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Phosphorylation and Activation of Epidermal Growth Factor Receptors in Cells Transformed by the *src* Oncogene

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Because functionally significant substrates for the tyrosyl protein kinase activity of pp60$^{v\text{-}src}$ are likely to include membrane-associated proteins involved in normal growth control, we have tested the hypothesis that pp60$^{v\text{-}src}$ could phosphorylate and alter the signaling activity of transmembrane growth factor receptors. We have found that the epidermal growth factor (EGF) receptor becomes constitutively phosphorylated on tyrosine in cells transformed by the *src* oncogene and in addition displays elevated levels of phosphoserine and phosphothreonine. High-performance liquid chromatography phosphopeptide mapping revealed two predominant sites of tyrosine phosphorylation, both of which differed from the major sites of receptor autophosphorylation; thus, the *src*-induced phosphorylation is unlikely to occur via an autocrine mechanism. To determine whether pp60$^{v\text{-}src}$ altered the signaling activity of the EGF receptor, we analyzed the tyrosine phosphorylation of phospholipase C-γ, since phosphorylation of this enzyme occurs in response to activation of the EGF receptor but not in response to pp60$^{v\text{-}src}$ alone. We found that in cells coexpressing pp60$^{v\text{-}src}$ and the EGF receptor, phospholipase C-γ was constitutively phosphorylated, a result we interpret as indicating that the signaling activity of the EGF receptor was altered in the *src*-transformed cells. These findings suggest that pp60$^{v\text{-}src}$-induced alterations in phosphorylation and function of growth regulatory receptors could play an important role in generating the phenotypic changes associated with malignant transformation.

The v-*src* oncogene product, pp60$^{v\text{-}src}$, is a 60-kDa tyrosyl protein kinase, and this enzymatic activity is essential to its ability to cause malignant transformation (52). Numerous cellular proteins become phosphorylated on tyrosine in *src*-transformed cells (9, 34, 35, 45), but the mechanism(s) by which these *src*-induced phosphorylations alter cellular behavior is unknown. Studies utilizing mutant forms of pp60$^{v\text{-}src}$ which retain kinase activity but are partially defective in their ability to cause phenotypic transformation suggest that only a limited subset of these tyrosine-phosphorylated proteins play an essential role in cellular transformation by *src* (10, 33, 35, 39, 57). It is widely suspected that the physiologically important substrates for pp60$^{v\text{-}src}$ will prove to be proteins which play a role in normal growth regulation, although it has been difficult to identify pp60$^{v\text{-}src}$-induced changes in phosphorylation and function of such regulatory proteins. It also is generally believed that at least some critical substrates for pp60$^{v\text{-}src}$ are associated with cell membranes, since cytosolic variants of pp60$^{v\text{-}src}$ are unable to transform cells (33, 39, 52, 57).

The best-characterized membrane-associated growth regulatory proteins are the transmembrane receptors for growth factors, and we have hypothesized (49) that pp60$^{v\text{-}src}$ might alter cellular regulation by phosphorylating and activating growth factor receptors. Indeed, we recently found that a 95-kDa cellular glycoprotein (49) whose tyrosine phosphorylation correlated tightly with phenotypic transformation in cells infected with various *src* mutants (35) is the β subunit of the receptor for insulinlike growth factor I (IGF-I) (36). Although neither the sites of phosphorylation nor consequences of this phosphorylation for intracellular signaling could be identified with the available reagents, this finding supports the hypothesis that *src*-induced changes in receptor phosphorylation and function might underlie some of the biological effects of *src* expression.

Because molecular and immunological tools to study the epidermal growth factor (EGF) receptor are widely available, we have been able to study in considerable detail the effects of pp60$^{v\text{-}src}$ on EGF receptor phosphorylation and function as a model for the effects of this oncogene product on transmembrane receptors generally. Like other tyrosine kinase receptors, the EGF receptor is subject to both trans- and autophosphorylation, and at least some of these phosphorylations have known regulatory consequences (4, 6, 14, 25, 28, 61, 63). This receptor displays three prominent sites of autophosphorylation, termed P1, P2, and P3, which correspond respectively to tyrosines 1173, 1148, and 1068 (18). Recently a fourth site of tyrosine phosphorylation at position 1086 has been described (29, 44), and certain mutated forms of the receptor reveal phosphorylation at tyrosine 992 (70). The major sites of autophosphorylation appear to be important for regulation of the receptor's interaction with exogenous substrates (2, 26, 27, 54, 61, 66). In addition, the EGF receptor is phosphorylated at threonine 654 by protein kinase C, a phosphorylation which inhibits receptor kinase activity (8, 14, 15, 30, 38, 40). Threonine 669 represents the major constitutive phosphorylation of the receptor, and this phosphorylation (along with that of serine 671) may be associated with specific substrate phosphorylations and with endocytosis (13, 24, 25). Phosphorylations also have been reported at serines 1046 and 1047 (12, 24), although the functional significance of these phosphorylations remains uncertain. Thus, the EGF receptor is multiply phosphorylated in vivo on tyrosine, threonine, and serine residues, and at least some of these phosphorylations play important roles in the regulation of receptor activity.

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In this study, we investigated whether expression of the $pp60^{v\text{-}src}$ tyrosine kinase affects the in vivo phosphorylation state and function of EGF receptors. For this work, we made use of a rat fibroblast cell line which contains a temperature-sensitive *src* kinase mutant (74), allowing us to turn the *src* kinase on and off rapidly by altering the temperature. These cells were infected with a retrovirus vector expressing a full-length cDNA encoding functional human EGF (hEGF) receptors (65, 66), allowing us to use human-specific immunological reagents for the EGF receptor and to obtain sufficient amounts of receptor for biochemical analysis. We found that expression of $pp60^{v\text{-}src}$ kinase rapidly induced substantial increases in the basal phosphorylation level of EGF receptors. Interestingly, the increases were found on all three phosphoamino acids: serine, threonine, and, most dramatically, tyrosine. High-performance liquid chromatography (HPLC) analysis of the tryptic phosphopeptides revealed that two predominant sites in the EGF receptor became tyrosine phosphorylated in *src*-transformed cells and that neither corresponded to the major sites of receptor autophosphorylation. To determine whether EGF receptor function was altered in the *src*-transformed cells, we asked whether phospholipase C-γ (PLC-γ) became tyrosine phosphorylated in cells coexpressing $pp60^{v\text{-}src}$ and the EGF receptor, since this tyrosine phosphorylation can be induced in response to EGF (43, 48, 67, 68) but does not occur when cells express only $pp60^{v\text{-}src}$. We found that PLC-γ was constitutively phosphorylated in cells coexpressing $pp60^{v\text{-}src}$ and the EGF receptor even in the absence of EGF, indicating that *src* expression induces the EGF receptor to utilize this signaling pathway constitutively. These findings support our hypothesis that *src* can affect intracellular signaling through the phosphorylation and activation of mitogen receptors.

MATERIALS AND METHODS

Materials. $^{32}P_i$ was from Dupont New England Nuclear (Boston, Mass.), receptor-grade EGF and ^{125}I-EGF were from Collaborative Research (Bedford, Mass.), and ^{125}I-protein A was from Amersham (Arlington Heights, Ill.). The hEGF receptor-specific monoclonal antibody used in immunoprecipitation experiments, which we refer to as OS1, was purchased as Ab 1 from Oncogene Science (Manhasset, N.Y.), and the antibody for fluorescence-activated cell sorting experiments, referred to here as R1, was from Amersham. The anti-EGF receptor peptide antiserum used to probe Western immunoblots, designated CR, was supplied by Cambridge Research Biochemicals (Wilmington, Del.). Antiserum against phosphotyrosine was prepared essentially by the method of Kamps and Sefton (33) as described by Reynolds et al. (58). Immunoprecipitation of PLC-γ was performed with a rabbit antiserum against a PLC-γ–TrpE fusion protein, obtained from Tony Pawson (University of Toronto), and Western blotting of PLC-γ was done with a monoclonal antibody pool obtained from Sue Goo Rhee (National Institutes of Health). Pansorbin was from Calbiochem (La Jolla, Calif.). Cell culture media and fetal bovine serum were from Hazleton (Lenexa, Kans.) or GIBCO (Grand Island, N.Y.). HPLC solvents were HPLC grade, and all buffers and salts were reagent grade. Other products were purchased from the suppliers noted below.

Cell culture. The cells studied were Rat-1 (hereafter referred to as R1) fibroblasts or derivatives thereof and were routinely maintained in Dulbecco modified Eagle medium plus 10% fetal bovine serum under 7.5% CO_2–92.5% air at 35 to 37°C unless specified differently. Growth of cells at restrictive temperature was at 39.3 to 39.5°C (hereafter referred to as 39°C). Details on the development and properties of R1/LA29, an R1 derivative cell line infected with a temperature-conditional *src* mutant (LA29) (53, 74), have been described (71). All experiments were performed by using cell cultures at or near confluency.

Expression of hEGF receptors in rat fibroblasts. R1 or R1/LA29 cells were infected with an amphotropic retrovirus expressing a full-length hEGF receptor cDNA under the control of the murine leukemia virus long terminal repeat (65, 66). Cells were cloned from the infected population by limiting dilution and screened for ^{125}I-EGF binding to the cell surface as previously described (72). They were further screened by morphological transformation in response to overnight treatment with EGF (50 ng/ml) and by incorporation of $^{32}P_i$ into receptor by metabolic labeling (assayed by immunoprecipitation). The R1-derived clone used in this study is termed R1/hER (for Rat-1 with hEGF receptor), and the R1/LA29 derivative is termed LA29/hER.

To measure internalization rates of the EGF receptor, cells were incubated with ^{125}I-EGF (72) for various lengths of time and then placed on ice; the surface ligand was stripped with 0.2 M acetic acid (pH 2.5)–0.5 M NaCl. Cells were then rinsed with phosphate-buffered saline (PBS) and solubilized for determination of internalized radioactivity.

Flow cytometric analysis. Nearly confluent cell cultures were incubated overnight in growth medium with 0.5% fetal bovine serum. Cells were then released from the culture dish with PBS containing 10^{-3} M EDTA and washed twice in saline; approximately 10^6 cells were suspended at 4°C in 100 μl of PBS plus 0.1% bovine serum albumin (BSA) and 5 μl (0.5 μg) of R1 antibody (which reacts with the extracellular domain of the EGF receptor but does not compete with EGF for binding). In some cases, cells were first incubated for 15 min with EGF before release from the dish. After a 30-min incubation with primary antibody, cell suspensions were washed three times in PBS and BSA, resuspended in 100 μl of PBS with a dilution of fluorescein-conjugated goat anti-mouse immunoglobulin G (12.5 μg in 5 μl; Jackson Immunoresearch, Bar Harbor, Maine), and incubated for 30 min. After a final series of washes, the relative fluorescence intensity was determined with an EPICS flow cytometer.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were done essentially as described previously (35, 36). For the quantitative immunoblotting shown in Fig. 1, various amounts of cell lysate up to 500 μg of protein were immunoprecipitated with excess OS1 antibody (2 μg) and Pansorbin (60 μl of a 10% suspension). The immunoprecipitates were electrophoresed on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted to nitrocellulose, and the transferred receptor was detected with the CR antipeptide antiserum at a 1:200 dilution followed by ^{125}I-protein A (1 μCi/ml). The radioactive receptor bands were localized by autoradiography and excised from the blot, and the amounts of receptor were quantified by using a gamma counter.

Preparation of ^{32}P-labeled EGF receptor. For metabolic labeling, cells were incubated for 12 to 14 h with 1 to 3 mCi of $^{32}P_i$ per ml in 90% phosphate-free Dulbecco modified Eagle medium plus 10% conditioned medium. Cultures designated +EGF were incubated for 2 min at room temperature with EGF at a final concentration of 500 ng/ml in the labeling medium immediately prior to lysis. Plates were rinsed once with ice-cold PBS, lysed in RIPA buffer (36), and immunoprecipitated with the OS1 antibody (see above).

Pellets were washed twice with RIPA containing 1 M urea, twice with RIPA containing 1 M NaCl, once with RIPA, and once with PBS.

^{32}P-autophosphorylation samples were prepared by lysing cells in HO buffer (RIPA without SDS), and hEGF receptor was precipitated with OS1 and Pansorbin. pp60$^{v\text{-}src}$ was immunoprecipitated with the EC10 anti-pp60 monoclonal antibody (53). Pellets were washed twice with HO buffer made to 1 M in NaCl, twice with HO, and once with PBS. The pellets were resuspended in 35 μl of a reaction mixture containing 20 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), 50 μM Na_3VO_4, 5 mM $MnCl_2$, 2 mM $MgCl_2$, and 10 μCi of carrier-free [γ-^{32}p]ATP (3,000 to 6,000 Ci/mmol; New England Nuclear) and incubated on ice for 30 to 45 min.

For phosphoamino acid analysis, hEGF receptor was extracted from pulverized gel slices with 0.05% trifluoroacetic acid (TFA) for 1 h at room temperature or was blotted onto an Immobilon-P membrane (Millipore). Phosphoamino acid analyses were done essentially as described previously (34, 45).

HPLC phosphopeptide analysis. The immunoprecipitated EGF receptor was stripped from the Pansorbin, acetylated by an adaptation of the procedure of Davis and Czech (15), and then purified on a 7.5% SDS-polyacrylamide gel. Only a single phosphorylated band was detectable by autoradiography of the wet, unfixed gel; this band was excised, washed in water, and digested with approximately 10 volumes of 50 mM NH_4HCO_3 containing 7.5 μg of tolylsulfonyl phenylalanyl chloromethyl ketone trypsin per ml at 37°C. After 6 to 12 h, a second addition of trypsin was made, and the incubation was continued for a total of approximately 24 h. The digestion solution was removed from the gel slices, the slices were extracted for an additional 8 h at room temperature with 50 mM NH_4HCO_3, and the extracts were combined. Greater than 95% of the radioactivity (usually >300,000 Cerenkov cpm) was released from the gel pieces by this treatment. The extracted peptides were lyophilized nearly to dryness in a Speed-Vac (Savant), redissolved in water, and lyophilized again. The sample was dissolved in 200 to 250 μl of 0.05% TFA, residual insoluble material was sedimented by centrifugation (>98% of the radioactivity remained in the supernatant), and peptides were resolved by reverse-phase HPLC, using a Vydac C_{18} column (4.6 × 250 mm) and gradient systems similar to those described previously (14, 15, 24). For separations using the low-pH system, the TFA concentration was reduced to 0.05% (vol/vol) and the slope of the acetonitrile gradient was reduced to 0.6%/min (between 5 and 45 min) to achieve the necessary resolution. Recovery of radioactivity was 80 to 95% for all analyses.

RESULTS

Effects of pp60$^{v\text{-}src}$ expression on EGF receptor phosphorylation in R1 cells. R1/LA29 cells are Rat-1 cells expressing a *src* gene mutant (*ts*LA29) that encodes a temperature-sensitive pp60$^{v\text{-}src}$ tyrosine kinase (74). These cells are phenotypically normal when cultured at the restrictive temperature for temperature-sensitive pp60$^{v\text{-}src}$ kinase activity (39°C) but become fully transformed when pp60$^{v\text{-}src}$ is activated by incubation at the permissive temperature (35°C). To determine whether pp60$^{v\text{-}src}$ activity had an effect on the phosphorylation state of EGF receptors, at various times following shift from restrictive to permissive temperature, endogenous EGF receptors were immunoprecipitated from R1/LA29 cells labeled with $^{32}P_i$. We observed a rapid *src*-induced increase in alkali-stable phosphorylation of the EGF receptor (data not shown). However, the low level of endogenous EGF receptors in these cells and the subsequent down-modulation of receptor gene expression after pp60$^{v\text{-}src}$ activation (71, 72) made it difficult to further characterize these phosphorylation changes. Therefore, we constructed R1 and R1/LA29 derivatives overexpressing the hEGF receptor.

Overexpression of hEGF receptors in R1 cells. R1 and R1/LA29 cells were infected with a recombinant murine amphotropic retrovirus containing a full-length cDNA encoding the hEGF receptor under the transcriptional control of the murine leukemia virus long terminal repeat (65, 66). After infection, clonal cell populations were obtained, and several that overexpressed functional hEGF receptors were identified by virtue of their ability to display elevated EGF binding and undergo changes in cell morphology after EGF treatment. Interestingly, none of the EGF receptor-overexpressing clones that we obtained from the R1/LA29 cells (almost a dozen in all) contained levels of EGF receptors as high as those derived from the normal R1 cells. Although we have not performed an extensive analysis of this phenomenon, it is possible that *src* expression limits the levels of EGF receptor expression that can be obtained.

Two overexpressor clones were selected for further study because of their relatively high EGF receptor levels and morphological responsiveness to EGF: R1/hER, derived from R1 cells, and LA29/hER, derived from R1/LA29 cells. On the basis of a modified Scatchard (60) analysis of ^{125}I-EGF binding (31), we estimate that the LA29/hER cells possess 50,000 to 75,000 hEGF receptors per cell and that the R1/hER cells possess five times that number (data not shown). The same estimate of relative EGF receptor expression in these cells was obtained by quantitative Western blot analysis (Fig. 1). The number of total receptors per cell, as determined by Western blotting, was not altered by growth of cells at either the restrictive or permissive temperature (Fig. 1).

The morphologies of LA29/hER cells under various conditions are shown in Fig. 2. These cells were phenotypically flat when incubated at the restrictive temperature (39°C) for pp60$^{v\text{-}src}$ activity but could be morphologically transformed either by incubation at the permissive temperature (35°C) or by addition of EGF. Morphological transformation could be induced by EGF, but not by temperature shift, in R1/hER cells (data not shown). That morphological transformation could be induced by EGF in the overexpressor cells, but not in the parental R1 or R1/LA29 cells, indicates that the overexpressed hEGF receptors are functional in these cells and that constitutive activation of these receptors is sufficient for morphological transformation.

Effect of pp60$^{v\text{-}src}$ on the phosphorylation state of hEGF receptors in LA29/hER cells. To determine whether *src*-induced phosphorylation changes such as were observed with the endogenous rat EGF receptor could also be detected with cells overexpressing the human receptor, a human-specific monoclonal antibody was used to immunoprecipitate hEGF receptors from LA29/hER cells. The cells had been grown at either the restrictive or the permissive temperature for pp60$^{v\text{-}src}$ activity and were either treated or not treated with EGF for 10 min prior to lysis. After immunoprecipitation, the receptors were separated on SDS-gels and the levels of receptor tyrosine phosphorylation were assessed by Western blots with antiphosphotyrosine antibodies. As expected, the EGF receptor became phosphory-

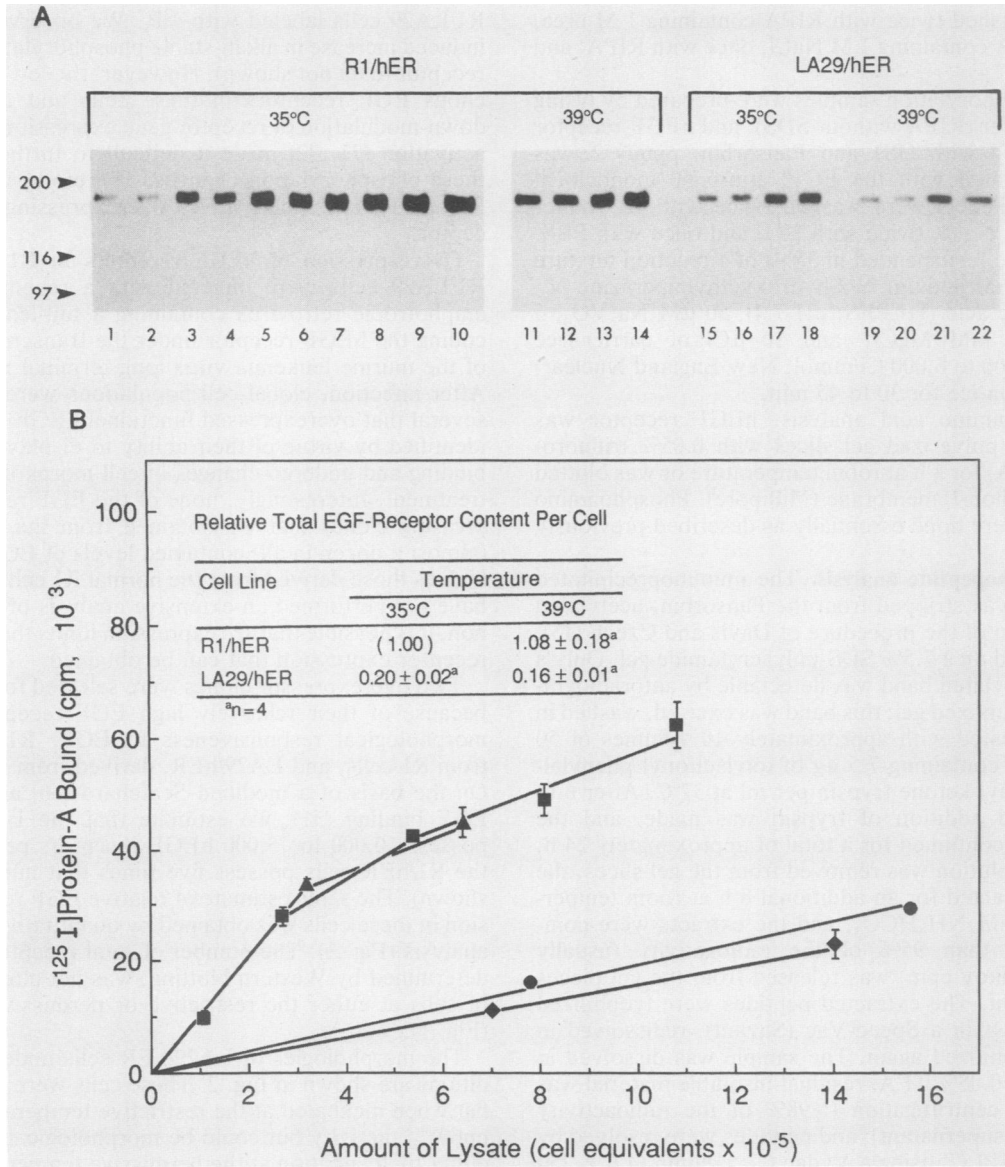

FIG. 1. Quantitation of relative EGF receptor levels by Western blot analysis. EGF receptors were immunoprecipitated from various amounts of cell lysate (in duplicate) and were then detected by immunoblotting as described in Materials and Methods. (A) Immunoblots; (B) quantitation of receptor levels, as determined by excising and determining the radioactivity of the receptor bands. Relative receptor levels normalized to the value obtained for R1/hER cells at 35°C are shown in the inset to panel B. R1/hER cells are Rat-1 cells overexpressing the hEGF receptor. LA29/hER cells express both the temperature-conditional pp60$^{v\text{-}src}$ and the hEGF receptor. Symbols in panel B: ■, R1/hER, 35°C; ▲, R1/hER, 39°C; ●, LA29/hER, 35°C; ◆, LA29/hER, 39°C.

lated on tyrosine when cells were treated with EGF (Fig 3A). However, in cells expressing active pp60$^{v\text{-}src}$ (35°C), the receptor was tyrosine phosphorylated even in the absence of EGF. When LA29/hER cells were shifted from the restrictive to the permissive temperature for pp60$^{v\text{-}src}$ activity, this receptor phosphorylation occurred as rapidly (within 0.5 h) (Fig. 3B), as did the restoration of pp60$^{v\text{-}src}$ autokinase activity (Fig. 3C).

To analyze the *src*-induced receptor phosphorylation in more detail, cells overexpressing the hEGF receptor were labeled to equilibrium with $^{32}P_i$ (12 to 14 h), and EGF receptors were isolated by immunoprecipitation with an excess of antireceptor antibody, followed by SDS-polyacrylamide gel electrophoresis (PAGE). Phosphorylation data for receptors isolated from the various cell types and conditions are shown in Fig. 4 and are expressed in terms of relative specific radioactivity; normalization was based on quantitation of the relative receptor levels in these cells (Fig. 1). These data confirm in a more quantitative manner the data shown in Fig. 3, that phosphorylation of the EGF receptor increases in response to either EGF addition or *src* expression. Expression of *src* without EGF addition led to a 2.5-fold elevation of receptor phosphorylation, a value nearly half that obtained by addition of saturating amounts of EGF (Fig. 4A). Addition of EGF to the *src*-transformed cells resulted in a further increase in receptor phosphorylation, approaching the level observed with the untransformed EGF-stimulated cells. The phosphorylation response to

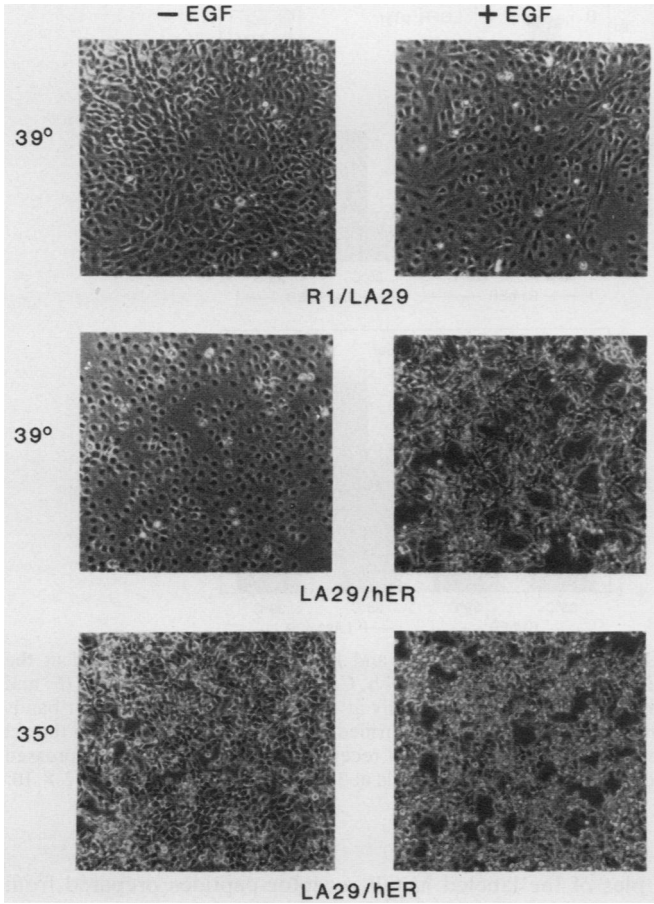

FIG. 2. Morphological properties of cells expressing temperature-conditional *src* and overexpressing the hEGF receptor. Cells were cultured under the conditions indicated for 24 h in the absence or presence of 50 ng of EGF per ml.

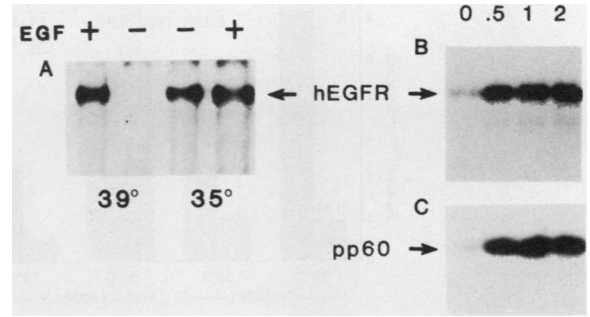

FIG. 3. Tyrosine phosphorylation of the hEGF receptor in cells expressing temperature-conditional *src*. Following the treatment described for each panel, cells were lysed and the human EGF receptor (hEGFR; A and B) or $pp60^{v\text{-}src}$ (C) was immunoprecipitated and resolved on 7.5% polyacrylamide gels. Tyrosine phosphorylation was detected by Western blot analysis using a polyclonal antiphosphotyrosine antibody. (A) Constitutive tyrosine phosphorylation in *src*-transformed cells. LA29/hER cells were cultured for 24 h at restrictive (39°C) or permissive (35°C) temperatures for $pp60^{v\text{-}src}$ activity. EGF treatment was for 10 min with 200 ng of EGF per ml. (B and C) Kinetics of receptor (B) and $pp60^{v\text{-}src}$ (C) tyrosine phosphorylation following shift of LA29/hER cells from 39 to 35°C. Time (in hours) is indicated above the lanes.

EGF was somewhat blunted in cells grown at the higher temperature, particularly in cells infected with the LA29 *src* mutant, and this was a consistent feature of these experiments.

The ^{32}P-labeled receptors were eluted from gel slices and subjected to partial acid hydrolysis, and the relative phosphoamino acid composition of these receptors was then determined by two-dimensional high-voltage electrophoresis (11, 45). Treatment with EGF increased phosphorylation of receptors on tyrosine as well as on threonine and serine, as expected (Fig. 4B to D). Interestingly, activation of $pp60^{v\text{-}src}$ also increased receptor phosphorylation, not only on tyrosine (as demonstrated by the immunoblots shown in Fig. 3) but on serine and threonine as well. $pp60^{v\text{-}src}$ activity increased receptor serine and threonine phosphorylation to a level more than half that obtained with EGF and increased tyrosine phosphorylation to a level approximately one-third that obtained by EGF addition to these cells. This represents a 12-fold stimulation of receptor tyrosine phosphorylation above the basal (nonstimulated, untransformed) level.

EGF receptor phosphorylation sites affected by $pp60^{v\text{-}src}$. To characterize the sites of *src*-induced elevation in EGF receptor phosphorylation, HPLC tryptic phosphopeptide mapping was performed on hEGF receptors immunoprecipitated from ^{32}P-labeled cells. The methodologies used to prepare and separate the phosphopeptides followed those previously published (14, 15, 18, 24) so that direct comparisons could be made with earlier reports. Phosphoamino acid analysis was performed on fractions containing the peaks of radioactivity.

The tryptic phosphopeptide profile obtained from an in vitro autokinase reaction using hEGF receptors from R1/hER cells revealed three major peaks of phosphotyrosine (Fig. 5A). These phosphopeptides displayed an elution profile nearly identical to that first described by Downward et al. (18); thus, maintaining their terminology, we refer to these peptides as P1, P2, and P3. The elution positions of these peptides were used as a reference for identifying phosphopeptides from hEGF receptors analyzed from in vivo-labeled cells.

R1/hER and LA29/hER cells were labeled to equilibrium with $^{32}P_i$, and phosphopeptide analysis was performed on the trypsin-digested receptors from these cells. A single phosphothreonine-containing phosphopeptide was detected in receptors from R1/hER cells that were not stimulated with EGF (Fig. 5B). This peptide eluted at the acetonitrile concentration expected for the peptide containing threonine 669 (13, 24). Several additional sites of phosphorylation were revealed in hEGF receptors obtained from R1/hER cells after treatment with EGF (Fig. 5C). These included peaks corresponding to the P1, P2, and P3 peptides seen with in vitro-phosphorylated receptors. Several minor phosphotyrosine-containing peptides were also observed, including two peaks at fractions 47 and 52, the region of the elution gradient in which the peptide containing tyrosine 1086 (P4) would be expected to elute (29, 44). The threonine 669 peptide was also present but was not resolved from the P2 peptide in this gradient system. Small peaks of phosphoserine were distributed throughout the profile, and there was also a prominent phosphoserine peak eluting just after P2.

In contrast to the results obtained with the R1/hER cells in the absence of EGF, the phosphopeptide profile of hEGF receptors from LA29/hER cells transformed by *src* (at 35°C) revealed multiple sites of phosphorylation in the absence of

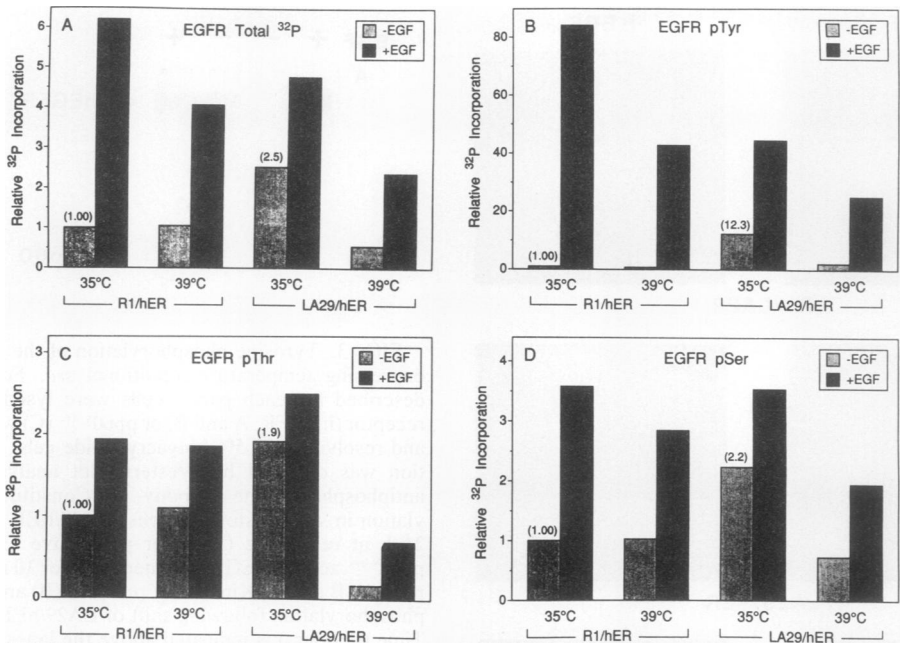

FIG. 4. Phosphorylation of the EGF receptor in response to *src*, EGF, or both. LA29/hER and R1/hER cells were cultured at the restrictive (39°C) or permissive (35°C) temperature for pp60$^{v\text{-}src}$ activity in the presence of $^{32}P_i$ for 14 h. Cells were stimulated with EGF, and the hEGF receptor was purified by immunoprecipitation and SDS-PAGE. Following autoradiography at 4°C, the radioactive receptor bands were excised from the gel and total radioactivity was determined by Cerenkov counting (A). The purified receptors were eluted from the gel slices and subjected to phosphoamino acid analysis (B to D). The data are normalized to equivalent receptor number (Fig. 1) and expressed as relative specific radioactivity in which the value for incorporation into receptor from R1/hER cells at 35°C, −EGF, is set at 1.0 (7.2×10^5 cells, 7,546 total cpm incorporated; pY, 4.1%; pT, 43.1%; pS, 52.7%).

ligand treatment (Fig. 5D). The most significant included one (which we term sPY1, for *src* phosphotyrosine 1) eluting very close to P1, around fraction 35, and another eluting around fraction 50, which we term sPY2 (asterisks in Fig. 5D). The level of sPY2 has varied from one experiment to another, but the relative amount of sPY1 has remained roughly constant, being approximately one-third of the radioactivity present in the threonine 669 peptide. Although the major phosphothreonine peptide (T669) was also present in the *src*-transformed but nonstimulated cells, phosphotyrosine-containing peptides at the elution positions corresponding to P1, P2, and P3 were barely detectable. Thus, the *src*-induced phosphorylations do not substantially induce phosphorylation on the major sites of in vitro receptor autophosphorylation.

When EGF was added to the *src*-transformed cells, P1, P2, and P3 phosphorylation became evident (Fig. 5E). These data appear to indicate that *src* expression does not substantially prevent ligand-stimulated receptor phosphorylation. However, it is important to note that since sPY1 and sPY2 phosphorylations are presumably substoichiometric (given that these phosphorylations are about one-third the level of the T669 phosphorylation), it is possible that the receptors in the transformed cells which display ligand-induced phosphorylation are a different population than the ones carrying the sPY1 and sPY2 phosphorylations. Thus, it is still possible that the *src*-induced phosphorylations inhibit EGF responsiveness, which would be consistent with the observation (Fig. 4) that ligand-induced tyrosine phosphorylation is somewhat less in the *src*-transformed cells than in the R1/hER cells.

To be certain that the *src*-induced phosphorylations were distinct from the major sites of autophosphorylation, samples of the labeled hEGF receptor peptides prepared from the autokinase reaction shown in Fig. 5A were mixed with the LA29/hER receptor peptides analyzed in Fig. 5D. When this mixed sample was resolved by HPLC, a clear separation of sPY1 from P1 was found (Fig. 6A), indicating that sPY1 is indeed a unique site of tyrosine phosphorylation. sPY2 could be resolved from the minor EGF-induced phosphopeptides at fractions 47 and 52, implying that sPY2 is distinct from P4. The separation of sPY1 and sPY2 from P1, P2, and P3 was also confirmed by using an acetonitrile gradient elution at pH 6.0 (24), which provides better resolution of these novel peptides from those previously reported and in addition resolves P2 from the T669 peptide (Fig. 6B). Again, the data demonstrate that the *src*-induced phosphorylations differ from the previously described autophosphorylations. These results exclude the possibility that the *src*-induced phosphorylations occur via an autocrine mechanism, which would be expected to result in phosphorylation of P1, P2, and P3.

EGF receptor physiology in *src*-transformed cells. Since expression of pp60$^{v\text{-}src}$ caused alterations in the phosphorylation state of EGF receptors, it was important to determine what effect, if any, these changes had on receptor physiology. To examine this, the EGF binding properties, internalization kinetics, and surface distribution of EGF receptors were examined in LA29/hER cells. Scatchard (60) and nonlinear regression analysis (31) of ^{125}I-EGF binding isotherms revealed no change in binding affinity and a modest (twofold maximum) decrease in total surface binding in cells cultured at 35 versus 39°C (data not shown). Since the ligand binding at 39°C includes a contribution from endogenous rat EGF receptors (which are expressed to a higher level at 39°C) (71, 72), the significance of this modest decrease is unclear. However, it is possible that a fraction of the

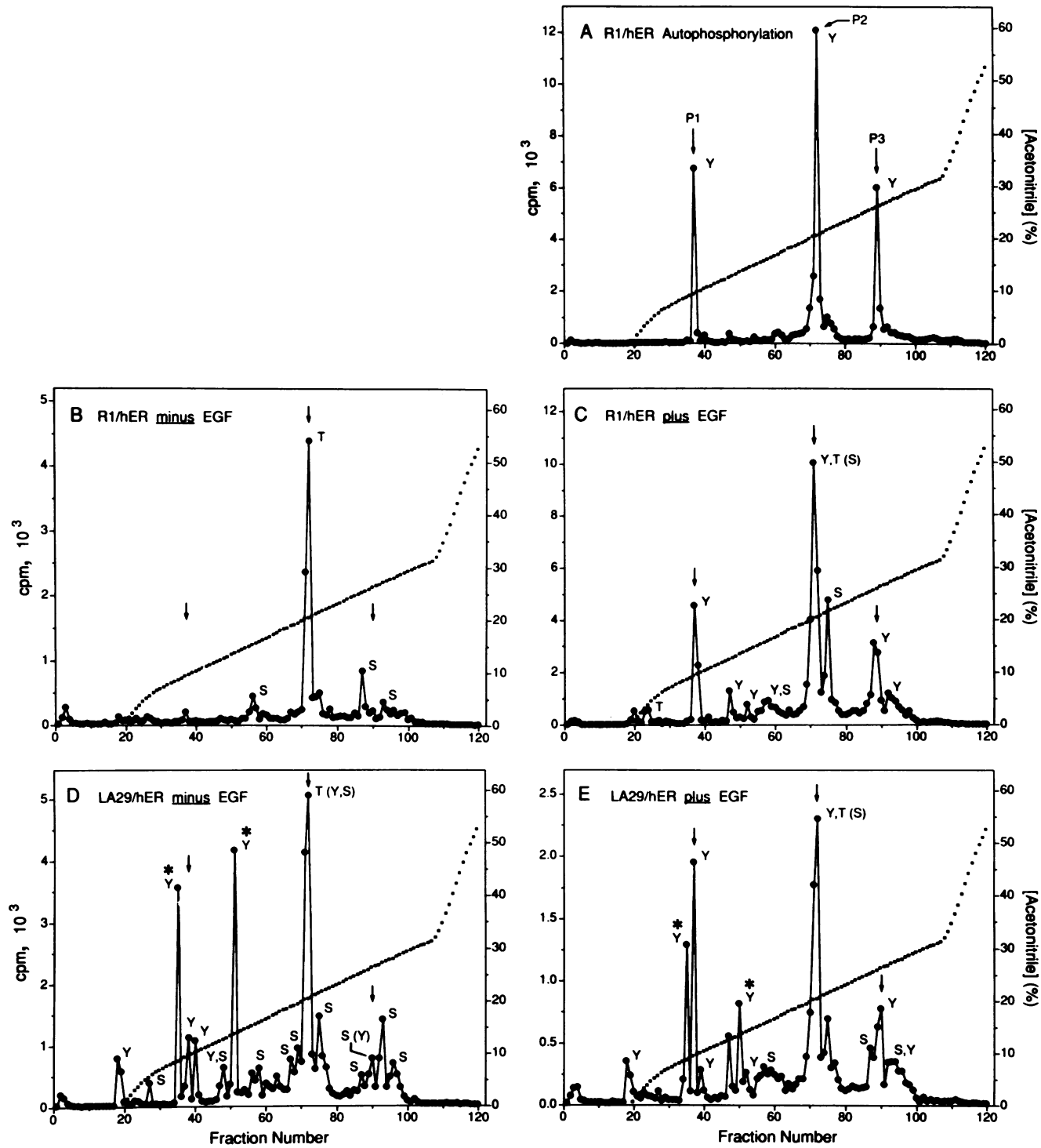

FIG. 5. Tryptic phosphopeptide mapping and phosphoamino acid analysis of EGF receptor phosphopeptides. EGF receptors were immunoprecipitated from $^{32}P_i$-labeled cells or were isolated from unlabeled cells and allowed to autophosphorylate in the presence of [γ-^{32}P]ATP. After electrophoresis on 7.5% polyacrylamide gels, the receptors were digested with trypsin and phosphopeptides were resolved by reverse-phase HPLC (0.05% TFA); fractions containing peaks of radioactivity (Cerenkov) were subjected to phosphoamino acid analysis. (A) Autophosphorylation; (B) in vivo basal phosphorylation, R1/hER cells, 35°C; (C) in vivo EGF-stimulated phosphorylation (R1/hER cells, 35°C plus EGF); (D) in vivo *src*-induced phosphorylation (LA29/hER cells, 35°C); (E) in vivo EGF stimulation of *src*-transformed cells (LA29/hER cells, 35°C plus EGF). The acetonitrile concentration (■) of each fraction (0.5 ml) was determined by conductivity. Arrows indicate elution positions of P1, P2, and P3. The principal *src*-induced peptides are marked with an asterisk. The phosphoamino acids present in each peak are identified by capital letters, with the minor components in parentheses. Each panel except B is representative of at least two independent experiments, and HPLC analysis was performed at least twice on the receptor isolated in each experiment. The values were not corrected to normalize for differences in amount, labeling, radiochemical decay or recovery of receptor in these different preparations and thus should not be compared between panels.

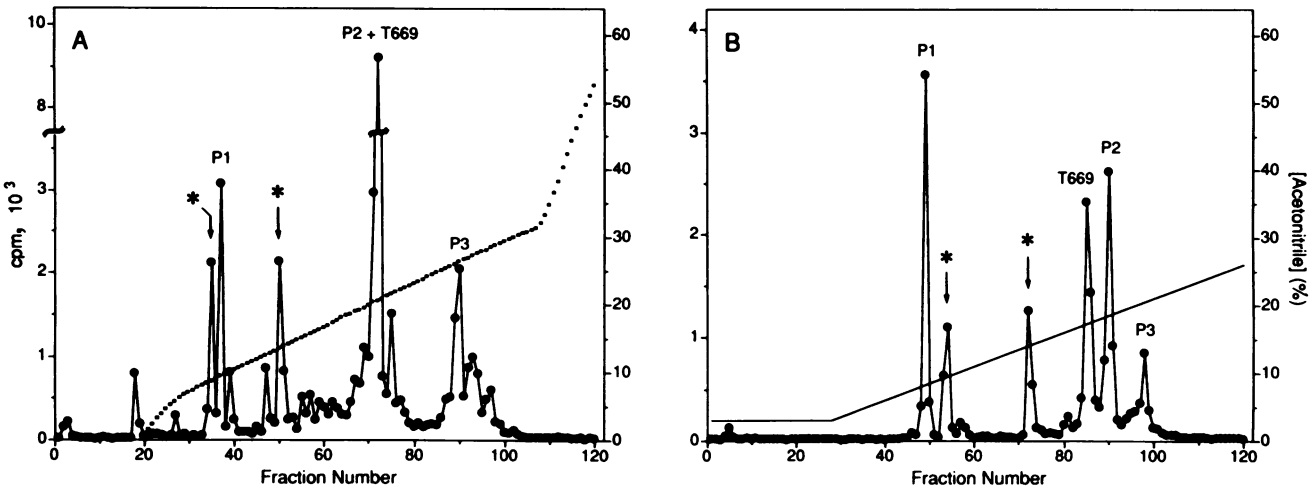

FIG. 6. Mixing experiments resolve *src*-induced phosphopeptides from EGF-induced phosphopeptides. EGF receptor phosphopeptides were generated as described in the legend to Fig. 5 and Materials and Methods. (A) Phosphopeptides generated following EGF receptor autophosphorylation were mixed with the EGF receptor phosphopeptides from unstimulated *src*-transformed cells and analyzed as for Fig. 5. (B) Known counts per minute of the following HPLC-purified peptides from the experiments shown in Fig. 5 were mixed: P1, P2, and P3 (Fig. 5A); T669 (Fig. 5B); and sPY1 and sPY2 (Fig. 5D). The mixture was then resolved by using an acetonitrile gradient at pH 6.0 as described by Heisermann and Gill (24), which provides better resolution of P2 from the T669 phosphopeptide and of sPY1 and P1.

receptors is in an internal pool in the *src*-transformed cells. By acid stripping cells at various times during incubation with ^{125}I-EGF, it was possible to approximate the rate at which ligand was being internalized. We could not detect a difference in ligand internalization rate induced by the expression of pp60$^{v\text{-}src}$ (data not shown). Flow cytometric analysis using an hEGF receptor-specific monoclonal antibody indicated that modal levels of hEGF receptor expression at the surface of LA29/hER cells were comparable at 39 and 35°C (Fig. 7), although at 35°C a small (15 to 20%) reduction in the surface expression of receptors and increase in heterogeneity within the population was noted. These effects could be due to differences in cell cycling between the cultures held at 39 versus 35°C or could be due more directly to the effects of *src* expression. At either temperature, treatment of these cells with EGF (15 min) caused a similar extent of receptor loss from the cell surface (twofold) at both temperatures, in agreement with the acid-strip measurements. These data demonstrate that the marked changes in phosphorylation of cellular substrate proteins caused by *src* are not accompanied by a generalized paralysis or marked stimulation of the EGF receptor with respect to ligand binding or ligand-mediated receptor internalization. There may, however, be a modest decrease in the percentage of the receptors displayed on the cell surface. As with the studies on ligand-induced phosphorylation (Fig. 5), the substoichiometric phosphorylation of the EGF receptor caused by pp60$^{v\text{-}src}$ leaves open the possibility that the population of receptors carrying the sPY1 and sPY2 phosphorylations could have larger differences in surface distribution and cycling kinetics than are revealed by these measurements of average receptor properties.

EGF receptor signaling in *src*-transformed cells. To determine whether the constitutive phosphorylation of the EGF receptor observed in *src*-transformed cells correlated with changes in receptor signaling, we first analyzed the in vitro kinase activity of the receptor in an immune complex kinase assay (data not shown). These experiments did not reveal clear-cut *src*-induced differences in receptor kinase activity,

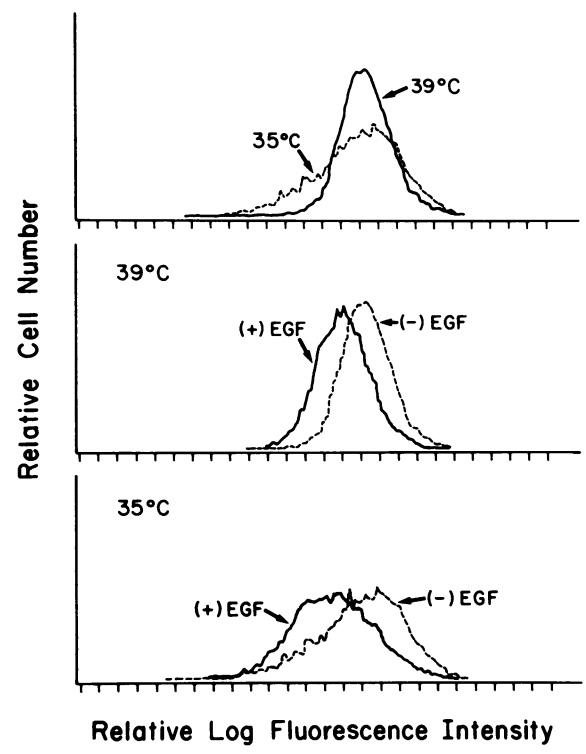

FIG. 7. Flow cytometric analysis of EGF receptor surface expression and internalization. LA29/hER cells grown at 35 or 39°C and either treated with EGF or left untreated were analyzed for surface EGF receptor expression as described in Materials and Methods. Each tic mark on the abscissa represents 10 channel numbers. Mean channel numbers were as follows: 39°C, 154; 39°C plus EGF, 141; 35°C, 150; 35°C plus EGF, 134; background, 75. Separate measurements determined that these values were all within the linear range of the instrument.

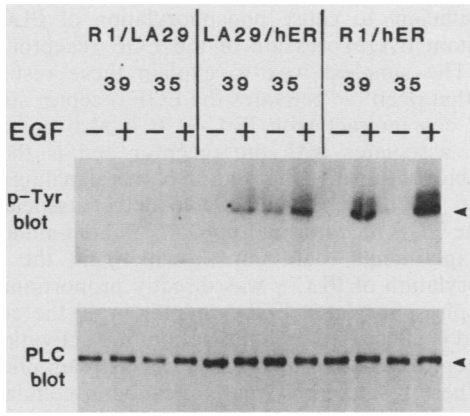

FIG. 8. Phosphorylation of PLC-γ in response to EGF and *src*. R1/LA29, LA29/hER, and R1/hER cells were cultured for 16 h at the restrictive (39°C) or permissive (35°C) temperature for pp60$^{v\text{-}src}$ activity. Cells were placed in serum-free medium with 0.1% BSA for 2 h, and then half of the cultures were stimulated with EGF for 10 min. Cells were lysed, and PLC-γ was immunoprecipitated from equal amounts of cell protein. After washing and resuspension in electrophoresis sample buffer, the immunoprecipitate was divided in half; both sample sets were then subjected to SDS-PAGE, one for determination of tyrosine phosphorylation levels by Western blotting with phosphotyrosine (p-tyr) antibodies (A) and the other for determination of PLC-γ levels by Western blotting with antibodies against PLC-γ (B). The arrows mark the position of PLC-γ. The coprecipitating band in lanes from EGF-stimulated R1/hER cells which migrates slightly above PLC-γ, at 170 kDa, is likely the EGF receptor (43, 48). This band is less apparent in immunoprecipitates from cells with lower levels of EGF receptors.

but under the conditions of the assay (which were designed to minimize cross-contamination with pp60$^{v\text{-}src}$) we also did not observe EGF stimulation of receptor kinase activity, a result previously noted with the isolated receptor (19). Although we also observed little evidence for increased receptor kinase activity in vivo (which would be expected to cause increased phosphorylation of P1, P2, and P3), we considered the possibility that receptor signaling might be limited as much by the binding of the receptor to specific substrates as by its kinase activity (2, 3, 42). We therefore examined the ability of the EGF receptor to cause phosphorylation of PLC-γ, which has been reported to be a substrate for the EGF receptor tyrosine kinase (42, 43, 48, 50, 67, 68) but not to be an in vivo substrate for pp60$^{v\text{-}src}$ (29a). Upon ligand-induced receptor activation and phosphorylation, PLC-γ transiently associates with the EGF receptor and becomes phosphorylated on tyrosine. It thus seemed possible that if the *src*-induced phosphorylations provided an alternative mechanism for receptor activation, PLC-γ could transiently associate with the EGF receptor in *src*-transformed cells and become phosphorylated even in the absence of ligand-induced receptor phosphorylation. Phosphorylation of PLC-γ could thus serve as an in vivo assay for functional activation of EGF receptors.

We therefore examined tyrosine phosphorylation of PLC-γ in *src*-transformed cells which also overexpressed the EGF receptor. PLC-γ was constitutively phosphorylated in these cells, even in the absence of EGF (Fig. 8). Cells expressing either temperature-conditional pp60$^{v\text{-}src}$ (R1/LA29) or the hEGF receptor (R1/hER) or both (LA29/hER) were cultured at either 35 or 39°C. They were then either left untreated or treated for 10 min with EGF. The cells were then lysed, and the degree of tyrosine phosphorylation of immunoprecipitated PLC-γ was determined by Western blotting with antiphosphotyrosine antibodies. Parallel blots with antibody against PLC-γ showed that the amount of PLC-γ was comparable in each set of lysates. In cells overexpressing the hEGF receptor, there was a pronounced increase in the tyrosine phosphorylation of PLC-γ in response to EGF, whether cultures were held at 35 or 39°C. In R1/LA29 cells, which express *src* but in which the hEGF receptor is not overexpressed, barely detectable levels of PLC-γ tyrosine phosphorylation were observed. We were not surprised that PLC-γ phosphorylation was not substantially increased in these cells even after EGF addition, since the level of endogenous receptors in these cells is quite low (71, 72). Thus, these cells serve as a negative control, demonstrating that PLC-γ cannot be phosphorylated in vivo by pp60$^{v\text{-}src}$ but that this phosphorylation requires an activated EGF receptor. In LA29/hER cells, which express both pp60$^{v\text{-}src}$ and the hEGF receptor, we found that phosphorylation of PLC-γ required addition of EGF when the cells had been cultured at 39°C. However, when cells were cultured at 35°C, the permissive temperature for pp60$^{v\text{-}src}$ activity, PLC-γ displayed constitutive phosphorylation on tyrosine even in the absence of EGF. In five independent experiments, the level of *src*-induced phosphorylation of PLC-γ in the LA29/hER cells ranged from 38 to 69% of that obtained by adding EGF to these cells (determined by densitometric scanning of the autoradiograms). This range is comparable to that observed for the *src*-induced tyrosine phosphorylation of the EGF receptor itself, relative to the level achieved following EGF stimulation. The lower level of EGF-induced PLC-γ tyrosine phosphorylation in the LA29/hER cells than in the R1/hER cells may reflect the fact that the level of EGF receptor is five times lower in the LA29/hER cells (Fig. 1). Thus, in all of these cases, the tyrosine phosphorylation of PLC-γ was directly proportional to the amount of phosphorylated EGF receptors in the cells. We interpret these results as indicating that the EGF receptor is constitutively activated in the *src*-transformed cells with respect to interaction with PLC-γ, a result which correlates with the novel *src*-induced phosphorylations that we report.

DISCUSSION

Phosphorylation of the EGF receptor. In this report, we demonstrate that expression of pp60$^{v\text{-}src}$ causes altered phosphorylation of the EGF receptor and that this phosphorylation occurs rapidly when cells expressing a temperature-sensitive pp60$^{v\text{-}src}$ kinase are shifted from the restrictive to the permissive temperature. Although most of the analysis reported here was performed on the hEGF receptor coexpressed with pp60$^{v\text{-}src}$ in Rat-1 cells, we found that the endogenous rat cell receptor also displays rapid *src*-induced phosphorylation.

We do not know whether tyrosine phosphorylation of the EGF receptor induced by *src* is due to pp60$^{v\text{-}src}$ directly or whether some other tyrosyl protein kinase serves as an intermediary. However, for the following reasons we think it is unlikely that the EGF receptor is phosphorylating itself consequent to an autocrine activation: (i) no evidence for the production of transforming growth factor α or EGF has been obtained in these cells despite extensive and sensitive examination (72), and (ii) the sites of tyrosine phosphorylation induced by EGF, either in vitro or in vivo, are distinct from the major ones induced by *src*.

The major *src*-induced phosphopeptides did not include P1, P2, or P3, which correspond to the predominant EGF-

induced tyrosine phosphorylations, but rather consisted of novel phosphopeptides termed here sPY1 and sPY2. Although we have not directly determined the stoichiometry of phosphorylations induced by pp60$^{v\text{-}src}$, we have found the magnitude of these phosphorylations to be more than one-half of the autophosphorylation on P1 which results when saturating amounts of EGF are added to the *src*-transformed cells and to be approximately one-third of the constitutive phosphorylation of T669. Since this is a level of phosphorylation close to that seen in response to ligand, it should represent a stoichiometry sufficient for substantial biological effects.

We have not yet determined the sequences of the sPY1 and sPY2 peptides. However, the relative elution position of sPY1 on reverse-phase HPLC in an acetonitrile gradient coincides with that predicted (47) for the tryptic peptide containing tyrosine 845, which is homologous to the pp60$^{v\text{-}src}$ autophosphorylation site (tyrosine 416). This is the only tyrosine-containing peptide in the cytoplasmic domain of the EGF receptor which would be predicted to elute earlier than P1 in this solvent system.

The increased phosphorylations seen in the *src*-transformed cells occurred on serine and threonine as well as on tyrosine. Thus, pp60$^{v\text{-}src}$ must activate cellular serine/threonine kinases which can phosphorylate the receptor, or else the tyrosine phosphorylation of the receptor renders it susceptible to phosphorylation by these other kinases. Reports of *src*-induced stimulation of protein kinase C or of phosphatidylinositol metabolism and turnover (which could activate protein kinase C) have previously been described (1, 7, 17, 21, 23, 32, 46, 51, 62, 64), and we have found (59a) that *src*-transformed cells display increased activity of MAP kinase (59), a serine/threonine-specific protein kinase (56). Thus, there is precedent for *src*-induced activation of serine and/or threonine-specific protein kinases.

EGF receptor function. Western blot analysis revealed that activation of pp60$^{v\text{-}src}$ by shifting LA29/hER cells from the restrictive to the permissive temperature did not significantly change the total number of human EGF receptors per cell. This finding indicates that degradation of the overexpressed human receptor is not accelerated by *src* expression, in agreement with our earlier analysis of endogenous receptors (71). We detected no significant effects of *src* expression on affinity of the EGF receptor for ligand or on EGF-induced receptor internalization, whether measured by EGF binding or by flow cytometry. However, LA29/hER cells displayed a 15 to 20% reduction in the percentage of receptors on the cell surface when cultured at the permissive temperature. It is important to note, however, that because the *src*-induced phosphorylations are likely to be substoichiometric, these measurements of average receptor properties may not reflect the properties of the population of receptors carrying the sPY1 and sPY2 phosphorylations.

To determine whether the signaling activity of the EGF receptor was altered in response to pp60$^{v\text{-}src}$, we examined the tyrosine phosphorylation of PLC-γ. This enzyme transiently associates with the EGF receptor and becomes tyrosine phosphorylated consequent to EGF stimulation (42, 43, 48, 50, 68, 69). The EGF receptor is able to directly phosphorylate PLC-γ in vitro (50), and only an active, phosphorylated EGF receptor can bind to and phosphorylate PLC-γ (42). Thus, phosphorylation of PLC-γ provides a suitable measure for activation of EGF receptor signaling. We found that PLC-γ was constitutively phosphorylated in cells transformed by *src* which were also expressing high levels of the EGF receptor. Expression of pp60$^{v\text{-}src}$ alone was insufficient to cause phosphorylation of PLC-γ, and concomitant overexpression of the EGF receptor was required. The simplest way to explain these results is to suggest that pp60$^{v\text{-}src}$ activates the EGF receptor so that the receptor can interact with PLC-γ. It is also possible that pp60$^{v\text{-}src}$ associates with the receptor and is the kinase responsible for the phosphorylation of this signaling enzyme. However, we have been unable to detect association between the EGF receptor and pp60$^{v\text{-}src}$ in coimmunoprecipitation experiments (data not shown). That the level of phosphorylation of PLC-γ was directly proportional to the level of phosphorylated EGF receptor in all the cell types examined is consistent with the notion that activation of the EGF receptor is necessary for this downstream phosphorylation. These results imply that *src* can alter cellular signaling by unscheduled phosphorylation of growth factor receptors. The results also point to a novel mechanism for receptor activation, one which occurs independently of the major sites of autophosphorylation.

Implications for transformation. It is clear that constitutive activation of the EGF receptor is sufficient to transform cells to a malignant state. This has been shown not only for the case of the *erb*-B oncogene, which is a mutationally activated form of the EGF receptor (20), but also for cells that overexpress normal or nonactivated forms of the EGF receptor to an extent which saturates or bypasses cellular desensitization mechanisms. Thus, addition of EGF to cells overexpressing the EGF receptor not only converts these cells to a transformed morphology, as shown here, but also renders them capable of anchorage-independent growth and tumorigenicity (16, 58, 65). Since we find that pp60$^{v\text{-}src}$ is capable of activating the EGF receptor with respect to at least one of its signaling activities, this provides an attractive mechanism by which pp60$^{v\text{-}src}$ and related tyrosine kinase oncogene products might transform cells: it is possible that this group of oncogene products alters cellular regulation, at least in part, by phosphorylating and activating membrane receptors. They may also directly phosphorylate and activate signaling molecules which function downstream from receptors, but since the receptor activation is sufficient for transformation, these additional substrates would be redundant.

We do not believe that the EGF receptor is a necessary substrate for transformation by pp60$^{v\text{-}src}$: cells that lack EGF receptors are capable of being transformed by *src* (72), and *src* expression down-modulates endogenous EGF receptor gene expression (70). However, we feel that the results reported here provide a useful paradigm for studying the interaction of pp60$^{v\text{-}src}$ with growth-regulatory receptors.

The significance of these results is increased by our recent findings which identified the β subunit of the IGF-I receptor as an in vivo substrate for pp60$^{v\text{-}src}$ (36). The phosphorylation of the IGF-I receptor correlated tightly with phenotypic transformation in cells expressing various *src* mutants (35), suggesting that the IGF-I receptor is a functionally significant substrate for pp60$^{v\text{-}src}$; whether it is an essential substrate remains to be determined. We also have found that the β subunit of the insulin receptor becomes constitutively phosphorylated on tyrosine in *src*-transformed cells by a nonautocrine mechanism (37a). Moreover, Yu et al. (75) have reported that pp60$^{v\text{-}src}$ can phosphorylate and activate the insulin receptor tyrosine kinase in vitro, indicating that this receptor phosphorylation can be catalyzed directly by pp60$^{v\text{-}src}$.

Therefore, we suggest that pp60$^{v\text{-}src}$ transforms cells at least in part by phosphorylating and activating a variety of

tyrosine kinase membrane receptors. Whether receptors of other classes also become phosphorylated remains to be determined. Since the tyrosine kinase receptors all have similar, overlapping activities, they represent a class of at least partially redundant substrates. Phosphorylation of any one of these receptors would thus be unnecessary, as we have found in the case of the EGF receptor.

It is widely suspected that $pp60^{v\text{-}src}$ transforms cells by constitutively performing activities which are performed in a regulated fashion by $pp60^{c\text{-}src}$. Could the receptor phosphorylation and activation reported here to be induced by $pp60^{v\text{-}src}$ also occur normally in cells expressing $pp60^{c\text{-}src}$? Such a suggestion is consistent with reports from Parsons and collaborators that overexpression of $pp60^{c\text{-}src}$ renders cells substantially more responsive to the mitogenic effects of EGF and other peptide growth factors (41, 52a, 73). Since the kinase activity of $pp60^{c\text{-}src}$ and related oncogene products appears itself to be regulated by transmembrane receptors (5, 22, 37, 55), it is possible that these proto-oncogenes function normally to communicate between heterologous receptors laterally in the plane of the membrane, thus helping to broaden the magnitude of responsiveness to individual agonists. Thus, we speculate that c-*src* and related proto-oncogenes serve normally as a medium for cross-talk between receptor systems and that v-*src* is oncogenic because of its unregulated interaction with these receptors.

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The first two authors contributed equally to this work. M.J.W. and W.J.W. initiated this research, and W.J.W. generated and characterized the overexpressor cell lines. D.M.P. with the assistance of D.L.F. examined the extent and sites of EGF receptor phosphorylation. M.J.W. with the technical assistance of Amy Hester-Osgood performed the studies on PLC-γ. We thank Douglas Lowy and Thierry Velu for the retrovirus expressing the hEGF receptor, H. Shelton Earp for antisera recognizing the rat EGF receptor, S. Parsons for antibody against $pp60^{src}$, and Tony Pawson and Sue Goo Rhee for antibodies against PLC-γ. We are especially indebted to Tony Hunter for pointing out to us that PLC-γ was not a substrate for $pp60^{v\text{-}src}$.

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