c-Src and cooperating partners in human cancer

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The proto-oncogene c-*src* is rarely mutated in human cancers, and when overexpressed in normal cells is non- or weakly oncogenic. These observations have raised doubts about the involvement of c-*src* in the etiology of human tumors. However, recent studies have shown that c-Src, a non-receptor tyrosine kinase, exhibits elevated protein levels and activity in numerous types of human cancers. Furthermore, it has been found to be a critical component of multiple signaling pathways that regulate proliferation, survival, metastasis, and angiogenesis. Because of its important role in these oncogenic processes, it represents a therapeutic target ripe for exploitation.

c-Src was first identified as the cellular form of v-Src, the transforming gene product of the avian tumor virus Rous sarcoma virus (Brown and Cooper, 1996). Unlike v-Src, however, c-Src is weakly oncogenic (Biscardi et al., 2000). The difference in pathogenic activity between these two proteins lies in their structure and regulation (Brown and Cooper, 1996; Xu et al., 1999a). c-Src is maintained in an inactive configuration by multiple intramolecular interactions. Mutations that capitalize on disrupting these restrictive intramolecular interactions result in constitutive activation, which is best demonstrated by the truncation of the C-terminal negative regulatory domain in v-Src.

Although v-Src causes tumors in chickens, it has not been found to be an etiological agent in human tumors. Recently, a C-terminally truncated c-Src that exhibits constitutive catalytic activity similar to v-Src was detected in small subsets of colon and endometrial cancers (Irby et al., 1999; Sugimura et al., 2000). Other studies, however, have failed to detect such mutations in colon cancer patients (Wang et al., 2000; Nilbert and Fernebro, 2000; Laghi et al., 2001), suggesting that genetic activation of c-Src is a rare occurrence that may be restricted to different ethnic groups.

More commonly found in colon cancer is elevated expression of wild-type (*wt*) c-Src. Indeed, elevated protein levels and/or catalytic activity of c-Src have been detected in a number of human cancers, including lung, skin, colon, breast, ovarian, endometrial, and head and neck malignancies (reviewed in Biscardi et al., 2000; Irby and Yeatman, 2000). Given the low capacity of *wt* c-Src for cellular transformation and the paucity of examples of mutational activation in human cancers, the involvement of c-Src in the etiology and progression of human cancers was doubted for many years. Recently, documentation of its increased protein expression and/or catalytic activity and a greater understanding of its function in cells has prompted investigators to hypothesize that c-Src may facilitate the action of other signaling proteins, rather than being a dominant transforming agent on its own. In fact, this hypothesis has been borne out by numerous examples.

Cooperative processes of c-Src

c-Src is a multifunctional protein involved in the regulation of a variety of normal and oncogenic processes, including proliferation, differentiation, survival, motility, angiogenesis, and functions of fully differentiated cells (reviewed in Thomas and Brugge, 1997). To carry out these activities, c-Src interacts with numerous cellular factors, including cell surface receptors (EGF family, CSF-1, PDGF, and FGF receptors, as well as integrins, cell-cell adhesion molecules, etc. [Biscardi et al., 2000; Irby and Yeatman, 2000; Owens et al., 2000; Moro et al., 2002]), steroid hormone receptors (Migliaccio et al., 1996, 2000; Boonyaratanakornkit et al., 2001), components of pathways regulated by heterotrimeric G proteins (Luttrell et al., 1999; Ma et al., 2000), STATs (Silva et al., 2003), focal adhesion kinase (FAK) (Kaplan et al., 1994), the adaptor proteins p130Cas (Burnham et al., 2000) and Shc (Sato et al., 2002), and many others. Rather than provide a comprehensive and cursory overview of how c-Src fulfills its many potential roles, we have chosen to focus this review on three representative partners (effectors) of c-Src, specifically, the epidermal growth factor receptor (EGFR) family, FAK, and steroid hormone receptors. Each of these effectors represents a different class of proteins and functions in unique signaling pathways for which the molecular nature and biological consequences of the interactions with c-Src have been investigated. Discussions of interactions between c-Src and other molecules can be found in review or research articles cited above.

c-Src and EGF receptor family members

Members of the EGFR family regulate differentiation, proliferation, survival, motility, and angiogenesis, events critical to cancer initiation and progression (Holbro et al., 2003). In addition to the EGFR itself, this family includes ErbB2, ErbB3, and ErbB4. In breast cancers, c-Src and members of the EGFR family are overexpressed in ∼70% of tumors, and in the majority of these tumors, c-Src is co-overexpressed with at least one member of the EGFR family (reviewed in Biscardi et al., 2000). This frequency suggests that the two families of tyrosine kinases may functionally and physically interact to promote breast cancer development. Indeed, in model systems and in human breast cancer tissues and cell lines that co-overexpress both c-Src and EGFR, the biological synergy between these two tyrosine kinases has been demonstrated (Biscardi et al., 2000). Similarly, c-Src activity is necessary for ErbB2-mediated anchorage-independent growth, motility, and survival (Karni et al., 1999; Belsches-Jablonski et al., 2001; R. Ishizawar and S. Parsons, submitted). However, the involvement of c-Src in ErbB3- or ErbB4-regulated cell processes is much less well understood.

Investigations into the molecular basis for the biological interactions between c-Src and members of the EGFR family have revealed that c-Src physically associates with activated receptors (Maa et al., 1995; Muthuswamy and Muller, 1995;
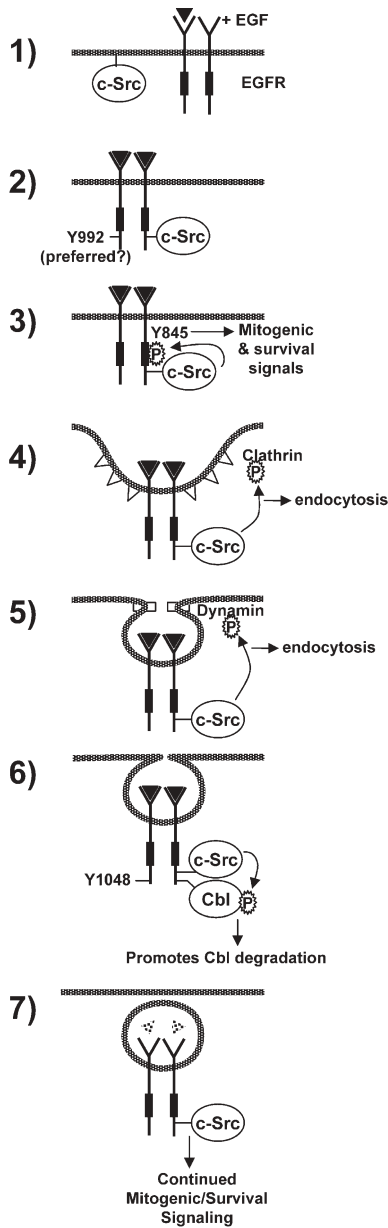
Figure 1. Physical and functional interactions between c-Src and the EGF receptor

Ligand activation of the EGFR results in physical association of c-Src with the EGFR (most likely via an SH2/pTyr interaction), transient activation of c-Src, phosphorylation of Tyr 845 on the EGFR by c-Src, and stimulation of mitogenic and survival pathways (see Figure 2). c-Src phosphorylation of clathrin and dynamin also enhances receptor internalization and endosomal signaling, while at the same time reducing receptor degradation by phosphorylating the ubiquitinating enzyme, Cbl, and inducing its degradation. This results in recycling of the receptor to the cell surface and renewed rounds of signaling. Panels 1–7 represent the various steps that are noted above, indicated on the figure, and discussed in more detail in the text.

Belsches-Jablonski et al., 2001), becomes transiently activated, and phosphorylates downstream targets (Muthuswamy et al., 1994; Osherov and Levitzki, 1994) (Figure 1). One target is the EGFR itself, which can be phosphorylated on multiple sites by c-Src, most notably Tyr 845 (reviewed in Biscardi et al., 2000).

Tyr 845 is situated within the activation loop of the catalytic domain of the EGFR in a position that is conserved among all receptor and nonreceptor tyrosine kinases. Autophosphorylation at this conserved site on other tyrosine kinase receptors is necessary for full catalytic and biological activity. In contrast, phosphorylation of Tyr 845 is mediated by c-Src, not by activated EGFR, and is not associated with modulating receptor autokinase activity or ability to activate SHC and Erk2. Nevertheless, Tyr 845 is required for mitogenesis, as substitution with phenylalanine creates a mutant EGFR that inhibits EGF-induced DNA synthesis. Together, these results suggest that the Erk2 pathway is not sufficient for EGF-induced proliferation and that other effectors downstream of pY845 are required. Indeed, several mediators of pY845 signaling have been identified, including STAT5b, a transcription factor involved in mitogenesis (Kloth et al., 2003), and cytochrome c oxidase subunit II (Cox II) (Boerner et al., 2004), a mitochondrially encoded protein that is involved in oxidative phosphorylation and postulated to regulate cytochrome c release during apoptosis. Thus, pY845 appears to activate at least two distinct signaling pathways, one that promotes EGF-induced cell proliferation through STAT5b and another that enhances cell survival through Cox II.

That phosphorylation of Tyr 845 is dependent upon the catalytic activity of c-Src rather than that of the EGFR suggests that c-Src can modulate lateral activation of the EGFR by extracellular stimuli other than EGF. Indeed, multiple extracellular factors, such as G protein-coupled receptor ligands, steroids, cytokines, extracellular matrix (ECM) proteins, ionizing radiation, ultraviolet light, and certain ions can transactivate the EGFR (Knebel et al., 1996; Prenzel et al., 2000; Wu et al., 2002). In many cases, this transactivation requires c-Src. For example, c-Src is necessary for integrins to associate with and transactivate the EGFR. This event results in phosphorylation of EGFR at multiple tyrosines, including Tyr 845 (Moro et al., 2002). c-Src also couples EGFR to GPCRs, as evidenced by the ability of Src-specific pharmacological inhibitors and the Y845F EGFR mutant to abrogate lysophosphatidic acid (LPA)-induced mitogenesis (Biscardi et al., 2000; Prenzel et al., 2000). Wu et al. (2002) reported that c-Src-dependent phosphorylation of Tyr 845 on the EGFR is necessary for Zn^{2+} activation of Ras. Taken together, these studies demonstrate that c-Src augments EGFR activity by integrating EGFR with other nonrelated membrane receptors and intracellular signaling molecules through pY845 (Figure 2).

Other substrates of c-Src include clathrin and dynamin (Wilde et al., 1999; Ahn et al., 2002), two proteins involved in internalization of multiple types of membrane receptors (including EGFR) (Figure 1). Clathrins assemble in a protein lattice to form the coated pits into which ligand-bound receptors are sorted and internalized. Dynamin governs separation of the endocytic vesicles from the plasma membrane. Phosphorylation of clathrin and dynamin by c-Src enhances the endosomal pool of activated receptors that continue to signal until degraded (Figure 1).

In addition to modulating internalization, c-Src appears to regulate EGFR degradation. Ligand-activated EGFR is ubiquitinated by Cbl, which promotes receptor endocytosis and degradation (reviewed in Thien and Langdon, 2001). In this process, c-Src facilitates the ubiquitination and proteasomal degradation of Cbl, thereby reducing levels of Cbl and retarding EGFR downregulation. These events promote receptor recycling back
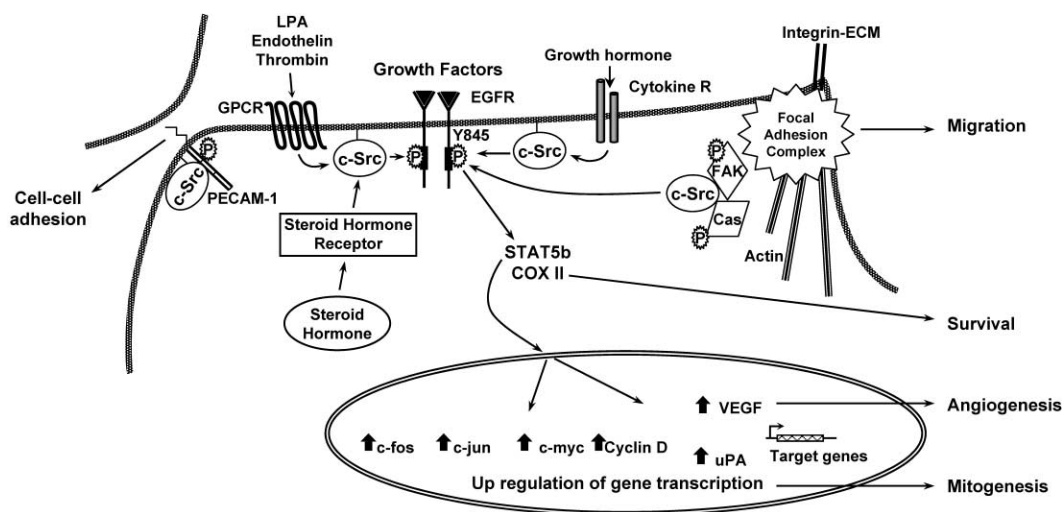
Figure 2. c-Src regulates biological processes that are critical for oncogenesis

As described in the text, c-Src regulates intracellular pathways that control proliferation, survival, cell-cell adhesion, migration, and angiogenesis. Some of these pathways utilize the EGF receptor and a specific site on the receptor that is phosphorylated by c-Src, i.e., Tyr 845. Others include proteins that regulate focal adhesion dynamics (FAK) and hormone-regulated events (sex steroid hormone receptors).

to the plasma membrane and extend EGFR signaling (Bao et al., 2003) (Figure 1).

c-Src and focal adhesion kinase

c-Src also interacts with focal adhesion kinase (FAK), a 120 kDa tyrosine kinase that is postulated to play a key role in cancer metastasis by modulating the formation and turnover of focal adhesions (reviewed in Parsons, 2003). Focal adhesions are dynamic intracellular structures that link the extracellular matrix (ECM) to the actin cytoskeleton through cell surface integrins (reviewed in Burridge and Chrzanowska-Wodnicka, 1996). As a component of focal adhesions, FAK interacts with multiple cellular proteins to translate integrin signaling into cell spreading, motility, and invasion. Exploitation of FAK activity in human tumors occurs through elevated expression, a situation that correlates with increased cancer cell motility, invasiveness, and proliferation (Owens et al., 1995; Parsons, 2003).

FAK and c-Src have been shown to form a transient, active complex following integrin engagement by ECM proteins or ligand stimulation of the EGF or PDGF receptors. These cell surface molecules interact with the N-terminal portion of FAK, which results in the autophosphorylation of FAK at Tyr 397. Multiple SH2-containing signaling molecules, such as c-Src, 85 kDa subunit of phosphoinositide-3-kinase, phospholipase Cγ, and Grb7, are recruited to pTyr 397 (reviewed in Parsons, 2003; Schlaepfer and Mitra, 2004). The association between c-Src and FAK results in activation of c-Src and the phosphorylation of FAK on Tyr 576, 577, 861, and 925, which enhances FAK kinase activity and generation of docking sites for Grb2 and other signaling proteins. The FAK/c-Src complex also phosphorylates the cytoskeletal adaptor proteins paxillin and Cas. Together with FAK, these molecules recruit and activate regulators of ERK, Jun kinase (JNK), and Rho signaling pathways, which modulate multiple gene expression events of both transcription factors and target proteins involved in cell motility and invasion (reviewed in Schlaepfer and Mitra, 2004). That the interplay between c-Src and FAK is important to these events is evidenced by results of Src and FAK pharmacological inhibitor studies and homozygous deletion and rescue experiments (Parsons, 2003; Schlaepfer and Mitra, 2004).

c-Src and sex steroid hormone receptors

Like peptide growth factors, sex steroid hormones, such as estrogen, progesterone, and androgen, influence a plethora of cellular functions, including mitogenesis, survival, and differentiation, and are known to be important for breast and prostate cancer progression. Receptors for these hormones are well recognized as ligand-dependent transcriptional activators that require hours to days for their effects to be manifest (Mangelsdorf et al., 1995). However, many recent reports describe rapid effects (within seconds to minutes) of these hormones on cell membrane/cytoplasmic signal transduction pathways that do not require changes in gene transcription or protein synthesis. For example, estrogen, progesterone, and androgen can stimulate the c-Src/p21ras/ERK pathway in breast and prostate cancer cells, respectively (Migliaccio et al., 1996, 1998, 2000). Considerable confusion remains, however, regarding the details of this activation and whether the pathway mediates steroid receptor-mediated cell proliferation. One group reports that progesterone activation of the ERK pathway via c-Src requires direct interaction of the progesterone receptor (PR) with the estrogen receptor (ER) (Ballare et al., 2003), while another group provides evidence that the PR interacts directly with c-Src (Boonyaratanakornkit et al., 2001). The latter findings are supported by an earlier study in which elevated c-Src activity correlated with the presence of PR in human breast cancer tissue but not with the presence of ER (Lehrer et al., 1989). That activated c-Src can phosphorylate the ER has been reported numerous times, but the functional significance of this phosphorylation to breast cancer progression remains uncertain (Arnold et al., 1997). Another report suggests that estrogen activation of the c-Src/ERK pathway is dependent upon the GPCR homolog, GPR30, which is required for transactivation of the EGF receptor and stimulation of downstream signaling cas-

cades through release of HB-EGF (Filardo et al., 2000). Finally, a scaffold protein (MNAR—modulator of nongenomic activity of ER) has been found to physically bridge ER and c-Src in a trimeric complex, resulting in activation of c-Src and enhanced ER transcription (Wong et al., 2002). While no one model can accommodate all the data, together, these studies suggest that steroid hormone receptors exist in multiprotein complexes that include c-Src, and that these complexes may play critical roles in the development of sex hormone-responsive cancers. Assessment of the requirement of the c-Src/steroid receptor pathways in cancer pathology is currently an active topic of investigation.

c-Src as a chemotherapeutic target

Because c-Src is a critical component of so many different processes that promote cancer progression, it is becoming recognized as a valid chemotherapeutic target. Strategies to inhibit c-Src include reducing its protein-protein interactions, protein stability, and catalytic activity. Within the first category, several small-molecule nonkinase inhibitors, such as AP22408, AP22161, and UCS15A, have been developed. AP22408 was designed to mimic the pTyr structure of binding proteins in complex with the SH2 domain of c-Src (Shakespeare et al., 2000). AP22408 exhibits an osteoclast-selective antibone resorptive activity, suggesting that SH2 domain inhibitors may be useful agents for the treatment of bone metastases (such as those found in breast and prostate cancers) and chemotherapy-induced osteoporosis. UCS15A, which disrupts SH3 domain-mediated protein-protein interactions (Sharma et al., 2001), blocks the interaction of the SH3 domain of c-Src to multiple proteins, including Sam68, Grb2, cortactin, and PLC-γ (Oneyama et al., 2002), suggesting that this inhibitor can target multiple pathways regulated by the c-Src SH3 domain. While inhibitors of c-Src protein-protein interactions hold promise for the future, their effectiveness in cancer cells has yet to be tested.

Triggering protein instability or preventing maturation of newly synthesized protein is another approach to diminishing c-Src activity. The chaperone Hsp90 guides the maturation of c-Src and other oncoproteins to a fully functional conformation and intracellular localization (Xu et al., 1999b). The ansamycin class of drugs disrupts association of Hsp90 with c-Src and shows promising, specific inhibition of cancer cells in Phase I trials (Neckers, 2002), suggesting that disruption of the maturation process of critical oncoproteins, including c-Src, may be an effective antitumor strategy.

During the maturation process, c-Src is posttranslationally modified at its N terminus by addition of a myristoyl moiety (Resh, 1994). This modification directs c-Src to cellular membranes and is required for the functional activity of the protein (Wilson et al., 1989). Interestingly, several colon cancer cell lines and gallbladder tumors exhibit elevated N-myristoyltransferase levels that correlate with poor prognosis (Rajala et al., 2000), suggesting that targeting the enzyme that mediates myristoylation may be another mechanism of inhibiting c-Src and other myristoylated signaling proteins involved in cancer progression.

ATP analogs, such as tyrophostins and pyrimidine compounds, directly inhibit the tyrosine kinase activity of c-Src and/or related kinases (Altmann et al., 2002). Two well-recognized agents among this class are STI571 (Imatinib/Gleevec), the BCR-Abl/c-kit inhibitor (Druker and Lydon, 2000), and ZD1839 (Gefitinib/Iressa), an EGFR inhibitor (Wakeling et al.,

2002). Surprisingly, in a small subset of non-small cell lung cancers, specific somatic mutations in the ATP binding pocket of EGFR have been found to confer not only a heightened aggressiveness to the disease but also greater sensitivity to ZD1839 inhibition (Paez et al., 2004; Lynch et al., 2004). These results make a strong argument for the design and use of highly specific inhibitors. Also in support of this approach is the idea that specific inhibitors of c-Src need to be developed that will avoid the generalized toxicity brought about by inhibiting other Src family members and their critical functions in normal cells. In contrast to these notions is the finding that resistance to STI571 (due to mutations in the catalytic domain of BCR-Abl) (Shah and Sawyers, 2003) can be overcome by the use of broad-spectrum Src family inhibitors (Shah et al., 2004). Thus, arguments can be made for both high- and low-specificity inhibitors, suggesting that trials are necessary in each case to assess their ultimate clinical effectiveness and appropriate use.

Because c-Src and its family members are critical mediators of multiple signaling pathways that regulate all stages of cancer progression (from initiation to metastasis) in multiple cell types, one can envision the use of c-Src inhibitors in a wide range of malignancies at all stages of disease. Their use as single agents or in combination with other targeted therapies, standard chemotherapies, or radiation may dictate their ultimate effectiveness and whether they function as cytostatic or cytolytic agents. In any event, there is great hope that the promising effects of some of the c-Src inhibitors in model systems will translate into greater benefits for patients undergoing cancer therapy.

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