BSA) except TPA, which was dissolved in dimethyl sulfoxide, and Na_3VO_4, which was dissolved in water. These agents were diluted 100- to 1,000-fold into cell media.

Biosynthetic labeling. ^{35}S labeling of cellular proteins was accomplished by incubating cells at 37°C with 0.15 or 1.3 mCi of Tran[^{35}S] label (>1,000 Ci/mmol; ICN Pharmaceuticals Inc.) for 16 to 22 h in methionine-free or methionine- and cysteine-free DMEM supplemented with 5% DMEM, 0.5% serum, and 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-OH (pH 7.4). For ^{32}P labeling, cells were incubated with 2.5 mCi of [^{32}P]orthophosphate (ICN) per ml for 14 to 20 h in phosphate-free DMEM supplemented with 5% medium, 0.5% serum, and 10 mM HEPES-OH (pH 7.4). For Na_3VO_4 experiments, the compound was included for the entire labeling period. Eight 6-cm dishes were labeled, each in a volume of 2.2 ml, for phosphopeptide mapping experiments of the N-terminal fragments of pp60$^{c\text{-}src}$.

Antisera and immunoprecipitation. Cells were rinsed once with cold phosphate-buffered saline, lysed in a 6-cm dish (volume, 1 ml) of cold RIPA buffer (10 mM sodium phosphate [pH 7.0], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 100 μM Na_3VO_4, 1% Trasylol [Mobay]) and clarified at 29,000 × *g* for 1 h at 4°C. pp60$^{c\text{-}src}$ was immunoprecipitated by incubating the lysate on ice for 30 min with 2 μl of a 1:100 dilution of ascites from hybridoma 327 (MAb 327) (41), generously provided by J. Bolen (National Institutes of Health), followed by a 30-min incubation on ice with 10 μl of affinity-purified rabbit anti-mouse immunoglobulin G (Cappel). DNase-free RNase A (200 μg/ml; Cooper Biochemicals) was added with MAb 327 for immunoprecipitation of ^{32}P-labeled proteins. After a third 30-min incubation on ice with 50 μl of a 10% solution of Formalin-fixed *Staphylococcus aureus* (Calbiochem-Behring), immune complexes were collected by centrifugation at 4°C over a 1-ml cushion of 10% sucrose in RIPA buffer and subsequently were washed three times with 1 ml of RIPA buffer. For two-dimensional gel electrophoresis or enolase kinase reactions, immune complexes were washed an additional two times with RIPA buffer lacking SDS and sodium deoxycholate. Proteins were released from immune complexes by incubation at 100°C for 3 min in 70 μl of one-dimensional sample buffer (50 mM Tris hydrochloride (pH 6.8), 10% glycerol, 2% SDS, 20% β-mercaptoethanol) or incubation at 37°C for 5 min in 80 μl of two-dimensional sample buffer (9.95 M urea, 4% Nonidet P-40, 2% ampholytes [pH range, 6 to 8], 0.1 M dithiothreitol, 0.3% SDS). Proteins were resolved in one dimension on SDS–15% polyacrylamide gels or in two dimensions as described previously (17, 25). Isoelectric focusing was performed with ampholytes (pH range, 3.5 to 10; LKB Instruments, Inc.) for 10,000 V × h. Proteins were detected by autoradiography (^{32}P) at 4°C with Kodak XAR or XS film or by fluorography (^{35}S) at −70°C with presensitized Kodak XAR film.

In vitro kinase reactions. Immune complexes of pp60$^{c\text{-}src}$ were washed once with kinase buffer (20 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid]-OH [pH 7.0], 10 mM $MnCl_2$) and suspended in 10 μl of kinase buffer. [γ-^{32}P]ATP in ethanol (3,000 Ci/mmol; Amersham Corp.) was dried under vacuum and resuspended in kinase buffer with acid-denatured enolase (15). This mixture was added to the immune complexes so that each reaction contained 20 μCi of [γ-^{32}P]ATP in a total reaction volume of 20 μl, with 0.2 μg of enolase. Certain reactions contained 2 μM of unlabeled ATP as well. Reactions were terminated by the addition of 2× one-dimensional sample buffer (see above) after 30 min at 30°C, and proteins were resolved on SDS–15% polyacrylamide gels. Gel bands were quantified by counting Cerenkov radiation and by densitometry.

Phosphatase treatment. Immune complexes from cells labeled with either [^{35}S]methionine or [^{32}P]orthophosphate were washed twice with 1 ml of SDS- and sodium deoxycholate-free RIPA and twice with 1 ml of phosphatase buffer (20 mM MES [morpholinoethanesulfonic acid]-OH [pH 5.5], 1 mM $MgCl_2$, 0.8 mM dithiothreitol, 4 μg of leupeptin per ml, 4 μg of soybean trypsin inhibitor per ml, 2% Trasylol) and resuspended in phosphatase buffer. Aliquots of the resuspended immunoprecipitates were incubated at 37°C for 1 h with 0.2 U of potato acid phosphatase (Boehringer Mannheim Biochemicals) in a total reaction volume of 50 μl, with or without 20 mM sodium phosphate as a control to inhibit phosphatase. Control reactions were performed in the absence of phosphatase and sodium phosphate. Reactions were terminated by the addition of 1 ml of RIPA buffer. Immune complexes were re-collected by centrifugation, and ^{35}S-labeled samples were analyzed by one- or two-dimensional gel electrophoresis. The extent of dephosphorylation was assessed by one-dimensional SDS-polyacrylamide gel electrophoresis of ^{32}P-labeled samples.

Peptide mapping and phosphoamino acid analysis. One-dimensional peptide maps were prepared on SDS–15% polyacrylamide gels with 100 ng of *S. aureus* V8 protease (Miles Laboratories, Inc.) as described previously (12). pp60$^{c\text{-}src}$ was extracted from the gels and subjected to tryptic digestion as described elsewhere (33). In certain experiments, pp60$^{c\text{-}src}$ was digested for an additional 10 h, after boiling in the presence of 10% β-mercaptoethanol and lyophilization, with two 10-μg samples of thermolysin at 55°C. Phosphopeptides were separated in two dimensions on 100-μm cellulose thin-layer plates by electrophoresis at pH 1.9 (acetic acid–88% formic acid–water, 156:50:1,794 [vol/vol]) for 30 min at 1 kV, at pH 8.9 (1% ammonium carbonate) for 20 min at 1 kV, or at pH 4.72 (*n*-butanol–pyridine–acetic acid–water, 2:1:1:36 [vol/vol]) for 40 min at 1 kV, followed by chromatography (*n*-butanol–pyridine–acetic acid–water, 75:50:15:60 [vol/vol]) (33). The phosphoamino acid contents of proteins and peptides were determined as described elsewhere (19, 33). Thin-layer electrophoresis was performed on a CBS Scientific apparatus.

RESULTS

Stimulation of pp60$^{c\text{-}src}$ by agonists of phosphatidylinositol turnover. We and others (26, 29, 51, 52, 62) have demonstrated previously that pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ are phosphorylated to high levels at a serine residue(s) in cells responding to treatment with tumor promoters or synthetic diacylglycerols. Furthermore, it was shown that purified protein kinase C, but not several other protein-serine/threonine kinases, phosphorylated pp60$^{c\text{-}src}$ at the same sites in vitro as were observed upon TPA treatment in vivo (29). If protein kinase C truly mediated TPA-induced phosphorylation of pp60$^{c\text{-}src}$, all natural agonists of protein kinase C should do so as well. To determine whether this was the case, we examined the phosphorylation state of pp60$^{c\text{-}src}$ in murine, rat, and human fibroblasts treated with a variety of hormones and mitogens with known effects on phosphatidylinositol turnover in the cell lines examined.

We first confirmed the mitogenicity of the hormone preparations we used by measuring their effects on [^{3}H]thy-

midine uptake into serum-starved fibroblasts (data not shown). ^{32}P-labeled quiescent fibroblasts were then treated briefly (5 to 10 min) with saturating doses of the hormones and mitogens, pp60$^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327 and SDS gel electrophoresis, and the sites of phosphorylation within pp60$^{c\text{-}src}$ were examined by two-dimensional peptide mapping. The tryptic phosphopeptide maps presented in Fig. 1 are a synopsis of these results. Only phosphopeptide maps from murine pp60$^{c\text{-}src}$ are presented, although the same results were obtained from experiments with rat and human cells (data not shown). pp60$^{c\text{-}src}$ from untreated cells yielded two predominant phosphopeptides containing S17 and Y527 (Fig. 1A). Phosphoamino acid analysis and various protease digestions of tryptic peptides indicated that the minor phosphopeptides (a, b, and c) (Fig. 1A) are related to the two major ones; peptide a contains Y527, while peptides b and c contain S17 (data not shown). Treatment with EGF and insulin, which do not stimulate phosphatidylinositol turnover in most fibroblast lines (2, 30, 38), did not change the level or sites of pp60$^{c\text{-}src}$ phosphorylation (Fig. 1B and C). However, agents which induce diacylglcerol production and lead to the activation of protein kinase C in fibroblasts (FGF [63], PDGF [1, 30], $PGF_{2\alpha}$ [42], serum [57, 64], Na_3VO_4 [43], vasopressin [4, 64], or TPA, which binds and activates protein kinase C [10, 44] led to the appearance of a new phosphopeptide containing phosphoserine (Fig. 1D through J and data not shown). We have determined previously that this phosphopeptide contains S12 (29). Our results suggested that the increase in N-terminal serine phosphorylation noted by Ryder and Gordon (58) following treatment with Na_3VO_4 is probably accounted for by S12 phosphorylation. Except for panel 1H, the phosphopeptide maps presented in Fig. 1 illustrate the level of S12 phosphorylation 10 min after the hormones were added to the cells. The largest effect on pp60$^{c\text{-}src}$ phosphorylation, however, probably occurs earlier. For example, maximal S12 phosphorylation following FGF treatment was achieved within 2 min and was no longer evident at 15 min (data not shown). This result suggests that phosphate at S12 is removed rapidly.

Although EGF is reported not to stimulate protein kinase C activity in most fibroblast lines (see, for example, references 2, 30, and 38), it does activate the phosphatidylinositol pathway in the human epidermal carcinoma cell line A431 (31, 59, 60). Phosphopeptide analysis indicated that phosphorylation of pp60$^{c\text{-}src}$ on S12 was also stimulated by EGF, as well as by bradykinin, another agonist of protein kinase C activation (31) in A431 cells (Fig. 2). The stimulation of pp60$^{c\text{-}src}$ phosphorylation by agents that activate protein kinase C has been found in a wide variety of cell types, including B lymphoid cells (39), pituitary cells, and neurons (our unpublished observations). In contrast to TPA, these agents result in transient and substoichiometric phosphorylation of S12 in pp60$^{c\text{-}src}$. This difference in kinetics can be explained by the transient, rather than prolonged, activation of protein kinase C by these agents and presumably by the restriction of protein kinase C activated by diacylglycerols to the plasma membrane (for a review, see reference 66), where not all pp60$^{c\text{-}src}$ molecules are located (55).

Enhancement of pp60$^{c\text{-}src}$ specific protein-tyrosine kinase activity by PDGF. All of the agents tested above were examined for their effects on pp60$^{c\text{-}src}$ immune-complex protein tyrosine kinase activity. After 5 min of growth factor treatments, pp60$^{c\text{-}src}$ was immunoprecipitated and incubated with the exogenous substrate enolase in the presence of [γ-^{32}P]ATP. Of the agents tested (EGF, insulin, FGF, PDGF, $PGF_{2\alpha}$, Na_3VO_4, vasopressin, serum, and TPA), only PDGF stimulated reproducibly pp60$^{c\text{-}src}$ protein kinase activity towards enolase (Fig. 3A and data not shown). None of the agents altered the amount of pp60$^{c\text{-}src}$ (data not shown). The maximal two- to threefold increase in activity occurred 5 to 10 min following PDGF treatment and returned to near basal levels within 2 h (Fig. 3B). In conjunction with our earlier study, these results suggest that S12 phosphorylation alone is insufficient to alter the immune-complex protein kinase activity of pp60$^{c\text{-}src}$ and that other changes mediated specifically by PDGF stimulate pp60$^{c\text{-}src}$ phosphotransferase activity in this assay.

PDGF induces the appearance of a second form of pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ phosphorylated at S12 exhibits a slight retardation in SDS gel mobility. All agents which induced S12 phosphorylation, except PDGF, simply caused the same reduced SDS gel mobility as did TPA (data not shown). PDGF treatment of ^{32}P-labeled quiescent fibroblasts resulted in the formation of a doublet of pp60$^{c\text{-}src}$ in SDS gels (see Fig. 3C). The doublet appeared within 2 min of PDGF treatment, was maximal at 5 to 10 min, and was nearly gone 2 h after treatment was initiated (Fig. 3C). These experiments were performed first with crude PDGF preparations and then repeated with pure PDGF preparations as well as a B-chain homodimer preparation expressed in yeast. Identical results were obtained. The more rapidly migrating major phosphoprotein of the doublet comigrated on one-dimensional SDS gels with pp60$^{c\text{-}src}$ from TPA-treated cells. The minor, more slowly migrating form (pp60$^{c\text{-}src+}$) appeared as if it were approximately 1 kilodalton (kDa) larger. Enhanced resolution of the doublet was obtained in later experiments by running the gels for twice as long (data not shown).

Generation of pp60$^{c\text{-}src+}$ by phosphorylation of a small subset of pp60$^{c\text{-}src}$ molecules. We could not detect the PDGF-induced doublet of pp60$^{c\text{-}src}$ on one-dimensional SDS gels of [^{35}S]methionine-labeled immunoprecipitates. This indicated that only a minor population of pp60$^{c\text{-}src}$ molecules had reduced SDS gel mobility. Therefore, to examine further the nature of the pp60$^{c\text{-}src}$ doublet, we resolved immunoprecipitates on two-dimensional gels. pp60$^{c\text{-}src}$ from untreated ^{35}S-labeled NIH 3T3 cells resolved into four isoelectric species termed forms 1 to 4 (Fig. 4A). *S. aureus* V8 protease analysis confirmed that these four proteins were pp60$^{c\text{-}src}$ and that the other proteins present in the two-dimensional gels were unrelated contaminants (data not shown). As we will discuss below, forms 1 to 4 arise from the same primary translation product by multiple phosphorylation events. After TPA treatment, only the two most acidic forms were evident, consistent with increased phosphorylation of S12 by protein kinase C (Fig. 4B). If ^{35}S-labeled pp60$^{c\text{-}src}$ immunoprecipitates were treated with potato acid phosphatase under conditions which removed >80% of the ^{32}P from ^{32}P-labeled samples, one major isoelectric species resulted (Fig. 4C). A mix of samples similar to those shown separately in Fig. 4A and C illustrated that this form was more basic than forms 1 to 4, and it was termed form 0 (Fig. 4D). That a more basic species resulted from dephosphorylation suggested that all pp60$^{c\text{-}src}$ molecules in NIH 3T3 cells normally contain at least one phosphate moiety. To confirm that this was the case, pp60$^{c\text{-}src}$ immunoprecipitates from untreated ^{32}P-labeled NIH 3T3 cells were resolved in two dimensions (Fig. 4E). Mixing ^{32}P- and ^{35}S-labeled immunoprecipitates indicated that the four ^{32}P-labeled species were forms 1 to 4 (data not shown).

PDGF treatment resulted in an enrichment of ^{35}S-labeled pp60$^{c\text{-}src}$ forms 3 and 4 (Fig. 4F). In addition to forms 1 to 4,

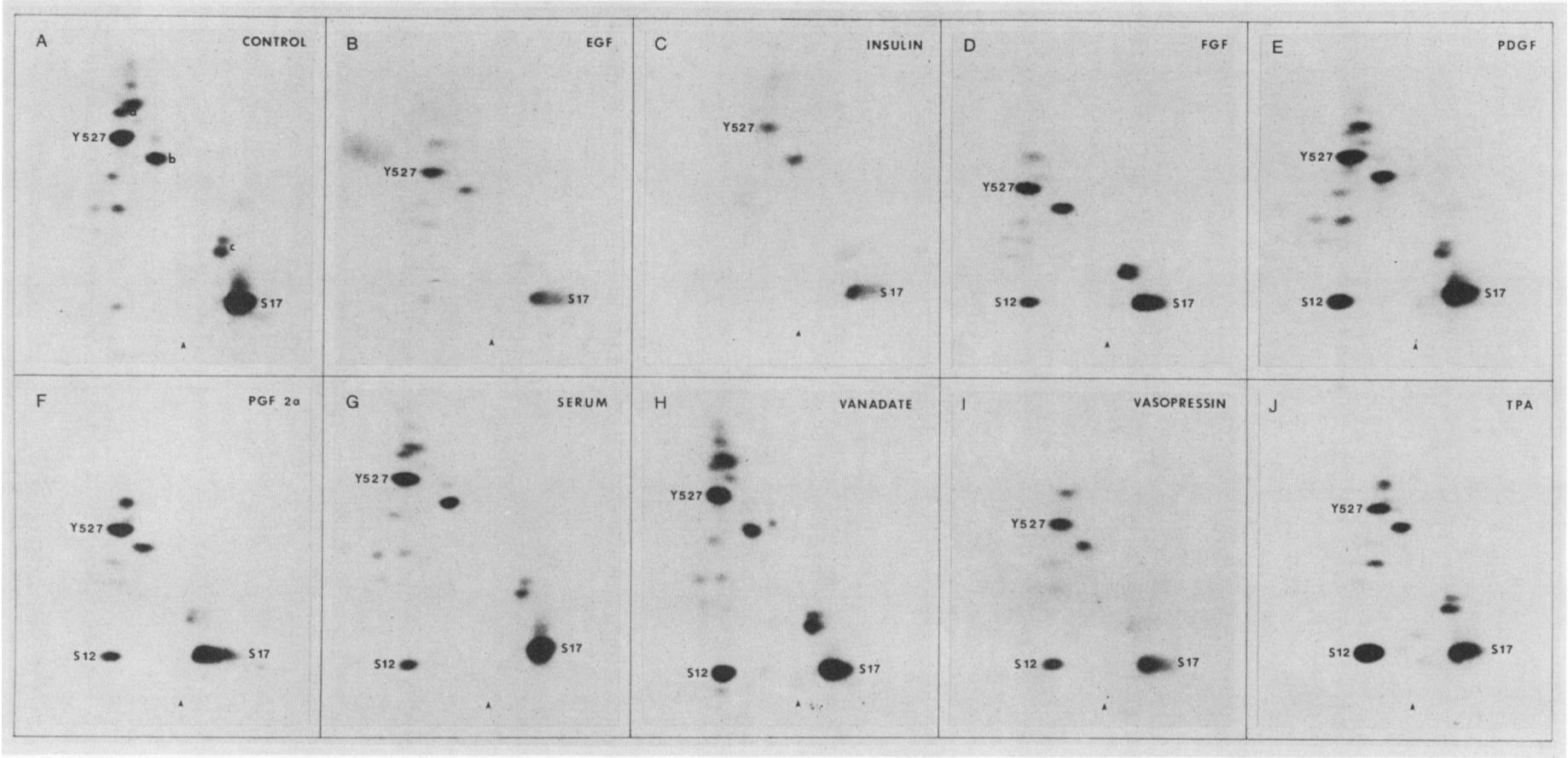


FIG. 1. Phosphopeptide maps of $pp60^{c\text{-}src}$ from fibroblasts. NR6 3T3 cells (A, E, and H) and Swiss 3T3 cells (B, C, D, F, G, I, and J) were labeled with [^{32}P]orthophosphate and treated for 10 min with 1 mM acetic acid–0.1% BSA (A), EGF (B), insulin (C), FGF (D), PDGF (E), $PGF_{2\alpha}$ (F), 10% serum (G), vasopressin (I), or TPA (J) at concentrations given in Materials and Methods, or they were labeled in the presence of 50 μM Na_3VO_4 (H). $pp60^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327, subjected to tryptic digestion, and separated in two dimensions as described in Materials and Methods. In each case, the origin is indicated with an arrowhead. Electrophoresis was performed at pH 8.9 in the horizontal dimension, with the anode on the left. Cerenkov counts per minute loaded onto thin-layer plates ranged from 150 to 800 cpm. The exposure times were 5 days (at −70°C with presensitized Kodak XAR5 film and intensifying screens).

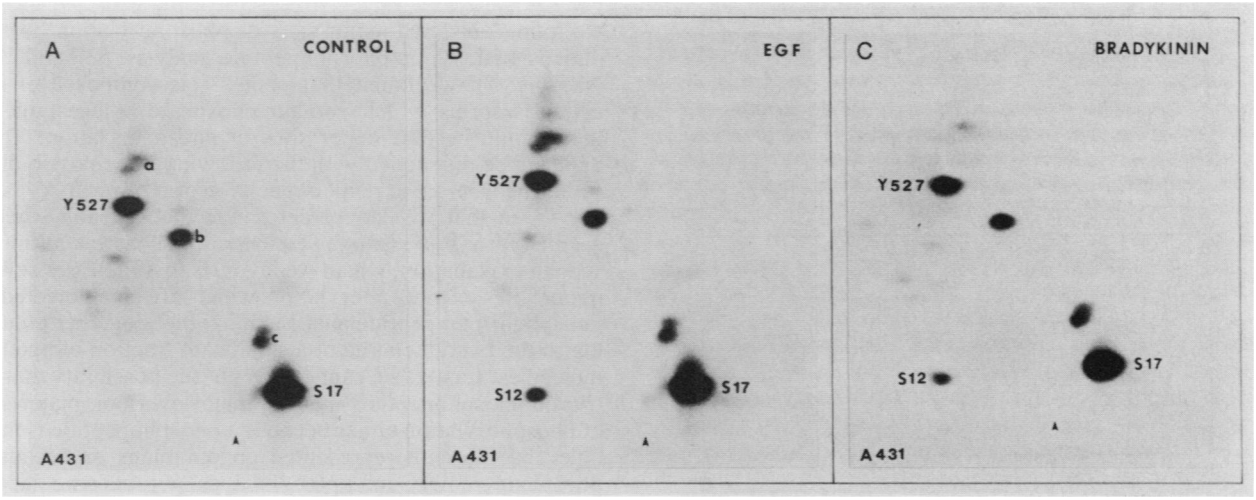


FIG. 2. Phosphopeptide maps of pp60$^{c\text{-}src}$ from A431 cells. A431 cells were labeled with [^{32}P]orthophosphate and treated for 10 min with 1 mM acetic acid–0.1% BSA (A), EGF (B), or bradykinin (C) at concentrations specified in Materials and Methods. pp60$^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327, subjected to tryptic digestion, and separated in two dimensions as described in Materials and Methods. In each case, the origin is indicated with an arrowhead. Electrophoresis was performed at pH 8.9 in the horizontal dimension, with the anode on the left. Cerenkov counts per minute loaded onto thin-layer plates were between 1,500 and 1,700 cpm. The exposure times (at −70°C with intensifying screens) were 2.5 days.

there were minor forms of pp60$^{c\text{-}src}$ from PDGF-treated cells which migrated above forms 3 and 4 (Fig. 4F); these minor forms were also observed in ^{32}P-labeled immunoprecipitates from PDGF-treated cells (Fig. 4G). pp60$^{c\text{-}src}$ isolated from PDGF-treated ^{35}S-labeled cells and subsequently treated with potato acid phosphatase yielded only one major isoelectric form with a single SDS gel mobility, form 0 (Fig. 4H). This demonstrated that the shifts in isoelectric and SDS gel mobilities of pp60$^{c\text{-}src}$ from PDGF-treated cells were most likely due to phosphate additions.

These experiments raise a question regarding the number of phosphate moieties on pp60$^{c\text{-}src}$ in resting cells. Although

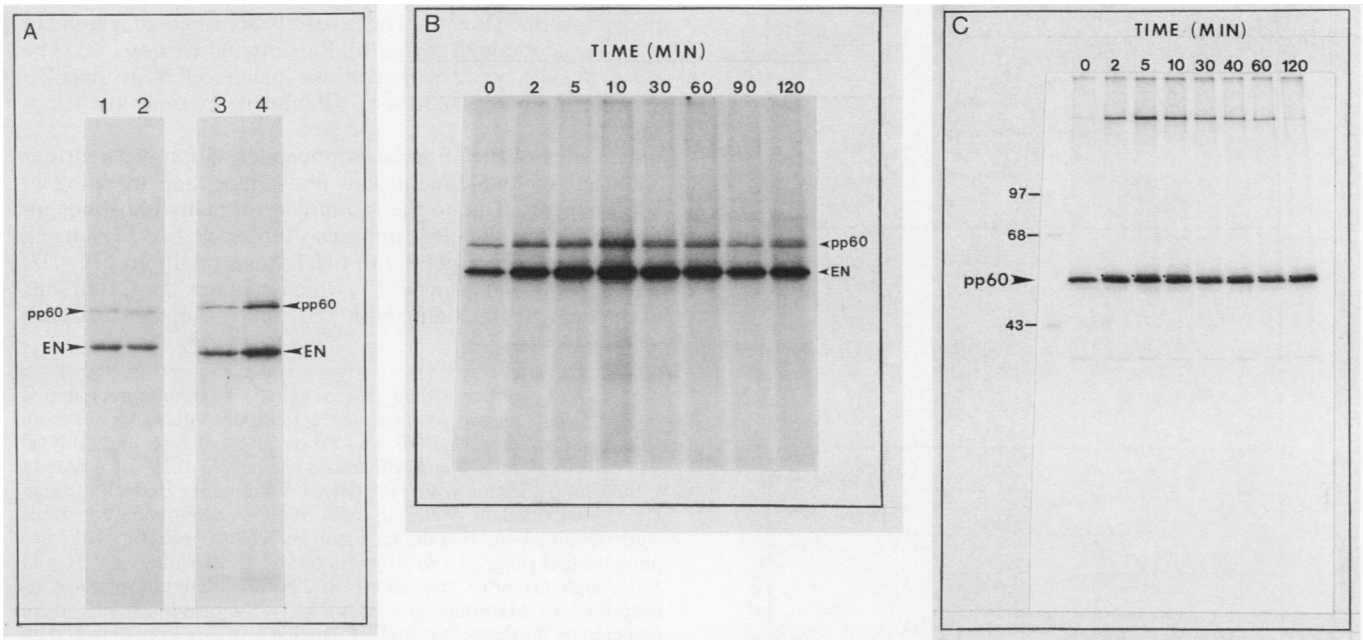
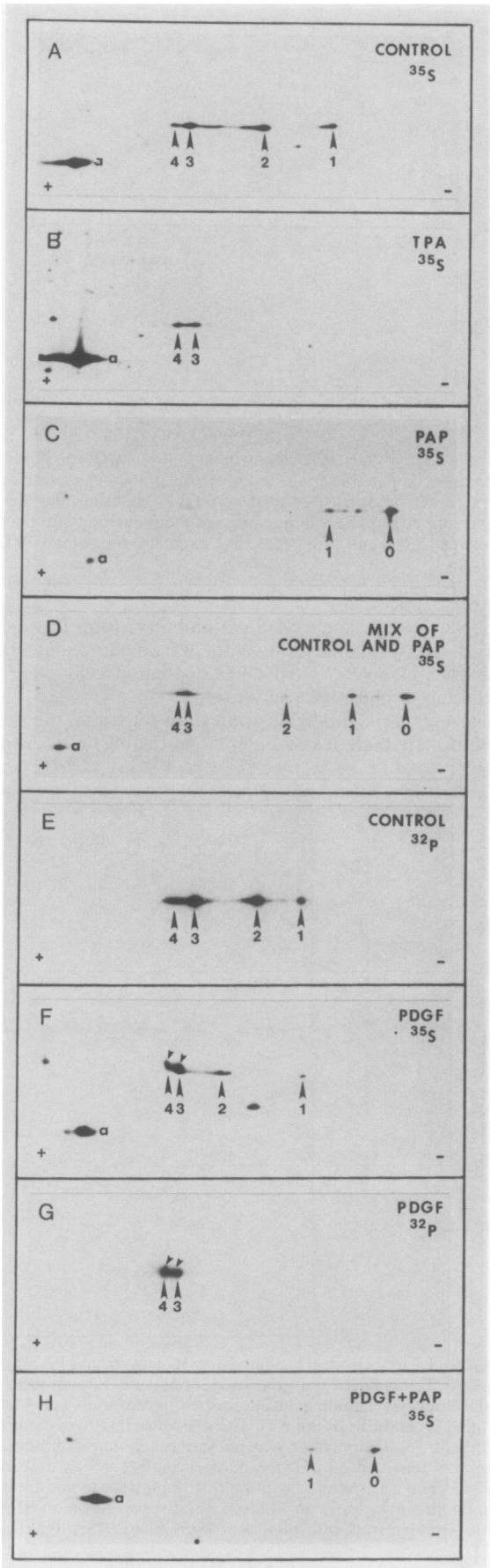


FIG. 3. Protein kinase activity of pp60$^{c\text{-}src}$ from PDGF-treated cells. (A) NIH 3T3 cells were treated for 10 min with TPA, dimethyl sulfoxide, or PDGF. pp60$^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327, incubated with the exogenous substrate enolase in the presence of [γ-^{32}P]ATP and 2 μM unlabeled ATP, and resolved on an SDS–15% polyacrylamide gel as described in Materials and Methods. pp60$^{c\text{-}src}$ was from cells treated with TPA (lane 1), dimethyl sulfoxide (lanes 2 and 3), and PDGF (lane 4). The exposure time was 3 days. EN, Enolase. (B) NIH 3T3 cells were treated for 0, 2, 5, 10, 30, 60, 90, or 120 min with PDGF. pp60$^{c\text{-}src}$ was immunoprecipitated with MAb 327 and incubated with enolase in the presence of [γ-^{32}P]ATP, and the mixture was resolved on an SDS-polyacrylamide gel as described in Materials and Methods. The exposure time was 16 h. EN, enolase. (C) NIH 3T3 cells were labeled with [^{32}P]orthophosphate and treated for 0, 2, 5, 10, 30, 40, 60, or 120 min with PDGF. pp60$^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327 and resolved on an SDS–15% polyacrylamide gel. The exposure time was 16 h. The molecular weight markers shown were phosphorylase *b* (97 kDa), BSA (68 kDa), and ovalbumin (43 kDa). pp60$^{c\text{-}src+}$ is indicated by dots.



we found only two major sites of phosphorylation by two-dimensional tryptic phosphopeptide analysis, S17 and Y527 (see Fig. 1), we found that pp60$^{c\text{-}src}$ is composed of four major isoforms which contain phosphate, suggesting that there could be four major sites of phosphorylation. These data can be reconciled with the following three possibilities. First, the isoelectric point of each isoform of pp60$^{c\text{-}src}$ is not known so that we cannot determine theoretically whether each isoform represents a sequential phosphate addition. A second explanation, which we favor, is that there are several minor sites of phosphorylation which are not detected by phosphotryptic peptide analysis but which together contribute to the overall charge of a significant fraction of pp60$^{c\text{-}src}$ molecules. Lastly, we cannot rule out the possibility that our tryptic phosphopeptide analysis might overlook major sites of phosphorylation either because a phosphopeptide is insoluble and thus not represented on our maps or because a phosphate moiety on pp60$^{c\text{-}src}$ does not become labeled detectably with ^{32}P due to very slow turnover.

Hyperphosphorylation of the N-terminus of pp60$^{c\text{-}src+}$ from PDGF-treated cells. The approximate locations of the PDGF-induced phosphorylation events in pp60$^{c\text{-}src}$ were determined by one-dimensional peptide mapping with *S. aureus* V8 protease. The fragments which contain the N terminus of pp60$^{c\text{-}src}$ (V1, V3, and V4) from PDGF-treated NIH 3T3 cells migrated as doublets (Fig. 5), indicating that the hyperphosphorylation occurred in the N-terminal region of the molecule. Phosphoamino acid analysis of the six N-terminal V8 fragments of pp60$^{c\text{-}src}$ from PDGF-treated cells demonstrated that the V1, V3, and V4 fragments contained only phosphoserine, while their more slowly migrating counterparts (V1$^+$, V3$^+$, and V4$^+$) contained phosphotyrosine as well as phosphoserine in a 1:8 ratio of phosphotyrosine to phosphoserine (Fig. 6). These results are in good agreement with those obtained earlier by Ralston and Bishop (53). The same results were obtained by using different pp60^{src}-specific antibodies and 4-h ^{32}P-labeling periods (data not shown).

The sites of PDGF-induced phosphorylation were further analyzed by two-dimensional phosphopeptide mapping of V8 fragments. Due to the insolubility of many phosphopeptides in water, the electrophoresis buffer we had been using was changed from pH 8.9 to pH 1.9 and finally to pH 4.72. The V2 fragment of pp60$^{c\text{-}src}$, which contains the C terminus of the enzyme, including both Y527 and Y416, was analyzed

FIG. 4. Two-dimensional gels of pp60$^{c\text{-}src}$ immunoprecipitates. pp60$^{c\text{-}src}$ was isolated by immunoprecipitation with MAb 327 from ^{35}S-labeled (A through D, F, and H) or ^{32}P-labeled (E and G) NIH 3T3 cells treated for 5 min with 1 mM acetic acid–0.1% BSA (A and C through E), 15 min with TPA (B), or 5 min with PDGF (F through H) as described in Materials and Methods. Immunoprecipitates were resolved by two-dimensional gel electrophoresis with ampholytes (pH range, 3.5 to 10), with (C and H) or without (A, B, and E through G) prior treatment with potato acid phosphatase as described in Materials and Methods. ^{35}S-labeled proteins were detected by fluorography, and ^{32}P-labeled proteins were detected by autoradiography, both at −70°C with presensitized Kodak XAR5 film. The exposure times were 14 days (A), 14 days (B), 21 days (C), 21 days (D), 3 days with an intensifying screen (E), 14 days (F), 5 days (G), and 21 days (H). Only relevant portions of the gels are shown. Exposures were chosen to demonstrate most clearly the migration of the modified forms and the relative isoelectric points of the various isoforms rather than equal recoveries. Indeed, although they are representative and reproducible, most of the gels shown were generated from different experiments. Small arrowheads indicate pp60$^{c\text{-}src+}$. Acidic proteins are on the left. a, Actin.

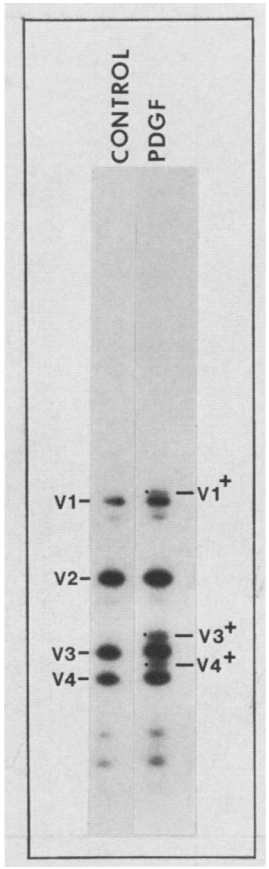


FIG. 5. Partial *S. aureus* V8 peptide analysis of pp60$^{c\text{-}src}$ from PDGF-treated cells. Gel bands containing pp60$^{c\text{-}src}$ isolated by immunoprecipitation with MAb 327 from ^{32}P-labeled NIH 3T3 cells treated for 5 min with 1 mM acetic acid–0.1% BSA or PDGF were subjected to partial V8 protease digestion as described in Materials and Methods. V8 protease fragments were detected by autoradiography. The exposure time was 2 days.

first. To isolate the V2 fragment of pp60$^{c\text{-}src+}$, pp60$^{c\text{-}src+}$ was cut from preparatory gels, turned 90°, and then rerun on a gel with V8 protease. One side of the resultant V2 fragment was therefore derived from pp60$^{c\text{-}src+}$, and the other side was derived from contaminating pp60$^{c\text{-}src}$. By tryptic phosphopeptide analysis of V2 fragments, it appeared that the ratio of Y527 phosphorylation to Y416 phosphorylation was very similar in pp60$^{c\text{-}src}$ from untreated cells and in both the faster- and slower-migrating phosphoproteins in the PDGF-induced doublet (Fig. 7A to C). This result suggested that the levels of Y527 and Y416 phosphorylation did not change detectably upon activation of pp60$^{c\text{-}src}$ phosphotransferase activity induced by PDGF.

We next examined the phosphorylation sites in the N-terminal region of pp60$^{c\text{-}src}$. In preliminary experiments, we examined the V1, V3, and V4 segments separately. However, V3 and V4 yielded phosphopeptide maps identical to those from V1. Therefore, we will present either the V1 phosphopeptide map as a representative or maps derived from the combination of the three fragments. The V1, V3, and V4 fragments from untreated cells yielded two major phosphopeptides (Fig. 7D). Phosphoamino acid analysis, Edman degradation, and various protease digestions of these tryptic phosphopeptides showed that both contained only S17 (data not shown). The V1, V3, and V4 fragments from PDGF-treated cells gave rise to the same S17-containing tryptic phosphopeptides and a new tryptic phosphopeptide containing S12 (Fig. 7E). The V1$^+$, V3$^+$, and V4$^+$ segments yielded the S17- and S12-containing tryptic peptides and two additional tryptic phosphopeptides termed peptides d and e (Fig. 7F). Peptides d and e contained only phosphoserine (Fig. 8A and B). Since we had demonstrated the presence of phosphotyrosine in the V1$^+$, V3$^+$, and V4$^+$ fragments (see Fig. 6D to F) and yet did not observe a phosphotyrosine-containing peptide, we reasoned that such a peptide must have been insoluble. Therefore, we digested the tryptic phosphopeptides of pp60$^{c\text{-}src}$ from PDGF-treated cells with thermolysin. The V1, V3, and V4 segments from trypsin-thermolysin double digests yielded two major phosphopeptides (Fig. 7G). By comigration with known phosphopeptides and Edman degradation (data not shown), these phosphopeptides were shown to contain S17 and S12. The V1$^+$, V3$^+$, and V4$^+$ fragments gave rise to the S17- and S12-containing phosphopeptides, a third major phosphopeptide (peptide f), and several minor phosphopeptides (Fig. 7H). Peptide f contained phosphoserine (Fig. 8C). Since both tryptic peptides d and e were cleaved by thermolysin and only one phosphoserine-containing peptide, peptide f, resulted from this cleavage, it seems likely that peptides d and e arise from incomplete tryptic cleavage and contain the same site of PDGF-induced serine phosphorylation. One of the minor phosphopeptides, peptide g, contained phosphotyrosine (Fig. 8D). The low yield of peptide g probably does not accurately reflect the stoichiometry of N-terminal tyrosine residues but rather the insolubility of the parent tryptic peptide and its resistance to digestion. These results demonstrate that PDGF treatment induces multisite phosphorylation of pp60$^{c\text{-}src}$ within its N-terminal 16 kDa and that there are most likely three targets for PDGF-induced phosphorylation: one unidentified serine residue, one unidentified tyrosine residue, and S12.

DISCUSSION

pp60$^{c\text{-}src}$ is constitutively phosphorylated at S17 and Y527. In this report, we show that all agonists of phosphatidylinositol turnover that we tested, in addition to direct activators

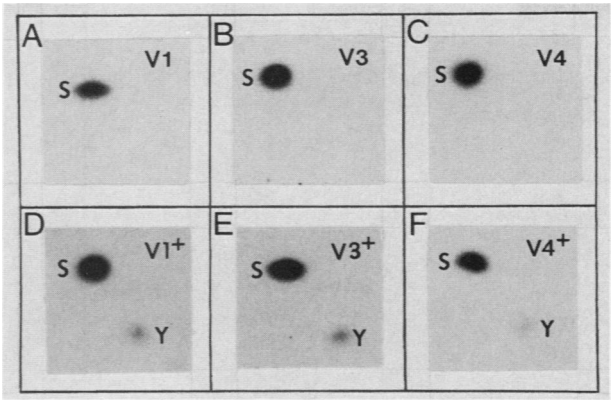


FIG. 6. Phosphoamino acid analysis of pp60$^{c\text{-}src}$ V8 protease fragments from PDGF-treated cells. V8 fragments similar to those shown in Fig. 5, lane 2, were subjected to partial acid hydrolysis, and the phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as described in Materials and Methods. Phosphoamino acids are from V1 (220 cpm) (A), V3 (140 cpm) (B), V4 (100 cpm) (C), V1$^+$ (250 cpm) (D), V3$^+$ (200 cpm) (E), and V4$^+$ (120 cpm) (F). The exposure times were 5 days (at −70°C with intensifying screens). S, Phosphoserine; Y, phosphotyrosine.

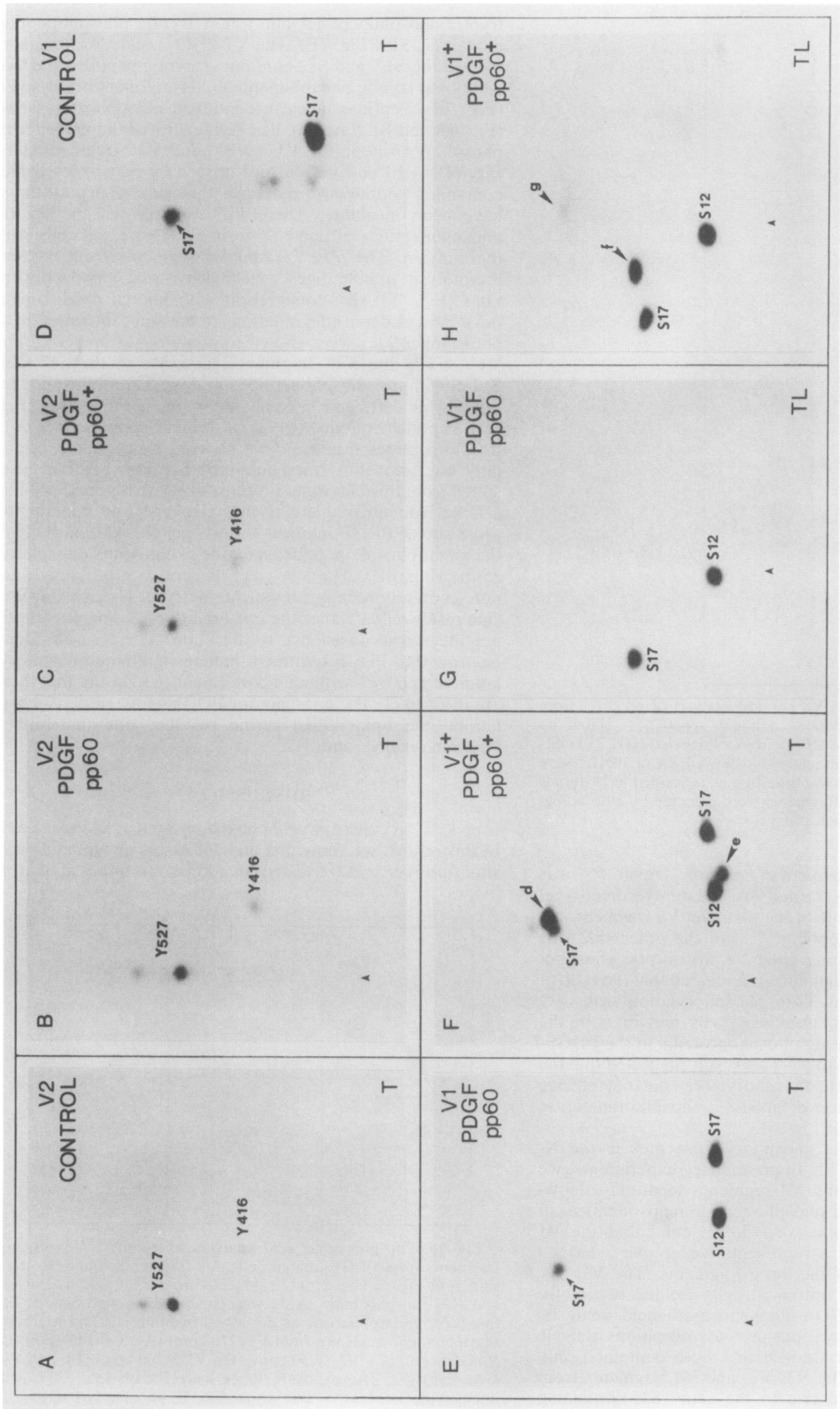


FIG. 7. Phosphopeptide maps of $pp60^{c\text{-}src}$ from PDGF-treated cells. V8 protease fragments similar to those shown in Fig. 5 were subjected to tryptic digestion (A through F) or tryptic and thermolytic double digestion (G and H). Phosphopeptides of the V2 fragment from untreated cells (A) and phosphopeptides derived from the V2 fragment of the faster-migrating form of $pp60^{c\text{-}src}$ (B) and $pp60^{c\text{-}src+}$ (C) from PDGF-treated cells are shown. The samples shown in panels B and C were separated from each other as described in the text. Phosphopeptides derived from the N-terminal V8 protease fragments from untreated cells (D), from the faster-migrating form of $pp60^{c\text{-}src}$ of PDGF-treated cells (E and G), and from $pp60^{c\text{-}src+}$ (F and H) are shown. Electrophoresis was performed at pH 1.9 (A through F) or pH 4.72 (G and H) in the horizontal dimension, with the anode on the left. Cerenkov counts per minute loaded onto thin-layer plates and exposure times were 230 cpm and 24 h (A), 150 cpm and 5 days (B), 50 cpm and 5 days (C), 100 cpm and 5 days (D), 800 cpm and 2 days (E), 725 cpm and 2 days (F), 130 cpm and 2 days (G), and 160 cpm and 2 days (H).

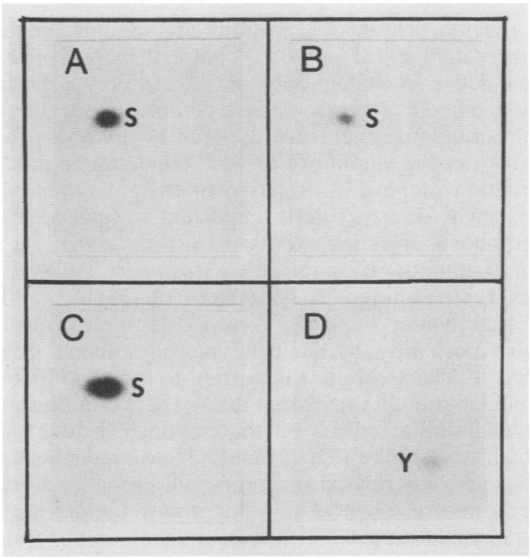


FIG. 8. Phosphoamino acid analysis of phosphopeptides. Phosphopeptides were removed from thin-layer plates and subjected to partial acid hydrolysis, and the phosphoamino acids were separated by two-dimensional thin-layer electrophoresis as described in Materials and Methods. Phosphoamino acids are from peptide d of Fig. 7F (A), peptide e of Fig. 7F (B), peptide f of Fig. 7H (C), and peptide g of Fig. 7H (D). The exposure times were 6 days (at −70°C with an intensifying screen). Cerenkov counts per minute loaded onto thin-layer plates were 60 cpm (A), 45 cpm (B), 145 cpm (C), and 30 cpm (D). S, Phosphoserine; Y, phosphotyrosine.

of protein kinase C, result in phosphorylation of pp60$^{c\text{-}src}$ on S12. This evidence is particularly important in the argument that pp60$^{c\text{-}src}$ is a physiological target of protein kinase C in light of recent phosphorylation studies of the glucose transporter. The glucose transporter is phosphorylated by protein kinase C in vitro at the same sites observed in vivo following TPA treatment (65). However, physiological activators of protein kinase C fail to induce this phosphorylation event (W. J. Allard, E. M. Gibbs, L. A. Witters, and G. E. Lienhard, Fed. Proc. **45**:1552, 1986), raising the possibility that another protein kinase mediates TPA-stimulated phosphorylation of the glucose transporter. This appears not to be the case with pp60$^{c\text{-}src}$ phosphorylation. The data presented here extend our earlier findings and confirm that pp60$^{c\text{-}src}$ behaves as a bona fide physiological substrate of protein kinase C.

Protein kinase C consists of several highly related isozymes which are found in all animal cells so far tested with overlapping but distinct distributions (for a review, see reference 66). The existence of protein kinase C isozymes has led to the speculation that each isozyme has a different substrate specificity or is activated by different agonists. We have examined the phosphorylation of pp60$^{c\text{-}src}$ in response to a large number of agonists and in a wide variety of cell types (this paper and our unpublished observations) and have not yet been able to separate the activation of protein kinase C from the phosphorylation of pp60$^{c\text{-}src}$ on S12. This indicates that pp60$^{c\text{-}src}$ is probably a common substrate of protein kinase C isozymes, although their affinities towards pp60$^{c\text{-}src}$ might vary.

Phosphorylation by protein kinase C appears to have no effect on the immune-complex protein kinase activity of pp60$^{c\text{-}src}$. We argued earlier (29) that an immune-complex protein kinase assay might not always reflect accurately the in vivo activity of pp60$^{c\text{-}src}$. Indeed, transforming mutants of both pp60$^{c\text{-}src}$ (6) and pp56^{lck} (Kurt Amrein, personal communication) exist now which exhibit no significant change in specific protein kinase activity in an immune-complex phosphotransferase assay yet clearly increase the phosphotyrosine content of cellular proteins. Since TPA and indirect activators of protein kinase C induce phosphorylation of a 42-kDa protein on tyrosine (3, 20, 27, 37), protein kinase C phosphorylation of pp60$^{c\text{-}src}$ might be involved in this event.

The results of PDGF treatment on pp60$^{c\text{-}src}$ activity are interpreted more easily. PDGF alone of the agents we tested (EGF, insulin, FGF, PDGF, vasopressin, serum, $PGF_{2\alpha}$, Na_3VO_4, and TPA) clearly enhances pp60$^{c\text{-}src}$ protein kinase activity two- to threefold. Activation of phosphotransferase activity is accompanied with the same kinetics by the appearance of a pp60$^{c\text{-}src}$ doublet in SDS gels. We showed by two-dimensional gel electrophoresis and phosphatase treatment that the more slowly migrating form of the doublet, pp60$^{c\text{-}src+}$, is generated by hyperphosphorylation of a small population of pp60$^{c\text{-}src}$ molecules. Hyperphosphorylation was restricted to the N-terminal region of the enzyme and occurred on both serine and tyrosine residues. These changes in pp60$^{c\text{-}src}$ are a direct consequence of PDGF action and are not due to a contaminating activity since partially purified human platelet PDGF, PDGF purified to homogeneity from human platelets, and PDGF produced as a B-chain homodimer in yeast all produced the same effects on pp60$^{c\text{-}src}$ protein kinase activity and phosphorylation.

In complete agreement with an earlier study of the effects of PDGF on pp60$^{c\text{-}src}$ phosphorylation (53), we found that at least one N-terminal tyrosine residue becomes phosphorylated following PDGF treatment. Those investigators, however, did not examine the phosphopeptides derived from the N-terminal region of pp60$^{c\text{-}src}$ and thus did not detect the serine phosphorylation events that occur as well. S12 becomes phosphorylated on nearly all pp60$^{c\text{-}src}$ molecules, and a subpopulation of molecules becomes phosphorylated on probably one other serine residue. This unknown serine was not phosphorylated by protein kinase C in vitro and was not observed in pp60$^{c\text{-}src}$ from TPA-treated cells (29). This implies that another protein-serine/threonine kinase, in addition to protein kinase C, is activated within 2 min of PDGF treatment. It will be interesting to determine the nature of this protein kinase, whether it is a substrate of the PDGF receptor, and what role it plays in mediating the cellular response to PDGF.

We endeavored to determine whether PDGF-induced phosphorylations on unknown serine and tyrosine residues in pp60$^{c\text{-}src}$ would occur following PDGF treatment in the absence of protein kinase C activation. A common method of testing whether protein kinase C activation is a prerequisite event to a particular phenomenon is to down-regulate the enzyme by long-term treatment with tumor promoters and then to examine the phenomenon in question. As determined by immunoprecipitation, we were unable to remove >60% of protein kinase C molecules from the murine fibroblast lines we used (J. R. Woodgett, unpublished observation). Since the active population that remained, approximately 40%, was sufficient to completely phosphorylate S12 in pp60$^{c\text{-}src}$, we have not been able to determine whether the novel phosphorylation events were dependent on protein kinase C stimulation.

The phosphorylation of pp60$^{c\text{-}src}$ on N-terminal tyrosine residues has been observed in other circumstances. pp60$^{c\text{-}src}$ complexed to the mT antigen of polyomavirus is phosphorylated on an N-terminal tyrosine(s) in Na_3VO_4-treated polyomavirus-transformed cells (67) and also in an immune-

complex protein kinase assay (7, 68). The site of this phosphorylation has not been definitively identified but is probably Y90 or Y92 (W. Yonemoto and J. Brugge, personal communication). pp60$^{v\text{-}src}$ can also be phosphorylated on unidentified N-terminal tyrosines in Na_3VO_4-treated cells (13) and in autophosphorylation reactions carried out at high ATP concentrations (14, 54). A major N-terminal phosphotyrosine-containing tryptic peptide from in vitro phosphorylated mT-pp60$^{c\text{-}src}$ complexes has been readily detected under the same conditions of peptide analysis we used (8). We have been unable to recover a similar phosphotyrosine-containing tryptic peptide from the N-terminal region of pp60$^{c\text{-}src}$ from PDGF-treated cells, but we have identified a tryptic-thermolytic peptide which contains phosphotyrosine. Thus, although we do not know the exact site of PDGF-induced tyrosine phosphorylation, we believe the tyrosine phosphorylation we have studied is not occurring at Y90 or Y92. An obvious question regarding the tyrosine phosphorylation we observed is whether it results from autophosphorylation, phosphorylation by the PDGF receptor, or phosphorylation by a third protein-tyrosine kinase. Experiments addressing this issue are in progress.

Of the several phosphorylation events in pp60$^{c\text{-}src}$, which, if any, is responsible for the increase in phosphotransferase activity? We studied first the phosphorylation state of Y527 in detail, since the level of Y527 phosphorylation can clearly control pp60$^{c\text{-}src}$ phosphotransferase activity. We were unable to detect any change in the level of phosphorylation at either Y527 or Y416 following PDGF treatment. Although we cannot be certain that a very small change would have been detectable, there is evidence in two other circumstances that pp60$^{c\text{-}src}$ immune-complex protein kinase activity can be increased without detectable changes in the levels of Y527 and Y416 phosphorylation. The neuron-specific form of pp60$^{c\text{-}src}$, produced by alternative splicing, is reported to have elevated specific activity (5, 9). Additionally, pp60$^{c\text{-}src}$ isolated from mitotic cells has new sites of serine and threonine phosphorylation, higher specific activity, and no reported change in Y527 or Y416 phosphorylation (11). Therefore, we feel that one or more of the novel PDGF-induced phosphorylation events occurring in pp60$^{c\text{-}src}$ is probably directly or indirectly responsible for increasing its specific activity. The results of our phosphatase experiments indicate that one or more of these novel phosphorylation events is clearly responsible for the retardation in SDS gel mobility of pp60$^{c\text{-}src}$ from PDGF-treated cells. In this context, it is notable that a pronounced retardation in SDS gel mobility has been observed in pp60$^{c\text{-}src}$ containing new sites of N-terminal phosphorylation isolated from mitotic cells (11).

We have shown by two-dimensional gel electrophoresis that only a minor subpopulation of pp60$^{c\text{-}src}$ molecules contains the new sites of PDGF-induced phosphorylation. Thus, the small overall change in specific activity following PDGF treatment probably reflects a very large change in the specific activity of that modified subset of pp60$^{c\text{-}src}$ molecules. Although elevated in vitro protein kinase activity probably reflects increased in vivo activity, we have no evidence that pp60$^{c\text{-}src}$ in PDGF-treated cells is activated. Since the PDGF receptor is a protein-tyrosine kinase itself, it will be difficult to separate phosphorylation events mediated directly by the receptor from any mediated by pp60$^{c\text{-}src}$.

We do not know why such a small fraction of pp60$^{c\text{-}src}$ molecules becomes highly phosphorylated following PDGF treatment. In cell lines which overexpress pp60$^{c\text{-}src}$, the ratio of modified to unmodified molecules is lower (our unpublished results). These observations are reminiscent of the situation with the mT-pp60$^{c\text{-}src}$ complex in polyomavirus-infected cells, in which only small subsets of both mT antigen and pp60$^{c\text{-}src}$ molecules are complexed together (23). In both situations, there are apparently unknown factors which restrict the amount of pp60$^{c\text{-}src}$ that can be modified. The location of pp60$^{c\text{-}src}$ relative to that of other protein kinases might be particularly important in limiting PDGF-induced phosphorylation events.

An intriguing question raised by this work is why PDGF appears to be unique in its effects on pp60$^{c\text{-}src}$. If the transient activation of pp60$^{c\text{-}src}$ by PDGF treatment occurs through a mechanism used in cell types other than fibroblasts and if pp60$^{c\text{-}src}$ activation is important to the transduction of extracellular stimuli, one would then expect other hormones to produce similar effects on the enzyme. Growth factors more highly related to PDGF than those we have tested and with more closely related receptors will probably be identified in the future. Such PDGF-like growth factors might be found to regulate pp60$^{c\text{-}src}$ activity in the same manner as does PDGF.

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LITERATURE CITED

1. **Berridge, M. J., J. P. Heslop, R. F. Irvine, and K. D. Brown.** 1984. Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. Biochem. J. **222**:195–201.
2. **Besterman, J. M., S. P. Watson, and P. Cuatrecasas.** 1986. Lack of association of epidermal growth factor-, insulin-, and serum-induced mitogenesis with stimulation of phosphoinositide degradation in BALB/c 3T3 fibroblasts. J. Biol. Chem. **261**:723–727.
3. **Bishop, R., R. Martinez, K. Nakamura, and M. J. Weber.** 1983. A tumor promoter stimulates phosphorylation on tyrosine. Biochem. Biophys. Res. Commun. **115**:536–543.
4. **Brown, K. D., J. Blay, R. F. Irvine, J. D. Heslop, and M. J. Berridge.** 1984. Reduction of epidermal growth factor receptor affinity by heterologous ligands: evidence for a mechanism involving the breakdown of phosphoinositides and the activation of protein kinase C. Biochem. Biophys. Res. Commun. **123**: 377–384.
5. **Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane.** 1985. Neurones express high levels of a structurally modified, activated form of pp60$^{c\text{-}src}$. Nature (London) **316**:555–557.
6. **Cartwright, C. A., W. E. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in carboxy-terminus regulatory domain. Cell **49**:83–91.
7. **Cartwright, C. A., M. A. Hutchinson, and W. Eckhart.** 1985. Structural and functional modification of pp60$^{c\text{-}src}$ associated with polyoma middle tumor antigen from infected or transformed cells. Mol. Cell. Biol. **5**:2647–2652.
8. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart.** 1986. Altered sites of tyrosine phosphorylation in pp60$^{c\text{-}src}$ associated with polyomavirus middle tumor antigen. Mol. Cell. Biol. **6**:1562–1570.
9. **Cartwright, C. A., R. Simantov, P. L. Kaplan, T. Hunter, and W. Eckhart.** 1987. Alterations in pp60$^{c\text{-}src}$ accompany differentiation of neurons from rat embryo striatum. Mol. Cell. Biol. **7**: 1830–1840.
10. **Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka.** 1982. Direct activation of calcium-activated,

phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257**:7847–7851.
11. **Chackalaparampil, I., and D. Shalloway.** 1988. Altered phosphorylation and activation of pp60$^{c\text{-}src}$ during fibroblast mitosis. Cell **52**:801–810.
12. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
13. **Collett, M. S., S. K. Belzer, and A. F. Purchio.** 1984. Structurally and functionally modified forms of pp60$^{v\text{-}src}$ in Rous sarcoma virus-transformed cell lysates. Mol. Cell. Biol. **4**:1213–1220.
14. **Collett, M. S., S. K. Wells, and A. F. Purchio.** 1983. Physical modification of purified Rous sarcoma virus pp60$^{v\text{-}src}$ protein after incubation with ATP/Mg^{2+}. Virology **128**:285–297.
15. **Cooper, J. A., F. S. Esch, S. S. Taylor, and T. Hunter.** 1984. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. J. Biol. Chem. **259**:7835–7841.
16. **Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter.** 1986. Tyr^{527} is phosphorylated in pp60$^{c\text{-}src}$: implications for regulation. Science **231**:1431–1433.
17. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. **1**:165–178.
18. **Cooper, J. A., and C. S. King.** 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60$^{c\text{-}src}$. Mol. Cell. Biol. **6**:4467–4477.
19. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
20. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1984. Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton proteins on tyrosine in quiescent chick cells. Mol. Cell. Biol. **4**:30–37.
21. **Cotton, P. C., and J. S. Brugge.** 1983. Neural tissues express high levels of the cellular *src* gene product pp60$^{c\text{-}src}$. Mol. Cell. Biol. **3**:1157–1162.
22. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**: 1471–1477.
23. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-*src* gene. Nature (London) **303**:435–439.
24. **Coussens, P. M., J. A. Cooper, T. Hunter, and D. Shalloway.** 1985. Restriction of the in vitro and in vivo tyrosine protein kinase activities of pp60$^{c\text{-}src}$ relative to pp60$^{v\text{-}src}$. Mol. Cell. Biol. **5**:2753–2763.
25. **Garrels, J. I.** 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J. Biol. Chem. **254**:7961–7977.
26. **Gentry, L. E., K. E. Chaffin, M. Shoyab, and A. F. Purchio.** 1986. Novel serine phosphorylation of pp60$^{c\text{-}src}$ in intact cells after tumor promoter treatment. Mol. Cell. Biol. **6**:735–738.
27. **Gilmore, T., and G. S. Martin.** 1983. Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature (London) **306**:487–490.
28. **Golden, A., S. P. Nemeth, and J. S. Brugge.** 1986. Blood platelets express high levels of the pp60$^{c\text{-}src}$-specific tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **83**:852–856.
29. **Gould, K. L., J. R. Woodgett, J. A. Copper, J. E. Buss, D. Shalloway, and T. Hunter.** 1985. Protein kinase C phosphorylates pp60^{src} at a novel site. Cell **42**:849–857.
30. **Habenicht, A. J., J. A. Glomset, W. C. King, C. Nist, C. Mitchell, and R. Ross.** 1981. Early changes in phosphatidylinositol and arachidonic acid metabolism in quiescent Swiss 3T3 cells stimulated to divide by platelet-derived growth factor. J. Biol. Chem. **256**:12329–12335.
31. **Hepler, J. R., N. Nakahata, T. W. Lovenberg, J. DiGuiseppi, B. Herman, H. S. Earp, and T. K. Harden.** 1987. Epidermal growth factor stimulates the rapid accumulation of inositol (1,4,5)-trisphosphate and a rise in cytosolic calcium mobilized from intracellular stores in A431 cells. J. Biol. Chem. **262**:2951–2956.
32. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
33. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
34. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low level of cellular protein phosphorylation by nontransforming overproduced p60$^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
35. **Kato, J.-Y., T. Takeya, C. Grandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming pp60$^{c\text{-}src}$ protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
36. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of pp60$^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49**:65–73.
37. **Kohno, M.** 1985. Diverse mitogenic agents induce rapid phosphorylation of a common set of cellular proteins at tyrosine in quiescent mammalian cells. J. Biol. Chem. **260**:1771–1779.
38. **L'Allemain, G., and J. Pouyssegur.** 1986. EGF and insulin action in fibroblasts: evidence that phosphoinositide hydrolysis is not an essential mitogenic signalling pathway. FEBS Lett. **197**:344–348.
39. **Ledbetter, J. A., L. E. Gentry, C. H. June, P. S. Rabinovitch, and A. F. Purchio.** 1987. Stimulation of T cells through the CD3/T-cell receptor complex: role of cytoplasmic calcium, protein kinase C translocation, and phosphorylation of pp60$^{c\text{-}src}$ in the activation pathway. Mol. Cell. Biol. **7**:650–656.
40. **Levy, J. B., H. Iba, and H. Hanafusa.** 1986. Activation of the transforming potential of pp60$^{c\text{-}src}$ by a single amino acid change. Proc. Natl. Acad. Sci. USA **83**:4228–4232.
41. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
42. **MacPhee, C. H., A. H. Drummond, A. M. Otto, and L. J. Jiminez de Asua.** 1984. Prostaglandin F2α stimulates phosphatidylinositol turnover and increases the cellular content of 1,2-diacylglycerol in confluent resting Swiss 3T3 cells. J. Cell. Physiol. **119**:35–40.
43. **Paris, S., and J. Pouyssegur.** 1987. Further evidence for a phospholipase C-coupled G protein in hamster fibroblasts. J. Biol. Chem. **262**:1970–1976.
44. **Parker, P. J., S. Stabel, and M. D. Waterfield.** 1984. Purification to homogeneity of protein kinase C from bovine brain—identity with the phorbol ester receptor. EMBO J. **3**:953–959.
45. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-*src* and chicken c-*src* in rat cells demonstrates qualitative differences between pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$. Cell **37**:131–139.
46. **Parsons, S. J., and C. E. Creutz.** 1986. pp60$^{v\text{-}src}$ activity detected in the chromaffin granule membrane. Biochem. Biophys. Res. Commun. **134**:736–742.
47. **Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton.** 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA **79**:973–977.
48. **Patschinsky, T., T. Hunter, and B. M. Sefton.** 1986. 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 pp60$^{v\text{-}src}$ of Prague Rous sarcoma virus. J. Virol. **59**:73–81.
49. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60$^{c\text{-}src}$. Cell **49**:75–82.
50. **Pruss, R. M., and H. R. Herschman.** 1977. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc. Natl. Acad. Sci. USA **74**:3918–3922.
51. **Purchio, A. F., L. Gentry, and M. Shoyab.** 1986. Phosphorylation of pp60$^{v\text{-}src}$ by the TPA receptor kinase (protein kinase C). Virology **150**:524–529.
52. **Purchio, A. F., M. Shoyab, and L. E. Gentry.** 1985. Site-specific increased phosphorylation of pp60$^{v\text{-}src}$ after treatment of RSV-transformed cells with a tumor promoter. Science **229**:1393–

1395.

53. **Ralston, R., and J. M. Bishop.** 1985. The product of the protooncogene c-*src* is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **82:** 7845–7849.
54. **Resh, M. D., and R. L. Erikson.** 1985. Characterization of pp60^{src} phosphorylation in vitro in Rous sarcoma virus-transformed cell membranes. Mol. Cell. Biol. **5:**916–922.
55. **Resh, M. D., and R. L. Erikson.** 1985. Highly specific antibody to Rous sarcoma virus src gene product recognizes a novel population of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ molecules. J. Cell Biol. **100:** 409–417.
56. **Reynolds, A. B., J. Vila, T. J. Lansing, W. M. Potts, M. J. Weber, and J. T. Parsons.** 1987. Activation of the oncogenic potential of the avian cellular *src* protein by specific structural alteration of the carboxy terminus. EMBO J. **6:**2359–2364.
57. **Rodriguez-Peña, A., and E. Rozengurt.** 1985. Serum, like phorbol esters, rapidly activates protein kinases C in intact quiescent fibroblasts. EMBO J. **4:**71–76.
58. **Ryder, J. W., and J. A. Gordon.** 1987. In vivo effect of sodium orthovanadate on pp60$^{c\text{-}src}$ kinase. Mol. Cell. Biol. **7:**1139–1147.
59. **Sahai, A., K. B. Smith, M. Panneerselvam, and D. S. Salomon.** 1982. Activation of calcium and phospholipid-dependent protein kinase by epidermal growth factor (EGF) in A431 cells: attenuation by 12-O-tetradecanoylphorbol-13-acetate (TPA). Biochem. Biophys. Res. Commun. **109:**1206–1214.
60. **Sawyer, S. T., and S. Cohen.** 1981. Enhancement of calcium uptake and phosphatidylinositol turnover by epidermal growth factor in A-431 cells. Biochemistry **20:**6280–6286.
61. **Smart, J. E., H. Oppermann, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop.** 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60$^{v\text{-}src}$) and its normal cellular homologue (pp60$^{c\text{-}src}$). Proc. Natl. Acad. Sci. USA **78:**6013–6017.
62. **Tamura, T., R. R. Friis, and H. Bauer.** 1984. pp60$^{c\text{-}src}$ is a substrate for phosphorylation when cells are stimulated to enter cycle. FEBS Lett. **177:**151–156.
63. **Tsuda, T., K. Kaibuchi, Y. Kawahara, H. Fukuzaki, and Y. Takai.** 1985. Induction of protein kinase C activation and Ca^{2+} mobilization by fibroblast growth factor in Swiss 3T3 cells. FEBS Lett. **191:**205–210.
64. **Vicentini, L. M., and M. L. Villereal.** 1984. Serum, bradykinin and vasopressin stimulate release of inositol phosphates from human fibroblasts. Biochem. Biophys. Res. Commun. **123:**663–670.
65. **Witters, L. A., C. A. Vater, and G. E. Lienhard.** 1985. Phosphorylation of the glucose transporter *in vitro* and *in vivo* by protein kinase C. Nature (London) **315:**777–778.
66. **Woodgett, J. R., T. Hunter, and K. L. Gould.** 1987. Protein kinase C and its role in cell growth, p. 215–340. *In* E. L. Elson, W. A. Frazier, and L. Glaser (ed.), Cell membranes: methods and reviews, vol. 3. Plenum Publishing Corp., New York.
67. **Yonemoto, W., A. J. Filson, A. E. Queral-Lustig, J. Y. J. Wang, and J. S. Brugge.** 1987. Detection of phosphotyrosine-containing proteins in polyomavirus middle tumor antigen-transformed cells following treatment with a phosphotyrosine phosphatase inhibitor. Mol. Cell Biol. **7:**905–913.
68. **Yonemoto, W., M. Jarvis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel.** 1985. Tyrosine phosphorylation within the amino-terminal domain of pp60$^{c\text{-}src}$ molecules associated with polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **82:**4568–4572.