The C Terminus of c-Src Inhibits Breast Tumor Cell Growth by a Kinase-independent Mechanism*

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Overexpression or increased activity of cellular Src (c-Src) is frequently detected in human breast cancer, implicating involvement of c-Src in the etiology of breast carcinomas. Curiously, overexpression of c-Src in tissue culture cells results in a weakly or non-transforming phenotype, indicating that it alone is not sufficient for oncogenesis. However, the protein has been demonstrated to potentiate mitogenic signals from transmembrane receptors. This report investigates the requirement for c-Src in breast cancer as a transducer and integrator of anchorage-dependent and -independent growth signals by utilizing the Src family pharmacological inhibitors, PP1 and PP2, or stable overexpression of the catalytically inactive c-Src mutant (K11032H11032 c-Src). Both methods of inhibiting endogenous c-Src diminished formation of soft agar colonies and tumors in nude mice.

The majority of the dominant-negative activity of K11032H11032 c-Src was mapped to the Src homology 2 (SH2) domain and C-terminal half of the molecule, but not to the Unique domain, Src homology 3 (SH3) domain, or the N-terminal half of K11032H11032 c-Src. Further analysis of the C terminus revealed that its ability to inhibit growth localized to the N-terminal lobe (N-lobe) of the catalytic region. These results underscore the requirement for c-Src to maintain the oncogenic phenotype of breast cancer cells and suggest that c-Src may be manipulated to inhibit cell growth by the direct disruption of its catalytic activity or the introduction of either the SH2 domain or the N-lobe of K11032H11032 c-Src.

The ubiquitously expressed tyrosine kinase c-Src1 is overexpressed or activated in human carcinomas of the breast, colon, lung, skin, esophagus, cervix, and gastric tissues, as well as in neuroblastomas and myeloproliferative disorders (1). Except in a rare subset of colon cancers (2), c-Src has not been found to be mutated in human tumors. The mechanism for the abnormal up-regulation of protein expression or catalytic activity of c-Src in cancers remains undefined. Attempts to address whether overexpression of c-Src alone induces cellular transformation have shown that the protein is non- or weakly oncogenic (3, 4).

Although overexpression of c-Src alone is insufficient, c-Src appears to promote the mitogenic and tumorigenic properties of other cellular proteins. For example, c-Src potentiates EGFR-induced anchorage-dependent and -independent growth in murine fibroblasts by mediating phosphorylation of the epidermal growth factor receptor (EGFR) at tyrosine 845 (Tyr-845) (5–7). c-Src has also been demonstrated to cooperate with G-protein-coupled receptors (8), platelet-derived growth factor receptor (9), colony-stimulating factor-1 receptor (10), and integrins (11) by facilitating proliferative signals from these various receptors through enhancement of p85 phosphatidylinositol 3-kinase, RasGAP, Shc, and phospholipase Cγ tyrosine phosphorylation (12–14). Regulation of receptor endocytosis and recycling by c-Src also augments receptor activity (15–18). Independent of its role in enhancing immediate downstream signals, c-Src has also been implicated in promotion of cell cycle progression by virtue of its cellular localization to the perinuclear and nuclear regions (19–22). Thus, despite its ineffectiveness as a single oncogenic agent, c-Src appears to have many opportunities to promote mitogenic and tumorigenic activity through other molecules.

Mutational analysis of c-Src has revealed its functional activity as well as defined its structural components. These studies have demonstrated that c-Src is composed of multiple domains. As ordered from its N to C termini, these domains include the membrane association, Unique, Src homology-3 (SH3), SH2, SH2-kinase linker, tyrosine kinase, and negative regulatory domains. The first 17-amino acid sequence of the membrane association domain, which is also known as the SH4 domain, undergoes post-translational myristoylation (23, 24), which targets c-Src to the plasma membrane in proximity with membrane receptors and cytoskeletal proteins, as well as to endocytic vesicles and perinuclear organelles (20, 23, 25). The Unique domain of c-Src, which is the least conserved region among Src family members, mediates protein interactions and is phosphorylated by protein kinase A, protein kinase C, and the Cdc2-cyclin complex (14, 26, 27). The SH3 and SH2 domains of c-Src interact with proteins containing proline motifs or phosphorylated tyrosine, respectively (28, 29). Together, the SH4, Unique, SH3, SH2, and the SH2-kinase linker comprise the N-terminal regulatory half of c-Src. The N terminus is involved in the regulation of the catalytic activity of c-Src and can functionally replace full-length wt c-Src in the proper formation of focal adhesions and cell spreading on fibronectin (30, 31). Interactions of the SH2 and SH3 domains of c-Src with p125 FAK, cortactin, and p130 Cas permit the N terminus to

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c-Src Is Required for Breast Tumor Growth

The tyrosine kinase and negative regulatory domains comprise the C-terminal half of c-Src, and each domain contains a site for tyrosine phosphorylation, Tyr-416 and Tyr-527, respectively, in chicken c-Src and Tyr-419 and Tyr-530 in human c-Src (36). Phosphorylation of Tyr-416 has been shown to enhance the activity of c-Src (36–38). In contrast, phosphorylation of Tyr-527 by C-terminal Src kinase or CHK, a related family member, inhibits c-Src via an intramolecular interaction between phospho-Tyr-527 and the SH2 domain (39–41). Auto-inhibition of c-Src also involves intramolecular interactions between the SH3 domain, a Type 2 polyproline sequence on the SH2-kinase linker, and the small N-terminal lobe (N-lobe) of the kinase domain (42–44). c-Src becomes fully activated upon dephosphorylation of Tyr-527, high affinity competitive SH2/SH3 protein interactions, and phosphorylation of Tyr-416. Phenylalanine substitution at Tyr-527 or SH2 or SH3 mutations that displace the autoinhibitory intramolecular interactions result in constitutive activation of c-Src and cellular transformation (36, 38, 45–48).

c-Src is emerging as a useful therapeutic target. The rationale for this assessment is based on its involvement in a variety of cellular growth-promoting pathways and its up-regulation in many human cancers. Other than overexpressing C-terminal Src kinase, several pharmacological means of inhibiting c-Src have been described. ATP analogs, such as P1P and P2P, competitively inhibit the tyrosine kinase activity of c-Src (49). Another means of reducing Src activity is treatment with agents such as herbimycin A and geldanamycin, which destabilize newly synthesized c-Src by preventing its association with Hsp90 (50, 51). Src-activated signaling pathways have also been attenuated by compounds, such as AP22161 and UCS15A, that interrupt protein-protein interactions with c-Src (52–54). In our assessment of c-Src as a therapeutic target, we demonstrate that inhibition of c-Src activity in several breast tumor cell lines by P1P/P2P and catalytically inactive c-Src (Kwt c-Src) results in reduced anchorage-dependent and -independent growth, in vitro and in vivo. We also explored which regions of Kwt c-Src were responsible for its dominant interfering activity. The ability of Kwt c-Src to act in a growth-inhibitory manner localized to the SH2 domain and the C terminus, both independently of kinase activity. Further analysis revealed that the N-terminal lobe of the catalytic domain contained the predominant inhibitory activity of the C terminus. These results provide evidence that c-Src is a critical component of human breast cancer progression and should be explored further as a therapeutic target in this disease. In addition, the data suggest a novel mechanism for disrupting Src-mediated cellular signaling.

MATERIALS AND METHODS

Constructs—A pcDNA3 vector (Invitrogen, Carlsbad, CA) encoding wt chicken c-Src was constructed by inserting a HindIII-EcoRI fragment containing the wt c-Src cDNA (from pRL plasmid, gift of J. T. Parsons, University of Virginia) into the corresponding HindIII-EcoRI site of the pcDNA3 vector. Kinase-inactive (Kwt c-Src) was isolated by PCR from pm430 plasmid and cloned into the HindIII-EcoRI site of the pcDNA3 vector. Individual c-Src domains were generated by PCR from either wt or Kwt (for C terminus) c-Src pcDNA3 vector templates and cloned into the XbaI-HindIII site of the pcDNA3.1 myc-tag vector. Specifically, sequences encoding the Unique domain (residues 1–84), the SH3 domain (residues 85–149), the SH2 domain (residues 150–260), the N terminus (residues 1–260), and the C terminus (residues 261–533) of the chicken c-Src protein were isolated. The first seven amino acids of the c-Src membrane-targeting sequence were added to the N terminus of the isolated SH3 and SH2 domains during the PCR reaction. The sequences of each construct were confirmed by DNA sequencing. Kwt c-Src and C terminus were confirmed to be kinase inactive in vitro kinase assay (data not shown).

Similarly, regions within the C terminus were generated by PCR from full-length kinase-inactive c-Src and cloned into the XbaI-HindIII site of the pcDNA3.1 myc-tag vector. One region developed from the C terminus was the catalytic domain of Kwt c-Src (Kwt domain, residues 261–519). The Kwt domain was further divided into two separate structural sections: the N-terminal lobe (N-lobe, residues 261–342) and the C-terminal lobe (C-lobe, residues 343–519). The negative regulatory region (residues 520–533) was also constructed but was not expressible. Additionally, myristoylated versions of the C terminus (Myr. C-term) and Kwt domain (Myr. Kwt domain) were constructed by attaching by the sequence of the first seven amino acids of c-Src to the N terminus during PCR.

Cell Lines—Maintenance of the breast tumor cell lines in culture has previously been described (6). MDA-MB-468 and MCF-7 cell lines, stably expressing either Kwt c-Src or c-Src domains, were derived by transfection of appropriate plasmids with Lipofectin (Invitrogen), selection by G418 resistance, and cloning by limiting dilution. Clonal populations were screened for expression of the construct by Western immunoblotting. The -fold overexpression of the domains was estimated by comparative Western blotting analysis, using a c-Src-specific antibody directed against amino acids 2–17.

Western Immunoblotting—Western blotting was performed as described previously (6, 55), using purified 2–17 mouse monoclonal anti-c-Src antibody (mouse) (Quality Biological, Gaithersburg, MD), c-Src and rabbit polyclonal anti-myc antibody (residues 409–420) (Upstate Biotechnology, Lake Placid, NY) to identify the myc-tagged c-Src domains.

Colony Formation in Soft Agar and Tumorigenicity—Anchorage-independent growth was measured as described previously (6). The indicated number of cells was plated in 60-mm dishes for each of the breast tumor cell lines. P1P (Calbiochem, San Diego, CA) was used at a final concentration of 10 μM and added to the culture every 2–4 days. 2- to 3-week-old colonies were stained for 20 h at 37 °C in a solution of iodonitrotetrazolium salt (1 μM/ml; Sigma) in water and counted using EagleEye analysis software (Stratagene, La Jolla, CA). The soft agar colony assay for the MDA-MB-468 cell clones expressing various c-Src domains include analysis of two separate clones for each cell type (468 U6, 468 U3, 468 D1, 468 D2, 468 N4, 468 N5, and the 468 C terminus as indicated. Assessment of tumor formation in Taconic nu/nu mice was performed as described previously (6).

Transient Transfections and BrdUrd Incorporation—A 50–70% confluent, 35-mm dish of cells was transfected with 20 μl of Lipofectin and 4 μg of plasmid DNA according to the manufacturer’s directions and incubated in a humidified, 37 °C, 5% CO2 atmosphere for 24 h. Transfected cells were allowed to recover overnight in growth medium containing 10% fetal bovine serum, after which they were incubated with 100 μM BrdUrd for 17 h. When indicated, P1P or P3P (Calbiochem) were added to the growth medium 24 h after transfection and again at the time of BrdUrd addition. Alternately, 50 μM of the inhibitors was added after transfection and not replenished thereafter. BrdUrd incorporation was assessed by treating fixed cells with 2 N HCl for 1 h at 37 °C to allow epitope recognition by the FITC-conjugated anti-BrdUrd mAb (1:15 dilution, Roche Applied Science). Expression of various c-Src domains was detected by coinuculation with a 1:500 dilution of rabbit anti-myc tag antibody, followed by a 1:1000 dilution of Texas Red-conjugated goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA).

Immunofluorescence—COS-7 cells were transiently transfected with plasmids encoding the various c-Src domains using Lipofectin as described above. The next day, cells were fixed and stained for expression as previously described (56), with either 1:1000 dilution of rabbit polyclonal myc-tag antibody to visualize the c-Src domains or 1:5000 dilution of EC10 mouse mAb (at an initial concentration of ~1 mg/ml) (57) to visualize either full-length wt or Kwt c-Src. This was followed by incubation with either 1:1000 dilution of Texas Red-conjugated goat anti-myc IgG or FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). For co-localization analysis, antibodies were used in combination.

Mitotic Visualization—MDA-MB-468 cells were transiently transfected with plasmids encoding GFP or various c-Src domains using Effectene (Qiagen, Valencia, CA) according to the manufacturer’s directions. Cells were allowed to recover 48 h post-transfection and then fixed. Expression of the carboxyl-terminally myc-tagged c-Src domains was detected by immunostaining with rabbit anti-myc tag antibody and FITC-conjugated donkey anti-rabbit IgG. DNA was stained with DAPI. The spindle assembly was detected by a mAb specific for tubulin, DM
TABLE I

| Cell line       | Tumor sizea |
|-----------------|-------------|
|                 | mm³         |
| MDA-MB-468      | 230 ± 40    |
| 468 K²⁻        | 100 ± 20    |
| 468 K⁴⁻        | 60 ± 10     |

a Mean tumor volume ± SE (expressed in mm³) of eight individual sites was measured at day 23 after subcutaneous injection of 10⁵ cells into nude mice.
but significant, inhibition on colony formation. Analysis of the C-terminal stable clones revealed a general correlation between levels of expression (Fig. 3B) and ability to inhibit anchorage-independent growth (Fig. 4A). As exemplified by the C-term5-stable clone, high overexpression of the C-terminal half of K-c-Src had a profound inhibitory effect on soft agar colony formation (Figs. 3B and 4A). In contrast, C-terminal expression at levels equal to or less than endogenous c-Src, as exemplified by the C-term5 and C-term13 clones, resulted in reduced capacity for growth inhibition (Fig. 4A). Two out of four additional clones expressing the C terminus at low levels demonstrated >50% inhibition of soft agar growth (data not shown). Therefore, the net effect of the C terminus appears to be inhibitory for anchorage-independent growth despite variability in expression among the clones. Furthermore, the dose-dependent inhibition observed with the C-terminal clones reproduces the effects of the full-length K-c-Src.

As a reflection of the growth-inhibitory activity of the C terminus, stable overexpression of this particular domain from K-c-Src was difficult to maintain. Therefore, we also investigated the effects of C terminus and the other c-Src domains on cell proliferation through transient transfection of the MDA-MB-468 cells. Cells positive by immunofluorescence for expression of the myc-tagged c-Src domains were scored for incorporation of BrdUrd into newly synthesized DNA. Fig. 4B shows that, similar to the results obtained from the soft agar assay, both the SH2 domain and C terminus significantly attenuated the ability of MDA-MB-468 breast cancer cells to incorporate BrdUrd, and again, the inhibitory effect of the SH2 domain on DNA synthesis was diminished in the context of the entire N terminus. Interestingly, the SH3 domain had little effect on BrdUrd incorporation, although it was capable of reducing soft agar colony formation. Therefore, it appears that the SH3 domain predominately influences anchorage-independent growth. Among all the domains of K-c-Src, the C terminus exhibited the greatest inhibition of BrdUrd incorporation (Fig. 4B).
The ability of the C terminus to inhibit DNA synthesis may explain why most of the stable clones we obtained from this construct expressed the protein at low levels and why such variable results with the soft agar assay were obtained. We also examined whether the inhibitory effects of the SH2 and C-terminal domains on cell proliferation resulted in cell death. The SH2- and C-term-expressing cells that lacked BrdUrd incorporation did not stain positively for TUNEL, an indicator of apoptosis (data not shown).

The C Terminus of c-Src Co-localizes with Full-length K⁻/H11002 c-Src—Because the C-terminal and full-length K⁻/H11002 c-Src negatively regulated anchorage-independent growth, we examined their intracellular localizations with the goal of gaining insights into their involvement in specific cellular processes, such as signal transduction from the plasma membrane or regulation of cell cycle progression from perinuclear regions (39). Localization of wt c-Src to perinuclear regions in microtubule-organizing centers, late endosomes, and focal adhesions has been described previously (19, 20, 25). COS cells were used for the immunolocalization studies, because the MDA-MB-468 cells have a severely rounded morphology and little visible cytoplasm. In COS cells both wild-type (wt) and K⁻/H11002 c-Src localized predominantly to punctate bodies in the perinuclear region, with some staining at the cell periphery (data not shown).

To determine whether the staining pattern of the C terminus was due to its lack of post-translational myristoylation, the myristoylation membrane-targeting sequence was added to the N terminus of the C terminus (Myr. C-term, Fig. 5A). We also sought to characterize further which portion of the C terminus mediated its growth-inhibitory effect and to assess the intracellular localization of the various domains within the C terminus. To this end, the catalytic domain of K⁻/H11002 c-Src (K⁻/H11002 domain) that lacked the negative regulatory region containing Tyr-527 was isolated and either left unmodified or tagged with the myristoylation sequence (Fig. 5A). Constructs encoding the N-terminal lobe (N-lobe) or the C-terminal lobe (C-lobe) of the catalytic region were also generated, using the crystal structure (42) as a guide.

The isolated domains were then assessed for expression and subcellular localization in COS cells as described above (Fig. 5B and data not shown). All regions from the C terminus of K⁻/H11002 c-Src localized in a manner similar to the full-length K⁻/H11002 c-Src whether they were myristoylated or not. Thus, membrane targeting did not appear to alter the subcellular localization of the C terminus or its various domains.

The Minimal Region from the C Terminus That Inhibits DNA Synthesis Localizes to the N-lobe of the K⁻/H11002 c-Src Catalytic Domain—Because the subcellular localization studies revealed no significant differences among the various domains, it re-

![Image](https://example.com/image.png)
mained unclear which region isolated from the C terminus was responsible for the growth-inhibitory effects of the C-terminal or full-length K c-Src. Therefore, the isolated C-terminal regions of K c-Src were examined for their ability to inhibit BrdUrd incorporation in cycling MDA-MB-468 breast cancer cells, and results were compared with the effects of serum withdrawal or treatment with the Src family kinase inhibitor, PP2, and its non-inhibitory analog, PP3. As shown in Fig. 6, PP2 treatment was the most effective inhibitor of BrdUrd incorporation, thereby becoming the reference for the other conditions targeting DNA synthesis. For example, media free of any serum-containing growth factors was also determined to reduce S phase entry relative to PP2 treatment.

Among the various isolated C-terminal regions of K c-Src, the N-lobe exhibited the greatest inhibitory effect on BrdUrd incorporation (Fig. 6). As a measure of its effectiveness, the N-lobe did not significantly differ from PP2 treatment, serum deprivation, or full-length K c-Src as a growth inhibitor (p value $> 0.10$), whereas the other portions of the C terminus of K c-Src were unable to inhibit to the same extent as PP2 treatment (p value $< 0.04$). Although not as competent a growth inhibitor as PP2 or N-lobe, both the C terminus, as demonstrated earlier in Fig. 4B, and the K c-domain were found to limit BrdUrd incorporation in $\sim 50\%$ of the cells. Addition of the myristoylation membrane-targeting sequence to either the C-terminal or K c-domain did not significantly alter their abilities to attenuate DNA synthesis (Fig. 6). Interestingly, the C-lobe had the lowest capacity for inhibiting DNA synthesis among all the C-terminal regions of K c-Src and had an effect comparable to PP3 (42 $\pm 18\%$ inhibition). The divergent effects on BrdUrd incorporation between the two lobes of the catalytic domain suggested that the majority of the inhibitory activity of K c-Src lies within the N-lobe.

As another measurement and to further confirm the growth-inhibitory effects of the N-lobe, we assessed cell cycle progression by visualizing chromatid formation and spindle assembly in mitosis with DAPI and monoclonal $\alpha$-tubulin antibody, respectively. MDA-MB-468 breast cancer cells were transiently transfected with vectors expressing myc-tagged regions within the catalytic domain of K c-Src, including C terminus or GFP (Fig. 7A). Quantification of the GFP control population indicated that $\sim 8\%$ of the MDA-MB-468 cells actively progress through mitosis under normal serum-augmenting growth conditions (Fig. 7B). Notably, expression of either the C terminus or N-lobe diminished the number of mitotic cells. This reduction in mitosis was significant and corresponded with the ability of both domains to inhibit BrdUrd incorporation. On the other hand, the C-lobe had minor effects on mitotic progression. This result may explain the minimal effects of the C-lobe on BrdUrd incorporation in comparison to the C terminus and N-lobe (Fig. 6). We also assessed whether the inhibitory effects of N-lobe on cell cycle progression induced cell death. The N-lobe, as well as the C terminus, did not induce propidium iodide uptake or positive TUNEL staining, both of which are indicators of cell death (data not shown). These results suggest that the C-terminal region of K c-Src facilitated growth arrest rather than cell death.

**DISCUSSION**

In support of c-Src as a component of oncogenic pathways, this report demonstrates that c-Src is required for the tumorigenic phenotype of several breast tumor cell lines. Both pharmacological Src kinase inhibitors and K c-Src overexpression attenuated the in vitro and in vivo tumorigenic growth of breast cancer cell lines. Although c-Src alone has only weak transforming potential, much evidence indicates that it cooperates with transmembrane receptors and intracellular transducers to enhance tumorigenesis (58). Pharmacological non-kinase inhibitors that target the ability of c-Src to interact with intracellular signaling molecules have been demonstrated to be effective inhibitors of c-Src-mediated bone resorption activity in osteoclasts (53, 54). However, these non-kinase inhibitors of c-Src have not been tested for their effects on the tumorigenic growth of breast cancer cells.

Based on the paradigm of non-kinase inhibitors, we attempted to determine which regions of K c-Src were responsible for inhibiting the anchorage-independent growth of breast cancer cells. We reasoned that the N terminus as a whole or the individual SH2 and SH3 domains within the K c-Src would be prime candidates for interfering with endogenous c-Src. The SH2 and SH3 domains of the N terminus have been shown to interact with many proteins, including dynamin (59), phosphatidylinositol 3-kinase (13, 14), phospholipase C- $\gamma$ (14), mitogen-activated protein kinase (14), and growth factor receptors (39, 58). Through association of the SH2 and SH3 domains with FAK (60) and p130CAS (33, 61), the N terminus of c-Src has been shown to be sufficient for reconstituting focal adhesion structures and cell spreading in c-Src null fibroblasts (30, 31). Although the N terminus restores focal adhesions and potentially promotes integrin signaling in a c-Src null background, we tested whether the isolated SH2 and SH3 domains could disrupt the activities of full-length endogenous c-Src in a normal background by competitively sequestering proteins involved in intracellular signaling pathways and cytoskeletal architecture. Indeed, we found that the SH2 domain partially inhibited both anchorage-independent growth and BrdUrd incorporation. Interestingly, the magnitude of inhibition by SH2 was reduced in the context of the entire N terminus. This finding could reflect either the added specificity provided by the N terminus or interference from other regions within the N terminus. With regard to the former, the isolated SH2 domain may recognize phosphotyrosines on non-client proteins and therefore hinder more signaling pathways than the N terminus. In comparison to the SH2 domain, the SH3 and Unique domains had only minor effects on either growth assay, suggesting that they do not contribute significantly to the inhibitory effects of K c-Src.
Surprisingly, the isolated C terminus of K\(^{-}\) c-Src inhibited both anchorage-independent growth and BrdUrd incorporation to a greater extent than the N terminus. Regions within the C terminus were isolated to further understand the mechanism of its growth-inhibitory properties. The K\(^{-}\) domain, which contains the catalytic region of K\(^{-}\) c-Src, was utilized to determine whether the negative regulatory domain was required to mediate growth-inhibitory activity. Under normal circumstances, phosphorylation of Tyr-527 in the negative regulatory domain autoinhibits c-Src through intramolecular interactions (42, 43), but it also has the potential to bind other SH2 or PTB domain-containing proteins. Moreover, the negative regulatory domain has been shown to interact with the Polyomavirus middle T antigen independently of Tyr(P)-527 (45). Although the negative regulatory domain has potential protein-protein interaction capabilities, the K\(^{-}\) domain was found to inhibit DNA synthesis to a similar extent as the C terminus. Furthermore, the C terminus was not tyrosine-phosphorylated by immunoblot analysis with an anti-phosphotyrosine antibody (data not shown). In addition, the ability of the C-terminal or the K\(^{-}\) domain to inhibit BrdUrd incorporation was unaffected by myristoylation. Together, these results indicate that the catalytic region of K\(^{-}\) c-Src contains the growth-inhibitory activity of the C terminus, without the need for the negative regulatory domain or membrane localization through myristoylation.

Given these results, the K\(^{-}\) domain was separated into two domains, the N-lobe and the C-lobe, based on the crystal structure (42). Interestingly, the N-lobe inhibited DNA synthesis to a greater extent than the C-terminal or K\(^{-}\) domain. In contrast, the C-lobe, which contains the A430V mutation, had reduced ability to diminish BrdUrd incorporation. By another measurement, both the C terminus and N-lobe prevented mitotic progression, whereas the C-lobe was much less effective. The mitotic arrest exhibited by either the C terminus or N-lobe paralleled the effects of a selective Src family tyrosine kinase inhibitor (62). Moreover, the growth-inhibitory activity of the N-lobe and the C terminus supported previous findings that myristoylation is not required for the mitotic activity of wild-type c-Src (63). Together, these data provide evidence that mitotic progression requires c-Src and that the growth-inhibitory activity of K\(^{-}\) c-Src localizes primarily to the N-lobe of the catalytic domain.

The SH2 and N-lobe domains of K\(^{-}\) c-Src inhibited cellular growth independently of apoptosis. It has been observed that the N-lobes from the catalytic regions of ErbB2 and the RET receptor mediate caspase cleavage of their respective kinase domains and subsequent apoptosis of cells expressing these kinases (64, 65). Interestingly, v-Src, the constitutively active oncogenic form of c-Src, has also been demonstrated to generate apoptotic signals in a caspase 3-dependent manner, when...
survival signals are inhibited (66). These considerations suggest that c-Src has apoptotic potential and that the N-lobe of its catalytic domain might induce not only mitotic arrest but also cell death. However, none of the regions from K\(^{-}\) c-Src, including the C terminus and N-lobe, induced cell death, as measured by either propidium iodide uptake or TUNEL staining (data not shown), suggesting that the N-lobe of c-Src mediates its inhibitory effects by a different mechanism.

The C-terminal and full-length K\(^{-}\) c-Src may obstruct cell proliferation by interfering with endosomal trafficking. Both the C-terminal and K\(^{-}\) c-Src localized to punctate structures in the perinuclear region of the cell. Structural domains within the C terminus also exhibited perinuclear localization. This punctate staining pattern is similar to the intracellular vesicle/late endosomal localization previously described for full-length c-Src (19, 25, 67). This localization reflects the involvement of c-Src in augmenting transmembrane activity by regulating endosomal uptake and recycling (15–18), as well as promoting Ras activation on endomembranes leading to cellular transformation (68). Moreover, c-Src has also been localized to microtubule-organizing centers and spindle poles during mitosis (25). By associating with microtubules and endosomal membranes, c-Src has the potential to regulate membrane trafficking within the cell (69–72). Therefore, K\(^{-}\) c-Src and the C terminus may disrupt the ability of c-Src to regulate compartmentalization of growth signals.

That the catalytic domain of K\(^{-}\) c-Src, particularly the N-lobe, may appropriate signaling molecules away from critical growth pathways is supported by findings that the kinase domains of at least three different tyrosine kinase families can bind intracellular proteins. In the cases of the EGF receptor and Janus (Jaks) families of kinases, these interactions have been demonstrated to enhance or inhibit the catalytic activity and/or cellular function of these kinases (73–77). With regard to the Src family, one study has found that the catalytic domain of c-Src interacts with \(\beta\)-arrestin, and expression of the K\(^{-}\) domain of c-Src blocks \(\beta\)-arrestin/c-Src-dependent G-protein-coupled receptor internalization and dynamin phosphorylation (78). Similarly, Shc and the trimeric G-proteins, Go, and Go, have been demonstrated to activate c-Src by associating with the catalytic domain of c-Src (79, 80). In addition, the catalytic domain of c-Src has been shown to associate with phospholipase D in an EGF-dependent manner to enhance cellular proliferation (81). The kinase domain of c-Src and related family members have also been observed to interact with EGFR and the erythropoietin receptor (82, 83). Therefore, overexpression of regions from the C terminus of K\(^{-}\) c-Src may block transduction of growth signals by affecting interactions of endogenous c-Src with other cellular signaling molecules.

In attempts to investigate these interaction possibilities, we examined immunoprecipitates of utr or K\(^{-}\) c-Src from cycling MDA-MB-468 cells for the presence of Shc, phospholipase D, and EGFR and were uniformly unsuccessful in observing a stable association among any of the signaling molecules and either form of c-Src. Our inability to reproduce these previously identified stable interactions may be a limitation of our model system (468 cells). However, in further attempts to address the mechanism whereby K\(^{-}\) c-Src mediates its inhibitory effects, we examined immunoprecipitates of K\(^{-}\) c-Src from 468 cells metabolically labeled with \[^{35}S\]methionine/cysteine for co-associating proteins. We found an ~85-kDa protein that uniquely and specifically co-associated with the full-length K\(^{-}\) c-Src and the C terminus but not with utr c-Src, the N terminus or its isolated domains, or the N-lobe or C-lobe of the C terminus (data not shown). The interaction between K\(^{-}\) c-Src and the 85-kDa protein was also observed in the ZR-75 breast cancer cell line. High scale purification and mass spectrometry revealed this protein to be Hsp90\(^{-}\).

Maturation of c-Src and v-Src has been demonstrated previously to be dependent on Hsp90\(^{-}\), and its interaction with the C termini of the Src molecules (51, 84). Similarly, ErbB2 has been shown to lose association with Hsp90\(^{-}\) and sensitivity to geldanamycin, an inhibitor of Hsp90\(^{-}\), when the kinase domain of ErbB2 is deleted (85). Therefore, the interaction of Hsp90\(^{-}\) with K\(^{-}\) c-Src and the C terminus appears to be real and specific. Whether stable interaction of K\(^{-}\) c-Src with Hsp90\(^{-}\) explains the growth-inhibitory activity of K\(^{-}\) c-Src cannot be definitively concluded, because the N-lobe does not associate with Hsp90\(^{-}\), despite its ability to inhibit cell cycle progression to an even greater extent than either the C-terminal or the K\(^{-}\) domain. In fact, Hsp90\(^{-}\) may sense that those proteins containing the single-site-inactivating mutation in the catalytic domain of K\(^{-}\) c-Src are misfolded or unstable, thereby associating with these proteins to prevent premature degradation. Therefore, although Hsp90\(^{-}\) was identified as a K\(^{-}\) c-Src co-associating protein, the possibility still exists that one or more other factors in regions of K\(^{-}\) c-Src shown to negatively affect DNA synthesis in human cancer cell lines.

In summary, this study provides evidence for the involvement of c-Src in the progression and maintenance of the biological activity of cancer cells. Perturbation of Src activity by either pharmacological agents or K\(^{-}\) c-Src inhibited anchorage-independent growth and tumor formation by breast cancer cells. Further analysis of K\(^{-}\) c-Src revealed that its inhibitory activity localized to the SH2 and C-terminal domains, the latter specifically to the N-terminal lobe of the kinase domain. These results demonstrate that the actions of endogenous c-Src can be inhibited by at least three different mechanisms: inhibition of its catalytic activity, introduction into cells of its isolated SH2 domain, and introduction of the isolated N-lobe of its catalytic domain. Together, these findings provide support for c-Src as a novel target for therapeutic interdiction in human cancers.

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