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Tyrosine Phosphorylation of a 120-Kilodalton pp60^{src} Substrate upon Epidermal Growth Factor and Platelet-Derived Growth Factor Receptor Stimulation and in Polyomavirus Middle-T-Antigen-Transformed Cells

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The monoclonal antibody 2B12 is directed toward p120, a 120-kDa cellular protein originally identified as a protein tyrosine kinase substrate in cells expressing membrane-associated oncogenic variants of pp60^{src}. In this report, we show that p120 was tyrosine phosphorylated in avian cells expressing membrane-associated, enzymatically activated variants of c-*src*, including variants having structural alterations in the *src* homology regions 2 and 3. In contrast, p120 was not tyrosine phosphorylated in cells expressing enzymatically activated, nonmyristylated pp60^{src}. Furthermore, p120 was tyrosine phosphorylated in avian cells expressing middle T antigen, the transforming protein of polyomavirus, as well as in rodent cells stimulated with either epidermal growth factor (EGF) or platelet-derived growth factor. Analysis of the time course of p120 tyrosine phosphorylation in EGF-stimulated cells revealed a rapid onset of tyrosine phosphorylation. In addition, both the extent and duration of p120 phosphorylation increased when cells overexpressing the EGF receptor were stimulated with EGF. Biochemical analysis showed that p120 (in both normal and *src*-transformed cells) was membrane associated, was myristylated, and was phosphorylated on serine and threonine residues. Hence, p120 appears to be a substrate of both nonreceptor- and ligand-activated transmembrane receptor tyrosine kinases and of serine/threonine kinases and is perhaps a component of both mitogen-stimulated and tyrosine kinase oncogene-induced signaling pathways.

Tyrosine phosphorylation of cellular proteins occurs in response to ligand stimulation of receptor tyrosine kinases (26, 28, 32, 36, 40, 47, 59, 62), upon the expression of activated oncogene-encoded protein tyrosine kinases (PTKs) (15, 16, 19, 21–23, 34, 43, 56), during agonist-stimulated secretion (12), upon platelet activation (13, 14, 18), and during cell cycle changes (10, 42). Recently, several tyrosine phosphoprotein substrates of both receptor and nonreceptor PTKs have been identified, including the *ras* GTPase-activating protein (GAP) (11, 26, 29, 41), phospholipase C-148 (PLC-γ) (32, 36, 37, 40, 46, 47, 61, 62), and Raf-1, a serine/threonine kinase (44, 45). Although the majority of cellular tyrosine phosphoprotein substrates of PTKs have not been identified to date, they have been detected and partially characterized with antibodies to phosphotyrosine (anti-pTyr) (17, 24).

Among the best-characterized nonreceptor PTKs is the product of the Rous sarcoma virus (RSV) *src* gene (reviewed in references 6 and 50). The *src* gene encodes a 60-kDa membrane-associated phosphoprotein, pp60^{src}, with an associated PTK activity that is required for the initiation and maintenance of cellular transformation (6, 50). Expression of viral *src* (v-*src*), or activated forms of cellular *src* (c-*src*), in chicken embryo (CE) cells induces the tyrosine phosphorylation of 15 to 20 phosphoproteins which can be detected by immunoblotting with anti-pTyr (19, 21, 25, 34, 53, 56). We previously have used anti-pTyr to purify tyrosine-phosphorylated proteins from CE cells expressing activated variants of *src* (24). Following immunization of mice with immunoaffinity-purified pTyr-protein mixtures, monoclonal antibodies (MAbs) to eight individual tyrosine-phosphorylated proteins were generated (25). One of these MAbs was directed to a protein of 120 kDa (p120) that was detected previously in whole-cell lysates on one-dimensional anti-pTyr immunoblots and defined by its tyrosine phosphorylation in CE cells expressing v-*src* or transforming, activated variants of c-*src* (34, 56). Here we report that p120, a myristylated, membrane-associated phosphoprotein, was tyrosine phosphorylated in cells expressing membrane-associated, enzymatically activated variants of c-*src* as well as in cells expressing the polyomavirus middle T antigen (mT). Furthermore, we show that p120 was tyrosine phosphorylated in response to stimulation of cells with two different mitogenic growth factors, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). These results indicate that tyrosine-phosphorylated p120 may be a component of cellular signaling pathways altered during oncogenic transformation as well as pathways stimulated during normal signal transduction.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Primary CE cells were prepared from 10-day chicken embryos (SPAFAS, Norwich, Conn.) (56, 57). Plasmids bearing the chicken c-*src* gene or mutated forms of c-*src* in a nonpermuted RSV vector (pRL) were transfected into cells as described previously (56, 57). Amphotropic Moloney murine leukemia virus (Mo-MLV) bearing the polyomavirus mT gene (designated mT/Mo-MLV) was used to infect CE cells. Rat-1 (rat fibroblast cell

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line), rat-HER (Rat-1/human EGF receptor [EGFR] overexpressor cell line), HIRc-B (Rat-1/human insulin receptor [INSR] overexpressor cell line), Rat-1/v-*src* (v-*src*-transformed Rat-1 line), and BALB/3T3 (mouse fibroblast cell line) were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum.

Immunoprecipitation and Western immunoblotting. Immunoprecipitates of p120 were prepared by lysis of cells in modified RIPA buffer containing proteinase and phosphatase inhibitors as previously described (21, 24, 56), followed by incubation of 500 μg of cellular protein with 5 μg of protein A-purified MAb 2B12 (25) at 0°C for 1 h. Immunocomplexes were recovered by the addition of 50 μl of protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) that had been preincubated with 5 μg of affinity-purified rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch, Bar Harbor, Maine), followed by gentle agitation for 1 h. The beads were washed in modified RIPA buffer three times, followed by one wash in TN buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl). The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, and incubated with 5 μg of MAb 2B12 or 2 μg of affinity-purified rabbit anti-pTyr per ml as described previously (24, 25).

Metabolic labeling, subcellular fractionation, and phosphoamino acid analysis. CE cells were labeled with $^{32}P_i$, [^{35}S] methionine, [^{3}H]myristic acid, or [^{3}H]palmitic acid (all from Dupont, NEN Research Products, Boston, Mass.) as previously described (2, 24, 55). Subcellular fractionation of CE cells was carried out by differential centrifugation of cell lysates prepared by hypotonic lysis as previously described (56). Two-dimensional phosphoamino acid analysis of p120 was performed as previously described (24).

Growth factor stimulation of cell lines. Cells were serum starved for 24 h as described previously (35) and then stimulated with Dulbecco modified Eagle medium containing EGF, PDGF, or insulin (Collaborative Research, Bedford, Mass.) for the time periods and concentrations indicated in the legends to Fig. 4 and 5. Cells were immediately lysed in modified RIPA buffer containing 1 mM EDTA and processed for immunoprecipitation and immunoblotting as described above.

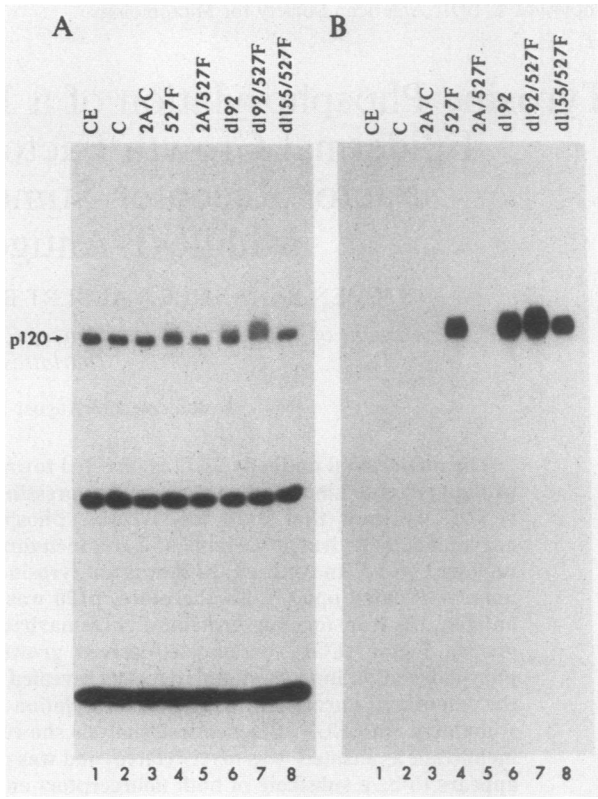

FIG. 1. Expression and tyrosine phosphorylation of p120 in CE cells infected with transforming and nontransforming *src* variants. (A) Level of p120 in infected CE cells. Extracts from CE cells (lane 1) or CE cells infected with RSV variants expressing c-*src* (lane 2), c-*src* 2A/C (lane 3), c-*src* 527F (lane 4), c-*src* 2A/527F (lane 5), c-*src* *dl*92 (lane 6), c-src *dl*92/527F (lane 7), and c-*src* *dl*155/527F (lane 8) were immunoprecipitated with MAb 2B12, and the resulting immune complexes were subjected to SDS-PAGE and immunoblotting with MAb 2B12. The bands at 50 and 25 kDa are the immunoglobulin G heavy and light chains, respectively. (B) Tyrosine phosphorylation of p120. An immunoblot identical to that shown in panel A was probed with anti-pTyr.

RESULTS

Expression and tyrosine phosphorylation of p120. We have previously described the derivation of a monoclonal antibody (MAb 2B12) directed to a 120-kDa protein that is tyrosine phosphorylated in RSV-transformed avian and rodent cells (25). Using MAb 2B12, we analyzed the levels and tyrosine phosphorylation of p120 in uninfected CE cells and cells expressing several oncogenic and nononcogenic *src* variants. The properties of these variants are summarized as follows: (i) c-*src* 527F contains an alteration of the C-terminal tyrosine residue (527Y) to phenylalanine (527F). This variant elicits transformation of avian and rodent cells and encodes a pp60$^{c\text{-}src}$ with an activated PTK (3, 27, 30, 34, 39, 52, 57); (ii) c-*src* 2A/527F contains an alteration of the N-terminal glycine residue (2G, the site of myristylation) to alanine and was expressed either as a double mutant with 527F or c-*src*, c-*src* 2A. The former variant encodes a nontransforming, enzymatically activated pp60^{src}, and the latter encodes a nontransforming, enzymatically down-regulated pp60^{src} (56); (iii) c-*src* *dl*92 and c-*src* *dl*92/527F have a deletion of residues 92 to 95 (within the A box [SH3 domain]) in either normal c-*src* or c-*src* 527F (50, 51) and encode *src* proteins that induce a partially transformed (fusiform) phenotype (53); and (iv) c-*src* *dl*155/527F has a deletion of residues 155 to 157 within the B box (SH2 domain) (50, 51) of c-*src* 527F and does not transform CE cells (55).

A single polypeptide of 120 kDa was detected when CE cell extracts were immunoprecipitated with MAb 2B12 and the subsequent immunoblots were probed with MAb 2B12 (Fig. 1A, lane 1). Overexpression of the c-*src* gene or several transforming or nontransforming *src* variants did not alter the steady-state level of p120 in these cells (lanes 2 to 8). The relative amount of p120 in these cells was unchanged when measured by immunoprecipitation of cells that had been metabolically labeled for 6 or 24 h with [^{35}S]methionine (data not shown). In addition, tyrosine-phosphorylated proteins of 120 kDa were selectively depleted from cell extracts by immunoprecipitation with MAb 2B12 (data not shown), showing that p120 was a major tyrosine-phosphorylated species in the 120-kDa region of the gel.

To investigate how alteration of putative functional domains of pp60^{src} influenced the tyrosine phosphorylation of p120, extracts were prepared from CE cells infected with *src* variants that confer different phenotypic properties to the infected cells (see above). p120 was immunoprecipitated

with MAb 2B12, and the immune complexes were analyzed by immunoblotting with anti-pTyr. Tyrosine phosphorylation of p120 was readily detected in CE cells expressing the oncogenic variant, c-*src* 527F, but not in cells expressing the activated, nontransforming, myristylation-deficient form of *src* encoded by c-*src* 2A/527F (Fig. 1B, lanes 4 and 5). Tyrosine phosphorylation of p120 was also observed in cells expressing the partially transforming, membrane-associated variants, c-*src* *dl*92 and c-*src* *dl*92/527F, as well as the nontransforming variant c-*src* *dl*155/527F (lanes 6 to 8). Tyrosine-phosphorylated p120 was not observed in uninfected CE cells or in cells expressing normal c-*src* or the nonmyristylated, nonactivated c-*src* variant 2A/C (lanes 1 to 3). These data indicated that tyrosine phosphorylation of p120 required both enzymatic activation and membrane association of pp60^{src}. However, p120 tyrosine phosphorylation was unaltered by mutations in the A (SH3) or B/C (SH2) regions of pp60^{src}.

Biochemical characterization of p120. Our earlier cell fractionation studies (56), coupled with the observation that tyrosine phosphorylation of p120 required the membrane association of pp60^{src}, suggested that p120 might be associated with cellular membranes. To confirm the localization of p120, uninfected CE cells or CE cells infected with c-*src* 527F were lysed in hypotonic buffer and subjected to cell fractionation (56), and MAb 2B12 was used to immunoprecipitate p120 from the individual subcellular fractions. Immunoblot analysis of p120 immune complexes with anti-pTyr (Fig. 2A) or MAb 2B12 (data not shown) demonstrated that the major portion of cellular p120 was associated with cellular membranes. A small amount of p120 was observed in the low-speed fraction, which was enriched for nuclear membranes and unbroken cells (Fig. 2A, lanes 1 and 2), while less than 10% was observed in the cytosol (lane 3). An identical pattern of p120 distribution was observed when MAb 2B12 was used to probe a Western blot of subcellular fractions from c-*src* or normal CE cells (data not shown). Using the conditions for subcellular fractionation reported here, we have found that other tyrosine-phosphorylated proteins in c-*src* 527F-transformed cells (e.g., p125 and p85) (25) were predominantly (>95%) cytosolic, whereas pp60^{src} was predominantly (>90%) membrane-associated (21a, 56).

In light of the apparent membrane localization of p120, we attempted to determine whether p120 was glycosylated. Both normal and c-*src* 527F-infected cells were labeled with [^{35}S]methionine in the presence of tunicamycin. Under these conditions, the electrophoretic mobility of metabolically labeled p120 was not altered by treatment with tunicamycin, whereas immunoprecipitated gp85^{env} exhibited an altered mobility (data not shown). These data indicated that p120 was not substantially glycosylated. However, consistent with its membrane localization, we found that p120 was labeled following incubation of CE or c-*src* 527F-infected cells with [^{3}H]myristic acid (Fig. 2B, lane 4) but not with [^{3}H]palmitic acid (data not shown). The amount of ^{3}H-labeled p120 observed in cells labeled with [^{3}H]myristic acid was lower than the amount of ^{3}H-labeled pp60^{src} immunoprecipitated with MAb EC10 (49) (Fig. 2B, lane 5). Quantitation of the relative levels of p120 and pp60^{src} in [^{35}S]methionine-labeled cell extracts, using MAb 2B12 (p120) or MAb EC10 (pp60^{src}), indicated an approximately 10-fold higher level of pp60^{src} than of p120. Thus, the amount of ^{3}H-labeled p120 observed in Fig. 2B was consistent with the overall lower level of p120 present in these cells, although we cannot rule out the possibility that only a portion of p120 was myristylated.

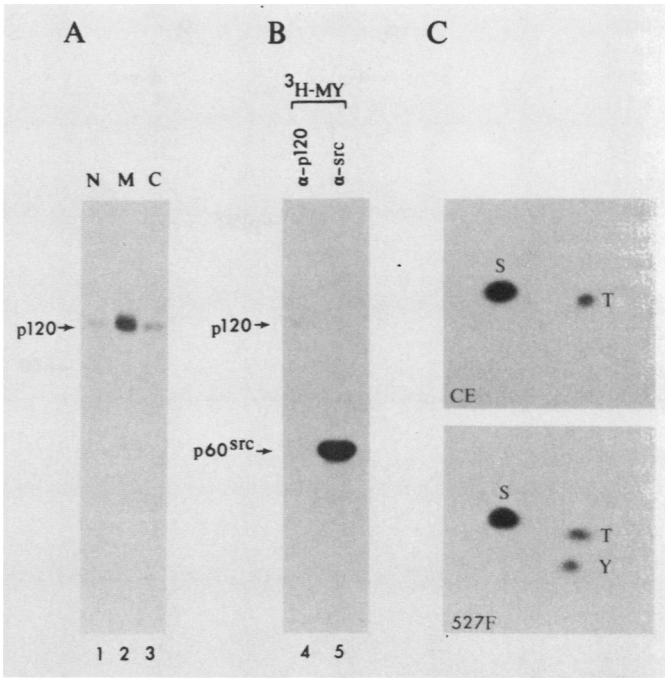

FIG. 2. Biochemical characterization of p120. (A) pTyr immunoblot. Subcellular fractions were prepared from CE cells infected with c-*src* 527F by hypotonic lysis and differential centrifugation (56). p120 was immunoprecipitated with MAb 2B12 from the nuclear (N), membrane (M), and cytoplasmic (C) fractions, and tyrosine-phosphorylated p120 was detected by probing immunoblots with anti-pTyr as described in Materials and Methods. (B) Myristylation of p120. c-*src* 527F-transformed CE cells were labeled with [^{3}H] myristic acid (^{3}H-MY), and p120 and pp60^{src} were immunoprecipitated with MAbs 2B12 (α-p120) and EC10 (α-src), respectively. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. The gel was exposed for 28 days. (C) Phosphoamino acid analysis of p120. CE cells (top) and c-*src* 527F-transformed CE cells (bottom) were labeled with $^{32}P_i$; immunoprecipitates of p120 were prepared with MAb 2B12, subjected to SDS-PAGE, extracted, acid hydrolyzed, and analyzed by two-dimensional thin-layer electrophoresis as described previously (24). Authentic phosphoamino acids were coelectrophoresed and identified by ninhydrin staining. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

The presence of phosphotyrosine on p120 in *src*-transformed cells was confirmed by labeling cells in vivo with $^{32}P_i$ and subjecting immunoprecipitated p120 to phosphoamino acid analysis (Fig. 2C). Both phosphoserine and phosphothreonine residues were detected in p120 immunoprecipitated from both CE and c-*src* 527F-infected cells. Phosphotyrosine was evident only in CE cells expressing pp60^{527F} (Fig. 2C). Thus, p120 is a membrane-associated phosphoprotein substrate of both serine/threonine and tyrosine kinases.

Tyrosine phosphorylation of p120 in polyomavirus mT-transformed cells. Since the expression of activated variants of pp60$^{c\text{-}src}$ resulted in tyrosine phosphorylation of p120, we determined whether p120 was tyrosine phosphorylated in CE cells expressing polyomavirus mT. Previous studies have shown that mT is complexed with 5 to 10% of endogenous pp60$^{c\text{-}src}$ molecules, as well as with c-*yes* and c-*fyn* proteins, in mT-transformed cells (1, 5, 7, 31, 33). In addition, pp60$^{c\text{-}src}$ in mT complexes is phosphorylated on Tyr-416, the site of autophosphorylation, suggesting that these mT-complexed pp60$^{c\text{-}src}$ molecules have elevated kinase activity (4). CE cells were infected with mT/Mo-MLV

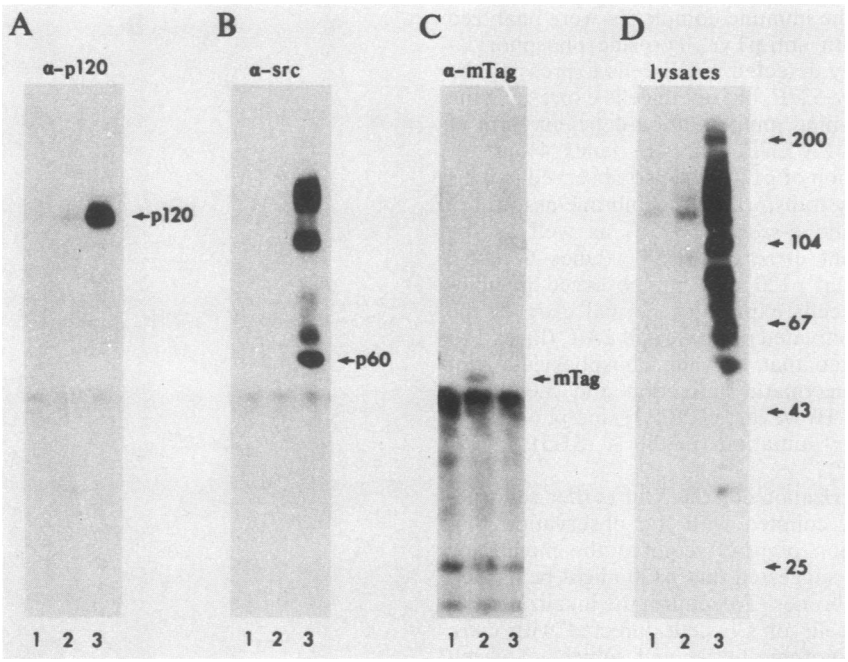

FIG. 3. Tyrosine phosphorylation of p120 in polyomavirus mT-transformed cells. CE cells were infected with mT/Mo-MLV (3), and the infected cells were cultured for 16 days. Lysates and immunoprecipitates were prepared from uninfected (lanes 1), mT/Mo-MLV-infected (lanes 2), and c-*src* 527F-infected (lanes 3) CE cells, analyzed by SDS-PAGE, and subjected to anti-pTyr immunoblotting. (A) p120 immunoprecipitated with MAb 2B12; (B) $pp60^{src}$ immunoprecipitated with MAb EC10; (C) mT immunoprecipitated with polyclonal anti-mT rabbit sera (α-mTag); (D) total cell lysates. Note that the higher-molecular-weight proteins present in panel B, lane 3, are the $pp60^{src}$-associated proteins, p130 and p110 (55). Positions of molecular size markers are indicated in kilodaltons on the right.

(3). Transformation was evident in the infected cultures at day 14, and p120 tyrosine phosphorylation was measured on day 16. Expression of mT in CE cells resulted in low but detectable tyrosine phosphorylation of p120 (Fig. 3A). The low level of tyrosine phosphorylation of p120 was consistent with the relatively small amount of activated, endogenous tyrosine kinase in complex with mT (5 to 10%) (1, 7) and was approximately 2 to 5% the level of tyrosine phosphorylation of p120 observed in cells expressing $pp60^{527F}$ (Fig. 3A, lane 3). Immunoprecipitation of $pp60^{src}$ from mT-infected cells revealed little detectable tyrosine-phosphorylated cellular *src* protein (Fig. 3B); however, this finding likely reflects the insensitivity of the anti-pTyr immunoblotting technique used in this experiment. Immunoprecipitation of mT from mT-transformed cells revealed readily detectable mT protein (Fig. 3B). Anti-pTyr immunoblot analysis of extracts from mT- and c-*src* 527F-infected cells readily demonstrated the difference in the levels of overall tyrosine-phosphorylated proteins in these cells (Fig. 3D). We conclude from these data that expression of mT results in the increased tyrosine phosphorylation of p120, presumably by activation of endogenous tyrosine kinases.

Tyrosine phosphorylation of p120 upon growth factor receptor stimulation. Several recent reports have demonstrated the interaction of growth factor receptors, including the EGFR and PDGF receptor (PDGFR), with the tyrosine phosphoproteins GAP, PLC-γ, and Raf-1 (reviewed in reference 59). These receptor-substrate interactions have included both physical association and tyrosine phosphorylation of substrates upon ligand stimulation of receptors (59). To investigate whether p120 was tyrosine phosphorylated upon stimulation of cells with EGF, we used Rat-1 cells and Rat-1 cells that overexpress either the human EGFR (rat-HER) (63a) or v-*src* (1a). Each of the cell lines was treated with EGF for 5 min and p120 was immunoprecipitated with MAb 2B12. Induction of p120 tyrosine phosphorylation was observed in rat-HER cells upon EGF stimulation (Fig. 4A, lanes 3 and 4); however, tyrosine phosphorylation of p120 was not detected in normal Rat-1 cells after EGF stimulation for 5 min (lanes 1 and 2). EGF did not affect the already high levels of tyrosine phosphorylation of p120 in Rat-1 cells expressing v-*src* (lanes 5 and 6). In addition, each of the Rat-1 cell lines exhibited the same relative level of p120, which was unaltered by EGF stimulation (Fig. 4B). Because tyrosine phosphorylation of p120 was virtually undetectable in Rat-1 cells following 5 min of EGF stimulation, the time course of p120 tyrosine phosphorylation was investigated in greater detail. Both Rat-1 and rat-HER cells were stimulated with EGF for various times up to 10 min (Fig. 4C and D), p120 was immunoprecipitated, and the tyrosine phosphorylation of p120 was assessed by using anti-pTyr immunoblotting. Densitometric analysis of the anti-pTyr immunoblots (Fig. 4C and D) revealed a rapid, albeit lower stimulation of p120 tyrosine phosphorylation in normal Rat-1 cells within 1 min (Fig. 4C), followed by a reduction of p120 phosphotyrosine signal, returning to basal levels by 5 min. In contrast, a parallel analysis of rat-HER cells showed a rapid induction of tyrosine phosphorylation of p120, with maximal phosphorylation by 1 to 2 min (Fig. 4D). No decrease in the steady-state level of p120 phosphorylation in rat-HER cells was detected within 10 min or after 1 h of EGF stimulation (data not shown). The maximum level of p120 tyrosine phosphorylation in rat-HER cells was approximately 30-fold greater than that observed in normal Rat-1 cells. This difference in tyrosine phosphorylation of p120 was comparable to the difference in the level of EGFR in the respective

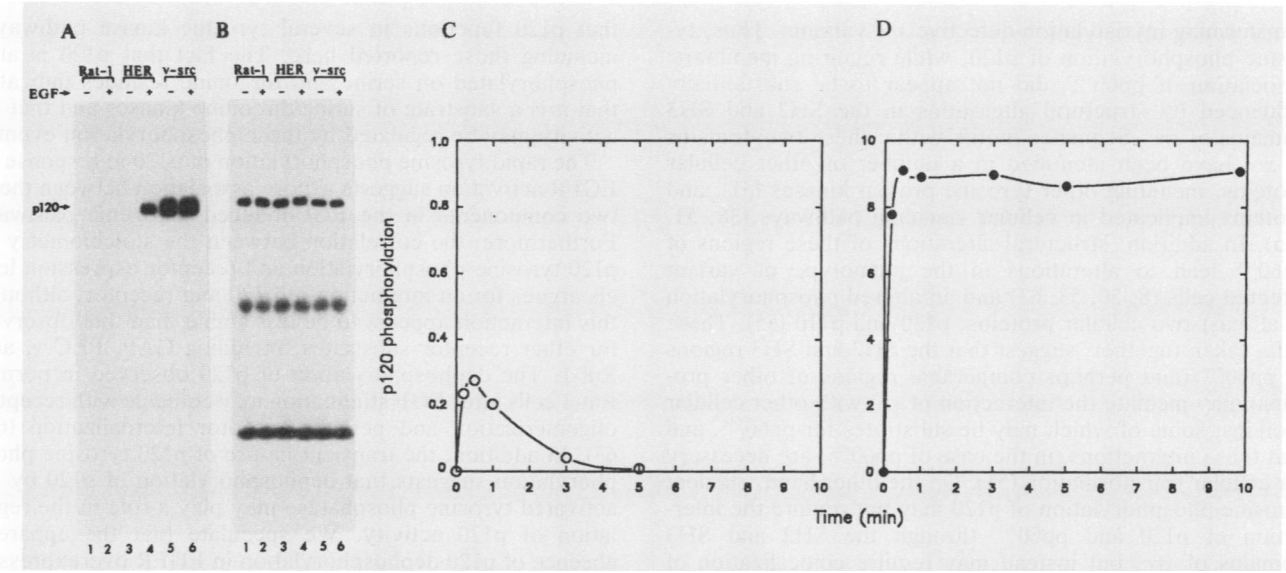

FIG. 4. EGF stimulation of p120 tyrosine phosphorylation. (A) Anti-pTyr immunoblot analysis of p120 immunoprecipitated from extracts of Rat-1 cells treated with EGF. Rat-1 (lanes 1 and 2), rat-HER (lanes 3 and 4), and Rat-1/v-*src* (lanes 5 and 6) cells were serum starved for 24 h and stimulated (+) or not stimulated (−) for 5 min with 200 ng of EGF per ml in Dulbecco modified Eagle medium supplemented with 0.1% bovine serum albumin as described in Materials and Methods. Immunoprecipitates of p120 were prepared as described for Fig. 1 and immunoblotted with anti-pTyr. (B) Levels of p120 in Rat-1, rat-HER, and Rat-1/v-*src* cells. An immunoblot identical to that shown in panel A was probed with MAb 2B12. (C) Time course of p120 tyrosine phosphorylation in Rat-1 cells. Cells were stimulated with EGF as described above for the indicated time points, and p120 immunoprecipitates were prepared and immunoblotted with anti-pTyr. The autoradiographs (multiple exposures) were analyzed by densitometry (Bio-Rad 620 video analyzer), the peaks were integrated, and the levels of p120 phosphorylation were expressed in arbitrary units. (D) Time course of p120 tyrosine phosphorylation in rat-HER cells, performed as described for panel C (note difference in scale).

cell lines. Rat-1 cells contain approximately 40,000 rat EGFR molecules, and rat-HER cells express greater than 500,000 human EGFR molecules (63a). The activation of these receptors by the addition of EGF to growth media was readily demonstrated by autophosphorylation of the receptors, detected by anti-pTyr immunoblotting (data not shown).

Stimulation of EGFR, PDGFR, and INSR with their respective ligands results in receptor autophosphorylation (28, 39, 59) and the tyrosine phosphorylation of cellular protein substrates (59). Stimulation of rodent cells overexpressing EGFR, PDGFR, or INSR with the respective growth factor for 5 min demonstrated that both EGF and PDGF, but not insulin, induced the tyrosine phosphorylation of p120 (Fig. 5). For cells stimulated with EGF and PDGF, analysis of p120 tyrosine phosphorylation at early and late time points revealed kinetics of stimulation similar to those in Fig. 4D. The significance of the difference in the level of p120 tyrosine phosphorylation between EGF and PDGF stimulation is unclear. Singh et al. (58) have reported that BALB/3T3 cells have 100,000 to 200,000 PDGFR molecules per cell. Hence, the differences in p120 tyrosine phosphorylation observed in response to EGF and PDGF may reflect differences in receptor number or intrinsic receptor kinase activity with respect to p120. The inability to detect tyrosine phosphorylation of p120 in response to insulin stimulation suggests that p120, like PLC-γ (47), may not be an efficient substrate of the INSR.

DISCUSSION

The experiments described in this report show that a myristylated 120-kDa membrane-associated tyrosine-phosphorylated protein, p120, is a substrate of pp60^{src}, mT-activated tyrosine kinases and is a tyrosine-phosphorylated component of at least two receptor-activated signaling pathways. Although tyrosine phosphorylation of p120 was mediated by both transforming and nontransforming variants of pp60^{src} which were membrane associated, p120 tyrosine phosphorylation was not observed in cells expressing non-

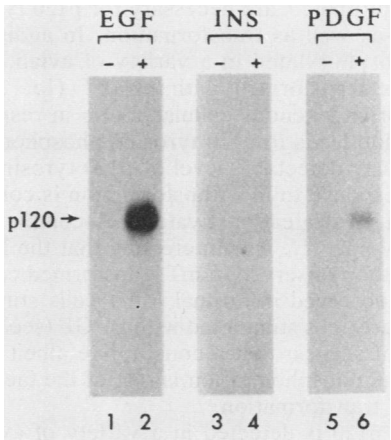

FIG. 5. Tyrosine phosphorylation of p120 by ligand activation of receptor PTKs. Rat-HER cells overexpressing the human EGFR (lanes 1 and 2) were serum starved for 4 h and either untreated (−) or treated (+) with 200 ng of EGF per ml for 5 min. Rat-1 cells overexpressing the human INSR, HIRc-B (lanes 3 and 4), were untreated (−) or treated (+) with 100 ng of insulin (INS) per ml for 5 min. BALB/3T3 cells (lanes 5 and 6) were untreated (−) or treated (+) with 40 ng of PDGF per ml for 5 min. Immunoprecipitates of p120 were prepared, subjected to SDS-PAGE, and immunoblotted with anti-pTyr as described in Materials and Methods.

transforming myristylation-defective *src* variants. Thus, tyrosine phosphorylation of p120, while requiring membrane association of pp60^{src}, did not appear to be substantially influenced by structural alterations in the SH2 and SH3 domains of *src*. Sequence motifs within these two domains of *src* have been identified in a number of other cellular proteins, including other tyrosine protein kinases (51), and proteins implicated in cellular signaling pathways (38, 51, 58a). In addition, structural alterations of these regions of pp60^{src} lead to alterations in the phenotype of variant infected cells (8, 20, 53, 63) and an altered phosphorylation of at least two cellular proteins, p130 and p110 (55). These data, taken together, suggest that the SH2 and SH3 regions of pp60^{src} (and perhaps comparable regions of other proteins) may mediate the interaction of *src* with other cellular proteins, some of which may be substrates for pp60^{src}, and that these interactions (in the case of pp60^{src}) are necessary for cellular transformation (51). On the other hand, efficient tyrosine phosphorylation of p120 may not require the interaction of p120 and pp60^{src} through the SH2 and SH3 domains of *src*, but instead may require colocalization of p120 and pp60^{src} via an alternative pathway. For example, orientation of the myristylated forms of p120 and pp60^{src} may require their association with a common membrane-bound myristylation receptor protein(s) similar to that described recently by Resh and Ling (54) for pp60^{src}. Transport to the membrane and stable association with membrane structures, including the myristyl receptor proteins, may represent an important aspect of the orientation of the *src* kinase and the p120 substrate. In this regard, substrates of pp60^{src} that appear to interact with *src* via the SH2 and SH3 domains (p110, p130 [55], GAP, and the GAP-associated p64 [1a, 11, 41, 48]) are cytosolic proteins. Thus, we would suggest that the association of pp60^{src} with membrane-associated and cytosolic substrates may be mediated by different mechanisms.

Several observations argue that tyrosine phosphorylation of p120 may be one of several events needed to initiate and maintain the transformed phenotype in *src*-expressing cells. As shown here, the enzymatic activation and membrane association of pp60^{src} are necessary for p120 tyrosine phosphorylation as well as transformation. In addition, p120 is tyrosine phosphorylated in a variety of avian, rodent, and human cells transformed with pp60^{src} (1a, 25). Finally, activation of endogenous cellular PTKs in response to mT transformation leads to p120 tyrosine phosphorylation. The low but clearly detectable level of p120 tyrosine phosphorylation in response to mT transformation is consistent with p120 being a substrate for activated, mT-complexed pp60$^{c\text{-}src}$ and possibly p62$^{c\text{-}yes}$. It is interesting that the level of p120 phosphorylation observed in mT-transformed cells was similar to that observed in normal Rat-1 cells stimulated with EGF or mouse cells stimulated with PDGF (see below). This finding might suggest that a constitutive albeit low level of p120 tyrosine phosphorylation is one of the factors contributing to mT transformation.

Although p120 is detected in a variety of avian, rodent, and human fibroblasts (1a, 25), recently we have observed that p120 is also present in several hematopoietic cell lines of B-cell and erythroid cell lineage (21a) as well as in developing neural tissue (14a). The steady-state level of p120 in CE cells was estimated from metabolic labeling experiments to be approximately 0.02 to 0.05% of total cellular protein, or about 300,000 to 700,000 molecules per cell. The abundance of p120 relative to the number of endogenous receptor and *src*-like tyrosine kinases is consistent with the possibility that p120 functions in several tyrosine kinase pathways, including those reported here. The fact that p120 is also phosphorylated on serine and threonine residues indicates that it is a substrate of serine/threonine kinases and that its activity may be regulated by these phosphorylation events.

The rapid tyrosine phosphorylation of p120 in response to EGFR activation suggests a close association between these two components in the EGF-induced mitogenic pathway. Furthermore, the correlation between the stoichiometry of p120 tyrosine phosphorylation and receptor expression levels argues for an interaction of p120 and receptor, although this interaction appears to be less stable than that observed for other receptor substrates, including GAP, PLC-γ, and Raf-1. The dephosphorylation of p120 observed in normal Rat-1 cells after EGF stimulation may coincide with receptor oligomerization and perhaps receptor internalization (64, 65). In addition, the transient nature of p120 tyrosine phosphorylation suggests that dephosphorylation of p120 by an activated tyrosine phosphatase may play a role in the regulation of p120 activity. We speculate that the apparent absence of p120 dephosphorylation in EGFR overexpressor cells may reflect reduced receptor internalization due to increased receptor load (64, 65) and consequent failure to activate the prerequisite tyrosine phosphatases. Alternatively, the steady-state tyrosine phosphorylation of p120 may relate to the capacity of EGFR overexpressor cells to constitutively phosphorylate p120 in response to the continuous presence of ligand. Interestingly, such EGFR overexpressor cells are capable of undergoing mitogen-induced transformation (9, 60). The ultimate identity of p120 will undoubtedly lead to a better understanding of the relationship between its tyrosine phosphorylation and its role in signaling and transformation pathways.

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REFERENCES

1. **Bolen, J. B., V. DeSeau, J. O'Shaughnessy, and S. Amini.** 1987. Analysis of middle tumor antigen and pp60$^{c\text{-}src}$ interactions in polyomavirus-transformed rat cells. J. Virol. **61**:3299–3305.

1a. **Bouton, A. H., S. B. Kanner, R. R. Vines, and J. T. Parsons.** Submitted for publication.

2. **Buss, J. E., and B. M. Sefton.** 1985. Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. J. Virol. **53**:7–12.

3. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.

4. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckert.** 1986. Altered sites of tyrosine phosphorylation in pp60$^{c\text{-}src}$ associated with the polyomavirus middle tumor antigen. Mol. Cell. Biol. **6**:1562–1570.

5. **Cheng, S. H., R. Harvey, P. C. Espino, K. Semba, T. Yanomoto, K. Toyoshima, and A. E. Smith.** 1988. Peptide antibodies to the human c-*fyn* gene product demonstrate pp59$^{c\text{-}fyn}$ is capable of complex formation with the middle-T antigen of polyomavirus. EMBO J. **7**:3845–3855.

6. **Cooper, J. A.** 1990. The *src* family of protein-tyrosine kinases,

p. 85–113. *In* B. E. Kemp (ed.), Peptides and protein phosphorylation. CRC Press, Inc., Boca Raton, Fla.
7. **Courtneidge, S., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-*src* cellular gene. Nature (London) **303**:435–439.
8. **DeClue, J. E., and G. S. Martin.** 1989. Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. J. Virol. **63**:542–554.
9. **Difiori, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger, and S. A. Aaronson.** 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Cell **51**:1063–1070.
10. **Draetta, G., H. Piwnica-Worms, D. Morrison, B. Druker, T. Roberts, and D. Bach.** 1988. Human cdc2 protein kinase is a major cell-cycle regulated tyrosine kinase substrate. Nature (London) **36**:738–744.
11. **Ellis, C., M. Moran, F. McCormick, and T. Pawson.** 1990. Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature (London) **343**: 377–381.
12. **Ely, C. M., K. M. Oddie, J. S. Litz, A. J. Rossomando, S. B. Kanner, T. W. Sturgill, and S. J. Parsons.** 1990. A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen-stimulated protein in fibroblasts. J. Cell Biol. **110**:731–742.
13. **Ferrell, J. E., and G. S. Martin.** 1988. Platelet tyrosine-specific protein phosphorylations regulated by thrombin. Mol. Cell. Biol. **8**:3603–3610.
14. **Ferrell, J. E., and G. S. Martin.** 1989. Tyrosine-specific protein phosphorylation is regulated by glycoprotein IIb-IIIa in platelets. Proc. Natl. Acad. Sci. USA **86**:2234–2238.
14a. **Flynn, D.** Unpublished data.
15. **Glenney, J. R.** 1989. Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. J. Biol. Chem. **264**:20163–20166.
16. **Glenney, J. R., and L. Zokas.** 1989. Novel tyrosine substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. J. Cell Biol. **108**:2401–2408.
17. **Glenney, J. R., L. Zokas, and M. P. Kamps.** 1988. Monoclonal antibodies to phosphotyrosine. J. Immunol. Methods **109**:277–285.
18. **Golden, A., and J. S. Brugge.** 1989. Thrombin treatment induces rapid changes in tyrosine phosphorylation in platelets. Proc. Natl. Acad. Sci. USA **86**:901–905.
19. **Hamaguchi, M., C. Grandori, and H. Hanafusa.** 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis with antiphosphotyrosine antibodies. Mol. Cell. Biol. **8**:3035–3042.
20. **Hirai, J., and H. E. Varmus.** 1990. Site-directed mutagenesis of the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of $p60^{c\text{-}src}$. Mol. Cell. Biol. **10**:1307–1318.
21. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ras*, and v-*erb* B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2**:305–315.
21a. **Kanner, S. B.** Unpublished data.
22. **Kanner, S. B., T. M. Gilmer, A. B. Reynolds, and J. T. Parsons.** 1989. Novel tyrosine phosphorylations accompany the activation of $pp60^{c\text{-}src}$ during chemical carcinogenesis. Oncogene **4**:295–300.
23. **Kanner, S. B., S. J. Parsons, J. T. Parsons, and T. M. Gilmer.** 1988. Activation of $pp60^{c\text{-}src}$ tyrosine kinase specific activity in tumor-derived Syrian hamster embryo cells. Oncogene **2**:327–335.
24. **Kanner, S. B., A. B. Reynolds, and J. T. Parsons.** 1989. Immunoaffinity purification of tyrosine-phosphorylated cellular proteins. J. Immunol. Methods **120**:115–124.
25. **Kanner, S. B., A. B. Reynolds, R. R. Vines, and J. T. Parsons.** 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc. Natl. Acad. Sci. USA **87**:3328–3332.
26. **Kaplan, D. R., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF β-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signalling complex. Cell **61**:125–133.
27. **Kato, J., Y. Hirota, N. Nakamura, N. Nakamura, and T. Takeya.** 1987. Structural features of the carboxy terminus of $p60^{src}$ that are required for the regulation of its intrinsic kinase activity. Jpn. J. Cancer Res. **78**:1354–1362.
28. **Kazlaukas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58**:1121–1133.
29. **Kazlaukas, A., C. Ellis, T. Pawson, and J. A. Cooper.** 1990. Binding of GAP to activated PDGF receptors. Science **247**: 1578–1581.
30. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary site of tyrosine phosphorylation. Cell **49**:65–73.
31. **Kornbluth, S., M. Sudol, and H. Hanafusa.** 1987. Association of the polyomavirus middle-T antigen with c-*yes* protein. Nature (London) **325**:171–173.
32. **Kumjian, D. A., M. I. Wahl, S. G. Rhee, and T. O. Daniel.** 1989. Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C. Proc. Natl. Acad. Sci. USA **86**:8232–8236.
33. **Kypta, R. M., A. Hemming, and S. A. Courtneidge.** 1988. Identification and characterization of p59*fyn* (a *src*-like protein tyrosine kinase) in normal and polyoma virus transformed cells. EMBO J. **7**:3837–3844.
34. **Linder, M. E., and J. G. Burr.** 1988. Nonmyristoylated $p60^{v\text{-}src}$ fails to phosphorylate proteins of 115-120 kDa in chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **85**:2608–2612.
35. **Luttrell, D. K., L. M. Luttrell, and S. J. Parsons.** 1988. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress $pp60^{c\text{-}src}$. Mol. Cell. Biol. **8**:497–501.
36. **Margolis, B., F. Bellot, A. M. Honegger, A. Ullrich, J. Schlessinger, and A. Zilberstein.** 1990. Tyrosine kinase activity is essential for the association of phospholipase C-γ with epidermal growth factor receptor. Mol. Cell. Biol. **10**:435–441.
37. **Margolis, B., S. G. Rhee, S. Felder, M. Mervic, R. R. Lyall, A. Levitski, A. Ullrich, A. Zilberstein, and J. Schlessinger.** 1989. EGF induces phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signalling. Cell **57**:1101–1107.
38. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332**:272–275.
39. **McClain, D. A., H. Maegawa, J. Levy, T. Hueckstadt, T. J. Dull, J. Lee, A. Ullrich, and J. M. Olefsky.** 1988. Properties of a human insulin receptor with a COOH-terminal truncation. J. Biol. Chem. **263**:8904–8911.
40. **Meisenhelder, J., P.-G. Suh, S. G. Rhee, and T. Hunter.** 1989. Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. Cell **57**:1109–1122.
41. **Molloy, C. J., D. P. Bottaro, T. P. Fleming, M. S. Marshall, J. B. Gibbs, and S. A. Aaronson.** 1989. PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature (London) **342**:711–714.
42. **Morla, A. O., and J. Y. J. Wang.** 1986. Protein tyrosine phosphorylation in the cell cycle of BALB/c 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA **83**:8191–8195.
43. **Morrison, D. K., P. J. Browning, M. F. White, and T. M. Roberts.** 1988. Tyrosine phosphorylations in vivo associated with v-*fms* transformation. Mol. Cell. Biol. **8**:176–185.
44. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF β-receptor. Cell **58**:649–657.
45. **Morrison, D. K., D. R. Kaplan, U. R. Rapp, and T. M. Roberts.** 1988. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. USA **85**:8855–8859.

46. **Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams.** 1990. Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with the PDGF receptor signaling complex. Mol. Cell. Biol. **10**:2359–2366.
47. **Nishibe, S., M. I. Wahl, P. B. Wedegaertener, J. J. Kim, S. G. Rhee, and G. Carpenter.** 1990. Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains. Proc. Natl. Acad. Sci. USA **87**:424–428.
48. **Parsons, J. T.** 1990. Closing the GAP in a signal transduction pathway. Trends Genet. **6**:169–171.
49. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{src} react with enzymatically active cellular pp60^{src} of avian and mammalian origin. J. Virol. **51**:272–282.
50. **Parsons, J. T., and M. J. Weber.** 1989. Genetics of *src*: structure and function of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. **147**:79–127.
51. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene **3**:491–495.
52. **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.
53. **Potts, W. M., A. B. Reynolds, T. J. Lansing, and J. T. Parsons.** 1988. Activation of pp60$^{c\text{-}src}$ transforming potential by mutations altering the structure of an amino terminal domain containing residues 90–95. Oncogene Res. **3**:343–355.
54. **Resh, M. D., and H.-P. Ling.** 1990. Identification of a 32K plasma membrane protein that binds to the myristylated amino-terminal sequence of p60$^{v\text{-}src}$. Nature (London) **346**:84–86.
55. **Reynolds, A. B., S. B. Kanner, H.-C. R. Wang, and J. T. Parsons.** 1989. Stable association of activated pp60^{src} with two tyrosine phosphorylated cellular proteins. Mol. Cell. Biol. **9**:3951–3958.
56. **Reynolds, A. B., D. J. Roesel, S. B. Kanner, and J. T. Parsons.** 1989. Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. Mol. Cell. Biol. **9**:629–638.
57. **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.
58. **Singh, J. P., M. A. Chaikin, and C. D. Stiles.** 1982. Phylogenetic analysis of platelet-derived growth factor by radio-receptor assay. J. Cell Biol. **95**:667–671.
58a. **Stahl, M. L., C. R. Ferenz, K. L. Kelleher, R. W. Kriz, and J. L. Knopf.** 1988. Sequence similarity of phospholipase C with the non-catalytic region of *src*. Nature (London) **332**:269–275.
59. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61**:203–212.
60. **Velu, T. J., L. Bequinot, W. C. Vass, M. C. Willingham, G. T. Merlino, I. Pastan, and D. R. Lowy.** 1987. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science **238**:1408–1410.
61. **Wahl, M. I., S. Nishibe, P.-G. Suh, S. G. Rhee, and G. Carpenter.** 1989. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc. Natl. Acad. Sci. USA **86**:1568–1572.
62. **Wahl, M. I., N. E. Olashaw, S. Nishibe, S. G. Rhee, W. J. Pledger, and G. Carpenter.** 1989. Platelet-derived growth factor induces rapid and sustained tyrosine phosphorylation of phospholipase C-γ in quiescent BALB/c 3T3 cells. Mol. Cell. Biol. **7**:2934–2943.
63. **Wang, H.-C. R., and J. T. Parsons.** 1989. Deletions and insertions within an amino-terminal domain of pp60$^{v\text{-}src}$ inactivate transformation and modulate membrane stability. J. Virol. **63**: 291–302.
63a. **Wasilenko, W. J., D. M. Payne, D. L. Fitzgerald, and M. J. Weber.** 1991. Phosphorylation and activation of epidermal growth factor receptors in cells transformed by the *src* oncogene. Mol. Cell. Biol. **11**:309–321.
64. **Wells, A., J. B. Welsh, C. S. Lazar, H. S. Wiley, G. N. Gill, and M. G. Rosenfeld.** 1990. Ligand-induced transformation by a noninternalizating epidermal growth factor receptor. Science **247**:962–964.
65. **White, M. F., J. M. Livingston, J. M. Backer, V. Lauris, T. J. Dull, A. Ullrich, and C. R. Kahn.** 1988. Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. Cell **54**:641–649.