Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6981–6985, July 1995
Cell Biology

Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: Implications for the etiology of multiple human cancers

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Communicated by Oscar L. Miller, Jr., University of Virginia, Charlottesville, VA, April 24, 1995 (received for review February 8, 1995)

ABSTRACT **c-Src is a nontransforming tyrosine kinase that participates in signaling events mediated by a variety of polypeptide growth factor receptors, including the epidermal growth factor receptor (EGFR). Overexpression and continual ligand stimulation of the EGFR results in morphological transformation of cells *in vitro* and tumor development *in vivo*. Elevated levels of c-Src and the EGFR are found in a variety of human malignancies, raising the question of whether c-Src can functionally cooperate with the EGFR during tumorigenesis. To address this issue, we generated c-Src/EGFR double overexpressors and compared their proliferative and biochemical characteristics to those of single overexpressors and control cells. We found that in cells expressing high levels of receptor, c-Src potentiated DNA synthesis, growth in soft agar, and tumor formation in nude mice. Growth potentiation was associated with the formation of a heterocomplex between c-Src and activated EGFR, the appearance of a distinct tyrosyl phosphorylation on the receptor, and an enhancement of receptor substrate phosphorylation. These findings indicate that c-Src is capable of potentiating receptor-mediated tumorigenesis and suggest that synergism between c-Src and the EGFR may contribute to a more aggressive phenotype in multiple human tumors.**

Cellular Src (c-Src) is a nontransforming cytoplasmic membrane-associated tyrosine kinase that is required for mitogenic signaling through multiple growth factor receptors, including the receptor for epidermal growth factor (EGF) (1–3). Elevated levels of the EGF receptor (EGFR) and its family members [which are also tyrosine kinases (4)] have been causally linked to a number of human malignancies, including prostatic, breast, bladder, colon, ovarian, and lung cancer (for review, see refs. 5 and 6). That EGFR can function as an oncogene has been shown by the ability of normal fibroblasts expressing high levels of the receptor to grow in soft agar and develop tumors in nude mice in an EGF-dependent manner (7, 8). Elevated c-Src activity has also been observed in many of the same types of tumors that express high levels of the EGFR, including carcinomas of the breast and colon (9–11). This codistribution raises the question of whether the two tyrosine kinases can cooperate in the genesis or progression of these diseases. To address this issue, we generated a panel of murine fibroblasts that overexpress either EGFR or c-Src alone or both EGFR and c-Src and characterized them for their growth and tumorigenic properties. We found that c-Src synergistically increased the oncogenic activity of the EGFR. Biochemical analysis was also carried out to investigate the mechanism(s) of the synergy.

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MATERIALS AND METHODS

Cell Lines. The derivation and characterization of the clonal C3H10T½ murine fibroblast cell lines used in this study, Neo (control), 5H (c-Src overexpressor), and IV5 (v-Src transfectant), have been described (1, 12). 5H cells express c-Src 20- to 30-fold greater than endogenous levels. NeoR1 and 5HR11 cells were derived by infection of Neo or 5H cells, respectively, with an amphotropic retrovirus encoding human EGFR (8). Saturation binding analysis (13) revealed that NeoR1 and 5HR11 cells expressed nearly equal levels of cell surface receptors [2.1×10^5 and 1.6×10^5 receptors per cell, respectively—approximately 40-fold greater than endogenous levels (12)]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum, antibiotics (penicillin, 10 units/ml; streptomycin, 10 μg/ml), and G418 (400 μg/ml).

Antibodies. EGFR-specific mouse monoclonal antibodies (mAbs), 291-3A and 291-4A, were obtained from a fusion of RBF/Dn immune spleen cells (The Jackson Laboratory) with cells of the myeloma line Fox-NY (HyClone). Mice were immunized with a bacterially expressed TrpE–human EGFR fusion protein, containing amino acids 793–1186 of the receptor. Epitopes for 3A and 4A mAbs were mapped to residues 889–944 and 1052–1134, respectively (14), and both mAbs were found to be of the IgG2a subclass. Q9 antibody was raised in rabbits against the C-terminal peptide of c-Src (residues 522–533) and exhibits a higher affinity for c-Src than for other Src family members (C. M. Ely, J. Litz, and S.J.P., unpublished data). Antibodies specific for phospholipase C-γ (PLC-γ) (pool of isozyme-specific mouse mAbs), phosphotyrosine [Tyr(*P*)] (mAb 4G10), and Src homology collagen (Shc) (rabbit) were purchased from Upstate Biotechnology (Lake Placid, NY).

[^{3}H]Thymidine Incorporation. Confluent cells in 24-well tissue culture dishes were starved of serum overnight and incubated in medium lacking serum but containing EGF (40 ng/ml) for various lengths of time. [^{3}H]Thymidine (1 μCi; 20 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was added to each well 30 min prior to the times indicated in the figure. [^{3}H]Thymidine incorporation was assayed by the procedure of Mosca *et al.* (15). Triplicate determinations were made at each time point.

Anchorage-Independent Growth. Approximately 10^5 cells were suspended in 0.5% agarose-containing DMEM (supplemented with 15% fetal calf serum ± EGF at 40 ng/ml) and layered over an agarose plug in a 60-mm dish. The agarose plug contained 1% agarose in DMEM (supplemented with 10% fetal calf serum). Cells were incubated for 3 weeks, during which time fresh medium ± EGF (40 ng/ml) was added to the

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; PLC-γ, phospholipase C-γ; Shc, Src homology collagen; Tyr(*P*), phosphotyrosine; mAb, monoclonal antibody.

plates every 3 or 4 days. Colonies composed of >6 cells were counted after 16 days.

Tumorigenicity. Approximately 10^7 cells in 0.15 ml of sterile PBS were injected subcutaneously into each hip of 28- to 42-day-old NIH III *nu/nu* male mice (Charles River Breeding Laboratories). Animals were observed twice weekly, and tumors were measured with the aid of a micrometer. No exogenous EGF was added.

Immunoprecipitation, Immunoblot Analysis, and *in Vitro* Kinase Assays. Methods for immunoprecipitations and immunoblot analysis have been described (16, 17). Primary antibodies were detected with either ^{125}I-labeled goat anti-mouse IgG (New England Nuclear) or ^{125}I-labeled protein A. c-Src was localized with ^{125}I-labeled mAb 327 (a gift of J. Brugge, Ariad Pharmaceuticals, Cambridge, MA). For kinase assays immunoprecipitates were prepared in and washed once with CHAPS buffer [10 mM CHAPS/50 mM Tris·HCl, pH 8.0/150 mM NaCl/2 mM EDTA/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/leupeptin (50 μg/ml)/0.5% aprotinin] and washed three additional times with HBS buffer (150 mM NaCl/20 mM Hepes, pH 7.4). Each kinase reaction was conducted in 20 μl containing 20 mM Pipes (pH 7.5), 10 mM $MnCl_2$, and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear) for 10 min at room temperature. Incubations were terminated by addition of sample buffer, and labeled products were resolved by SDS/PAGE and visualized by autoradiography.

Metabolic Labeling of Cells. For metabolic labeling, LA 29 cells [Rat-1 fibroblasts co-expressing the EGFR and v-Src (18)] were incubated overnight with carrier-free [^{32}P]orthophosphate (ICN; 0.5 mCi/ml) in 90% phosphate-free DMEM/10% conditioned medium. Labeled cells were rinsed twice with ice-cold PBS and lysed in CHAPS buffer, and extracts were immunoprecipitated with 3A and 4A antibody.

Two-Dimensional Tryptic Phosphopeptide Analysis. Immunoprecipitates of *in vitro* or *in vivo* ^{32}P-labeled EGFR were resolved by SDS/PAGE. The EGFR was localized by autoradiography, excised from the gel, and digested with trypsin as described by Boyle *et al.* (19). Phosphotryptic peptides were separated by electrophoresis at pH 1.9 (first dimension) and ascending chromatography (second dimension) on thin layer plates.

RESULTS

Synergism Between EGFR and c-Src in Mitogenesis and Tumorigenesis. To compare the relative rates and extents of DNA synthesis induced by EGF in Neo, 5H, NeoR1, and 5HR11 cells, a time course analysis of [^{3}H]thymidine incorporation was carried out. Fig. 1 shows that while the rate of DNA synthesis was very similar in all cell lines (reaching a maximum at 18–20 hr of stimulation), the extent of DNA synthesis was greatest in 5HR11 double overexpressors. At 18 hr after stimulation, 5HR11 cells exhibited levels of DNA synthesis that were ≈30-fold, ≈10-fold, and ≈3-fold above those in Neo, 5H, and NeoR1 cells, respectively, indicating a synergistic interaction between the EGFR and c-Src.

To assess EGF-dependent transformation, the ability of each cell line to form colonies in semisolid medium in the presence or absence of EGF was tested. As demonstrated in Table 1, a cooperative effect between c-Src and the EGFR could again be seen, wherein ≈10-fold and ≈4-fold increases in numbers of colonies were observed with 5HR11 cells as compared to 5H and NeoR1 cells, respectively. A similar potentiation was observed when an independently derived set of single and double c-Src/EGFR overexpressor clones were examined (L. K. Wilson and S.J.P., unpublished data).

To assess tumorigenicity, nude mice were inoculated with Neo, 5H, NeoR1, or 5HR11 cells. Table 1 shows that pinhead-sized tumors (1.0–2.0 mm in diameter) were found in

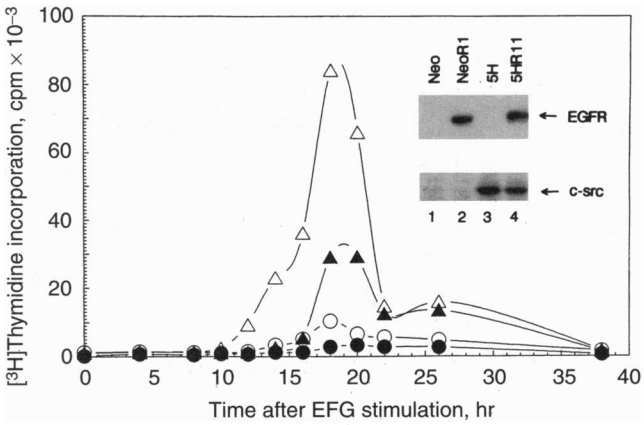

FIG. 1. c-Src potentiation of EGF-induced [^{3}H]thymidine incorporation in EGFR overexpressors. Density-arrested Neo, 5H, NeoR1, and 5HR11 cells were serum-starved overnight and incubated with EGF (40 ng/ml) for the indicated times. Incorporation of [^{3}H]thymidine was quantified. ●, Neo; ○, 5H; ▲, NeoR1; △, 5HR11. (*Inset*) Lysate protein (100 μg) from the indicated cells was examined by direct Western immunoblot analysis for EGFR and c-Src protein levels.

40–50% of mice inoculated with 5H or NeoR1, whereas 100% of 5HR11-inoculated mice developed tumors with diameters up to 1 cm in <2 weeks. These results represent the most striking demonstration of synergism between c-Src and the EGFR of the three tests performed.

***In Vivo* Association Between c-Src and Activated EGFR.** Src family kinases have been shown to stably associate with activated platelet-derived growth factor and colony-stimulating factor I receptors (20, 21). These findings raise the possibility that one mechanism by which c-Src could potentiate the tumorigenic capabilities of the EGFR would be to bind the receptor and in some manner augment its activity. However, *in vivo* complexes between endogenous c-Src and activated EGFR have been difficult to detect. We speculated that the failure to isolate such complexes may be due to their transient and unstable nature. In an attempt to circumvent this problem, stimulated and nonstimulated cells were lysed in buffers containing a mild detergent (CHAPS). c-Src or the EGFR was then immunoprecipitated from the lysates, and the immunoprecipitates were subjected to *in vitro* kinase assays. In receptor immunocomplexes, only EGFR was detected from cells expressing the human receptor (i.e., NeoR1 and 5HR11 cells) and c-Src did not appear as a coprecipitating phosphorylated substrate in either case. However, Fig. 2 demonstrates that c-Src immunocomplexes from 5HR11 cells contained a coprecipitating protein of 170 kDa that became radiolabeled during the kinase reaction (Fig. 2*D*). This protein was specifically coprecipitated by c-Src antibodies (data not shown) and was

Table 1. c-Src potentiation of tumorigenicity in EGFR-overexpressing cells

Cell line	No. of colonies		Tumorigenicity	
	- EGF	+ EGF	Tumor size	Tumor frequency
Neo	0	0	0	0/10
5H	0	35 ± 3	+	4/8
NeoR1	4	82 ± 10	+	3/8
5HR11	3	339 ± 15	+++++	8/8
1V5	2×10^4	ND	ND	ND

Values for number of colonies are the mean ± SEM obtained from three experiments of each cell type. For tumor size, each + corresponds to 1.0–2.0 mm, measured 2 weeks after subcutaneous injection of 10^7 cells at each site. ND, not determined. Tumor frequency is expressed as number of mice with tumors/total number of mice.

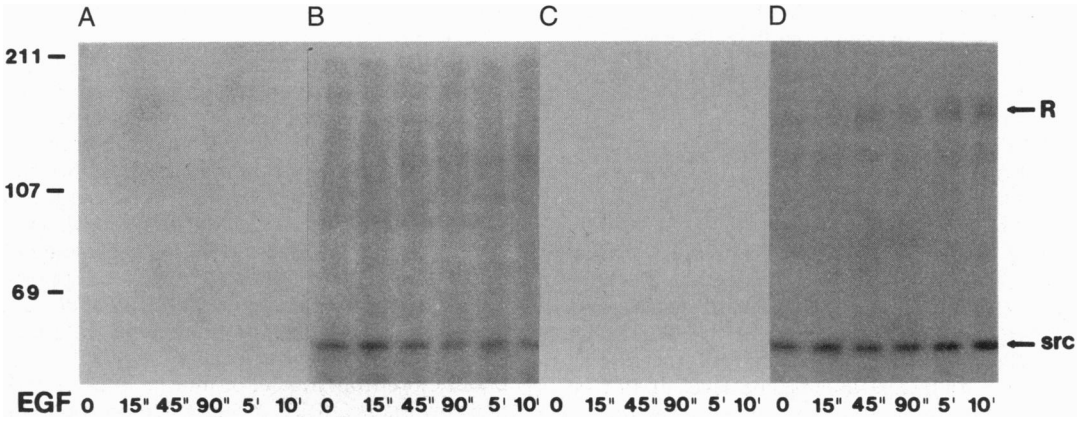

FIG. 2. *In vivo* association between EGFR and c-Src. Equal amounts (500 μg) of cell lysate prepared from nonstimulated cells or cells stimulated with EGF (100 ng/ml) for the indicated times were immunoprecipitated with the Q9 c-Src antibody. Immunocomplexes were subjected to an *in vitro* kinase reaction, and labeled proteins were analyzed by SDS/PAGE and autoradiography. (*A*) Neo control cells. (*B*) 5H cells. (*C*) NeoR1 cells. (*D*) 5HR11 cells. Upon longer exposure of autoradiograms, c-Src was detected in *A* and *C*.

absent from c-Src immunocomplexes prepared from Neo control (Fig. 2*A*), 5H (Fig. 2*B*), and NeoR1 (Fig. 2*C*) cells. The phosphorylation of this 170-kDa protein was EGF-dependent and occurred within 15 sec of treatment. Incorporation of radiolabel increased throughout the depicted time course (Fig. 2*D*) and up to 60 min of EGF stimulation (data not shown). From results of an experiment, 1–5% of the total receptor was estimated to coprecipitate with c-Src.

Src-Dependent *in Vitro* and *in Vivo* Phosphorylation of EGFR. To confirm that the 170-kDa protein that was coprecipitated with c-Src was EGFR, the *in vitro*-labeled 170-kDa protein (designated src/R) was excised from the gel and subjected to two-dimensional tryptic phosphopeptide analysis. Its peptide map was compared to that of the EGFR (designated R) that had been precipitated from NeoR1 cells after EGF stimulation and phosphorylated *in vitro*. The bulk of the tryptic phosphopeptides of these two proteins was identical, as shown in Fig. 3 (compare *A* and *B*), indicating that the 170-kDa protein that coprecipitated with c-Src was indeed the EGFR. However, two spots (peptides 0 and 3) seen in maps of Src/R (Fig. 3*B*) were not found in maps of R (Fig. 3*A*). These results indicate that the presence of c-Src resulted in the *in vitro* phosphorylation of the EGFR at two distinct sites that did not represent known EGFR autophosphorylation sites.

Wasilenko *et al.* (18) reported that in Rat-1 cells coexpressing the EGFR and v-Src, unique *in vivo* tyrosyl phosphorylated peptides of the EGFR were detected. To determine whether the EGFR from v-Src-transformed cells was phosphorylated on peptides 0 and 3 *in vivo*, EGFR was immunoprecipitated from metabolically ^{32}P-labeled Rat-1 v-Src/EGFR coexpressors and digested with trypsin. The resulting phosphopeptides were analyzed either alone (Fig. 3*C*) or as a mixture with *in vitro*-labeled Src/R tryptic peptides (Fig. 3*D*). Peptide 0 was phosphorylated *in vivo* in v-Src-transformed cells, providing evidence for the same phosphorylation occurring in the c-Src/EGFR double overexpressors. Peptide 3 was not detected in the EGFR from v-Src-transformed cells and could represent an *in vitro* anomaly, a c-Src-specific site, or a peptide from another protein, such as the HER-2/*neu* receptor.

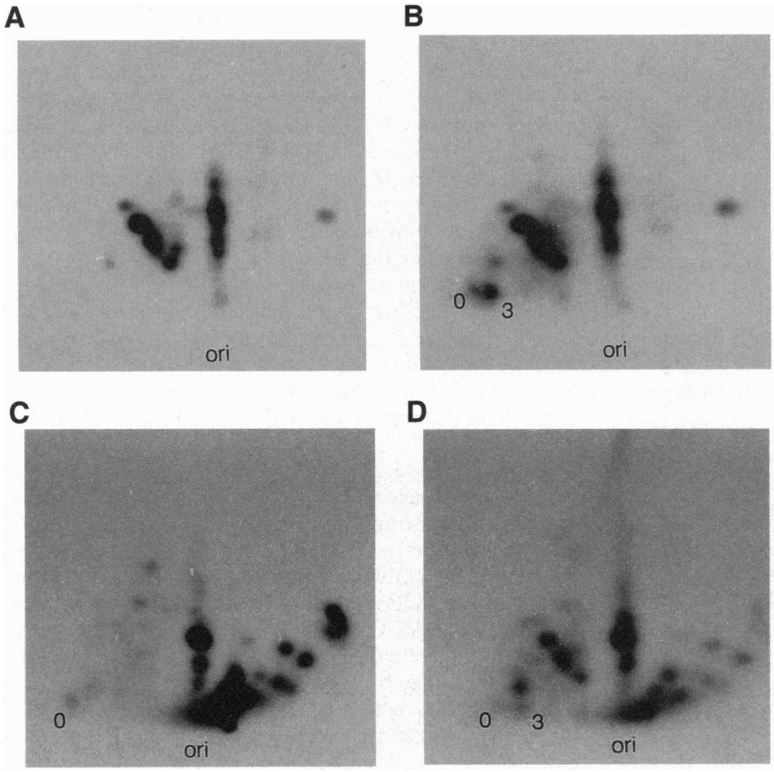

FIG. 3. Phosphotryptic peptides of EGFR radiolabeled *in vitro* and *in vivo*. *In vitro* ^{32}P-labeled EGFR was obtained from EGFR immunocomplexes from NeoR1 cells (*A*) or c-Src immunocomplexes from 5HR11 cells (*B*) stimulated with EGF (100 ng/ml) for 30 min and *in vivo* ^{32}P-labeled EGFR was prepared from Rat-1 fibroblasts coexpressing EGFR and v-Src (*C*). After SDS/PAGE, EGFR was recovered from gels and trypsinized. Phosphopeptides were analyzed in two dimensions by electrophoresis and chromatography on TLC plates. (*D*) Mixture of peptides in *B* and *C*. A constant amount of radioactivity (1000 Cherenkov cpm) of each sample was analyzed in *A–D*. ori, Origin. Spots 0 and 3 represent additional sites of tyrosyl phosphorylation in the src-associated EGFR.

Enhanced Phosphorylation of EGFR Substrates in c-Src/EGFR Double Overexpressors. To determine whether c-Src-dependent phosphorylation of the EGFR could result in enhanced phosphorylation of downstream signaling molecules, we examined the *in vivo* tyrosyl phosphorylation of PLC-γ and (Shc) (4, 22) in single and double overexpressors prior to and after EGF stimulation. Fig. 4*A* shows that tyrosyl phosphorylation of PLC-γ reached its peak 30 min after stimulation in both NeoR1 and 5HR11 cells but was enhanced in 5HR11 double overexpressor cells at 10, 30, and 60 min. Since similar amounts of PLC-γ were detected in all lysates, the elevated Tyr(*P*) content of PLC-γ in 5HR11 cells indicated an increased EGF-dependent tyrosine kinase activity in 5HR11 cells compared to NeoR1 cells, presumably accounted for by activity of the receptor itself. In four experiments, the level of PLC-γ tyrosine phosphorylation in 5HR11 cells ranged from 1.5- to 4-fold greater than that in NeoR1 cells. Fig. 4*B* shows that the level of Shc tyrosyl phosphorylation was also elevated in 5HR11 cells compared to the other cell lines. Thus, two substrates, preferentially phosphorylated by the EGFR, were found to be more highly phosphorylated when c-Src was overexpressed along with the receptor than when the receptor or c-Src was overexpressed alone.

DISCUSSION

The results described in this report demonstrate that elevated levels of c-Src, the nonreceptor tyrosine kinase, potentiate the oncogenic capacity of the EGFR. Since the EGFR and the expression level or the activity of c-Src are elevated in a common subset of human cancers (especially in breast and colon carcinomas), and high EGFR levels are associated with a poor prognosis in some types of breast cancer (5, 6, 9–11), our finding suggests that a synergism between c-Src and EGFR may contribute to malignant progression in human disease.

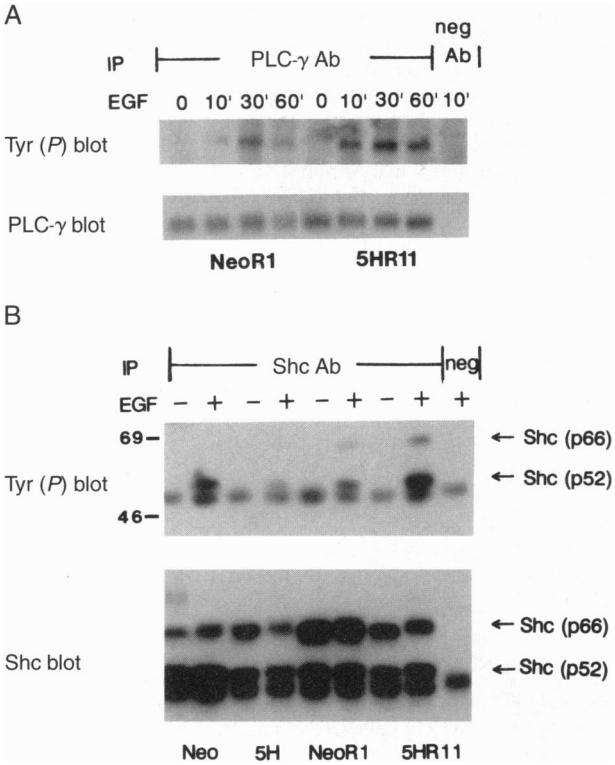

FIG. 4. Elevated tyrosyl phosphorylation of PLC-γ and Shc in cells coexpressing EGFR and c-Src. (*A*) PLC-γ. Equal amounts (500 μg) of lysate prepared from nonstimulated or EGF-stimulated (100 ng/ml) NeoR1 and 5HR11 cells were immunoprecipitated with PLC-γ antibody and examined by immunoblot analysis with either Tyr(*P*) or PLC-γ antibodies. (*B*) Shc. Lysate (500 μg) from nonstimulated or stimulated (2 min) Neo, NeoR1, 5H, or 5HR11 cells was immunoprecipitated with Shc antibody and examined by immunoblot analysis with either Tyr(*P*) or Shc antibody. IP, immunoprecipitation.

Results presented in this report also suggest a mechanism for the synergism between c-Src and the EGFR. This involves formation of a heterocomplex between c-Src and the activated EGFR, the appearance of a distinct tyrosyl phosphorylation on the receptor, and increased tyrosyl phosphorylation of receptor substrates. Enhanced signaling is presumed to result in an increase in cell proliferation. Evidence supporting this model comes from multiple studies. Luttrell *et al.* (23) and Muthuswamy *et al.* (24) have reported coprecipitation of the EGFR or HER-2/neu with the isolated c-Src SH2 domain or endogenous c-Src from human or murine breast tumor cells. Weber and coworkers (18, 25, 26) have demonstrated that the insulin-like growth factor I receptor and EGFR are constitutively phosphorylated on tyrosine and activated in cells transformed by v-Src. Wasilenko *et al.* (18) have further shown that the receptor contains two sites of tyrosine phosphorylation that are dependent upon the presence of v-Src. That c-Src can play a proactive role in tumor development has been demonstrated by Guy *et al.* (27), who showed that mice transgenic for the polyoma virus middle-sized tumor antigen require c-Src for tumor development. Thus, these findings support the model that c-Src can contribute to the malignant phenotype of cells overexpressing the EGFR by associating with and mediating the phosphorylation of the receptor, events that are postulated to result in hyperactivation of the receptor.

What is the nature of the association between c-Src and the EGFR? Activated EGFRs have been shown to form heteromeric complexes with multiple signaling and bridging molecules via SH2–Tyr(*P*) interactions (4, 22). *In vitro* affinity precipitation (23) and overlay experiments (M.-C.M. and S.J.P., unpublished data) show that the isolated c-Src SH2 domain can bind activated EGFR specifically and directly *in vitro*. These findings and previous studies from this laboratory demonstrating that the c-Src SH2 domain is required for EGF-dependent mitogenesis (1) suggest that the interaction between EGFR and c-Src *in vivo* may be direct. However, several factors also suggest that the binding may be indirect or, if direct, of a low affinity or short-lived. (*i*) In 10T½ cells, we can detect only a small portion (1–5%) of the receptor in complex with c-Src. (*ii*) We are able to visualize the complex consistently only when low-stringency detergents are employed, such as CHAPS. (*iii*) High levels of both kinases are required to demonstrate their associations. These observations are consistent with a complex that might easily dissociate during isolation, and the low number of receptors that we are able to detect may actually represent a minimal estimation of the total number that associate with c-Src *in vivo*.

Demonstration of phosphorylation of the receptor on a distinct site in c-Src immunocomplexes raises the question as to which kinase is responsible for the phosphorylation. One obvious candidate is c-Src itself. Investigations into the identity of the phosphorylated residue on peptide 0 indicate that Tyr-845 is the target (M.-C.M., T.-H.L., J. Shannon, M. J. Weber, and S.J.P., unpublished data). The amino acid sequence surrounding this residue bears 50% homology with the region encompassing the Src autophosphorylation site, Tyr-416 (28), suggesting that Tyr-845 is in a context favorable for phosphorylation by c-Src. Arguing against c-Src as the critical kinase is our inability to detect alterations in c-Src kinase activity after EGF treatment in any of the four cell lines used for this study (M.-C.M. and S.J.P., unpublished data).

An alternative candidate for the kinase that phosphorylates the receptor may be the receptor itself. Precedents for autophosphorylation with resulting hyperactivation include Src and the insulin receptor, both of which contain homologues of Tyr-845 (28, 29). Finally, the distinct tyrosyl phosphorylation

of the receptor may be mediated by a third unidentified kinase present in the c-Src–EGFR immunocomplexes. EGFR family members, such as HER-2/neu (4, 30), other Src family members, or the JAK kinases (31), are likely possibilities, and all have been reported to be involved in EGFR-mediated signaling. Regardless of its identity, our results indicate that the specific tyrosine phosphorylation of the receptor is dependent on the presence of c-Src.

Other mechanisms involving c-Src could also contribute to the transformed phenotype of c-Src/EGFR double overexpressors. For example, overexpression of c-Src could potentiate interaction of the EGFR with other family members or affect receptor internalization. Association of c-Src with the receptor could also alter its substrate specificity, permitting phosphorylation of receptor substrates, such as PLC-γ and Shc. It is equally likely that c-Src-dependent EGF-independent events could affect the malignant properties of the double overexpressors. For example, p75/p85 cortactin and p190RhoGAP are two proteins [described as preferred c-Src substrates (16, 17)] that play roles in actin-based cytoskeletal reorganization that accompanies mitogenesis and transformation (32, 33). Thus, c-Src could increase the tumorigenic properties of EGFR overexpressors by multiple mechanisms.

We thank D. Lowy for the gift of the amphotropic virus expressing human EGFR, M. Payne for Q9 c-Src antibody, and members of the S.J.P. (especially J. Biscardi), T. Parsons, and M. Weber laboratories for technical help and useful discussions throughout the course of these studies. This work was supported by Grant CA 39438 from the National Cancer Institute of the National Institutes of Health (S.J.P.) and a fellowship from the Leukemia Society of America (M.-C.M.).

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