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c-Src trafficking and co-localization with the EGF Receptor promotes EGF ligand-independent EGF Receptor Activation and Signaling

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Abstract

c-Src is a non-receptor tyrosine kinase that associates with both the plasma membrane and endosomal compartments. In many human cancers, especially breast cancer, c-Src and the EGF Receptor (EGFR) are overexpressed. Dual overexpression of c-Src and EGFR correlates with a Src-dependent increase in activation of EGFR, and synergism between these two tyrosine kinases increases the mitogenic activity of EGFR. Despite extensive studies of the functional interaction between c-Src and EGFR, little is known about the interactions in the trafficking pathways for the two proteins and how that influences signaling. Given the synergism between c-Src and EGFR, and the finding that EGFR is internalized and can signal from endosomes, we hypothesized that c-Src and EGFR traffic together through the endocytic pathway. Here we use a regulatable c-SrcGFP fusion protein that is a bona fide marker for c-Src to show that c-Src undergoes constitutive macropinocytosis from the plasma membrane into endocytic compartments. The movement of c-Src was dependent on its tyrosine kinase activity. Stimulation of cells with EGF revealed that c-Src traffics into the cell with activated EGFR and that c-Src expression and kinase activity prolongs EGFR activation. Surprisingly, even in the absence of EGF addition, c-Src expression induced activation of EGFR and of EGFR-mediated downstream signaling targets ERK and Shc. These data suggest that the synergy between c-Src and EGFR also occurs as these two kinases traffic together, and that their colocalization promotes EGFR-mediated signaling.

1. Introduction

c-Src is a member of a family of membrane bound tyrosine kinases that regulate cell growth, differentiation and adhesion [1,2]. c-Src is initially synthesized on soluble ribosomes in the cytosol and is co-translationally modified with myristate on its N-terminus. Fatty acylation of c-Src influences its ability to bind cellular membranes and consequently affects its intracellular distribution and signaling capabilities [3].

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c-Src is a critical signal transducer for the EGF receptor tyrosine kinases (EGFR) [4]. Considerable biological synergy exists between c-Src and the EGFR [5]. In many human cancers, particularly breast cancer, c-Src and members of the human epidermal growth factor receptor (HER) tyrosine kinases, specifically HER1/EGFR and HER2/neu, are both highly overexpressed [6]. Dual overexpression of c-Src and EGFR occurs in some breast cancer cell lines and this has been correlated with Src-dependent increases in activation of and/or phosphorylation of EGFR downstream effectors. c-Src activity is required for ErbB2-mediated induction of cell growth and motility [7,8]. Overexpression of both c-Src and EGFR leads to synergistic activation of EGF induced mitogenesis and tumor formation [9,10]. c-Src binds to and phosphorylates EGFR on Tyr845; this phosphorylation event is required for EGF-mediated mitogenesis [9,11]. The synergism between c-Src and EGFR serves to upregulate the mitogenic activity of EGFR downstream effectors involved in tumorigenesis, as well as increasing EGFR internalization. Thus, obtaining a clearer understanding of c-Src subcellular localization and its relationship with EGFR signaling is pivotal in understanding the mechanisms underlying malignant cell signaling.

Ligand addition induces EGFR endocytosis [12]. Although it was originally assumed that only plasma membrane localized receptors would signal, it is now known that internalized EGFR can continue to signal from endosomes [13]. Given that endosomally localized EGFR is capable of signaling, and that c-Src phosphorylation of EGFR is important for EGFR signaling, it is logical to hypothesize that c-Src may share a trafficking pathway with EGFR that allows the activated receptor to signal from endosomes. Several lines of evidence indicate that c-Src interacts with the endosomal machinery. First, c-Src associates with endosomal membranes in fibroblasts [14]. Other Src family kinases have also been shown to interact with endosomes and/or lysosomes in other cell types [15–19]. Second, c-Src colocalizes with dynamin and γ -adaptin, proteins involved in clathrin-coated pit endocytosis [20]. c-Src phosphorylates dynamin and clathrin in EGF stimulated cells; phosphorylation promotes endocytosis and increases the pool of activated, internalized receptors in endosomes [21,22]. Third, c-Src regulates the rate of EGFR downregulation [23]. Upon EGF binding, EGFR is ubiquitinated by the ubiquitin ligase Cbl and degraded. Overexpression of c-Src increases Cbl ubiquitination and degradation, thereby resulting in less Cbl-mediated EGFR degradation and increased levels of EGFR [24]. Thus, it is possible that c-Src endocytoses along with internalized receptors, and that this event promotes receptor-mediated signaling from internal organelles.

Recent studies have indicated that different trafficking pathways are used by different Src family kinases en route to the plasma membrane. For example, Lck, Lyn and Hck traffic through the Golgi and the secretory pathway, whereas Src and Fyn do not [15,25–28]. Surprisingly, the trafficking pathway of native c-Src remains largely unknown. Imaging studies have revealed the presence of c-Src in both endosomes and the plasma membrane [14], but the mechanisms regulating the distribution between these two locations are only starting to be understood. It was recently shown that upon PDGF stimulation of cells, an internal pool of c-Src is activated and transported to the plasma membrane and cytoskeleton by RhoB containing endosomes [29]. c-Src kinase activity has also been shown to regulate RhoD-dependent endosome movement [30].

To date, little is known about the mechanism of c-Src trafficking from the plasma membrane to intracellular compartments. Multiple venues exist for internalization from the plasma membrane into the cell interior, including clathrin mediated endocytosis, caveolin-mediated endocytosis, clathrin and caveolin-independent endocytosis, phagocytosis and macropinocytosis [31,32]. Previous studies have documented that v-Src expression induces constitutive macropinocytosis – an actin-driven, clathrin-independent endocytic process promoting the uptake of fluid-phase solute [33–35]. Here we show that c-Src internalizes via constitutive macropinocytosis from the plasma membrane and that this movement is dependent

on c-Src kinase activity. c-Src and the activated EGFR co-localized and trafficked together during EGF stimulation, and EGF activation was prolonged in a c-Src kinase-dependent manner. We also report that c-Src can promote and sustain EGFR activation even in the absence of EGF ligand and that this results in enhancement of downstream EGFR signaling.

2. Materials and Methods

2.1 Antibodies and reagents

Antibodies were purchased from the indicated suppliers: polyclonal rabbit anti-EGFR, anti-cSrc (N-16), anti-ERK2 (C-14), anti-phospho-ERK (Y204), and anti-phospho-EGFR (Y1173) and mouse monoclonal anti-phospho ERK (E-4), Santa Cruz Biotechnology Inc. (Santa Cruz, CA); Rabbit anti-phospho-c-Src (Y416), and anti-phospho-EGFR (Y1173), Cell Signaling Technology (Danvers, MA); mouse monoclonal anti-Rac, anti-EEA1, BD Transduction Laboratories (San Diego, CA); anti-CD63, Cymbus Biotechnology Ltd. (Hampshire, UK); Alexa Fluor conjugated anti-mouse, anti-rabbit, and anti-goat secondary, Molecular Probes-Invitrogen (Carlsbad, CA); goat anti-mouse Cy5, Jackson ImmunoResearch Labs (Westgrove, PA); anti-EGFR (LA1) neutralizing antibody, Millipore (Billerica, MA). Texas-red phalloidin, Alexa Fluor 647 Dextran (10000Da), and Alexa Fluor 647 EGF were purchased from Molecular Probes -Invitrogen. EGF was purchased from Calbiochem-Oncogene Research Products (La Jolla, CA).

2.2 Plasmid Construction

Full-length chicken c-Src was amplified by PCR with the sense primer 5' ATAGGAATTCATGGGGAGCAGCAAGAGCAAGCC 3' and the antisense primer 5' GACCGGTGGATCCCGTAGGTTCTCTCCAGGCTGGTA 3'. The PCR products and the pEGFP-N1 plasmid (Clontech) were digested with EcoRI and BamHI. Digested PCR products were ligated into pEGFP-N1 and the construct was confirmed by DNA sequencing. Point mutants of c-SrcEGFP were generated using the QuikChange mutagenesis kit (Stratagene). K295R c-SrcEGFP (kinase-dead) was generated using the following sense and antisense primers: 5' ACCAGAGTGGCCATACGGACTCTGAGCCCGGC 3', and 5' GCCGGCCTTCAGAGTCCGTATGGCCACTCTGGT 3'. Y527F c-SrcEGFP (constitutively active) was generated using the following sense and antisense primers: 5' TCGACAGAGCCCCAGTTCAGCCTGGAGAG 3', and 5' CTCTCCAGGCTGGAAGTGGGGCTCTGTCTGA 3'. Point mutants (A206K, L221K, and F223R) to render EYFP monomeric were made in the p-EYFP-N1 plasmid according to earlier studies using the QuikChange mutagenesis kit (Stratagene). The following sense and antisense primers were used: A206K 5' CACTACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAAC 3', 5' GTTGGGGTCTTTGCTCAGCTTGGACTGGGTGCTCAGGTAGTG 3', and L221K/F223R 5' CGCGATCACATGGTCTTGAAGGAGCGCGTGACCGCCGCCGGGATC 3', 5' GATCCCGGCGGCGGTACGCGCTCCTTCAGGACCATGTGATCGCG 3'. Monomeric c-SrcEYFP was constructed by digesting c-SrcEGFP and monomeric pEYFP-N1 (mEYFP) plasmid with EcoRI and BamHI, and by ligating c-Src into mEYFP.

2.3 Cell culture and transfection

COS-1 and COS-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. NIH 3T3 stable cell lines [36] expressing c-Src were maintained in DMEM/10%FBS supplemented with 0.5 g/L Geneticin (Invitrogen). Plasmid DNA amounts of 2–6 µg were used for transfections in 60 mm or 100 mm diameter dishes.

2.4 Western Immunoblotting

Cells were lysed in 1X RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% TritonX, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF, 1 mM NaVO₄) containing 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 250 mg/ml 4-(2-aminoethyl) benzene sulfonyl fluoride. Lysates were cleared by centrifugation at 55000 rpm at 4°C for 15 minutes. Protein concentration was measured using the DC Protein Assay (BioRad) and gel samples were prepared to ensure loading of equal protein amounts. Samples were run on 10% SDS-PAGE and Western immunoblotting was performed with the appropriate antibodies. Proteins were detected using the appropriate secondary antibodies conjugated to horseradish peroxidase and enhanced chemiluminescence. Western blots were scanned and quantitated using MacBas v2.0.

2.5 Pulse-Chase Stability

COS-1 cells were transfected as required and were split the next day into 60mm dishes. Cells were starved 48 hours post-transfection for 1 hour in DMEM supplemented with 2% dialyzed FBS and without cysteine and methionine. Cells were pulsed for 7 minutes with DMEM (- Cys/-Met) containing 50 mCi/mL of 35S Translabel at 37°C, and then chased for various timepoints as needed in DMEM containing 10% FBS and an excess of unlabeled cysteine and methionine. At each timepoint, cells were brought to ice, and lysed in 1X RIPA buffer. Lysates were cleared by centrifugation at 55000 rpm at 4°C for 15 minutes. cSrc immunoprecipitation was performed by incubating lysates overnight at 4°C with Protein A/G Agarose beads (Santa Cruz Biotechnology Inc.) using a rabbit polyclonal anti-Src antibody produced in the Resh lab. Samples were centrifuged and washed in successive rounds, resuspended in 2X SDS-PAGE sample buffer and boiled prior to loading on 10% SDS-PAGE gel. After SDS-PAGE, gels were soaked for 30 minutes in 1 M sodium salicylate at room temperature, dried for 1 hour, and then placed in a Phosphor screen for 1–3 days. Phosphor screens were scanned and analyzed using a Molecular Dynamics Storm PhosphorImager and IQ Mac imaging software.

2.6 Indirect Immunofluorescence

COS-1 or COS-7 cells were split 24 hours post-transfection onto 25 × 25 cm coverslips and placed in 6-cm dishes. 48 hours post-transfection, cells were rinsed twice with PBS, fixed with 4% formaldehyde/PBS at room temperature for 15 minutes. Cells were washed twice prior to permeabilization with 0.2% Triton X-100 for 5 min. Cells were rinsed again, blocked in 3% BSA/PBS for 30 minutes followed by 1h incubation at room temperature in primary antibodies as indicated. After 4 washes lasting 5 minutes each in PBS, cells were incubated for 45 minutes in fluorescently conjugated secondary antibodies as required for detection. For nuclear staining, cells were incubated in Hoechst dye, and then rinsed 4 times for 5 minutes in PBS before being mounted onto microscope slides using Prolong Gold Antifade reagent (Molecular Probes). In EGF stimulation experiments, transfected cells were split onto coverslips 24-hours post-transfection and then serum-starved overnight. The next day, cells were pulsed +/- 20 ng/mL of EGF for 10 minutes at 37°C, brought on ice, acid-washed (pH 4.5), and then washed twice for 5 minutes in PBS. Cells were then chased in serum-free DMEM for various timepoints as indicated; cells at the 0 minute timepoint remained on ice and were fixed immediately. Antibody staining was performed as described above. In some experiments, cells were pre-incubated for 1 hour at 37°C with 10 mg/mL anti-EGFR neutralizing antibody LA1 prior to EGF stimulation. 30–40 cells were imaged for each condition. Laser scanning confocal microscopy was performed as described previously [37] on a Zeiss LSM510 confocal microscope equipped with an Axiovert 100M inverted microscope using a 63X, 1.2-numerical-aperture water immersion lens for imaging. Colocalization was quantitated using MetaMorph software v6.0 (Molecular Devices).

2.7 Time-Lapse Microscopy

Cos-7 cells were split 24 hours post-transfection into 0.17 mm diameter black Delta T dishes suitable for live-cell imaging. Cells were transferred into a buffer containing Hank's Balanced Salt Solution containing 10mM hepes (pH 7.4) and were treated as required and placed on a Perkin-Elmer Spinning Disk microscope equipped with 60X, 1.4-numerical-aperture oil immersion lens. Live imaging was performed at 37°C.

3. Results

3.1 c-SrcGFP is a bona fide reporter of c-Src

To facilitate the study of c-Src trafficking in cells, a c-Src GFP fusion construct was generated. Since the N-terminus of c-Src contains the myristoylation site that governs its association to membranes [3], GFP was fused to the C-terminus of the protein. A previous study revealed that fusion of GFP directly to the C-terminus of c-Src generates a constitutively activated c-Src that is no longer capable of undergoing negative regulation [38]. We therefore created a seven amino acid linker between the C-terminal residue of c-Src and the N-terminal residue of GFP. This c-SrcGFP construct was transfected into COS-1 cells and its subcellular distribution was compared to that of c-Src. Earlier reports demonstrated that c-Src localized to endosomes [14], and at the cell periphery [39]. Likewise, c-SrcGFP expressed in COS-1 cells was distributed at the perinuclear region and at the plasma membrane (Figure 1A). COS-1 cells expressing c-Src and c-SrcGFP were lysed, and analyzed by SDS-PAGE and Western blotting with anti-c-Src antibody. As depicted in Figure 1B, expression levels of c-Src and c-SrcGFP were equivalent.

We then compared the stability of c-SrcGFP to that of c-Src. Pulse-chase metabolic labeling using ³⁵S-labeled cysteine and methionine revealed that c-Src and c-SrcGFP have half-lives of 9.6 and 9.5 hours respectively, indicating that both proteins have similar stabilities (Figure 1C). Lastly, we tested whether fusion of GFP to c-Src abrogated its ability to be regulated during autoactivation. Lysates derived from COS-1 cells expressing c-Src, c-SrcGFP, constitutively activated c-Src (Y527F c-Src) or constitutively activated c-SrcGFP (Y527F c-SrcGFP) were analyzed by immunoblotting with anti-PY416 antibody, which detects activated c-Src phosphorylated at Tyr 416, and total c-Src (Figure 1D). c-Src and c-SrcGFP exhibited similar ratios of activated/total c-Src, indicating that fusing GFP to the C-terminus of c-Src did not result in artificial activation of c-Src kinase activity. In contrast, Y527F c-Src and Y527F c-SrcGFP were both activated to 7- and 8-fold higher levels than their wildtype counterparts, respectively. Taken together, these results demonstrate that c-SrcGFP is localized and regulated in a manner identical to c-Src, and is thus a valid reporter of c-Src localization and function.

3.2 Intracellular c-Src localizes to endocytic compartments and is activated

Earlier studies reported that c-Src was localized to the Golgi apparatus and endosomal membranes, but did not identify the endosomal compartments that contained c-Src. Using indirect immunofluorescence, we observed that c-SrcGFP colocalized with both early endosomal antigen 1 (EEA1), a marker for early endosomes, and CD63, a marker for late endosomes and the multivesicular body (MVB) (Figure 2A). Quantitation using MetaMorph software revealed that the extent of colocalization of the perinuclear pool of c-SrcGFP was 17% and 25% for EEA1 and CD63 respectively (Figure 2B). In contrast, no colocalization of c-SrcGFP with anti-mannosidase II, a cis-medial Golgi marker, was observed (data not shown).

We next examined the localization of activated c-SrcGFP within cells. Most of the activated c-SrcGFP (67%) was present at the cell surface at sites of membrane ruffling (Figure 2C). In a subset of cells (33%), activated c-SrcGFP was localized both at the plasma membrane and at the membranes of enlarged intracellular vesicles (Figure 2C). Cells expressing a kinase-dead

(KD) c-SrcGFP fusion protein were devoid of signal for activated c-Src, confirming the specificity of the anti-pY416 antibody.

3.3 c-Src undergoes constitutive macropinocytosis from the plasma membrane

The presence of distinct populations of activated c-Src and total c-Src in perinuclear vesicles as well as the plasma membrane led us to question how the distribution between the two locations was achieved. A recent report demonstrated that c-Src can traffic from RhoB-positive endosomes to the plasma membrane, and that PDGF stimulation causes c-Src activation during its transit [29]. However, it is not known how or if c-Src traffics from the plasma membrane to intracellular sites and whether this event occurs via the endocytic pathway. To address this question, time-lapse confocal imaging experiments were performed on live cells expressing c-SrcGFP. COS-7 cells were used for live-cell imaging experiments, since these cells are flatter and more homogeneous than COS-1. c-SrcGFP was distributed at both the plasma membrane and the perinuclear region, as was observed in COS-1 cells. Time-lapse imaging revealed the formation of enlarged vesicles from the plasma membrane at sites resembling membrane ruffles (Figure 3A). With time, c-SrcGFP-enriched plasma membrane ruffles altered shape, circularized, and formed a closed vesicle containing a c-Src-enriched tail that protruded from the vesicle. The tail was then shed, and the vesicle started shrinking and slowly moved towards the perinuclear region of the cell. Vesicles ranged from 2–5 μm in diameter (Figure 3A).

Both the size of the vesicles and their formation from the plasma membrane at sites resembling membrane ruffles suggested that c-SrcGFP was trafficking via macropinocytosis, a clathrin-independent endocytic pathway. Three methods were then used to assess macropinocytosis: uptake of the fluid phase marker, Dextran; co-localization with markers for membrane ruffles; and co-localization with F-actin, since membrane ruffling and macropinosome formation are actin-dependent processes. First, c-SrcGFP expressing COS-1 cells were subjected to a thirty-minute pulse of fluorescently-labeled Dextran. The cells were fixed and visualized by confocal imaging. Dextran uptake was clearly observed in c-SrcGFP vesicles emanating from plasma membrane ruffles (Figure 2D). Second, co-staining with antibody to Rac1 revealed that c-SrcGFP colocalized with Rac-enriched membrane ruffles. Third, significant colocalization between c-SrcGFP and Texas-Red phalloidin, which stains F-actin, was detected at plasma membrane ruffles (Figure 2D). In some cells, c-Src and actin also co-localized at the membranes of enlarged vesicles. To determine whether c-Src tyrosine kinase activity was required for macropinocytosis, KD c-SrcGFP was expressed in cells and visualized in time-lapse confocal imaging experiments. KD c-SrcGFP localized to sites of plasma membrane ruffling. However, with time, the ruffles receded into the cytoplasm without forming vesicles (Figure 3B). Taken together, these data strongly suggest that c-SrcGFP traffics from the plasma membrane to internal membranes via macropinocytosis and that this movement is dependent on c-Src catalytic activity.

3.4 c-Src traffics with activated EGFR

To date, there has been little characterization of the underlying mechanisms governing macropinocytosis. It is known that EGF induces both Rac-mediated membrane ruffling and macropinocytosis, and that EGFR internalizes via macropinocytosis in addition to classical clathrin-dependent pathways. In addition, several lines of evidence point to functional synergism between c-Src and EGFR. c-Src modulates EGFR internalization from the cell surface, and potentiates EGFR-driven signaling. We therefore hypothesized that c-Src and EGFR would traffic and signal together.

To address this question, GFP, c-SrcGFP, and KDc-SrcGFP were expressed in COS-1 cells, which contain abundant amounts of endogenous EGFR. Serum starved cells were incubated for 10 minutes with EGF, chased for various timepoints in media lacking EGF, fixed, and

stained with an antibody directed against activated EGFR (anti-pEGFR, Y1173). At the zero chase timepoint, activated EGFR was observed at the cell surface and intracellularly in GFP and KDC-SrcGFP cells. In c-SrcGFP-expressing cells, strong colocalization between activated EGFR and c-SrcGFP was evident at cell surface ruffles and inside the cell. In the perinuclear region, we observed that EGFR and c-SrcGFP colocalized at punctae as well as at enlarged vesicles reminiscent of macropinosomes (Figure 4A).

EGFR down-regulation requires approximately 2–3 hours for internalized EGFR to be targeted to the lysosome for degradation [12,40]. In cells expressing GFP or KD c-SrcGFP, the fluorescence intensity for activated EGFR was clearly diminished at the 2 hour chase point, consistent with EGFR downregulation. However, c-SrcGFP expressing cells still retained a prominent pEGFR signal (Figure 4B). Both c-Src and activated EGFR colocalized at the plasma membrane and in intracellular locations. Neighboring cells that did not get transfected with c-SrcGFP served as a built-in control; these cells had no pEGFR signal remaining at the 2 hour timepoint. (Figure 4B). The specificity of the anti-pEGFR antibody was confirmed by including a blocking peptide targeted against the pEGFR epitope during primary antibody incubation. No pEGFR signal was detected (Figure 4C). These findings indicate that c-Src and EGFR co-localize and traffic together during EGF stimulation, and that the presence of c-Src prolongs EGFR activation in a c-Src kinase dependent manner.

3.5 c-Src induces EEA1 clustering in EGF-dependent manner

EGFR signals both from the plasma membrane and also from endosomes [13,41]. Additionally, EGFR and the downstream signaling adaptors Grb2 and Shc have been shown to colocalize both at the plasma membrane, and in endosomes [42–44]. Given our observation that c-Src and EGFR traffic together, we asked what influence c-Src had on the endocytic pathway during EGFR activation.

COS-1 cells were pulsed for 10 minutes with EGF and chased for either zero, 30, and 120 minutes, and the distributions of EEA and CD63 were monitored. In cells expressing GFP, EEA1 staining was evident in a punctate distribution spread throughout the entire cell. In contrast, the majority of c-SrcGFP expressing cells exhibited clustering of EEA1-positive endosomes into the perinuclear region. When c-Src kinase activity was abolished, EEA1 clustering was no longer observed, indicating that c-Src catalytic activity is important for early endosome localization during EGF stimulation (Figure 5A). Quantitation revealed that approximately 42% of c-SrcGFP expressing cells had induced clustering of EEA1 positive endosomes after a 10 minute EGF pulse, compared with 18% and 25% in GFP and KDC-SrcGFP expressing cells. After two hours, EEA1 endosomes remained clustered in 58% of c-SrcGFP expressing cells, compared to 29% and 26% for GFP and KDC-SrcGFP expressing cells, respectively (Figure 5C). Similar experiments were performed using indirect immunofluorescence against CD63 (Figure 5B). No c-Src-dependent endosomal clustering was observed for CD63-positive compartments upon EGF stimulation. These data suggest that clusters of c-SrcGFP and EEA1-positive endosomes serve as hubs for EGF-mediated signaling.

3.6 c-Src colocalizes with activated EGFR in a ligand-independent manner

To further study the synergy between c-Src and EGFR, we examined cells expressing GFP or c-SrcGFP in the absence of EGF stimulation. As expected, no signal for pEGFR was detected in GFP expressing cells. In sharp contrast, a strong pEGFR signal was detected specifically in cells expressing c-SrcGFP, even in the absence of EGF addition. c-SrcGFP and activated EGFR remained together throughout the cell both at the plasma membrane and intracellularly (Figure 5E). Cells expressing KDC-SrcGFP did not possess any activated EGFR in the absence of EGF addition. Two lines of evidence support the contention that the signal for pEGFR detected in the c-SrcGFP expressing cells is specific. First, neighboring nuclei representing untransfected

cells did not have activated EGFR. Second, pre-treatment of cells with a blocking peptide directed against the anti-pEGFR antibody completely blocked the pEGFR signal (Figure 5E).

The results depicted in Figure 5E suggested that c-Src sustained EGFR activation in a ligand-independent manner. To test this hypothesis, COS-1 transfected cells were pre-incubated with an excess of LA1, a neutralizing anti-EGFR antibody that binds to the ligand-binding domain on the extracellular side of EGFR, thus preventing EGF binding to EGFR. We first verified that LA1 addition neutralized the anti-EGFR signal under our conditions. GFP-transfected cells were pre-incubated with LA1, pulsed for ten-minutes with EGF, and then fixed and stained for activated EGFR. Little to no activated EGFR was detected in cells that were pre-incubated with the neutralizing antibody (Figure 5F) compared to control cells (Figure 4A). Next, the experiment was performed in the absence of EGF in c-SrcGFP expressing cells. Activated EGFR colocalized with c-SrcGFP at the cell surface and in intracellular vesicles in both the absence or presence of LA1 (Figure 5F). Thus, the presence of the anti-EGFR neutralizing antibody failed to prevent EGFR activation. These data suggest that EGFR activation by c-Src is independent of EGF ligand.

To assess whether the presence of a c-Src-dependent EGFR signal was representative of a functionally active and signaling-competent receptor, we tested activation of downstream effectors in the EGFR signaling pathway. For these experiments, we used NIH3T3 fibroblasts that were stably expressing either c-Src or control vector. Phosphorylation levels of ERK and Shc – two known effectors in EGFR-mediated signaling – were evaluated by Western immunoblotting of whole cell lysates. As depicted in Figure 5D, activation of ERK and Shc was enhanced in c-Src expressing cells in the presence of EGF (lanes 3 and 4). Moreover, a 2-fold increase in phosphorylated ERK and Shc was also observed in c-Src-expressing cells compared to control, even in the absence of EGF stimulation (Figure 5D, lanes 1 and 2 of each blot). Taken together, these results point to a c-Src-mediated enhancement of EGFR activation and downstream signaling in the absence of exogenously added EGF ligand.

4. Discussion

4.1 Real time live imaging reveals that c-Src internalizes from the plasma membrane via macropinocytosis

In this study, we investigated the inter-relationship between c-Src trafficking and c-Src mediated signal transduction. A key element was the design of a c-Src-GFP fusion protein that retains the properties of wild type c-Src. Others have reported that fusion of GFP directly to the c-Src C-terminus results in formation of a constitutively activated Src-GFP fusion protein [38]. Here we show that inclusion of a 7 amino acid linker between the C-terminus of c-Src and the N-terminus of GFP generates a fusion protein with properties identical to that of c-Src: c-SrcGFP had the same half-life and was localized and regulated to the same extent as c-Src. This regulatable construct enabled us to accurately follow the trafficking of authentic c-Src and to simultaneously determine the localization of both active and inactive c-Src.

Most of the active c-SrcGFP was present at the plasma membrane in membrane ruffles (Fig 2C), while most of the inactive c-SrcGFP localized to the perinuclear region. The latter population likely represents c-SrcGFP in early and late endosomes (Fig 2A, B), as well as c-SrcGFP associated with other intracellular membranes [14,18,45]. The differential enrichment of activated c-SrcGFP at the plasma membrane vs inactive c-Src in endosomes is consistent with the findings of Sandilands et al, who reported an increasing gradient of c-Src activation from the cell interior out to the plasma membrane in response to LPA or PDGF treatment [29].

In fixed cells, we were also able to capture a subpopulation of intracellular, activated c-SrcGFP that apparently had internalized in macropinosomes (Fig 3). Real time, live imaging of c-SrcGFP revealed that c-Src present at the plasma membrane undergoes internalization via macropinocytosis. This finding agrees with previous studies using v-Src and activated c-Src [34,35,38]. The current study emphasizes the importance of c-Src tyrosine kinase activity for this event; kinase-dead c-SrcGFP formed membrane ruffles that did not circularize or internalize into vesicles. Macropinocytosis occurs both constitutively and in response to growth factor stimulation [31,33] and requires the small G proteins Rac and Rab34 [46,47] as well as second messengers such as phosphoinositide 3-kinase and phospholipase C [48]. Our findings here, as well as those presented by others [38], indicate that c-Src not only is present on macropinosomes but also contributes to their formation via its tyrosine kinase activity.

Entry via macropinocytosis explains the accumulation of c-Src in vesicular structures in the perinuclear area of the cell. Greater than half of the intracellular population of c-Src was present in structures that did not stain with markers for either early or late endosomes. We propose that some of this internal pool represents c-Src that has internalized via macropinocytosis. Other studies have documented that macropinocytosis is an internalization event that is distinct from clathrin mediated endocytosis [31–33]. In A431 cells, macropinosomes remain as discrete intracellular vesicles that do not fuse with either early or late endosomes or with lysosomes [33,49]. In contrast, a recent study reported that c-Src containing macropinosomes fuse with late endosomes and lysosomes [38]. Our data suggests that at least 50% of the intracellular pool of c-Src is distinct from early and late endosomes, and some of this population may represent c-Src that has internalized via macropinocytosis. The remainder of the intracellular c-Src pool could represent newly synthesized c-Src, c-Src that had internalized via other mechanisms, and/or c-Src containing macropinosomes that had fused with early or late endosomes. Additionally, cell fractionation experiments revealed that not all c-Src is membrane-bound (data not shown). Approximately 30% of intracellular c-Src resides in the cytosol, most likely in an inactive state, as shown by the confocal immunofluorescent experiments performed in this study and by others [29].

4.2 c-Src and the activated EGFR traffic together: implications for synergy in signaling

EGFR internalization from the plasma membrane is an important means of regulating the duration and location of receptor mediated signal transduction. In the absence of ligand, EGFR constitutively internalizes from and is recycled back to the cell surface [12]. When EGF is added, the receptor primarily enters the cell from coated pits via clathrin mediated endocytosis [12,50]. However, a second, clathrin-independent route has been described for EGFR which involves internalization via caveolae [50]. Here we provide evidence that, upon EGF addition, EGFR co-localizes and co-internalizes along with c-Src. Due to technical limitations of the available antibodies, we were unable to perform the three-color analysis necessary to definitely identify the internalized, co-stained structures as macropinosomes. However, the well documented ability of EGF to induce macropinocytosis [33,49], as well as presence of both c-Src and activated EGFR in membrane ruffles and enlarged, internal vesicles (Figs 3 and 4), strongly suggests that at least some of the co-trafficking of c-Src and EGFR occurs via macropinocytosis. It is also possible that EGFR internalization, along with that of c-Src, continues to occur via clathrin-mediated endocytosis (see below).

Overexpression of c-SrcGFP caused prolonged retention of internalized, activated EGFR. This was also reflected in enhanced phosphorylation of pERK and Shc in EGF treated c-Src expressing cells, compared to cells expressing basal, endogenous levels of c-Src (Fig 5D). One explanation for the hyperactivation of EGFR signaling is that the presence of increased levels of c-Src diverts some of the activated EGFR to a macropinocytosis internalization pathway. If the internalized EGFR containing macropinosomes remain distinct from endosomes and

lysosomes, this would enable EGFR to evade degradation and to continue to signal from intracellular locations. Alternatively, it is possible that overexpressed c-Src interferes with EGFR trafficking generated via clathrin-mediated endocytosis. We noted that c-SrcGFP expression induced perinuclear clustering of EEA1-positive endosomes. Interestingly, another group recently reported that overexpression of the early endosomal protein GRIF1 causes perinuclear clustering of EEA-1 positive early endosomes, retention of internalized EGF, and inhibition of EGFR degradation [51]. GRIF1 was proposed to regulate trafficking of internalized EGFR from early to late endosomes [51]. If c-Src induced early endosome clustering prevents EGFR progression to late endosomes, this could also account for prolonged EGFR activation and signaling. Regardless of whether the intracellular c-Src/EGFR containing structures are current or past macropinosomes or endosomes, our data strongly imply that the presence of elevated levels of c-Src protein and kinase activity generate internal, endosomal signaling platforms that promote prolongation and hyperactivation of EGF-mediated EGFR signaling.

4.3 EGF-Independent co-localization and signaling by EGFR

Remarkably, activated EGFR was detected in c-Src overexpressing cells even in the absence of EGF addition. The signal for activated pEGFR was specific, as it was completely blocked by preincubation with a blocking peptide directed against the anti-pEGFR antibody. Since the cells were serum starved, there were no other exogenously added growth factors present. The pEGFR signal was not due to transactivation and/or release of endogenous EGF because addition of a neutralizing antibody that prevents EGF binding to EGFR had no effect on the pEGFR signal in c-Src overexpressing cells. While we cannot exclude the possibility that release of other EGF-like ligands might have been induced, it is clear that c-Src overexpression leads to EGFR activation and enhanced signaling even in the absence of exogenous EGF.

5. Conclusions

In this study, we establish that c-Src can internalize into cells via macropinocytosis and that this process is dependent on c-Src tyrosine kinase activity. When cells are stimulated with EGF, c-Src and the activated EGFR co-localize and co-internalize. Overexpression of c-Src results in retention of activated EGFR within the cell and hyperactivation of downstream EGFR-mediated signaling. Moreover, even in the absence of EGF addition, EGFR is activated and basal signaling is elevated 2-fold in c-Src overexpressing cells. We conclude that co-localization of EGFR with c-Src accounts for the synergy in signaling between these two tyrosine kinases. This is likely to be particularly relevant in physiologic situations, such as breast cancer, where both c-Src and EGFR are overexpressed.

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Figure 1. c-SrcGFP is a bona fide reporter of wild-type c-Src

A) COS-1 cells expressing c-SrcGFP were stained with Hoechst (blue) and imaged for fluorescence (left) and DIC (right). **B)** COS-1 cells transfected with either empty vector, or vectors encoding c-Src or c-SrcGFP were lysed and subjected to immunoblotting with anti-Src antibody. Blots were stripped and reprobed with anti-actin to verify equal protein loading. This is a representative blot of an experiment performed 3 times in duplicate. **C)** COS-1 cells transfected with vectors encoding either c-SrcGFP (left) or c-Src (right) were pulse labeled with ³⁵S-Translabel and then chased as described in Materials and Methods. **D)** Lysates from COS-1 cells transfected with vectors encoding either c-Src, Y527F c-Src, c-SrcGFP, or Y527F c-SrcGFP were probed by immunoblotting with anti-c-Src and anti-pY416 c-Src antibodies. The ratios of phosphorylated (activated) c-Src/total c-Src were computed after quantitation of the Western blots using MacBas. Results are from a representative experiment performed in duplicate.

Figure 2. Subcellular localization of c-SrcGFP and activated c-SrcGFP

A) Indirect immunofluorescence of COS-1 cells expressing c-SrcGFP (green) using antibodies specific for EEA1 (red, top panel), and CD63 (red, bottom panel). Scale bar, 10 μ m. **B)** Quantitation of c-SrcGFP colocalization with EEA1 and CD63 on a single-cell level using Metamorph. Colocalization measurements were computed after drawing a region of interest around the perinuclear region of the cell. Data represent mean colocalization values $\pm$ SEM for $n=2$ experiments for each condition (20 cells/experiment). **C)** Indirect immunofluorescence was performed on COS-1 cells expressing c-SrcGFP and KDC-SrcGFP (left panels, green) with anti-PY416 antibody (middle panels, red). In 67% of cells imaged, activated cSrc was primarily localized at the plasma membrane, while 33% of cells revealed an internal pool of activated cSrc. Scale bars: 10 μ m for cSrcGFP, 20 μ m for KDC-SrcGFP. **D)** COS-1 cells expressing c-SrcGFP (green, left panels) were stained with the fluorescent marker Dextran (magenta, middle) or with anti-Rac and anti-actin antibodies (red, middle) and indirect immunofluorescence was detected by confocal imaging. Arrows indicate areas of dextran uptake (white) by c-SrcGFP vesicles, or point to areas of colocalization (yellow) with Rac and actin. Scale bars, 10 μ m.

Figure 3. c-SrcGFP traffics from the plasma membrane to intracellular sites via macropinocytosis
A) Still frames captured from time-lapse confocal microscopy of live COS-7 cells expressing c-SrcGFP. Arrows indicate vesicle formation from plasma membrane ruffles. Data are representative of approximately 15–20 cells imaged using time-lapse confocal microscopy. Scale bar, 20 μ m. **B)** Still frames captured from time-lapse spinning disk microscopy of COS-7 cells expressing KDcSrcGFP (green). Arrows indicate areas of KDc-SrcGFP concentrated at the plasma membrane and indicate the movement of ruffles into the cell without vesicle formation. Data are representative of 7 cells imaged using time-lapse microscopy. Scale bar, 20 μ m.

Figure 4. c-SrcGFP traffics with activated EGFR

A, B) Confocal images of COS-1 cells expressing GFP, c-SrcGFP, or KDc-SrcGFP (green). Serum-starved cells were stimulated with a 10-minute pulse of EGF (20ng/mL) as described in Methods, and were fixed immediately (A) or chased for 120 minutes at 37°C (B). Cells were stained for endogenous phospho-EGFR (Y1173, red). Scale bars (A): 10 μ m for GFP and KDc-SrcGFP, 20 μ m for c-SrcGFP. Scale bars (B): 20 μ m for GFP and c-SrcGFP, 10 μ m for KDc-SrcGFP. **C)** Confocal images of Cos-1 cells expressing cSrcGFP (green). Serum-starved cells were pre-incubated with blocking peptide against anti-pEGFR (Y1173) antibody. Cells were stimulated with a 10-minute pulse of EGF (20ng/mL), fixed immediately and stained for endogenous phospho-EGFR (Y1173, not detectable). Scale bars, 10 μ m.

Figure 5. c-Src induces EEA1 clustering during EGF stimulation and colocalizes with activated EGFR in a ligand-independent manner

A–C) COS-1 cells expressing GFP, cSrcGFP, or KDcSrcGFP (green) were serum-starved, stimulated with a 10-minute pulse of EGF (20ng/mL) and chased for 0, 30, and 120 minutes. Cells were fixed and stained for the indicated marker protein. Scale bars, 10 μ m. **A)** Staining of endogenous EEA1 (red). **B)** Staining of endogenous CD63 (red). **C)** Quantitation of the percentage of cells that exhibited clustered EEA1 staining patterns. The images were scored qualitatively, as having clustered or non-clustered red signals, by two individuals who were blinded to experimental conditions. 30–40 cells were imaged for each condition. **D)** Serum-starved NIH3T3 stable cell lines expressing c-Src or empty vector were unstimulated or were

treated with or without EGF as previously described. Cell lysates were analyzed by Western blotting with anti-pERK and anti-ERK antibodies (left). Alternatively, cells were lysed and immunoprecipitated with anti-Shc antibody followed by Western blotting with anti-pTyr or anti-Shc antibodies. Representative immunoblots of four experiments performed in duplicate are depicted, with fold-activation levels of ERK and Shc indicated. **E)** Serum starved COS-1 cells expressing GFP, cSrcGFP, or KDc-SrcGFP (green) were fixed and stained for endogenous phosphoEGFR (red), with or without preincubation with an anti-pEGFR blocking peptide. Blue indicates Hoechst staining for nuclei. **F)** Serum-starved COS-1 cells expressing GFP or c-SrcGFP (green) were pre-incubated in the absence or presence of an anti-EGFR neutralizing antibody (LA1). Cells were either treated or not treated with EGF, fixed and stained for endogenous phosphoEGFR (red). Blue indicates Hoechst staining for nuclei.