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c-Src Tyrosine Phosphorylation of Epidermal Growth Factor Receptor, P190 RhoGAP, and Focal Adhesion Kinase Regulates Diverse Cellular Processes

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Contents

I. Introduction

Protein−tyrosine kinases regulate signaling pathways for a broad spectrum of cellular processes including responses to growth factors, neurotransmitters and hormones, activation of the immune response, regulation of cell−cell and cell−extracellular matrix interactions, as well as development, oncogenesis, and angiogenesis (reviewed in refs 1−7). The aberrant over- or underexpression of protein−tyrosine kinases or mutations that induce alterations in the regulation of protein−tyrosine kinase activity can lead directly to the perturbation of any of these processes. Thus, an understanding of the structure and function of this important class of protein kinases and an elucidation of the molecular signaling events mediated by these proteins are important not only for deciphering critical pathways regulated by them but also for designing new strategies to inhibit or block the action of these kinases in abnormal or pathological situations. Insights into the roles that tyrosine kinases play in diverse cellular processes have emerged from many avenues of research, but an understanding of their biological functions and molecular mechanisms of action in growth regulation and cancer are most well-studied. Thus, this review focuses on the roles of tyrosine kinases, particularly the Src family of tyrosine kinases in oncogenesis.

The link between tyrosine kinases and cancer has its origins in the studies of Peyton Rous, who discovered a virus (Rous sarcoma virus) that induces tumors in chickens[8] and morphologically transforms fibroblasts in tissue culture.[8] It was later discovered that a specific gene contained within the virus is responsible for its tumorigenic potential. This gene is termed Src (for sarcoma) and found to encode a protein tyrosine kinase. In Nobel Award-winning work that followed several decades later, Varmus, Bishop, and colleagues (reviewed in ref 9) discovered that the viral Src oncogene (acronym v-Src) arose from the capture and mutation of a normal cellular gene (c-Src, a proto-oncogene) during the viral infection process. This finding fostered the concept that during normal development proto-oncogenes encode critical regulators of cell growth and differentiation.

Since this discovery, it has been speculated that the protein tyrosine kinase family of enzymes (and particularly the Src family) may contribute to the development of human malignancies. However, evidence supporting this hypothesis has been slow to evolve, largely because early emphasis was placed on examining human tumors for mutations or genetic alterations in proto-oncogenes encoding these enzymes. Such alterations were speculated to generate oncogenic variants of the proto-oncogenes. Surprisingly, mutations in genes encoding tyrosine kinases proved relatively uncommon in human tumors, suggesting that they may not be as important as originally thought. Subsequent investigations, however, discovered that multiple tyrosine kinases are overexpressed or posttranslationally activated (frequently

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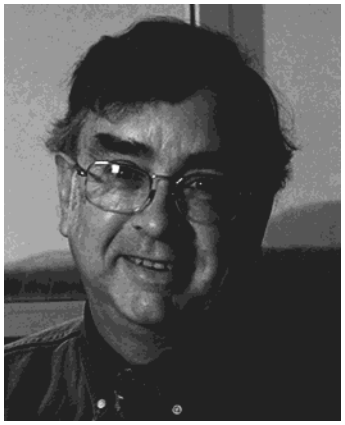
Jill K. Slack received her B.S. degree in Biology from the State University of New York at Albany and M.S. and Ph.D. degrees in Microbiology, Immunology, and Molecular Genetics from Albany Medical College. Her doctoral studies investigated the contribution of cellular adhesion to the regulation of gene expression. She is currently a postdoctoral fellow with J. Thomas Parsons, where she is studying the role of adhesion and growth factor-mediated signaling pathways in the regulation of cell migration.

by phosphorylation or dephosphorylation) in cancerous vs normal tissue, providing support for their involvement in development of human neoplasms. Tyrosine kinases found to be so modified include those that modulate growth, adhesion, invasion, and motility, properties of cells that are important for the development and progression of cancer. They fall into two general categories: Receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases. RTKs are relatively large proteins (~100−500 kDa) that span the plasma membrane, with an extracellular portion that binds a polypeptide growth factor and an intracellular catalytic domain that conveys the growth signal to the interior of the cell. Nonreceptor tyrosine kinases include several families of enzymes that localize to the cytoplasm or are tethered to intracellular membranes via lipid modifications. This review focuses on one family of the nonreceptor tyrosine kinases, the Src family, and three of its intracellular targets, all of which are thought to play important roles in the development and progression of the



J. Thomas Parsons received his B.A. degree in Chemistry from DePauw University and his Ph.D. degree in Biochemistry from Duke University. Following postdoctoral studies at St. Louis University and the University of Zurich, Switzerland, where he studied DNA and RNA tumor viruses, respectively, he joined the faculty of the Department of Microbiology at the University of Virginia Medical Center. There he continued his investigations into RNA tumor viruses, particularly Rous sarcoma virus, and its mechanisms of tumorigenesis. He is credited with the discovery of focal adhesion kinase (FAK), a substrate of Src and a critical component of cell focal adhesions. He is currently the F. Palmer Weber Medical Research Professor and Chairman of the Microbiology Department at Virginia, where he continues his investigations into the functions of FAK in cellular adhesion, migration, and invasion.

Sarah J. Parsons received her B.A. degree in Biology and Chemistry from Valparaiso University and her Ph.D. degree in Microbiology from Duke University. Following leave to raise a family, she conducted postdoctoral studies in tumor immunology at the University of Virginia Medical Center, where she was later appointed to the faculty. Her research concerns the role of the proto-oncogene product, c-Src, in normal cellular function and in human malignancies. She was named a Stohlmam Scholar of the Leukemia Society of America and is currently Professor of Microbiology and Associate Director of the Cancer Center at the University of Virginia. Her studies focus on molecular mechanisms of breast and prostate cancer progression.

malignant phenotype. These targets or substrates are the epidermal growth factor receptor (EGFR), p190 RhoGAP, and focal adhesion kinase (FAK). Emphasis is placed on describing the specificity and functional consequences of phosphorylation of these substrates by c-Src or other members of the Src family. Two of the targets are themselves protein tyrosine kinases (EGFR and FAK), while the third catalyzes hydrolysis of the γ phosphate from GTP bound to the Rho protein. The EGFR transmits growth signals to the nucleus of stimulated cells, while p190RhoGAP and FAK regulate actin cytoskeleton dynamics that are important in the processes of cell division and migra-

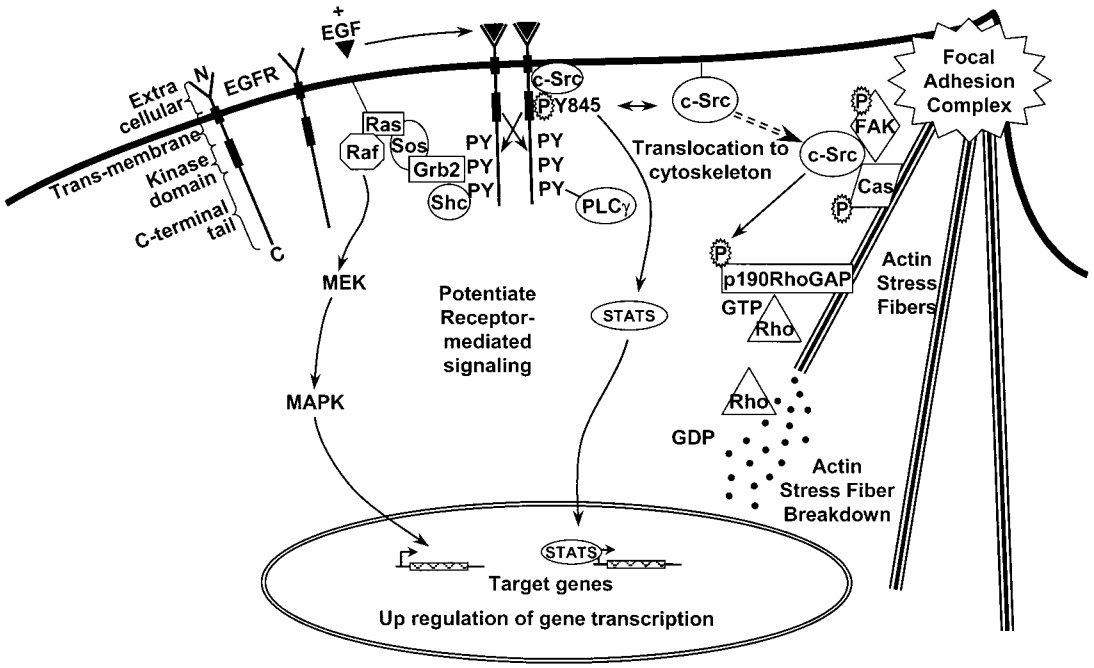


Figure 1. Targets of c-Src and their potential roles in mitogenesis, transformation, and migration. c-Src associates with and phosphorylates the ligand-activated EGFR on Tyr845, thereby potentiating downstream signaling from the receptor, particularly through the STAT family of transcription factors. In a reciprocal fashion, activated receptors induce translocation of c-Src to the cytoskeleton, where it phosphorylates several substrates, including FAK and p190RhoGAP. These substrates are central to the regulation of actin cytoskeleton rearrangements and thus signals that control morphological transformation, cell cycle progression, and migration. The various domains of the EGFR are noted: extracellular, transmembrane, kinase, and C-terminal tail. Upon autophosphorylation, tyrosine residues on the C-terminal tail serve as binding sites for SH2-containing signaling molecules, such as Shc, Grb2, and PLCγ, which in turn become activated and transmit signals that culminate in gene transcription and cell division. See text for further details.

tion, respectively (See Figure 1). Each substrate is discussed independently, following an introduction to Src and its mechanism of action and regulation.

II. c-Src Structure, Function, and Role in Human Cancers

A. c-Src Structure and Mechanisms of Regulation

c-Src is the cellular, nontransforming progenitor of v-Src, the oncoprotein encoded by the chicken retrovirus, Rous sarcoma virus. At least nine other proteins with similar overall structure comprise the Src family of tyrosine kinases.[1,10] Some are ubiquitously expressed (such as c-Src, c-Yes, Fyn, and Lyn), while others are restricted to specific cell types (such as Lck, Hck, and Fgr in hematopoetic cells). c-Src is a 60 kDa protein that is composed of seven domains: an N-terminal membrane association domain (also termed the SH4 domain), a "Unique" domain, SH3 and SH2 domains, an SH2-kinase linker domain, a catalytic domain (or SH1 domain), and a negative regulatory domain (Figure 2). (The SH designation denotes "Src homology" and reflects the overall conservation of amino acid sequences among Src family members.) Within the cell, c-Src localizes to the cytoplasmic faces of the plasma membrane and membranes of endosomes and secretory vesicles[11−13] as well as to the actin cytoskeleton (reviewed in refs 1 and 10). It is tethered to membranes via the Myristylation or SH4 domain by the combined action of an N-terminal, covalently linked myristate moiety, salt bridges between basic amino acids in the N-terminus of of the molecule and phosphates of the membrane phospholipid backbone, and noncovalent interactions with integral or associated membrane proteins.[13] Membrane localization of c-Src is required for its ability to participate in growth factor receptor-mediated signaling in normal cells[14] and for v-Src to malignantly transform cells (reviewed in ref 15). The nature of Src interactions with the actin cytoskeleton is varied and complex and is thought to involve third party molecules, such as FAK (discussed below).

The function of the Unique domain is not well understood. However, as its name implies, the Unique domain exhibits the greatest sequence divergence among Src family members and is speculated to specify protein−protein interactions that are unique to individual members of the family. It also contains many sites of serine/threonine phosphorylation that are mediated by cdc2 cyclin kinase, protein kinase A, and protein kinase C (reviewed in ref 10). The functional consequences of these phosphorylations is unclear at the present time, but it is thought that they may regulate catalytic activity or binding to specific cellular proteins. The SH3 and SH2 domains have been clearly documented to mediate the binding of c-Src with other signaling proteins. SH3 domains are found in many signaling molecules and bind polyproline sequences of defined specificity on target proteins. The SH3 domain of Src is composed of ~60 amino acids that assume a globular conformation, one side of which has a slightly depressed hydrophobic surface with an acidic cluster at one end. Results from the use of combinatorial peptide or phage

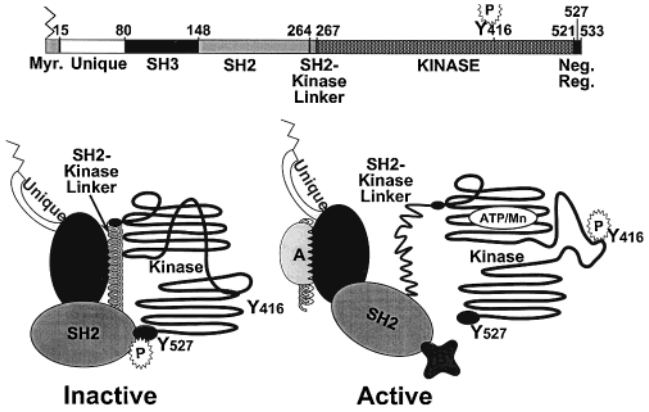


Figure 2. Structure of c-Src. As a linear molecule, the relationship between the various domains of c-Src can be seen. Numbers above the linear molecule denote amino acid residues in sequence from N- to C-terminus. The lower half of the figure is a schematic of the molecule based on crystallographic analyses of Src family members, Lck, Hck, and Src, each lacking the Myristylation and Unique domains.[23−25] Active and inactive configurations of the protein are depicted, with the black oval representing the SH3 domain, the dark gray oval representing the SH2 domain, the "spring" structure representing the SH2-kinase linker, the "stacked" folds representing the upper (N-terminal) and lower (C-terminal) lobes of the kinase domain, the stippled chain representing the activation loop with Y416, and the small black bulb representing Y527. A and B are signaling molecules with polyproline and phosphotyrosine moieties that bind the SH3 and SH2 domains, respectively. See text for further explanation of factors that regulate the active and inactive states of the enzyme.

display libraries indicate that the Src SH3 domain preferentially binds peptides that contain a RPLP-PLP motif, with the arginine contacting the acidic cluster[16,17] (reviewed in ref 10). This interaction displays a weak affinity in the micromolar range. The Src SH2 domain is an oblong, globular structure of ~100 amino acids that binds phosphotyrosine on specific cellular proteins. In vitro peptide binding studies suggest that molecules containing a pYEEI sequence are preferred binding partners of the Src SH2 domain and that this binding occurs with high affinity[18] (reviewed in ref 10). The isoleucine in the pY+3 position is the most critical for binding and positions itself in the hydrophobic pocket. The acidic residues at pY+1 and pY+2 rest on the surface of the domain. Sequences immediately upstream or downstream of either the polyproline motif (that binds the SH3 domain) or the pYEEI motif (that binds the SH2 domain) are also thought to contribute to the specificity of binding. Because many different signaling molecules contain SH2 or SH3 domains, defining which residues confer specificity is a major question in the field.[19] Interestingly, several documented binding partners of the Src SH3 and SH2 domains contain sequences that are identical or very similar to the motifs identified in the combinatorial screens, providing support for the potential biological importance of the motifs.[10]

The Src kinase domain is the region responsible for the catalytic activity of the molecule. It mediates the transfer of γ phosphate from ATP to tyrosine residues on substrate proteins, with preference for tyrosines that are imbedded in peptides with an EEIY motif. Isoleucine in the −1 position appears to be the most critical residue for efficient catalysis[20] (reviewed in ref 10). The Src kinase domain is a region that is well conserved among all tyrosine kinases and contains many subdomains that are highly related to serine/threonine kinases, including the ATP binding pocket.[21] The kinase activity of c-Src is regulated in large part by a short domain at the extreme C-terminus of the molecule. This negative regulatory region harbors a tyrosine residue that becomes phosphorylated (Tyr530 in human c-Src; Tyr527 in chicken c-Src) by another tyrosine kinase, termed C-terminal Src kinase or CSK.[22] Phosphorylated Tyr527/Tyr530 (pTyr527) is capable of binding its own SH2 domain in a manner that inhibits kinase activity without physically blocking the catalytic site, as shown in Figure 2.[23−25]

Binding of tyrosine-phosphorylated cellular proteins to the SH2 domain is thought to destabilize the intramolecular pTyr527/SH2 domain interaction and induce a conformational change that results in enzymatic activation. Structural studies have revealed that the SH2 and SH3 domains collaborate in their binding of respective protein partners, thereby cooperatively influencing the activity of the enzyme.[26] Furthermore, crystallographic analysis has shown that sequences just N-terminal to the catalytic domain (termed the SH2-kinase linker) comprise a loop structure that functions as a "pseudo" SH3 binding site.[23−25] Together, the intramolecular phosphotyrosine/SH2 and linker/SH3 interactions direct a conformation that presses the linker (and residues of the SH2 and SH3 domains themselves) against the backbone of the catalytic domain, thereby contributing to inhibition of kinase activity. As with the SH2 domain, binding of signaling proteins to the SH3 domain is thought to release the constraints of the linker/SH3 interaction on the kinase domain, resulting in activation of the enzyme.

The kinase domain itself is comprised of two lobes that form a cleft for substrate interaction (Figure 2).[25] The upper or N-terminal lobe binds ATP/Mg^{2+} or ATP/Mn^{2+} and functions as the phospho-donor complex. Connecting the upper and lower lobes is an "activation" loop that is modeled to assume a structured configuration in the inactive state, partially blocking the entrance of ATP into its binding pocket. This loop is thought to flip into an unstructured and highly mobile state upon catalytic activation, thereby allowing substrate/ATP/enzyme interaction. However, the exact nature of the "active state" is not known. (The reader is referred to a separate paper on the structure of tyrosine kinases in this issue.) The activation loop comprises a highly conserved subdomain that is found in all other tyrosine kinases. It contains a Tyr residue (Tyr416 in chicken c-Src and Tyr419 in human c-Src) that becomes phosphorylated upon activation.[21] Phosphorylation of this residue in Src and or its homologues in other tyrosine kinases facilitates but is not absolutely required for catalytic activity (reviewed in refs 1 and 10).

Mutation of Tyr527 to phenylalanine or deletion of the C-terminal regulatory domain (which occurs in v-Src) results in a constitutively active protein that

phosphorylates target proteins in an unregulated fashion and induces cellular transformation and oncogenesis.[27−30] In normal cells, c-Src is nononcogenic or only weakly so, even when it is overexpressed.[31,32] However, under certain cellular conditions (growth factor stimulation or binding to extracellular matrix—outlined below), the enzyme can become activated either via dephosphorylation of pTyr527 or by binding of signaling proteins to the N-terminal half of the protein or a combination of the two events. Activation is transient, and c-Src, in contrast to v-Src, is thought to respond to negative controls by rephosphorylation of Tyr527 and/or by the release of binding proteins and the resumption of intramolecular interactions. It has been the conjecture of many investigators that the transient nature of c-Src activation in cells often prevents detection of changes in activity. In fact, the possibility exists that only a small conformational change is necessary for biologically relevant catalysis to occur, if the substrate is properly positioned near the catalytic cleft. Thus, another "regulator" of c-Src activity may well be its intracellular localization and, at a finer level, its appropriate juxtaposition to substrate within a signaling complex. Identification of c-Src substrates and proteins that bind its SH2 and SH3 domains and investigations into their effects on c-Src structure are critical for further understanding the roles c-Src and its family members play in biological processes.

B. Evidence for the Involvement of c-Src in Human Cancers

Many lines of evidence suggest a role for c-Src in the genesis and progression of multiple types of human cancer, including carcinomas of the breast, lung, colon, esophagus, skin, parotid, cervix, and gastric tissues, as well as neuroblastomas and myeloproliferative disorders. This evidence is both genetic and biochemical in nature and has been generated by studies of cultured tumor cell lines and surgically generated tumor tissue (reviewed in ref 2). With the identification of the first proto-oncogenes came a plethora of studies examining the genomic content of multiple human tumors for deletions, amplifications, and diverse rearrangements in chromosomes containing protooncogenes. For the most part, these studies identified few if any gross changes in the c-Src gene, which maps to the q arm of chromosome 20. These findings led many investigators to conclude that c-Src plays a minor (if any) role in the genesis of human tumors. Finally, in 1999, a group of researchers at the Moffitt Cancer Center in Tampa, FL, found a C terminal deletion in the c-Src gene in 12% of patients with advanced human colon cancers.[33] This deletion occurs at amino acid residue 531 just downstream of the negative regulatory site pTyr530 in human c-Src and renders the protein constitutively active. Additional ways to modulate c-Src activity are through failure of CSK (c-Src kinase) to transphosphorylate Y530 or by increased dephosphorylation of pY530 by phosphotyrosine phosphatases (see below). However, the majority of the evidence pointing to an involvement of c-Src in human tumors comes through studies that measure protein levels and specific enzyme activities in various cell lines and tumor tissues. The results indicate that c-Src activity is frequently elevated in cancer samples above normal levels (ranging from 2- to 100-fold above normal and occurring in ~70% of the cases in breast cancer). This high enzymatic activity is accompanied by a high protein level, yielding little or no change in specific activity.[34−40] Such alterations occur in early to middle stages of tumor progression and are maintained or increased throughout progression to metastasis. These studies are suggestive of an involvement of c-Src in human oncogenesis but offer few if any clues as to its role.

Molecular engineering of cells to overexpress c-Src was found to be insufficient to transform murine fibroblasts in culture or to sustain tumor growth in intact animals.[31,32,41] However, expression of mutated, dominant interfering forms of c-Src showed that c-Src is required for growth factor-induced mitogenesis,[14,42] and studies in transgenic mice have demonstrated that c-Src is necessary for induction of mammary tumors by the polyomavirus middle T oncogene.[43] These findings suggest that c-Src may function to promote growth of tumor cells by participating in or augmenting mitogenic signaling pathways that are initiated by extracellular growth factors or intracellular oncogenes. Furthermore, cells which have been genetically engineered to lack the Src family members, c-Src, c-Yes, and Fyn, are poorly adhesive to extracellular matrix and defective for migration, properties of neoplastic cells that are critical for anchorage-independent growth and metastases.[44] Two proteins which regulate dynamic remodeling of the actin cytoskeleton in this process are known substrates for c-Src, i.e., FAK and p190RhoGAP. Thus, two prominent roles of c-Src appear to emerge with regard to malignant transformation. One is to enhance growth by augmenting signals immediately downstream of tyrosine kinase receptors, and the other is to affect morphogenetic properties of the cell by phosphorylating proteins that modulate the actin cytoskeleton (Figure 1). Each of these roles is discussed in greater detail below.

III. Epidermal Growth Factor Receptor (EGFR) as a Substrate for c-Src

A. Epidermal Growth Factor Receptor (EGFR) in Human Cancer

Because of its tethering to intracellular membranes (including the plasma membrane), c-Src and several of its family members are positioned to interact, both physically and functionally, with transmembrane growth factor receptors. Indeed, at least five different subfamilies of RTKs have been shown to physically associate with c-Src or various family members following ligand binding to the receptor. These RTKs include the epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, colony stimulating factor-1, and hepatocyte growth factor families of receptors (reviewed in ref 2). Each receptor consists of an extracellular ligand-binding domain that is unique to each family, a transmembrane segment, a tyrosine kinase catalytic domain, and a carboxy-

terminal region that contains sites of tyrosine autophosphorylation (see Figure 1). Binding of ligand causes dimerization of the receptor, activation of tyrosine kinase activity, and (trans) autophosphorylation of specific C-terminal tyrosine residues,[45,46] which in turn serve as docking sites for a variety of signaling molecules that contain SH2 domains.[19] Examples of such molecules are phospholipase C γ (PLC γ), phosphatidylinositol-3 kinase (PI-3 kinase), GTPase-activating protein of Ras (p120 RasGAP), phosphotyrosine phosphatases (PTPases), Janus kinases/signal transducers and activators of transcription (JAK/STATS), adapter proteins (including Shc, Grb, Nck), and members of the c-Src family of tyrosine kinases.[45,47] Molecules bound to the receptor become tyrosine phosphorylated and activated. Signals are subsequently transmitted from these molecules to the nucleus via several pathways, including the JAK/STAT and the Grb2/SOS/Ras/ Raf/MEK/ MAP kinase cascades[48,49] (see below for a more detailed explanation of the JAK/STAT pathway and other papers in this issue for a complete description of the MAP kinase pathway). After tyrosine phosphorylation and activation, members of the STAT and MAP kinase families translocate from the cytoplasm to the nucleus and induce changes in gene expression, which bring about a variety of functional outcomes, such as mitogenesis, morphogenesis, and motility.

The human EGFR is the prototype of a family that consists of four known members. It plays a variety of roles in normal development (reviewed in ref 2). The link between EGFR and human cancer initially came from studies by Velu et al.,[50] who demonstrated that cultured fibroblasts overexpressing EGFR become transformed when they are grown in the continuous presence of EGF. Subsequent studies found that EGFR is overexpressed in a variety of human cancers, including benign skin hyperplasia, glioblastoma and carcinomas of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung.[51] Approximately 30% of human breast tumors overexpress EGFR, and this overexpression is correlated with a loss of estrogen responsiveness and a poorer prognosis.[52,53] It is estimated that approximately 70% of human breast tumors overexpress one or more of the EGFR family members.[53−55] In most cancers, except glioblastomas, the EGFR is overexpressed as an intact molecule, harboring no mutations. In glioblastomas, however, and in a subset of other cancers, gross deletions in the ligand binding domain have been sustained, rendering the receptors constitutively active[56,57] (reviewed in ref 2). Such alterations have been causally linked to glioblastoma development. In other cancer types, much evidence suggests that full-length EGFR is involved in later stages of the disease and may play roles in growth and metastases.[58]

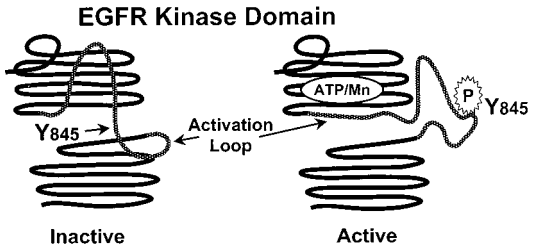
B. Biological Synergism between c-Src and Human EGFR

One striking finding emerges when one examines the overexpression patterns of c-Src and EGFR in human cancers, i.e., that both tyrosine kinases are co-overexpressed in many of the same tumor types. Such coexpression suggests that c-Src and EGFR may collaborate to regulate the genesis and/or progression of certain human cancers. In a direct test of this question, it was found that dual overexpression of c-Src and EGFR in a mouse fibroblast model system leads to synergistic increases in EGF-induced DNA synthesis, soft agar colony formation, and tumor formation in nude mice when compared with cells that express only c-Src or EGFR.[41] This enhanced oncogenesis correlates with EGF-induced physical association of c-Src with the EGFR, increased phosphorylation of the receptor substrates, Shc and PLC γ, and the phosphorylation of two novel tyrosine residues on the receptor, which have been identified by tryptic phosphopeptide mapping to be Tyr 845 and Tyr 1101.[59] Stover et al.[60] showed that activated Src can phosphorylate EGFR at Tyr 891 and Tyr 920 in vitro and that these sites can mediate binding of the SH2 domains of PI-3 kinase and Src itself. These same sites have been found to be phosphorylated in several colorectal carcinomas and MCF7 breast cancer cells, from which c-Src and PI-3 kinase can be coimmunoprecipitated with EGFR. Such findings support the idea that bidirectional interactions between c-Src and EGFR occur.

Interestingly, co-overexpression of both EGFR and c-Src also occurs in a subset of human breast cancer cell lines and breast tumor tissues.[36] Like the mouse fibroblast model, human breast tumor cell lines that co-overexpress c-Src and EGFR display EGF-induced complex formation between c-Src and EGFR, the appearance of Tyr 845 and Tyr 1101 phosphorylations on the c-Src-associated receptor, and increased phosphorylation of the EGF effectors, Shc and MAP kinase, as well as increased tumor size in nude mice when compared with the majority of cell lines that do not overexpress these tyrosine kinases or express only one of the pair. Enhanced MAP kinase and MEK activity have also been found in human breast tumors that overexpress both c-Src and EGFR.[61,62] These results suggest that c-Src and EGFR act synergistically and that this interaction is manifested by increased signaling through EGFR, unregulated growth, and tumorigenesis. Because complex formation between EGFR and c-Src can be detected only under conditions of mutual overexpression, disruption of this complex could provide the basis for novel therapeutic approaches.

C. Molecular Mechanism of c-Src/EGFR Synergism

Clues to understanding the molecular mechanism of the biological synergy between c-Src and EGFR are likely to be found in the novel phosphorylations on the EGFR (Tyr 845 and Tyr 1101) that are mediated by c-Src. Tyr 1101 is located in the carboxy-terminal tail of the receptor, amidst the major autophosphorylation sites. Its phosphorylation suggests that it may form the binding site for a novel signaling molecule, but to date such a molecule has not been identified. Thus, little is known about the biological significance of Tyr 1101 phosphorylation. Tyr 845 is located in the activation loop of the receptor's cata-



Alignment of Y845 Sequence Homologs:

EGF receptor	GLAKLLGAEEKEYHAEGGKVPIK	(Y845)
Neu/Erb-B2	GLARLLDIDETEYHADGGKVIPI	(Y882)
CSF-1 Receptor	GLARDIMNDSNYVVKGNARLPV	(Y807) (proliferation)
HGF Receptor	GLARDMYDKEYYSVHNKTGAKL	(Y1235/1236) (migration)
FGF Receptor	GLARDIHHIDYYKKTTNGRLPV	(Y653/654) (proliferation, neurite outgrowth)
PDGF-β Receptor	GLARDIMRDSNYISKGSTYLPL	(Y825) (proliferation)
Insulin Receptor	GMTRDIYETDYYRKGGKGLLPV	(Y1162/1163) (sugar uptake)
c-Src	GLARLIEDNEYTARQGAKFPI	(Y416)

Figure 3. Topology of Tyr845 and its homologues. Tyr 845 is located in the activation loop of the catalytic domain of the EGFR, in a highly conserved subdomain that has functional homologues in other tyrosine kinases, as indicated. Upon ligand binding, the activation loop is modeled to flip into a configuration that permits access to and stabilization of ATP and substrate, as described by Groenen et al.[185] Tyr845 is unique in that it is not an autophosphorylation site, as are homologues of Tyr 845 in other receptors, such as CSF-1, HGF, FGF, etc. Tyr845 is phosphorylated by c-Src.

lytic domain and is particularly intriguing because its homologues are found in a variety of receptor and nonreceptor tyrosine kinases (Figure 3).[21] In fact, substitution of Phe for the Tyr 845 homologues in other receptors renders them catalytically impaired and defective in downstream signaling.[63−65] In virtually all RTKs and nonRTKs examined to date, the Tyr 845 homologue is autophosphorylated. In contrast, Tyr 845 in the EGFR has never been identified as an autophosphorylation site either in vitro or in vivo. Failure to detect such a phosphorylation was interpreted to mean either that the site was not phosphorylated or that its phosphorylation was extremely short-lived. Ultimately, the ability to detect this phosphorylation in intact cells was found to be dependent on inhibiting intracellular PTPases,[59] giving support to the idea that the phosphorylation of Tyr 845 is rapidly turned over.

Recently, it was shown that phosphorylation of Tyr 845 is dependent upon the catalytic activity of c-Src, suggesting that c-Src directly phosphorylates this site.[66] Further evidence for this hypothesis is provided by the findings that Tyr 845 is phosphorylated in vitro to high stoichiometry when immune complexes of c-Src (that contain the co-immunoprecipitated receptor) are incubated in buffer containing ATP/Mn^{2+}. Similarly, peptides derived from the receptor that contain Tyr845 are phosphorylated by partially purified Src to a near 1:1 ratio of phosphate to peptide. Interestingly, the sequences immediately surrounding Tyr 845 do not conform strictly to that of an "optimal" Src phosphorylation site. However, a decapeptide containing this site bears 50% homology to the v-Src autophosphorylation site, Tyr 416, providing further evidence that Tyr 845 is phosphorylated by c-Src in situ. Thus, the unique characteristic of Tyr 845 appears to be that its phosphorylation is mediated by c-Src, whereas homologues in other receptor tyrosine kinases are phosphorylated by the receptors themselves. Why the EGFR has acquired this added level of regulation is unclear, but clues to this puzzle are beginning to be revealed and are described below.

Other examples of tyrosine kinases, whose conserved activation loop tyrosines become phosphorylated in trans (by nonhomologues kinases), have emerged in the past few years. Kinase-defective Lck, ectopically expressed in cells that lack endogenous Lck or that lack other Src family kinases, is detectably phosphorylated at Tyr 394, the Tyr 845 homologue.[67] These results suggest the existence of a ubiquitous activator of Src family tyrosine kinases. Serine/threonine kinases also have conserved activation loop phosphorylation sites, except that the phosphorylated residues are serines or threonines instead of tyrosines.[21] Transphosphorylation of Thr229 in the catalytic domain of p70 ribosomal S6 kinase by phosphoinositide-dependent kinase 1 (PDK1),[68] of Thr256 and Ser 422 in the serum and glucocorticoid-inducible kinase (SGK) by PDK1,[69] and of Ser177 and Ser181 in IκB kinase kinase β (IKKβ) by MEKK1or NIK[70] have been described. In these cases, phosphorylation of the critical serine/threonine residues is required for full activity of the enzymes as is phosphorylation of the homologous tyrosine residues in tyrosine kinases.

Cells expressing kinase-inactive c-Src not only fail to support phosphorylation of Tyr 845, but also display a drastically decreased ability to grow in soft agar in the presence of EGF and to form tumors in nude mice, suggesting that phosphorylation of Tyr 845 may be critical for mitogenesis and transformation. Indeed, in mouse fibroblasts that express either elevated or normal levels of c-Src, expression of a Tyr 845 Phe mutant form of EGFR results in a reduction in EGF-, serum-, and lysophosphatidic acid (LPA)-induced DNA synthesis. Furthermore, no stable cell lines expressing high levels of the mutant receptor can be generated, suggesting that the Tyr845Phe variant of EGFR functions in a dominant interfering manner with the endogenous EGFR and inhibits outgrowth of the cells bearing the mutant receptor.[59,66] Thus, c-Src-mediated phosphorylation of Tyr 845 appears to be necessary for mitogenic responses that emanate from the EGFR.

The finding that serum- and LPA-induced DNA synthesis are affected by the Tyr845Phe mutation suggests that other families of cell-surface receptors may mediate their effects in part through the EGFR. LPA, a major mitogen in serum, is known to activate a Gi-coupled receptor, a member of the serpentine family of receptors that traverses the plasma membrane seven times and mediates downstream signaling events through heterotrimeric G proteins (composed of α, β, and γ subunits).[71] Several laboratories have recently reported that activation of certain G-protein-coupled receptors (GPCR) can trigger phosphorylation of the EGFR receptor as well as activation of its downstream effectors, Shc and MAP kinase, and that this activation is dependent on c-Src kinase activity.[46,72,73] Ullrich and colleagues[74] have

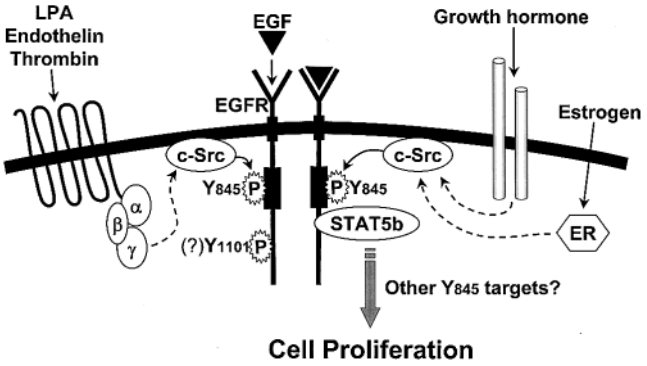


Figure 4. Phosphorylation of Tyr845 acts as a central conduit for multiple receptor families. Ligands for G-protein couple receptors (such as thrombin, endothelin, and LPA), a cytokine receptor (growth hormone), and steroid receptor (estrogen) all trigger the phosphorylation of Tyr845 on EGFR in a c-Src-dependent manner. Mutation of Tyr845 to phenylalanine ablates induction of DNA synthesis by these receptors, suggesting that relaying signals through c-Src and the EGFR is critical for cell cycle progression and DNA synthesis to occur in response to activation of a variety of heterologous receptors.

evidence to suggest that transactivation of the EGFR by GPCRs involves proheparin binding-EGF (proHB), a precursor of a variant of EGF that upon proteolytic processing binds and activates EGFR. GPCR stimulation rapidly induces expression of proHB-EGF and a metalloproteinase that is thought to be involved in the cleavage of membrane-bound proHB-EGF and formation of the active molecule. HB-EGF then binds and activates the EGFR, stimulating signaling cascades that regulate cell cycle progression and cell division. In addition to GPCRs, stimulation of the growth hormone cytokine receptor (a heterodimeric cell surface receptor) has been found to induce EGFR phosphorylation via Janus kinase 2 (JAK2) (reviewed in ref 75). Recent work in our laboratory (Biscardi et al., unpublished data) has demonstrated that treatment of mouse fibroblasts with different GPCR ligands (thrombin, endothelin, and LPA) or with the cytokine, growth hormone, induces increases in overall tyrosine phosphorylation of EGFR as well as in Tyr 845 phosphorylation (Figure 4). Interestingly, the kinase activity of c-Src is also required for phosphorylation of Tyr 845 via these alternate receptors, and mitogenesis is dependent on the phosphorylation of Tyr 845. Fibroblasts that express the Tyr845Phe variant of EGFR are impaired in their ability to synthesize DNA in response to these stimuli (Figure 4). Similarly, the Tyr845Phe mutant abolishes estrogen-stimulated DNA synthesis in the estrogen-responsive MCF7 human breast cancer cell line. (Estrogen receptor is a transcriptional transactivator that binds the lipophilic ligand, estrogen.) Following ligand binding, the receptor dimerizes and activates transcription of specific genes.[76] However, platelet-derived growth factor receptor activation is not affected by the Tyr845Phe mutation, indicating that the variant EGFR does not act as a general inhibitor of mitogenesis. Taken together, these findings suggest that the EGFR plays an important, perhaps widespread, role in mediating the cell's response to an array of external signals and that the c-Src-mediated phosphorylation of EGFR Tyr845 appears to be a critical event in this process. Such a role may offer an explanation for the unique dependence of the EGFR on c-Src for phosphorylation of Tyr845. In contrast to other RTKs, which autophosphorylate their Tyr845 homologues, c-Src can function as an intracellular intermediary between other classes of receptors and EGFR by phosphorylating Tyr845, thereby enhancing the array of signaling pathways that can be activated by any one receptor.

Although mutation of Tyr845 has profound effects on the cell's ability to respond mitogenically to EGF, many of the downstream targets of EGFR continue to be phosphorylated or activated to the same extent as they would be by a wild-type receptor. These effectors include Shc, MAP kinase, STAT3, and PLC γ[66] (Tice et al., unpublished results). This is a totally unexpected finding since MAP kinase in particular has been implicated as the major transducer of mitogenic signals from RTKs. The intrinsic kinase activity of the Tyr845Phe mutant also appears to be unchanged as does its ability to associate with c-Src.[66] However, recent evidence produced in a collaborative effort between the laboratories of S. Parsons and C. Silva (Kloth et al., submitted for publication) suggests that STAT5b may be a physiologically relevant effector of pTyr845.

The STATs are a family of transcription factors (STATs 1, 2, 3, 4, 5a, 5b, 6) that are activated at the plasma membrane by tyrosine phosphorylation in response to signals from cytokine and growth factor receptors (reviewed in refs 77 and 78). Tyrosine phosphorylation results in STAT dimerization, nuclear translocation, and binding of STAT dimers to consensus DNA sequences (elements) that are located upstream of regulated genes. Binding of STAT dimers to these elements activates transcription, which is a presumed precursor to cell division.

Increasing evidence indicates that STAT proteins are involved in the process of oncogenesis.[79,80] Two laboratories have shown that STAT3 is required for v-Src transformation,[81,82] while deGroot et al.[83] demonstrated that active STAT5 is necessary for the soft agar growth of BCR-Abl transformed leukemia cells. Recent studies[84] have also indicated a direct role for c-Src in the activation of STAT proteins. For example, c-Src was shown to mediate the EGF stimulation of STATs 1, 3, 5a, and 5b in murine fibroblasts engineered to overexpress the EGFR and in A431 human carcinoma cells, which endogenously express high levels of EGFR. In contrast, another group[85] has described a role for c-Src in the tyrosine phosphorylation (but not the transcriptional activation) of STAT5a and STAT5b in a COS cell transfection model. Constitutive STAT3 signaling, which has been observed in breast cancer cell lines, is regulated by the Src and JAK tyrosine kinases and participates in growth regulation of these cells.[86,87]

Our recent studies (Kloth et al., submitted for publication) indicate a role for the STAT proteins in signaling pathways that are activated in murine fibroblasts and breast cancer cells co-overexpressing c-Src and EGFR. We have shown that c-Src tyrosine kinase activity is required for maximal transcriptional activation of STAT5b by EGF and that phos-

phorylation of Tyr 845 is required for the tyrosine phosphorylation of STAT5b. Furthermore, a dominant interfering form of STAT5b inhibits DNA synthesis in response to EGF. These studies suggest a model whereby EGFR/c-Src co-overexpression and EGF stimulation lead to the phosphorylation of Tyr845, the recruitment and activation of STAT5b, enhanced STAT5b transcriptional activity, and increased DNA synthesis. Interestingly, STAT5b does not become tyrosine phosphorylated upon GPCR or estrogen receptor stimulation and MAP kinase activation by these receptors is unaltered by the presence of the Tyr845Phe variant of the EGFR. These findings suggest that STAT5b and MAP kinase are either not required or insufficient to transmit growth signals initiated by GPCRs or estrogen receptor, even though these receptors require phosphorylation of Tyr845 on the EGFR for their mitogenic capabilities (Biscardi et al., unpublished results). Such results suggest that substrates other than STAT5b and Shc/MAP kinase mediate critical growth signals from GPCRs and the estrogen receptor.

D. Summary

Substantial evidence is accumulating to indicate functional synergism between the nonreceptor tyrosine kinase c-Src and the EGFR in promoting the development and progression of human cancers that simultaneously overexpress these proteins. Recently, one mechanism by which this synergy occurs has been uncovered. It involves the EGF-dependent association of c-Src with EGFR and phosphorylation of the receptor by c-Src on residues Tyr845 and Tyr1101. The functional consequences of Tyr1101 phosphorylation are unknown, but phosphorylation of Tyr845 is required for EGF-induced DNA synthesis and activation of members of the STAT family of transcription factors, particularly STAT5b, but not activation of Shc or MAP kinase. Whether the STATs are the predominant mediators of Tyr845-dependent mitogenesis or whether there are other mitogenic signaling pathways that emanate from phosphorylated Tyr845 remains to be determined. Surprisingly, Tyr845 phosphorylation has also been found to be an intermediate in mitogenic signaling from heterologous receptors, such as GPCRs, cytokine receptors, and the estrogen receptor. Thus, EGFR, and specifically phosphorylation of Tyr845 by c-Src, appears to play an important, perhaps widespread role in mediating growth responses to an array of external signals that all contribute to the neoplastic phenotype.

IV. P190 RhoGAP as a Substrate for c-Src

A. Structure of P190 RhoGAP

p190 RhoGAP (p190) was initially identified in v-Src-transformed cells as a highly tyrosine-phosphorylated protein that coprecipitated with the GTPase-activating protein (GAP) for p21Ras, p120 RasGAP.[88,89] Structurally, p190 consists of an N-terminal GTP-binding domain, a middle domain that contains the major site of tyrosine phosphorylation

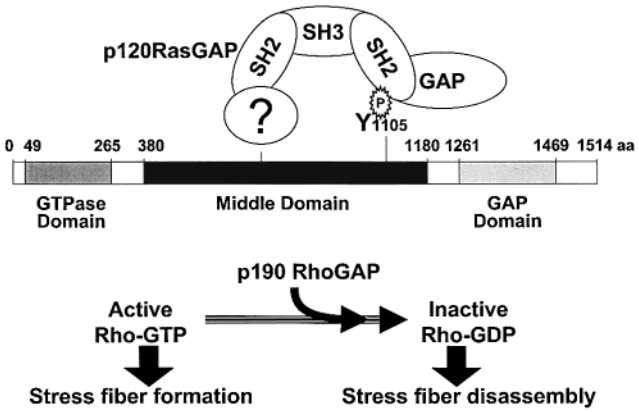


Figure 5. Schematic diagram of p190RhoGAP and its association with p120RasGAP. p190 consists of an N-terminal GTP-binding domain, a middle domain, and a C-terminal GAP domain that is specific for the Rho family of small GTPases. Tyr1105 (Y1105) in the middle domain is the major site of tyrosine phosphorylation, and c-Src is the kinase responsible for its phosphorylation. The interaction of p190 and p120 is mediated by the two SH2 domains of p120 which bind to tyrosine-phosphorylated residue 1105 and some other unknown region in the middle domain (indicated by the question mark, ?). The amino acid residues that delineate the boundaries for the domains are indicated above the figure. The lower panel depicts the role of Rho in regulating actin stress fiber dynamics. In the GTP-bound state, Rho induces actin stress fiber assembly, while in the GDP-bound state, it permits disassembly. P190 RhoGAP catalyzes the conversion of RhoGTP to RhoGDP, thereby promoting stress fiber disassembly.

(Tyr1105) and mediates binding to p120 RasGAP, and a C-terminal GAP (GTPase-activating protein) domain which has been shown to inactivate the Rho family of small GTPases (Figure 5).[90−96] The biological role of the GTP-binding domain of p190 is unknown, although it has been shown to bind to GTP and responds to a GAP-like activity.[90,97] The function of the middle domain was also not known until Tyr1105 was shown to be the major in vivo and in vitro site for tyrosine phosphorylation.[91] In addition, the middle domain contains five SH3 domain-interactive PXXP motifs, but binding partners for these PXXP motifs have not yet been identified. The presence of the major site of tyrosine phosphorylation and the PXXP motifs suggest that the middle domain is a protein−protein interaction domain. That p190 serves as a regulator of Rho was suggested by the presence of a GAP domain, which is conserved among other RhoGAPs, including the breakpoint cluster region protein, n-chimerin, and p50 RhoGAP.[93,95,98]

B. Regulation of Actin Cytoskeletal Events by Rho Family of Small GTPases

The Rho family of GTPases functions in a number of cellular events, including actin cytoskeletal arrangements, cell cycle progression, transformation, and apoptosis.[99−103] Specifically, the Rho family mediates changes in the actin cytoskeleton in response to growth factors and cell adhesion.[104] This family of small GTPases (~21 kDa) is comprised of many members which include Rho, Rac, and Cdc42.[105] Rho-GTP induces the formation of actin stress fibers and focal adhesions, Rac-GTP regulates cortical actin

polymerization and induces lamellipodia and membrane ruffling, while Cdc42-GTP induces filopodia formation.[106−109] In vivo, p190 RhoGAP activity has been shown to be specific for Rho, as microinjection of the p190 GAP domain into cells causes actin stress fiber disassembly but does not affect membrane ruffling.[93] p190 is thought to induce actin stress fiber disassembly by inactivating Rho (Figure 5). This is accomplished through activation of Rho's intrinsic GTPase activity, which results in the conversion of Rho-GTP to Rho-GDP. Since active Rho-GTP is required for actin stress fiber assembly, generation of inactive Rho-GDP is presumed to lead to actin stress fiber disassembly (Figure 5). A number of downstream effectors of Rho family members have been identified. For a summary of Rho family targets and how actin filament assembly and disassembly are regulated by them, the reader is referred to several reviews.[105,110]

Actin cytoskeleton dynamics are important for adhesion, motility, and cell cycle progression. Treatment of cells with the actin-disrupting drug cytochalasin D has been shown to inhibit DNA synthesis induced by EGF as well as other growth factors.[111−113] These findings suggest that actin cytoskeletal integrity is needed for cell cycle progression into S phase. However, the molecular relationship between actin disassembly and cell cycle progression has not been well defined. Later stages of the cell cycle, such as mitosis, also involve changes in the actin cytoskeleton, particularly during cytokinesis.[114] Since morphological transformation by v-Src and growth factor stimulation of cells (which involves c-Src) both induce alterations in the actin cytoskeleton, it has been postulated that c-Src plays a key role in regulating actin dynamics that are important for cell growth and proliferation.[115.116]

C. Tyrosine Phosphorylation of P190 by c-Src

The hypothesis that p190RhoGAP may mediate some of the effects of c-Src on the actin cytoskeleton was derived from experiments which identified p190 as a c-Src substrate. Chang et al. [116,117] demonstrated that the level of tyrosine phosphorylation of p190 correlated directly with the level of c-Src in cells and not with growth factor (EGF) stimulation. Specifically, p190 tyrosine phosphorylation was enhanced when wild-type c-Src (K^+ c-Src) was overexpressed in C3H10T1/2 murine fibroblasts and decreased below normal levels in cells overexpressing kinase-defective c-Src (K^- c-Src). A kinetic analysis of p190 localization in Neo control, K^+ c-Src overexpressors, and K^- c-Src overexpressors using fluorescence microscopy revealed that p190 is diffusely distributed throughout the cytoplasm in all three types of resting fibroblasts. Upon EGF stimulation, p190 rapidly and transiently condenses into wavelike arcs in the cytoplasm that radiate away from the nucleus. In K^+ c-Src overexpressors, p190 arc formation is accelerated, whereas in K^- c-Src overexpressors, p190 arc formation is delayed as compared to the Neo control cells. The additional finding that the time course for maximal actin stress fiber disassembly induced by EGF closely parallels the kinetics for the redistribution of p190 into the arcs in each of the three cell lines suggested that the localization of p190 into arcs is important for actin stress fiber disassembly. Although these correlative studies suggested a role for c-Src phosphorylation of p190 in regulating EGF-induced actin cytoskeletal rearrangements, they did not directly demonstrate that tyrosine phosphorylation of p190 is required for such events.

To directly determine if c-Src phosphorylation of p190 is necessary for EGF-induced actin stress fiber disassembly in C3H10T1/2 murine fibroblasts, a p190 structure/function analysis was performed in our laboratory. In these experiments, GST-tagged fusion proteins of the isolated p190 GAP domain (aa 1261−1469), the combined wild-type middle domain/GAP protein (aa 380−1469), or a Tyr1105Phe middle domain/GAP mutant (aa 380−1469) were microinjected into Neo cells, and injected cells were then stimulated with EGF. Microinjection of the GAP domain alone into normal fibroblasts caused constitutive actin stress fiber disassembly in the absence or presence of EGF stimulation. In contrast, when the wild-type middle domain/GAP protein was injected, constitutive disassembly of actin was ablated and EGF regulation was restored (i.e., inducible and transient disassembly). These results suggest that the p190 middle domain negatively regulates the GAP domain in vivo[186] and serves as the structure that receives the disassembly signal from the EGFR. Further studies, wherein the Tyr1105Phe middle domain/GAP was injected, demonstrated that phosphorylation of Tyr1105 is a positive regulator of GAP activity and required for EGF-induced actin disassembly. Thus, two signals appear to be necessary for growth factor-regulated actin disassembly, c-Src phosphorylation of p190 and growth factor (EGF) treatment. Additional studies suggested that EGF treatment is needed to remove a negative regulator of the RhoGAP domain from the middle domain and that phosphorylated Tyr1105 serves as a docking site for SH2-containing proteins.

A candidate for one of the proteins that binds Tyr1105 and may regulate p190 RhoGAP activity is p120 RasGAP. The interaction between p190 and p120 has been fully characterized and is speculated to link the Ras and Rho signaling pathways. In fact, binding of p190 to p120 inhibits the RasGAP activity of p120, which results in increased levels of GTP-bound Ras. Elevated Ras-GTP levels in turn lead to enhanced mitogenesis.[117−119] However, how might p120 RasGAP binding to p190 affect p190 RhoGAP actvity? The amino-terminus of p120 contains a tandem arrangement of SH2−SH3−SH2 domains that have been proposed to mediate binding to p190 (Figure 5). Structure-function analyses of p120 RasGAP have demonstrated that the two SH2 domains synergistically bind tyrosine-phosphorylated p190.[118,120,121] These findings suggest that the interaction of p190 with p120 may be mediated by dual phosphotyrosine sites on p190. Results from mutagenesis and transient overexpression studies led Hu and Settleman[92] to propose that complex formation between p190 and p120 requires phosphorylation

of Tyr1087 and Tyr1105 in p190 and that the dual p-Tyr-SH2 interaction results in a conformational change in p120 that exposes the SH3 domain of p120 to additional binding proteins (Figure 5). Interestingly, both Tyr1087 and Tyr1105 of p190 are in a YXXPXD motif that is thought to be recognized by the SH2 domain of p120.[122] However, tryptic phosphopeptide and mass spectrometry analyses of p190 from either Neo control or C3H10T1/2 murine fibroblasts overexpressing c-Src (K^+ c-Src) revealed that only Tyr1105 is phosphorylated in vivo and that the overall phosphorylation pattern of p190 does not change appreciably upon growth factor stimulation.[91] Furthermore, coimmunoprecipitation of p190 and p120 is observed when p190 is tyrosine phosphorylated at low or even undetectable levels.[91,92,117,120] Together, these observations suggest that mechanisms other than phosphotyrosine-SH2 binding may regulate the interaction of p190 with p120. In particular, these data indicate that if the interaction of p190 and p120 is direct, then one of the SH2 domains of p120 is binding in a phosphotyrosine-independent manner. Since p190 is also heavily serine phosphorylated, it has been suggested that phosphoserine residues may contribute to the binding of p190 and p120.[91] In fact, there are numerous examples of phosphoserine-dependent interactions with SH2 domains.[123−129] Furthermore, in vitro binding of the purified p120 SH2/3/2 protein of p120 RasGAP to phosphorylated Tyr1105 of the p190 GSTmiddle domain/GAP protein does not alter p190's RhoGAP activity.[186] This finding leads to the speculation that additional proteins, perhaps SH3-containing proteins (that bind the PXXP motifs of p190) may be recruited to the p120/p190 complex by EGF and induce conformational changes in the p190 middle domain that affect RhoGAP activity.

The concept that c-Src regulates p190 RhoGAP activity and thus Rho-mediated actin dynamics is supported by numerous correlative reports in the literature. Although in the murine C3H10T1/2 fibroblasts system, EGF stimulation does not alter the level of tyrosine phosphorylation of p190,[91,116] for some ligands, tyrosine phosphorylation of p190 increases upon stimulation. For example, engagement of β1 integrins by plating cells on specific extracellular matrix proteins causes an increase in p190 tyrosine phosphorylation that is dependent on c-Src.[130,131] Furthermore, Arthur et al.[130] demonstrated that increases in p190 tyrosine phosphorylation by c-Src correlate with an increase in immunoprecipitated p190 RhoGAP activity. These findings suggest that tyrosine phosphorylation of p190 may be regulated differently, depending upon the ligand/receptor pathways that are activated. One potential mechanism for this specificity may be preferential activation of different p190 family members. The p190 family consists of at least two members, p190A and p190B.[95,132] P190B is 51% homologous with p190A and appears to be involved in β integrin receptor signaling. Although the proteins share only partial identity, distinguishing between their biological actions is problematic due to lack of specific antibodies. Recently, experiments performed by Chiarugi et al.[133] have identified a low molecular weight protein tyrosine phosphatase (LMW−PTPase) that dephosphorylates p190, which they suggest inactivates p190 RhoGAP activity. Such findings further support the idea that tyrosine phosphorylation of 190 is regulatable. Interestingly, the activity of the LMW−PTPase itself appears to be modified by c-Src phosphorylation. Studies performed by Fincham et al.[134] also demonstrate a positive correlation between tyrosine phosphorylation of p190 by v-Src and actin stress fiber disassembly. The finding that expression of an activated form of RhoA can suppress the v-Src induced cytoskeletal disruption implicates p190 as a candidate effector of Src-induced cytoskeletal disruption.

D. Summary

In summary, the RhoGAP activity of p190 appears to be regulated in part by c-Src phosphorylation of Tyr1105. The model derived from the microinjection studies[186] is depicted in Figure 6. According to this model, c-Src phosphorylation of p190 is necessary but not sufficient to induce actin stress fiber disassembly. An additional signal is required from the EGFR. The nature of this signal is not known but is the focus of work in progress.

The function of p190 in cells is probably much more complicated than indicated by the actin cytoskeletal studies. Results of additional studies suggest that upon transient overexpression of full length p190, the GTP-binding domain of p190 participates in the regulation of cell shape[96] (B. Dukes et al., manuscript in preparation). Recent studies performed in our laboratory suggest that p190 mediates cell cycle progression or cell survival signals independently of its GAP domain (B. Dukes et al., manuscript in preparation). Together, these findings indicate that p190 may play multiple roles in cell signaling.

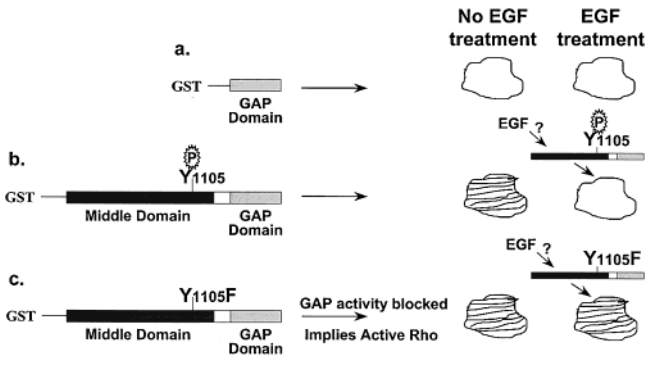


Figure 6. Model for regulation of EGF-induced actin cytoskeletal dynamics by p190 domains. (A) Microinjection of the GAP domain of p190 causes constitutive actin stress fiber disassembly in the absence or presence of EGF stimulation, but the fusion of the middle domain to the GAP domain prevents constitutive disassembly and restores EGF regulation to the process (B). That phosphorylation of Tyr1105 by c-Src is necessary for actin stress fiber disassembly is shown by the inability of EGF to induce disassembly when the Tyr1105Phe middle domain/GAP mutant is microinjected (C). The figure depicts cells with (striated) and without (clear) actin stress fibers.

V. Focal Adhesion Kinase (FAK) as a Substrate for c-Src

A. Src and FAK as Regulators of Adhesion Signaling

Normal cellular growth control, which reflects a balance of proliferation, differentiation and death, is a carefully regulated process that responds to environmental cues, contributed largely by serum growth factors, and adhesive influences, provided by the extracellular matrix (ECM). Adhesion of cells to the ECM is mediated by the integrin family of heterodimeric receptors.[135,136] Engagement of integrin receptors with their extracellular ligands leads to the formation of well-defined structures (termed focal adhesions) that link the ECM with cytoplasmic actin cytoskeleton (Figure 7).[136,137] It is now clear that these sites of ECM-integrin adhesion are in fact, dynamic structures, varying in size and organization.[138,139] These adhesions are comprised of a number of abundant cytoskeletal proteins (for example, vinculin, talin, alpha-actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the Src family and focal adhesion kinase (FAK).[136,140] Recruitment of protein tyrosine kinases to focal adhesions leads to their activation and the subsequent tyrosine phosphorylation of multiple focal complex-associated proteins including FAK, paxillin and Cas. Inhibitor studies provide evidence that tyrosine phosphorylation plays an important role in the overall organization of adhesion complexes and their dynamic regulation.[136,137]

B. FAK/c-Src Binding and Activation

The protein tyrosine kinase FAK was originally identified as a protein whose tyrosine phosphorylation increased upon transformation of cells with v-Src.[141] FAK is a structurally distinct nonreceptor protein tyrosine kinase comprised of a central catalytic domain, flanked by an N-terminal domain that has sequence similarity with band 4.1 proteins and a C-terminal domain that mediates numerous protein−protein interactions (Figure 8). In normal embryonic fibroblasts, attachment and spreading of cells on ECM proteins leads to the rapid recruitment of FAK to the focal adhesion complex, its concurrent phosphorylation on tyrosine, and activation.[142−146] These events require integrin clustering.[147] The targeting of FAK to adhesion complexes is mediated by an approximately 100 amino acid domain within the C-terminus, termed the Focal Adhesion Targeting (FAT) region (Figure 8). Sequences within the FAT domain are both necessary and sufficient to target FAK to adhesion complexes,[148] and the integrity of this region is essential for FAK signaling[149,150] (Figure 8). FAK has also been shown to bind the short cytoplasmic domains of β_1 and β_3 integrins in vitro.[151] These observations suggest a mechanism whereby integrin clustering stimulates FAK recruitment to nascent focal complexes, oligomerization of FAK and subsequent transphosphorylation.

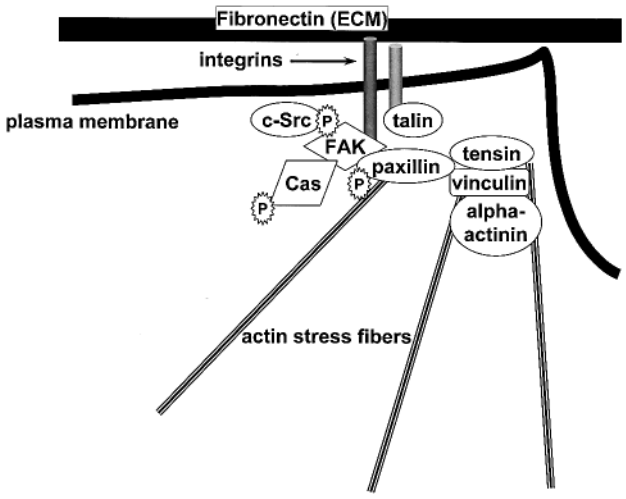


Figure 7. Focal adhesion. Transmembrane integrin receptors link components of the extracellular matrix (ECM), including fibronectin, to the internal cytoskeleton consisting of actin microfilaments. This link is facilitated by several protein−protein interactions involving paxillin, tensin, vinculin, talin, and α-actinin. The focal adhesion also contains nonreceptor tyrosine kinases, FAK and Src, that become phosphorylated and activated upon integrin engagement. Activated FAK and Src subsequently phosphorylate Cas, a multifunctional adapter protein, and paxillin. See text for additional details.

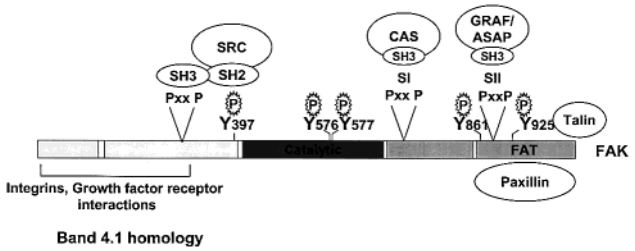


Figure 8. Schematic representation of the organization of the domains of focal adhesion kinase. The N-terminal domain shares similarity with Band 4.1 proteins and directs interactions with integrins and growth factor receptors. The central domain is the catalytic domain. The C-terminal domain contains sites for multiple protein−protein interactions. SI denotes Site I, an interaction site with the SH3 domain of Cas. Tyr397 is the major site of autophosphorylation and a site of interaction with the SH2 domain of Src. FAT denotes the region required for focal adhesion targeting. Paxillin interacts with sequences that overlap the FAT domain. Additional sites of tyrosine phosphorylation are indicated.

Cell spreading on ECM proteins results in the tyrosine phosphorylation of FAK on tyrosine 397 (Tyr397) (Figure 8) and the formation of stable complexes with Src family members.[152,153] Phosphorylation at Tyr397 correlates with increased catalytic activity of FAK.[145,154] and is required for the subsequent adhesion-dependent tyrosine phosphorylation of other focal complex-associated proteins such as paxillin and Cas.[155−159] The phosphorylation on Tyr397 creates a high affinity binding site recognized by the SH2 domain of Src family kinases.[152] The sequences surrounding Tyr397 (...YAEI...) are highly conserved in FAK proteins of all species and closely resemble the sequences comprising the consensus Src SH2 binding site, YEEI.[18] Additional experiments have revealed that a consensus Src SH3 binding site residing upstream of Tyr397 functions as a ligand for the SH3 domain of Src.[160] A mutation in FAK that destroys the Src SH3 binding reduces the efficiency

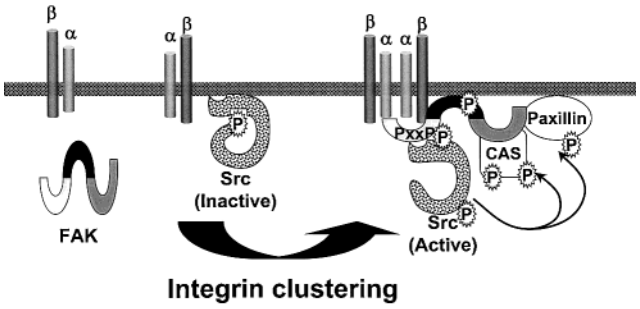


Figure 9. Schematic representation of the proposed interactions of FAK and Src. The large black arrow denotes the activation of Src and FAK in response to integrin clustering. The small arrows denote FAK/Src-mediated phosphorylation of Cas and paxillin. See text for additional details.

of adhesion-induced FAK tyrosine phosphorylation and the tyrosine phosphorylation of the focal adhesion protein paxillin.[160] Finally, binding of Src to FAK leads to the hypophosphorylation of the negative regulatory site of Src (Tyr527) and the coordinate increase in tyrosine phosphorylation of paxillin.[155,161] FAK mutants deficient in Src binding (e.g., Tyr397-Phe) fail to effectively induce the translocation of Src to focal adhesion structures.[161] These observations suggest a mechanism for Src activation by which the binding of Src SH3 and SH2 domains to phosphorylated Tyr397 and the upstream Src SH3 binding region serves to relieve the intramolecular constraints within Src, thereby promoting autophosphorylation of Src, an increase in Src catalytic activity,[152,161] and the concomitant dephosphorylation of the Src regulatory site of phosphorylation, Tyr527 (Figure 9).

C. c-Src/FAK Substrates

Activation of the FAK−Src complex is central to regulation of downstream signaling pathways that control cell spreading, cell movement, cell proliferation and cell survival.Several focal adhesion proteins, including FAK itself, Cas, and paxillin, are substrates of the bipartite FAK/c-Src kinase complex.[154−159,162−164] The FAK/c-Src kinase complex stimulates phosphorylation of FAK on tyrosines 576/577, 861, and 925. Tyrosines 576/577, located in the kinase domain, are Src substrates.[154] In vitro phosphorylation of these sites is enhanced by the presence of Src. In addition, a kinase defective mutant of FAK that is incapable of binding ATP is phosphorylated at Tyr576/577 by Src to the same extent as wild-type FAK. Phosphorylation of these sites stimulates maximal FAK kinase activity. Finally, FAK mutants lacking Tyr576/577 fail to restore cell migration to FAK null cells. Thus, by analogy with other tyrosine kinases including Src, phosphorylation of these sites likely stabilizes FAK in an active conformation to facilitate interaction with substrates.

Src phosphorylation of FAK on Tyr861[162] creates a potential binding site for SH2 domain-containing proteins, although to date, none have been identified. Phosphorylation of Tyr925 has been observed in v-Src transformed cells, generating a docking site for Grb2.[163] The interaction with Grb2 leads to the recruitment of SOS and activation of the Ras-MAPK signal transduction pathway.[163]

The identification and characterization of FAK binding partners has provided important information about how FAK serves to mediate signaling from adhesion complexes. Two adhesion complex proteins, talin and paxillin, have been identified as FAT binding partners[165−167] (Figure 8). Paxillin binding to FAK is mediated by sequences which significantly overlap the region required for focal adhesion targeting.[166,167] Because paxillin has been shown to bind directly to the cytoplasmic domains of integrin receptors[151,168] as well as to the focal adhesion protein vinculin, paxillin (or paxillin-like proteins) may function as the "docking partner" for FAK in adhesion complexes. The interaction of FAK with paxillin leads to phosphorylation of paxillin following integrin-mediated adhesion.[155] FAK phosphorylates paxillin on Tyr118[164] while Src mediates paxillin phosphorylation on Tyr31.[155] Phosphorylation of these sites creates binding sites for the adapter protein Crk.[155] Mutation of these sites to phenylalanine blocks Crk binding and impairs cell motility[169] (although a recent report[170] contradicts this observation).

The C-terminal domain of FAK is rich in protein−protein interaction sites. A proline-rich sequence designated Site I (Figure 8) provides the major binding motif recognized by the SH3 domain of Cas, a multi-functional linker protein.[157,158,171] Upon integrin clustering, Cas is localized to adhesion complexes and is phosphorylated on tyrosine[156−158,171,172] by both FAK and Src. FAK phosphorylates Cas on tyrosines in the sequence ...YDYVHL... located in the C-terminal domain of Cas.[156] Phosphorylation of this site creates a binding site for the SH2 domain of Src while a proline rich sequence located approximately 20−30 amino acids upstream binds the SH3 domain of Src, resulting in activation of additional Src molecules in focal adhesions.[173] Src phosphorylates Cas on up to 15 YXXP motifs located within the substrate binding domain of Cas.[174] Following phosphorylation of these tyrosines, the adapter protein Crk binds[158,174] and initiates a signaling cascade that leads to the activation of the GTPase Rac, a key regulator of cell motility.[175,176] Mutants in FAK lacking the binding site for Cas are compromised in signaling to downstream effectors and show defects in cell migration.[177] In addition, mutants of Cas lacking the substrate binding domain are defective in migration.[178] Thus, CAS is a key component of focal adhesions and required for cell movement.

D. Summary

Cellular adhesion regulates growth and motility, at least in part, by signaling through FAK/Src tyrosine kinase complexes. The ability of cells to evade this pathway results in oncogenic transformation. Furthermore, alterations in adhesion-dependent signal transduction regulate tumor metastasis by conferring typically nonmotile cells the ability to detach from native ECM and migrate to distal sites. The pronounced alterations in cell shape and cell adhesion exhibited by v-Src transformed cells point to the role of Src in regulating elements of cytoskel-

etal organization.[15] FAK, initially identified as a substrate for oncogenic Src has emerged as a key regulator of integrin signaling.

In addition, elevated FAK expression and activity have been observed in a number of human cancer cell lines with increasing tumorigenic potential.[179−184] Therefore, understanding the mechanisms whereby these kinases propagate adhesion-dependent regulatory signals is important for understanding defects that arise during malignant transformation.

VI. Glossary

EGF	epidermal growth factor
GAP	GTPase activating protein
FAK	focal adhesion kinase
ER	estrogen receptor
GPCRs	G protein-coupled receptors
STAT	signal transducer and activator of transcription
PLCγ	phospholipase C gamma
Shc	Src and collagen homologous protein
SH2	Src homology 2 domain
SH3	Src homology 3 domain
GTPase	guanine nucleotide triphosphatase
MAP kinase	microtubule-associated protein kinase
MEK	MAP-ERK kinase
PI-3 kinase	phosphatidyl inositol-3 kinase
ECM	extracellular matrix

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