Overexpression of Cellular Src in Fibroblasts Enhances Endocytic Internalization of Epidermal Growth Factor Receptor

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Previous studies have demonstrated a requirement for the nonreceptor tyrosine kinase, cellular Src (c-Src), in epidermal growth factor (EGF)-induced mitogenesis and a synergistic interaction between c-Src and EGF receptor (EGFR) in tumorigenesis. Although endocytic internalization of EGFR may be thought to attenuate EGF-stimulated signaling, recent evidence suggests that signaling through Ras can be amplified by repeated encounters of endosome-localized, receptor-Shc-Grb2-Sos complexes with the plasma membrane, where Ras resides almost exclusively. Based on these reports, we examined EGFR trafficking behavior in a set of single and double c-Src/EGFR C3H10T½ overexpressors to determine if c-Src affects basal receptor half-life, ligand-induced internalization, and/or recycling. Our results show that overexpression of c-Src causes no change in EGFR half-life but does produce an increase in the internalization rate constant of EGFR/EGFR complexes when the endocytic apparatus is not stoichiometrically saturated; this effect of c-Src on EGFR endocytosis is negligible at high receptor occupancy in cells overexpressing the receptor. In neither case are EGFR recycling rate constants affected by c-Src. These data indicate a functional role for c-Src in receptor internalization, which in turn could alter some aspects of EGFR signaling related to mitogenesis and tumorigenesis.

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane receptor tyrosine kinase that binds a number of related peptide growth factors, including epidermal growth factor (EGF) and transforming growth factor-α. Binding of ligand to EGFR induces transphosphorylation on several C-terminal tyrosyl residues, which upon phosphorylation bind the SH2 domains of various signaling molecules, including Shc, Grb2, PLCγ (1, 2), and Shp2 (3). Association of these molecules with the EGFR results in their activation, either via changes in subcellular localization (thereby bringing them into juxtaposition with their substrates) or by tyrosyl phosphorylation (inducing conformational changes and enzymatic activation).

These activated molecules then initiate signaling cascades that culminate in a variety of responses, including cell division (4). EGFR has been linked to the process of oncogenesis as well as to normal mitogenesis. The chicken proto-oncogene for EGFR was one of the first cellular genes found to be transduced by a retrovirus and shown to function as an oncogene (see for example Ref. 5). Increased expression of EGFR is seen in many human tumors, including glioblastomas/neuroblastomas and carcinomas of the breast, bladder, kidney, stomach, ovary, prostate, and lung (6–10), suggesting a role for EGFR in the etiology of these diseases. In some experimental cell systems, overexpression of EGFR by itself leads to malignant transformation when cells are grown in the continuous presence of EGF (11, 12).

Cellular Src (c-Src) is a 60-kDa nonreceptor tyrosine kinase, the first proto-oncogene identified in humans. A role for c-Src in EGF-mediated responses has been demonstrated by a number of studies. First, overexpression of wild-type c-Src in C3H10T½ murine fibroblasts expressing normal levels of receptor (3–10 × 10³ receptors/cell) potentiated EGF-directed mitogenesis, whereas overexpression of dominant negative variants of c-Src not only ablated the enhanced mitogenic response but also interfered with the endogenous response (13, 14). These findings indicated that c-Src is required for EGF-induced mitogenesis, a conclusion substantiated by Roche (15). In subsequent studies, Maa et al. (16) reported that overexpression of both c-Src and EGFR in 10T½ cells (1–3 × 10³ receptors/cell) resulted in synergistic increases in EGF-dependent growth and tumor formation in nude mice.

The mechanism(s) by which c-Src influences the biological action of the EGFR are not entirely elucidated, although several studies have provided clues to the problem. c-Src has been found associated with EGFR in breast cancer cell lines (17), in fibroblasts overexpressing both c-Src and the receptor (16), and in A431 cells (19). In fibroblasts, this association is EGF-dependent and results in the appearance of two novel tyrosyl phosphorylations on the receptor and increased phosphorylation of receptor substrates in vivo (16). These findings suggest that c-Src potentiates the action of the EGFR by binding to it and inducing its phosphorylation, resulting in hyperactivation of the receptor and enhanced downstream signaling. Several studies have attempted to localize the site of c-Src binding to the receptor, using in vitro peptide competition approaches (17, 20, 21). Tyr-992 is the most consistently noticed site, but other Tyr residues have also been implicated, suggesting that c-Src may bind multiple sites. Activation of c-Src following EGF stimulation of fibroblasts overexpressing EGFR has also been reported (22, 23), suggesting that the two tyrosine kinases are capable of mutual regulation.
HINTS also exist that c-Src may influence endocytic trafficking of EGFR. A significant fraction of c-Src in fibroblasts has been found associated with endosomes (24), and the SH3 domain of c-Src is capable of binding and activating dynamin (25), a GTPase that localizes to the plasma membrane and is a regulator of endocytosis (26–28). Bergeron and co-workers (reviewed in Ref. 29) have evidence that selective and regulated signal transduction from receptor tyrosine kinases can continue within the endosome. Finally, Wang and Moran (30) recently reported the requirement for the signal transducer, Grb2, in endocytosis of EGFR.

With these factors in mind, we undertook an examination of single and double c-Src/EGFR overexpressors to determine if c-Src had an effect on endocytic internalization and recycling of EGFR. We have obtained the new finding that c-Src increases the rate of endocytosis of EGF-EGFR complexes until the endocytic apparatus is saturated and becomes rate-limiting. c-Src has no effect on EGF-EGFR complex recycling. In the context of previous findings, our results suggest that, among multiple mechanisms, c-Src may potentiate the action of EGFR by enhancing complex internalization, possibly amplifying signaling through an endosome-associated intermediate.

EXPERIMENTAL PROCEDURES

Materials—EGF was isolated from mouse submaxillary glands by Dr. Steve Wiley at the University of Utah (31) and was subsequently iodinated with 125I (Amersham) using Iodobeads (Pierce) following the manufacturer’s protocol. Specific activities were generally ~150,000 cpm/ng. Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline, L-glutamine, penicillin/streptomycin, and fetal bovine serum were all acquired from Sigma. Geneticin/G418 sulfate and trypsin EDTA were obtained from Life Technologies, Inc. Isoton II used in cell counting was manufactured by Coulter Diagnostics (Hialeah, FL). Tissue culture flasks used for routine cell culture were obtained from Corning Costar. All plates used for trafficking and proliferation experiments were Falcon (Franklin Lake, NJ) tissue culture plates.

Cell Lines and Passage—Derivation and characterization of the cell lines used have been described previously (13, 14, 16). Essentially, 10T1⁄2 cells were first transfected with a plasmid expressing a neomycin-resistance gene only (Neo), plasmids expressing a neomycin-resistance and a full-length wild-type avian c-src gene (5H), or plasmids expressing both a neomycin-resistance and a kinase-defective avian c-src gene. The kinase-defective c-Src protein harbors an Ala-Val substitution at residue 430 in the kinase domain and is designated hereafter as 430. The Neo and 5H cell lines were infected with an amphotropic retrovirus expressing a full-length human EGF receptor cDNA to produce the NR and 5HR cell lines. 5H and 5HR cells contain equal levels of c-Src (approximately 20- to 30-fold above endogenous), and NR and 5HR express comparable numbers of surface EGF receptor (2–3 × 10^5/cell). Cells were routinely passaged in DMEM supplemented with 10% fetal bovine serum and 0.4 mg/ml Geneticin/G418 sulfate and were not allowed to reach confluence during passage, to avoid spontaneous transformation. Pre-confluent 75 cm^2 tissue culture flasks were split 1:5 every 2 days. Cells were frozen in regular passage medium supplemented with 10% Me2SO and 25% fetal bovine serum.

Basal Receptor Half-life—Cells were grown to ~80% confluence, washed three times in prewarmed phosphate-buffered saline, and incubated for 30 min in methionine-deficient DMEM (ICN, Costa Mesa, CA) supplemented with 5% dialyzed fetal bovine serum. Cells were labeled in 100 μCi/ml L-[35S]methionine (NEN Life Science Products) for 1 h, washed three times in prewarmed phosphate-buffered saline, and chased in DMEM medium containing 200-fold excess unlabeled methionine and 30 μg/ml cyclohexamide (Sigma) for 4, 7.5, 12, 15, or 22 h. Cells were lysed in radioimmune precipitation buffer (150 mM NaCl, 50 mM Tris base, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 0.5% aprotinin, 12.5 μg/ml leupeptin, 1 mM sodium vanadate), and then EGF was immunoprecipitated, resolved by SDS-polyacrylamide gel electrophoresis, and quantified by Molecular Dynamics PhosphorImager.

Trafficking Parameters—Cells were grown to confluence (150–300 cells/mm²) on 35- or 60-mm plates in normal medium and then switched to DHB (DMEM with 20 mM Hepes and 1 mg/ml bovine serum albumin) containing 2 mM L-glutamine approximately 30 min before each experiment.

**FIG. 1.** Comparison of surface and internal counts of (A) EGFR (NR) and (B) c-Src/EGFR (5HR) overexpressors as a function of time. EGF was added to the cells at t = 0. Closed circles, surface counts; open circles, internal counts.
placed at 37 °C in prewarmed DHB. The DHB was aspirated, and excess cold EGF was added at regular time intervals. At each time point, the unlabeled EGF was aspirated at 0 °C, and the cells were washed six times with ice-cold WHIPS buffer. Acid strip with urea was used to remove any surface-bound ligand, and internalized ligand was determined by solubilizing cells in 1N NaOH at 37 °C for 1 h. The recycling rate constant could be computed from the slope of a plot of ln Ci versus t (32), assuming that ligand degradation was negligible. To assess the amount of degraded ligand, the unlabeled EGF chase was collected rather than aspirated and passed over a G-15 Sephadex column (34).

RESULTS

Previous work has shown an association of c-Src with both EGFR (16–19) and endosomal membranes (24). These associations, along with the increase in mitogenesis observed in cells overexpressing c-Src upon incubation with EGF (13), suggest a mechanism of action for c-Src in mitogenic signaling events that involves EGFR trafficking. To determine whether trafficking parameters are affected by c-Src, the basal half-life of EGFR and EGF-induced internalization and recycling rate constants were measured as described under “Experimental Procedures.”

C3H10T1/2 murine fibroblasts overexpressing EGFR alone (NR) or overexpressing both c-Src and EGFR (5HR) were examined for effects of c-Src on the basal EGFR half-life by [35S]methionine pulse-chase analysis. Fig. 2 shows that EGFR in the NR cells and 5HR cells exhibit turnover half-times of approximately 20 and 22 h, respectively, in the absence of EGF. Thus, c-Src overexpression has little or no effect on intrinsic EGFR turnover.

EGF-induced endocytic rate constants (k_e) for EGFR were measured for the following panel of C3H10T1/2 fibroblasts: neomycin-resistant only (Neo), c-Src overexpressors (5H), kinase-defective c-Src overexpressors (430), EGFR overexpressors (NR), and c-Src/EGFR double overexpressors (5HR). Representative internalization data are shown in Fig. 3. The endocytic rate constants are simply the slopes of the lines shown. Panels A and B represent rates at a low number of surface complexes (<300/cell). It is clear that both of the cell lines overexpressing c-Src (5HR and 5H) had increased endocytosis rates compared to their respective counterparts. Panel C demonstrates that the c-Src effect was not seen at all levels of surface complexes. The number of surface complexes in panel C is 2 orders of magnitude higher than those in A or B. For this circumstance, the cells overexpressing c-Src had the same endocytosis rate as those with endogenous levels.

The entire range of endocytic rate constants measured for the various cell lines is shown in Fig. 4. Fig. 4A shows that as the average number of surface EGF-EGFR complexes drops below 5,000, the endocytic rate constant for the 5HR cell line increased dramatically, whereas the rate constant for the NR cell line reached a plateau. Neo and 5H cell lines also exhibited a difference in internalization rate constants in this region (Fig. 4B); the average k_e value for the Neo cell line was 0.042 ± 0.016, whereas the average value for the 5H cell line was 0.096 ± 0.014. These data demonstrate that c-Src can cause an increase in EGFR internalization rate. The EGFR internaliza-
ition rate constant in the 430 cell line is somewhat lower than for the Neo cell line (Fig. 4B) but not with statistical significance (k_e = 0.023 ± 0.014). At high receptor occupancy levels above 5,000 surface complexes/cell, endocytic rate constants for 5HR and NR were identical due to saturation of the endocytic apparatus (35, 36). Because of the low level of EGFR expressed in the 5H and Neo cell lines, we were unable to saturate the endocytic machinery.

Note, comparing Fig. 4A with 4B, that the internalization rate constants for the Neo and NR cell lines exhibit different averages at low receptor occupancy. This is most likely due to differences in the overall average C_s values between the two cell lines. Since Neo therefore exhibits a larger average C_s (data not shown), the internalization rate constant should be slightly lower than for NR.

To check for specificity of the c-Src effect, endocytic rate constants for the 5HR and NR cell lines should be measured for a different ligand/receptor pair. The transferrin receptor has been previously shown to internalize independently of receptor occupancy and to be unaffected by the presence of saturating levels of EGF, indicating that transferrin receptor internalization follows a different mechanism than that of EGFR (35).

Accordingly, we tested the effect of c-Src on the internalization rate constant of transferrin receptor. The 5HR and NR cell lines were found to internalize transferrin similarly, exhibiting rate constants of 0.25 ± 0.03 and 0.30 ± 0.03 (n = 4), respectively. Thus, the effect of c-Src appears to be specific.
acid strip or insufficient cold chase, resulting in reinternalization of labeled ligand. Analysis of pulse-chase data neglects any internalization of labeled ligand; the presence of excess unlabeled ligand and the light acid strip removes any labeled ligand from the internalization pool. The analysis used to calculate the recycling rate constant from internalization data takes the endocytic rate constant into account, resulting in the calculation of a higher recycling rate constant.

To determine if differences in recycling were hidden by assay insensitivity, steady-state values for the numbers of internal and surface EGF-EGFR complexes \((C_i, C_s)\) were measured for all four cell lines. Fig. 6, panels A and C, show that at all concentrations of surface complexes, the 5HR cell line exhibited a higher ratio of \(C_i\) to \(C_s\) than the NR cell line, consistent with its increased EGFR internalization rate constant and unchanged EGFR recycling rate constant. This trend remains for concentrations of ligand that saturate the endocytic machinery, which is not consistent with the observed endocytic rate constants. This implies there could indeed be some differences in recycling, rate or sorting fractions at high occupancy. There could also be an effect due to degradation by 3 h. There were no significant differences between the 5H and Neo cell lines, even though their internalization rate constants differed (Fig. 6, panels B and D). Since the internalization rate constants were lower for the 5H and Neo cell lines compared with the 5HR and NR lines, it was not surprising that the effect of endocytic dynamics on steady-state receptor distribution was minimal for the former (32). To fully answer this question, assays more sensitive than any currently available would need to be developed.

**DISCUSSION**

Our data demonstrate a new functional role for c-Src in EGFR trafficking dynamics, specifically with respect to receptor endocytosis and steady-state levels of internalized receptor. Increased rate constants for EGFR internalization were observed in c-Src overexpressors relative to control cells expressing endogenous levels of c-Src. Internalization rates were found to be increased until the endocytic machinery became saturated at high levels of EGF-EGFR complexes. Increased internalization rates were also reflected in the larger steady-state pool of internalized EGFR in cells overexpressing both EGFR and c-Src compared with cells overexpressing EGFR alone.

Our experimental methodology for studying EGF-induced EGFR internalization and recycling has relied on using \(^{125}\text{I}\)-labeled EGF to follow EGF-EGFR complexes. EGF-EGFR complexes have been found to remain largely associated during endosomal sorting (37), and previous work has demonstrated consistency of this methodology with other approaches to quantifying EGFR trafficking processes (33).

The mechanism by which c-Src affects EGFR internalization is not known, but an examination of the endocytic process yields some possible clues. Ligand-activated EGFRs are recruited into clathrin-coated pits by an unknown component of
the endocytic apparatus. A candidate for such a component is adaptin (38). At low levels of ligand-receptor complexes, c-Src may function by potentiating the activity of such a recruitment factor. At high complex numbers, this factor becomes limiting and is no longer available to be recruited or activated by c-Src to bind additional complexes. A similar hypothesis has been proposed as an explanation for the differential affinity of EGFR mutants for coated pits (39). In this scenario, it was suggested that the affinity of the receptor for coated pits depends upon the binding of the receptor to a specific internalization component (IC) and the binding of the receptor-IC complexes to coated pits. In the work discussed here, c-Src might be affecting the rate of receptor-IC complex formation.

Whether physical association between c-Src and EGFR is required for this process or whether c-Src acts independently of the receptor or in concert with other tyrosine kinases is unknown. In the absence of EGF, c-Src overexpression does not affect EGFR half-life. This finding suggests that physical association between c-Src and EGFR induced by EGF is important for the effect of c-Src on EGFR internalization. The requirement of a physical complex for the effect of c-Src on EGF-induced tumorigenesis has previously been suggested (16). Internalization of the constitutively internalized transferrin receptor is not affected by the presence of c-Src. This implies that the increased internalization of EGFR due to c-Src has a certain specificity. In addition, there is the question of possible involvement of other tyrosine kinases or even other receptors, such as ErbB2, in the internalization process. Investigating the presence and effect of any other components is a question for future work.

In addition to the recruitment factor or internalization component, c-Src may affect the function of dynamin. Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). Dynamin is a GTPase that is critical for the formation and release of the endocytic vesicles from the plasma membrane (26–28). 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