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ROLE OF C-SRC TYROSINE KINASE IN EGF-INDUCED MITOGENESIS

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TABLE OF CONTENTS

1. ABSTRACT

c-Src, the prototype of the cytoplasmic, membrane-associated, non-receptor tyrosine kinases, is a co-transducer of mitogenic signals emanating from a number of tyrosine kinase polypeptide growth factor receptors. Examples of such receptors include those that bind the platelet-derived growth factor (PDGF), colony stimulating factor-1 (CSF-1), and epidermal growth factor (EGF). Investigations into the mechanisms by which c-Src contributes to receptor signaling suggest that interactions between the two proteins are bidirectional, i.e., that c-Src can bind, phosphorylate, and activate the receptor, and vice versa. The consequences of these interactions appear to be enhanced phosphorylation of specific substrates. Delineating which cellular proteins are substrates of which tyrosine kinase and determining the consequences of tyrosine phosphorylation on the function of specific substrates are the goals of current investigations.

Utilizing the murine C3H10T½ fibroblast model, in which a panel of wild type and mutant c-Src/EGF receptor overexpressors has been studied for temporal and spatial second messenger responses to EGF, distinctions between substrates of c-Src and the EGF receptor and the effects of tyrosine phosphorylation on substrate function are beginning to emerge. In the 10T½ model, preferred substrates of c-Src are almost exclusively comprised of those molecules that associate with the actin cytoskeleton or with focal adhesions, such as cortactin, p190RhoGAP, and p130CAS, while preferred substrates of the EGF receptor include the receptor itself, SHC, phospholipase C-gamma and p62DOK. While the major mitogenic signaling pathway is thought to proceed directly from the receptor (through SHC/GRB2/SOS/Ras/Raf/MEK/MAPkinase/Elk1), more evidence is accumulating to suggest that proteins involved in regulating the actin cytoskeleton (such as c-Src substrates) also participate in mitogenesis, either as unique transducers of growth signals and/or as monitors of anti-apoptotic conditions (substratum attachment). How c-Src may contribute to the EGF mitogenic response through tyrosine phosphorylation of or association with its specific substrates is discussed.

Cellular Src (c-Src), prototype for a family of intracellular membrane-associated tyrosine kinases, is required for

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mitogenesis initiated by multiple growth factor receptors, including the receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), colony stimulating factor-1 (CSF-1), and the basic fibroblast growth factor (bFGF). C-Src is also overexpressed and/or activated in many of the same human carcinomas that overexpress members of the EGF receptor (EGFR) family, suggesting that the two types of tyrosine kinases can cooperate during the genesis of human tumors. This review focuses on the role of c-Src in EGF-dependent mitogenesis and tumorigenesis, i.e., on the interactions between c-Src and the receptor and on identification of c-Src substrates, their functions, and the effects of tyrosine phosphorylations on their functions. A synopsis of other mitogenic and signaling systems is also included for comparative purposes.

2. INTERACTION OF C-SRC WITH MITOGENIC RECEPTORS

2.1 Platelet-derived Growth Factor

The PDGF receptor (PDGFR) is a 185 kDa transmembrane tyrosine kinase that becomes enzymatically activated following ligand binding and autophosphorylates at multiple sites in the cytoplasmic domain of the molecule. These phosphorylated tyrosine residues serve as docking sites for a variety of signal transducers, including the adaptor proteins, GRB2, Nck, and SHC, Raf-1 (a serine/threonine kinase), p120RasGAP (a GTPase activating protein for Ras), PI_3 kinase (phosphatidylinositol-3 kinase), SHP2 (a tyrosine phosphatase) and PLC-gamma (phospholipase C-gamma) (1-3). The first evidence that c-Src and its family members were involved in PDGF signaling was provided by Ralston and Bishop (4), who observed that c-Src becomes activated upon PDGF stimulation. Kypta *et al.* (5) later demonstrated that not only c-Src, but also c-Fyn and c-Yes, are activated in a PDGF-dependent manner and become transiently associated with the receptor following PDGF treatment. Association between Src family kinases and the receptor is believed to involve phosphotyrosine (pTyr)-SH2 interactions, since the SH2 domain of c-Fyn is required for binding to the receptor *in vitro* (6), and mutation of two tyrosine phosphorylation sites in the juxtamembrane region of the receptor, Y579 and Y581, results in a reduction in both PDGF-induced c-Src activation and binding to the receptor *in vivo* (7). These data and results from *in vitro* peptide binding studies (8) suggest that Y579 and Y581 directly mediate binding of c-Src to the PDGFR, although it is not clear whether c-Src or the receptor phosphorylates these sites. The integrity of sequences surrounding Y857 of the PDGFR (the major autophosphorylation site in the kinase domain that is homologous to Tyr 416 of Src) is also important for the binding of c-Src, since mutation of this residue significantly reduces association of the two kinases. However, Y857 is not believed to be the direct site of interaction (9).

Activation of c-Src by PDGF is transient and accompanied by the appearance on c-Src of two novel serine phosphorylations in the N-terminal 16 kDa fragment (S12 and an unidentified S residue; 10), and a novel tyrosine phosphorylation, Y138 (11). Y138 is located in the SH3 domain of c-Src, and *in vitro* peptide binding experiments (11) suggest that phosphorylation of Y138 may alter the ability of the SH3 domain to associate with other signaling molecules through SH3/polyproline interactions. Mutation of Y138 generates a c-Src molecule that is inhibitory for PDGF-induced DNA synthesis (12, 13), suggesting that the SH3 domain is required for mitogenesis. That Src family members as a group are required for PDGF-initiated transit of cells through G1 to S phase is supported by the inhibitory effects of the introduction of kinase inactive c-Src or an antibody specific for the C-terminal domain of Src family members on BrdU incorporation (14).

Interaction of c-Src with the PDGFR appears to have consequences not only for c-Src but also for the receptor. Hansen *et al*. (15) have shown that Tyr 934 in the kinase domain of the PDGFR is phosphorylated by c-Src both *in vitro* and *in vivo*, and mutation of this site to phenylalanine and expression in intact cells results in a decreased mitogenic signal and an increase in chemotaxis and motility. In addition, the PDGF-stimulated tyrosine phosphorylation of PLC-gamma is enhanced in mutant receptor cells compared with wild-type (*wt*) receptor cells. These data suggest that phosphorylation of Y934 by c-Src positively regulates mitogenesis, while negatively regulating cell movement, possibly via a pathway that involves PLC-gamma.

2.1 Colony Stimulating Factor

The receptor for CSF-1, c-Fms, is a transmembrane molecule with strong homology to the PDGFR (16). CSF-1 is released by osteoblasts in response to treatment with parathyroid hormone (PTH) (17) and stimulates the proliferation, differentiation and survival of cells of the mononuclear phagocytic lineage (18). Osteoclasts respond to CSF-1 by rearranging actin and undergoing cytoplasmic spreading (17), a phenomenon shared with a variety of cell types in response to polypeptide growth factors. Interactions of signaling proteins with c-Fms are much less well-characterized as compared to other receptor tyrosine kinases. PI_3 kinase has been demonstrated to associate with the receptor, but this association is not required for CSF-1-stimulated cell growth (19). Upon CSF-1 stimulation of macrophages (which normally express c-Fms) or mouse fibroblasts (which are engineered to express c-Fms), Src family members associate with the receptor and become activated (20). That binding between Src and the receptor may be mediated by pTyr/SH2 interactions is suggested by the finding that a GST-fusion protein of the SH2 region of c-Fyn complexes with the receptor *in vitro*. *In vivo* association can be reduced by mutation of the receptor at Tyr 809, a major phosphorylation site in the kinase domain that is homologous to Tyr 416 in Src. Roche *et al.* (21) have demonstrated that CSF-1-induced DNA synthesis requires Src family members, by microinjecting a C-

terminally directed antibody that inhibits Src family tyrosine kinase activity *in vitro*. Though not as thoroughly studied as the PDGFR, these data suggest that the interaction of c-Fms with c-Src is regulated in much the same fashion as c-Src with the PDGFR and that the interaction has similar effects on the binding partners.

2.3 Fibroblast Growth Factor

The association of c-Src with the basic FGF receptor (bFGFR) appears to be cell type-specific. For example, in NIH3T3 cells c-Src has been shown to co-precipitate with FGFR in an FGF-dependent fashion. This association may be mediated by the SH2 domain of c-Src, since GST fusion proteins of the SH2 domain of v-Src precipitate FGFR (22). In lung capillary endothelial cells, FGF stimulation leads to an increase in c-Src autophosphorylation activity but no complex formation between c-Src and FGFR. In porcine aortic endothelial cells engineered to express the FGFR, no complex between c-Src and FGFR is seen, and FGF stimulation results in a down-modulation of c-Src kinase activity (23). These results suggest the involvement of cell-type-specific factors which mediate interactions between the receptor and c-Src and regulate c-Src activity. These results also raise the question of whether c-Src binding to other receptors may be influenced by third party proteins.

2.4 Epidermal Growth Factor Receptor and family members

The EGFR is a 170kDa transmembrane receptor tyrosine kinase that dimerizes, phosphorylates multiple residues in its cytoplasmic domain, and binds and/or phosphorylates a variety of signaling proteins in a ligand-dependent fashion, similar to the PDGF, CSF-1, and bFGF receptors. Many of the binding proteins and substrates of the EGFR are the same as for the PDGFR, including GRB2, SHC, p120RasGAP, PLC-gamma, and PI_3 kinase (for review 24-27). Tyrosine phosphorylation of PLC-gamma initiates actin rearrangement, Ca^{2+} mobilization from intracellular stores, and activation of protein kinase C (28, 29), while changes in inositol phosphate metabolism and increases in anti-apoptotic signals are brought about by tyrosine phosphorylation of PI_3 kinase (2). Proliferative signals are transduced through SHC and/or GRB2/SOS (2) to Ras and the MAP kinase cascade, which regulates gene expression via phosphorylation of transcription factors. The treatment of cells with EGF, therefore, stimulates multiple, parallel signaling pathways that are thought to coalesce to initiate cell division. These pathways are depicted in figure 1.

2.4.1 Requirement for c-Src in EGF-induced mitogenesis

A major goal of our laboratory has been to determine if c-Src is required for EGF-induced cell proliferation, and if so, to elucidate its role. Our studies were initiated by examining c-Src from a variety of rodent and avian cells for alterations in specific kinase activity following EGF stimulation. While EGF-dependent activations of c-Src were detected in several experiments, such changes were not consistently observed (30), prompting us to test directly in intact cells the notion that c-Src is involved in mitogenesis. Thus, c-Src was overexpressed in C3H10T½ murine fibroblasts (a cell line that exhibits tight contact inhibition, a very low frequency of spontaneous focus formation in monolayer, and normal levels of EGFR), and transfectants were tested for their responsiveness to EGF, using [^{3}H]-thymidine incorporation as a measure of progression through the cell cycle. We found that clonal cell lines overexpressing varying levels of c-Src ranging from 2-30 times above endogenous exhibit EGF-induced DNA synthesis that is 2-5 fold higher than in normal cells (30). Such cell lines are indistinguishable from normal, parental 10T½ cells in morphology and saturation density, and unable to form colonies in soft agar. Receptors in c-Src overexpressors are present in nearly equal numbers and exhibit similar affinities for ligand as receptors on control cells. Cell lines overexpressing structurally-altered forms of c-Src (kinase inactive, SH2-domain defective, and myristylation-defective) fail to potentiate the EGF response as observed with wildtype c-Src. In fact, all the c-Src mutants act in a dominant negative fashion (31), indicating that c-Src is required for signaling through the EGFR. This finding was corroborated by Roche, *et al.* (21), using microinjection of antisera to Src family members or kinase inactive c-Src cDNA and measuring EGF-induced DNA synthesis by BrdU incorporation.

Chronic stimulation of cells overexpressing the EGFR results in colony formation in soft agar and tumor formation in nude mice (32). To determine if c-Src affects EGF-induced tumorigenesis as it does EGF-induced mitogenesis, cell lines which overexpress the human EGFR (HER1) alone or in the presence of overexpressed c-Src were generated from the 10T½ parent and examined for levels of EGF-dependent [^{3}H]-thymidine incorporation, growth in soft agar and tumor formation in nude mice. In this model system, expression of both HER1 and c-Src results in a synergistic increase in growth and tumorigenicity, as compared to cell lines overexpressing c-Src or HER1 alone. The differences in the tumorigenicity of the various cell lines is striking, with the double c-Src/HER1 overexpressors forming tumors of greater than 1.0 cm in diameter at each injection site within one week, and the single c-Src or HER1 overexpressors forming only pinhead-sized tumors at only 50% of the injection sites (33).

The mechanism of the synergism between HER1 and c-Src is not fully understood at this time, but formation of a stable complex mediated by SH2/pTyr interactions may play a role. Using antibodies specific to c-Src, an EGF-dependent, *in vivo* c-Src/HER1 complex can be detected in double overexpressors but not in single c-Src or HER1 overexpressors (33), and only when mild detergent conditions are used for extraction. These results suggest that the association is weak or indirect. Co-immunoprecipitation of c-Src with HER1 has also been observed in human carcinoma cell lines (30, 34-36). That the interaction may be direct is suggested by the finding that GST fusion proteins containing the c-Src SH2 domain both precipitate the receptor from crude

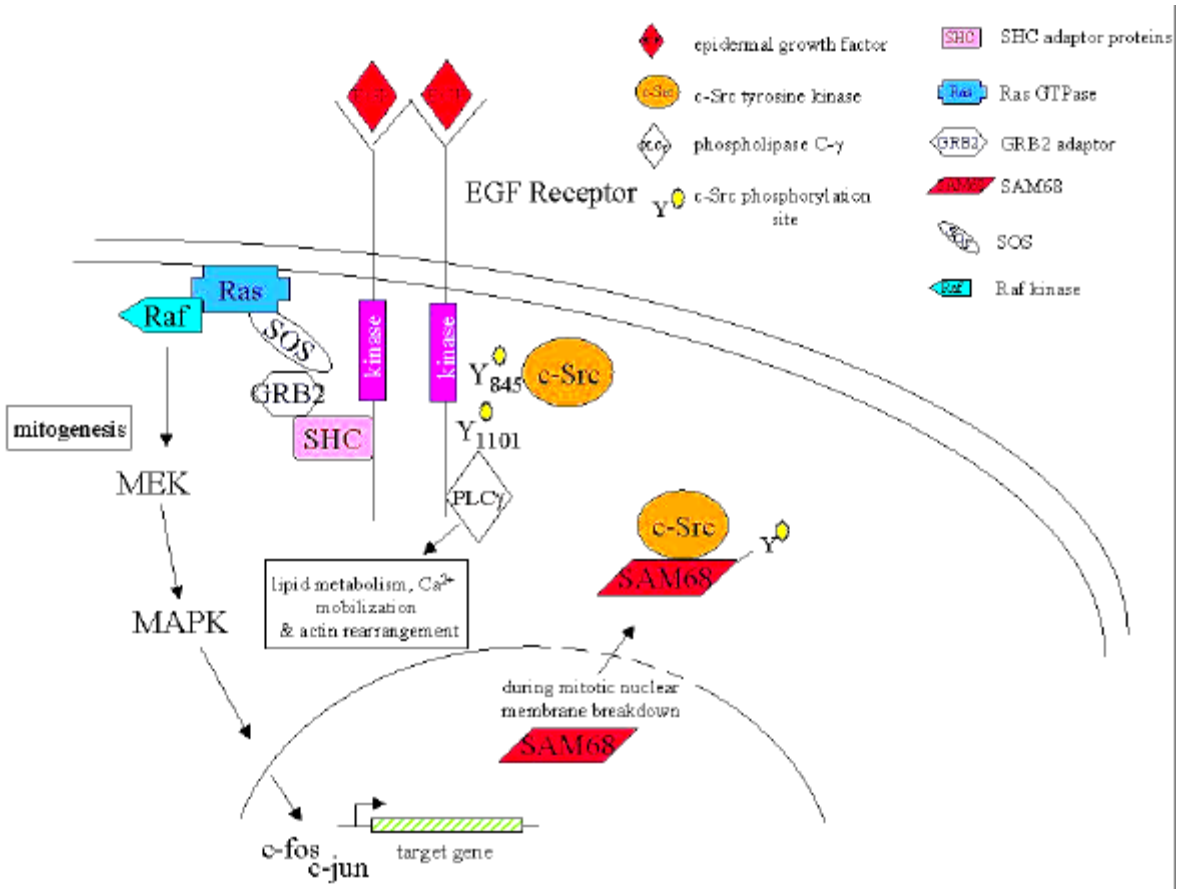

Figure 1. Postulated effect of c-Src on EGF mitogenic pathways. Two signaling pathways of the EGF receptor are depicted, namely, those involving PLC-gamma and MAP kinase. Tyrosine residues 845 and 1101 of the EGF receptor are phosphorylated by c-Src, and phosphorylation of Y845 is believed to augment receptor tyrosine kinase activity, as manifested by increased phosphorylation of the receptor targets, PLC-gamma and SHC.

cellular lysates and bind the receptor directly in a Far Western assay (37, Biscardi *et al*., 1997, in preparation). Using a panel of truncated receptors (38) or a panel of peptides to compete the precipitation of the receptor by a GSTc-SrcSH2 fusion protein (37, 34) other investigators have identified Y992 of the receptor as a potential site of c-Src SH2 binding, while Stover *et al*., (34) have implicated Y891 and 920 as additional sites. These results suggest that c-Src might bind multiple sites on the receptor.

Are there immediate consequences on c-Src of this association? Several investigators (39, 40) have reported an EGF-dependent activation of c-Src kinase activity in cells overexpressing HER1. However, results from our laboratory in both the 10T½ mouse model and human carcinoma cell lines, as well as from others (41), have indicated no reproducible, significant activation of c-Src, in the presence or absence of EGF treatment. However, Biscardi *et al*. (1997, in preparation) have evidence in the 10T½ model that HER1, in complex with c-Src, gains two novel sites of tyrosine phosphorylation, which have been identified as Y845 and Y1101 (figure 1). Both sites are phosphorylated *in vitro* and *in vivo* and require the presence of c-Src (Biscardi *et al.*, 1997, in preparation). Phosphorylation of Y845 requires a kinase active c-Src, suggesting that c-Src phosphorylates Y845 directly, while phosphorylation of Y1101 requires the presence but not the kinase activity of c-Src (Tice *et al*., 1997, in preparation). This finding suggests that c-Src either recruits another tyrosine kinase into the complex or induces HER1 to phosphorylate Y1101. Y845, located in the kinase domain of HER1, is the Src Y416 homologue. When Y416 of Src is phosphorylated, the enzyme becomes hyperactivated, which suggests that phosphorylation of Y845 by c-Src may result in a hyper-activation of receptor kinase activity. Indeed, the HER1 substrates, SHC and PLCγ, are more highly phosphorylated in the HER1/c-Src double overexpressors than in either of the single overexpressors, providing evidence for the receptor hyper-activation hypothesis (33). Sato *et al*.(42) have also observed phosphorylation of Y845 in HER1 from A431 cells, and Stover *et al.* (34) have identified Y891

and Y920 as additional phosphorylation sites of c-Src.

2.4.2 Involvement of HER family members and c-Src in the etiology of human breast cancer

A survey of the literature yields much evidence for potential functional interactions between HER1 or its family members (HER2, 3, and 4) and c-Src in human breast tumor progression. Overexpression and/or gene amplification of HER1 and HER2 is seen in approximately 20-30% of human breast tumors, and overexpression of HER2 has been correlated with poor patient prognosis (43, 44). Furthermore, c-Src has been found to be elevated in 70/70 breast tumors examined (45). The increases in protein level and/or activity of both HER family members and c-Src in a significant proportion of human breast tumors suggest that the two families of molecules might functionally interact in human tumors, as they do in 10T½ cells. To the extent feasible, this hypothesis has been tested by Biscardi *et al.* (36) in a panel of 14 human breast tumor cell lines and three tissue samples from patients. A direct correlation between levels of c-Src/HER family member overexpression, complex formation, phosphorylation of Y845 and Y1101, relative levels of SHC phosphorylation, and tumor formation in nude mice (with several exceptions) were observed.

Taken together, the *in vivo* and *in vitro* data from the 10T½ mouse fibroblasts and the breast cancer cell lines suggest a physical and functional association of HER1 and c-Src and a potential synergism between the two kinases in human breast tumor progression. However, clinical data suggest that overexpression of HER1 occurs as a late event in breast tumor progression, while overexpression of HER2 occurs as an intermediate event. These findings argue that HER1/c-Src interactions are more important in later stage tumor progression, whereas HER2/c-Src interactions (if they occur) are more important in mid-stage tumor formation and progression.

2.4.3 c-Src and HER2 (erbB2/*neu*) Receptor

HER2 (erbB2/*neu*) is a 185kDa transmembrane protein that is highly homologous to HER1 (80%) and the rat *neu* gene (46, 47). When mutated at a single residue in the transmembrane domain, the rat *neu* gene becomes oncogenic (48), but no such mutations have been found in human cancers, even when HER2 is overexpressed (44). Activation of the HER2 kinase domain appears to occur by homodimerization under conditions of overexpression (48, 49). At the present time, downstream targets of HER2 appear indistinguishable from those of HER1 and include SHC (50), PLC-gamma (51), and p120RasGAP (51). In addition, activation of the MAP kinase pathway via GRB2/SOS/Ras has been demonstrated in breast cancer cell lines that overexpress HER2 (52).

In spite of the identification of downstream targets, no specific ligand for HER2 has yet been found that could account for its frequent activation in human breast cancers. Heregulin (which was at first thought to be the ligand for HER2) was found to bind HER3 and HER4, inducing not only homodimerization but also heterodimerization with HER2 (HER3/HER2 or HER4/HER2) (53-56). Heterodimerization appears to be the predominant complex responsible for the trans-phosphorylation of the receptors and initiation of cellular signaling (49, 54, 57, 58). Stimulation of cells with EGF also causes heterodimerization between HER1 and HER2 (in addition to homodimerization of HER1) (49, 57, 59, unpublished observations, Belsches and S. Parsons), thus providing a potential mechanism by which the two receptors share downstream targets.

The question under investigation at the present time is whether c-Src is a co-transducer of signals through HER2 as it is through HER1. Does c-Src enhance the inherent oncogenic potential of HER2? Because HER2 is so abundantly and frequently overexpressed in human tumors, as is c-Src, these question carry greater significance. Luttrell *et al.* (37) showed that HER2 can be precipitated by the GSTc-SrcSH2 fusion protein from extracts of the human breast carcinoma cell line, SKBR3, suggesting that stable complexes may also form between c-Src and HER2 *in vivo*. Our laboratory has detected *in vivo* HER2/c-Src complexes in three of fourteen human breast tumor cell lines and in three of thirteen tumor tissues (Belsches *et al.*, 1997, in preparation), and Muthuswamy and Muller (60) have shown association between c-Src and HER2 in mammary tumors from HER2 transgenic mice. In addition, we have found that while constitutive association between c-Src and HER2 can be detected in serum-starved breast cancer cells, the amount of HER2 which co-immunoprecipitates with c-Src is augmented when the cells are stimulated with EGF. In contrast, heregulin stimulation of the cells does not increase the amount of the c-Src/HER2 complex above constitutive levels. These data suggest that the association between HER2 and c-Src may be augmented by activation of HER1 and HER1/HER2 heterodimerization and not through HER3 or HER4. Further studies on HER2/c-Src interactions are currently underway.

3. EFFECT OF C-SRC ON IMMEDIATE-EARLY EVENTS FOLLOWING EGF STIMULATION OF C3H10T½ CELLS

3.1 Small molecule second messengers

Binding of ligand to the EGF receptor results in a variety of immediate-early events, including receptor dimerization, autophosphorylation, and internalization (2). In some cell lines, Ca^{2+} influx (61, 62), alkalinization (63), production of inositol phosphates (64), and increases in cyclic AMP (65) also accompany receptor activation. In the C3H10T½ cells described above, EGF treatment fails to trigger detectable alterations in the levels of any of these second messengers in either control or c-Src overexpressors (both expressing only endogenous levels of receptor), suggesting that they are not contributing significantly to the EGF mitogenic signal in these cells (66). However, tyrosyl phosphorylation of multiple cellular proteins are affected by c-Src overexpression, both in an EGF-dependent and -independent manner (66).

3.2 Tyrosine phosphorylation of cellular proteins

In EGF-treated fibroblasts and epithelial cells many proteins, in addition to the receptor itself, are rapidly phosphorylated on tyrosine either by the receptor or by other EGF-responsive tyrosine kinases (2). That these tyrosine phosphorylations are important for EGF mitogenic signaling is evidenced by the fact that a catalytically active receptor is required for progression through the cell cycle (67-69) and that microinjection of antiphosphotyrosine antibodies into cells ablates some early EGF responses (68). Three prime questions facing the field of signal transduction today are, what proteins are phosphorylated on tyrosine in response to which ligand, which tyrosine kinases are mediating the phosphorylations, and what effect does tyrosine phosphorylation have on the function of the substrate?

In the cases of the tyrosine kinase receptors discussed above, where evidence for involvement or even requirement for the Src family of tyrosine kinases in receptor-mediated mitogenesis is available, which tyrosine kinase mediates the phosphorylation of which substrate becomes a critical question, and one that is not so easily answered. The situation becomes even more complicated when members of other tyrosine kinase families are also involved in the receptor signaling cascades. Examples of additional families include the JAK (70), FAK (71), and AKT (72) families. In EGF-induced cascades in fibroblasts, evidence for involvement of all three of these families has been forthcoming (67, 73, 74), in addition to the receptor and Src families. An even greater level of complexity arises when one considers that tyrosine kinases can phosphorylate one another and affect one another's activities (as is the case with c-Src and the PDGF/EGF receptors). Our laboratory approached this issue by focusing on c-Src as a co-factor in EGF-evoked responses. As described above, we demonstrated that the tyrosine kinase activity of c-Src is required not only for the ability of overexpressed wt c-Src to potentiate EGF mitogenesis but also for the ability of the endogenous receptor in the context of endogenous levels of wt c-Src to faithfully propagate the growth signal (31). In light of this requirement, we reasoned, at least some of the EGF-induced tyrosyl phosphorylations of cellular proteins were likely to be mediated by c-Src.

3.3 EGF receptor-specific and c-Src-specific substrates

Multiple proteins, in addition to the receptor itself, become phosphorylated on tyrosine in response to EGF. They include ezrin (75), a structural protein associated with the actin cytoskeleton, HER2 (76), phospholipase C-gamma (PLC-gamma) (64, 77), annexins (Ca2+-binding proteins) (78, 79), c-Cbl, an adapter protein with multiple protein interaction domains (80), and unidentified proteins of various electrophoretic mobilities. In 10T½ fibroblasts we examined the spectrum of protein tyrosyl phosphorylation in EGF-stimulated cell lines overexpressing *wt* and mutant forms of transfected chicken c-Src (and containing endogenous levels of receptor), using antibodies specific for pTyr in the Western immunoblotting procedure. We found that overexpression of *wt* c-Src (i) augmented the EGF-dependent tyrosyl phosphorylation of all cellular proteins normally detected after EGF stimulation, predominantly those of 170 (EGF receptor), 120-130, 100, 75, 62, and 57 kDa, (ii) enhanced the basal, unstimulated tyrosyl phosphorylation of a subset of these proteins (120-130, 100, and 75 kDa), and (iii) increased and prolonged the EGF-dependent tyrosyl phosphorylation of the same subset of proteins. Furthermore, overproduction of structurally altered forms of c-Src exclusively reduced or ablated the phosphorylation of the 120-130, 100, and 75 kDa proteins. Based on these findings, we have described the 120-130, 100, and 75 kDa proteins as preferred c-Src substrates and the 170, 62, and 57 kDa proteins as preferred EGF receptor substrates (66).

A number of these proteins have been identified by depleting whole cell extracts of a single pTyr-immunoreactive band with specific antibodies. By such means the substrates designated as preferred receptor substrates were determined to be the receptor itself (170 kDa), the p120RasGAP-associated p62DOK (81, 82), and SHC (57 kDa) (83, 84, J.-H. Chang, M.-C. Maa, and S. J. Parsons, unpublished). In contrast, only one of the preferred c-Src substrates has been definitively identified using the antibody-depletion approach. p75 is cortactin (85), a cortical actin binding protein that was first discovered as a v-Src substrate (86, 87). The identities of the other c-Src substrates (p100 and p120-130) are still under study. P100 remains unidentified, while investigations into the p120-130 band have revealed that it is comprised of several proteins, including p125FAK (88) and p130CAS (89). The characterization, phosphorylation by c-Src, and studies investigating the effect of tyrosyl phosphorylation on cortactin, p125FAK, and p130CAS function are discussed in more detail below.

Further investigations into the identity of proteins in the 120-130 kDa range uncovered no more substrates for c-Src in that size category, but did reveal another substrate, p190RhoGAP (84). P190RhoGAP was discovered as a c-Src substrate when we asked whether p120RasGAP might be a component of the complex of tyrosine phosphorylated proteins in the p120-130 kDa group. Immunoprecipitations of p120RasGAP followed by Western immunoblotting showed that p120RasGAP was not tyrosine phosphorylated in c-Src overexpressors or in control cells, either in the presence or absence of EGF. Rather, a protein of 190 kDa, that co-precipitated with RasGAP, was found to be more highly phosphorylated on tyrosine in c-Src overexpressors than in control cells (84). Tyrosyl phosphorylation of p190 was not altered by EGF stimulation, indicating it was a preferred substrate of c-Src. Other laboratories had shown that under conditions of EGFR overexpression (90-92) and longer exposure to ligand (30 min or greater), p190 can become tyrosyl phosphorylated, even when c-Src levels are low. However, the kinetics of phosphorylation are slower than those of the more preferred EGF receptor substrates, suggesting that the phosphorylation may regulate a

function of p190 that is unique to cells with very high levels of receptor. Our laboratory has therefore focused on c-Src/P190RhoGAP interactions in cells expressing normal levels of EGFR, and our findings will be discussed in more detail below.

3.4 Receptor internalization

As will be discussed more below, all of the c-Src substrates identified in the 10T½ model appear to have functional interactions with the actin cytoskeleton. One process critical to receptor signaling and involving the actin cytoskeleton is receptor internalization, an event normally linked to receptor degradation and cessation of signaling (93). In collaboration with the laboratory of Dr. Doug Lauffenburger at MIT we investigated whether receptor internalization was altered by c-Src overexpression, postulating that c-Src would enhance mitogenesis by inhibiting internalization and prolonging receptor signaling. To our surprise we found that c-Src overexpression increases rather than decreases the rate of receptor internalization following EGF treatment of 10T½ fibroblasts. (See figure 2.) Interestingly, the increased rate of internalization is not accompanied by measurable changes in receptor recycling but rather by increases in steady-state pools of internalized, activated receptors. Overexpression of kinase inactive c-Src reduces these processes to normal levels or even partially interferes with them (94).

How might this scenario contribute to the enhanced cell proliferation seen in cells that overexpress *wt* c-Src? Recent evidence (95, 96) suggests that EGF/EGFR complexes continue to signal in the endosomal compartment. Bergeron and colleagues (97) have discovered the existence of receptor/Shc/GRB2/SOS complexes in endosomes, which may continue to elicit growth-promoting signals following internalization, perhaps via repeated encounters with Ras at the plasma membrane. By increasing the rate of receptor internalization and the steady state level of internalized receptors, c-Src may promote the frequency of interactions between endosomes and the plasma membrane, thereby augmenting mitogenic and tumorigenic signaling.

The mechanism by which c-Src affects EGFR internalization is at this time unknown, but an examination of the endocytic process yields some possible clues. Ligand-activated EGFR are recruited into clathrin-coated pits by an unknown component of the endocytic pathway. Candidates for such a component are adaptin (98) and GRB-2 (99). c-Src might affect the rate of association between these molecules and the receptor. Another possibility is dynamin (100-102). Dynamin is a GTPase that is critical for the formation and release of the endosome from the plasma membrane. Dynamin has also been shown to bind the SH3 domain of c-Src *in vitro*, raising the possibility that c-Src might interact with dynamin *in vivo*. Such evidence has recently been obtained in PC12 cells by Foster-Barber and J. M. Bishop (Personal Communication). These findings lead us to speculate that overexpression of c-Src could result in the activation or recruitment of a larger pool of dynamin than in cells expressing normal levels of c-Src.

A third possibility also exists, i.e., that in c-Src overexpressors, the EGFR is internalized by mechanisms not involving clathrin-coated pits. One alternative mechanism is via caveolae - small invaginations of the plasma membrane that have been implicated in the transcytosis of macromolecules across capillary endothelial cells, the uptake of small molecules, interactions with the actin-based cytoskeleton, and the compartmentalization of certain signaling molecules, including G-protein coupled receptors, heterotrimeric G proteins, and members of the Src family of tyrosine kinases (103, 104). Caveolae contain a specific protein, termed caveolin, which is a substrate for v-Src in v-Src transformed cells (105) and has also been found to co-purify with c-Src in normal cells (103, 104, 106, 107) c-Src could potentiate EGF internalization via the unconventional caveolae pathway. Whether under these circumstances caveolae can transmit internal growth signals as is speculated for endosomes is a subject for future investigation. Another consideration should be kept in mind. To date, no evidence exists for a requirement for tyrosine phosphorylation in the caveolin/c-Src interaction or for that matter in the dynamin/c-Src interaction, yet the catalytic activity of c-Src is required for enhanced receptor internalization and complex accumulation. This scenario leaves open the possibility that an unidentified substrate(s) of c-Src is also critical for these events.

4. c-Src AND THE CYTOSKELETON

Of the c-Src substrates or binding proteins that have been identified in the 10T½ model, all are conceptually connected to the actin cytoskeleton (figure 3). These include, p125FAK, p130CAS, p190RhoGAP, and cortactin. Within cells, P125FAK and P130CAS localize to focal adhesions, which serve as anchoring sites for the internal actin cytoskeleton to the external substratum through integrins. P190RhoGAP regulates the small GTP binding protein, Rho, which in turn regulates actin stress fiber assembly and disassembly (108). Cortactin localizes to cortical actin in intact cells functions of c-Src in mitogenesis are mediated through the actin cytoskeleton. How c-Src signals via its substrates to contribute to the mitogenic response is a subject under intense investigation in our and other laboratories. A short summary of our current understanding of the functions and regulation of these proteins by c-Src follows (figure 3).

4.1 Focal adhesion kinase

P125FAK is a cytoplasmic tyrosine kinase that localizes to focal adhesions and contributes to the processes of integrin-mediated cell spreading and migration (reviewed in 109). Both of these processes involve continual remodeling of the actin cytoskeleton.

Abundant evidence links c-Src to these processes, but its role (especially in cell spreading) may be largely non-catalytic, since defects in spreading of

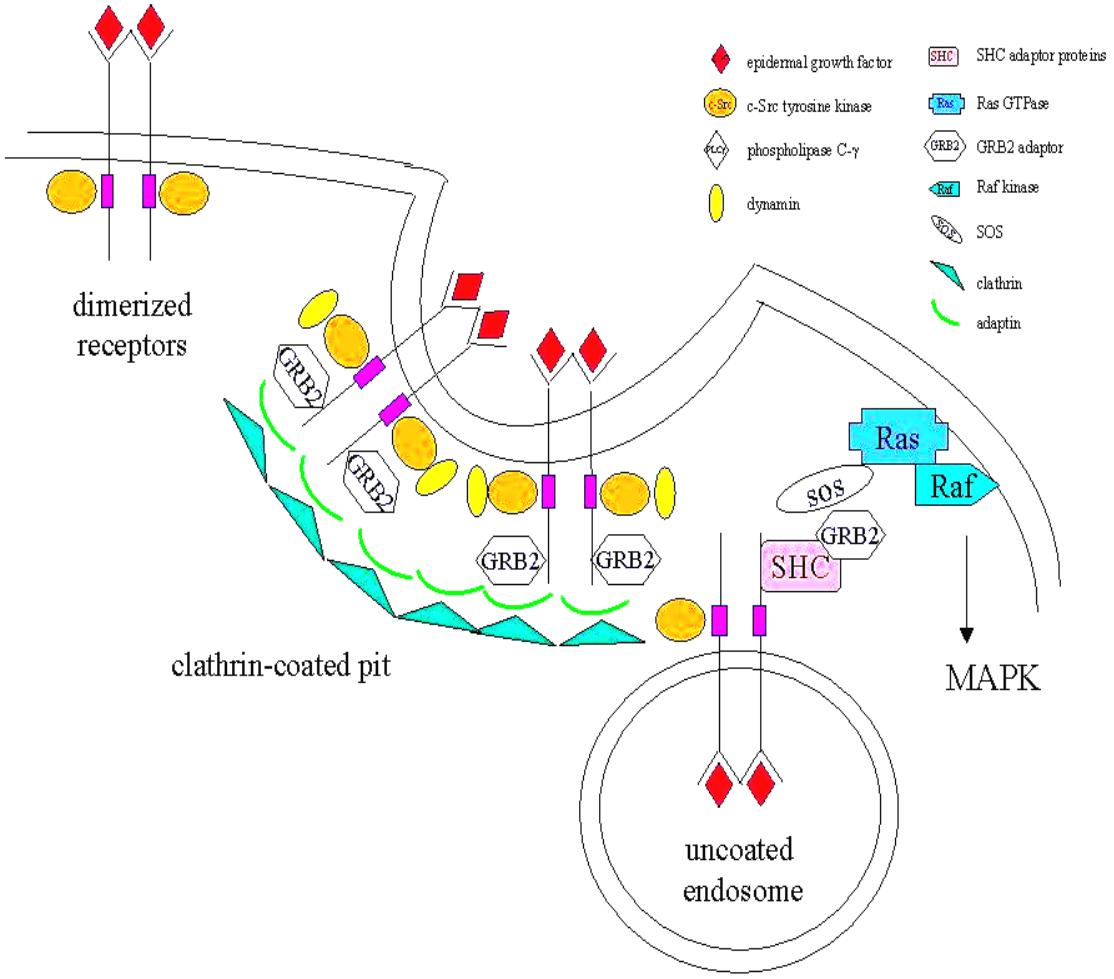

Figure 2. Effect of c-Src on receptor internalization. EGF receptor dimerization, activation and internalization is depicted. Localized regions of the plasma membrane recruit activated receptors into clathrin-coated pits and invaginate to form clathrin-coated vesicles. Uncoating of the vesicles results in formation of endosomes, from which the receptors continue to signal. C-Src increases both the rate of receptor internalization and the steady-state pool of internalized receptors, thereby increasing opportunities for signaling from the endosome to occur, before the decision is made to degrade the receptor or recycle it to the membrane. Two postulated targets of c-Src in this process are adaptin and dynamin.

fibroblasts derived from c-Src null mice can be restored by the SH2 and SH3 domains but not by the catalytic domain of c-Src (110). Motility requires the coordinated activation of both growth factor and adhesion receptor signaling (111), in ways that are poorly understood. Since c-Src is required for the action of several growth factors that induce motility (such as EGF and PDGF) (109), c- Src/FAK interactions may be important in this process as well. How FAK participates in EGF-dependent mitogenic signaling is unexplored at this point, except perhaps to function as an adherence checkpoint. Currently, our data support only a minor role for c-Src/FAK interactions in EGF-induced growth.

In unstimulated 10T½ cells the basal level of FAK tyrosyl phosphorylation is only slightly elevated in c-Src overexpressors as compared to controls (1-1.5 fold), and no increase in FAK tyrosyl phosphorylation is observed within 30 min of EGF stimulation. These findings suggest that FAK may be only a weak substrate for c-Src in 10T½ fibroblasts and is contraindicatory of a role for FAK in mitogenic potentiation by c-Src. However, if the functional interaction between FAK and c-Src is largely non-catalytic, one would not expect FAK tyrosyl phosphorylation to be affected much by c-Src overexpression, thereby leaving the questions unanswered as to whether FAK/c-Src interactions contribute to mitogenesis and further, whether adhesion/motility are coupled to mitogenesis.

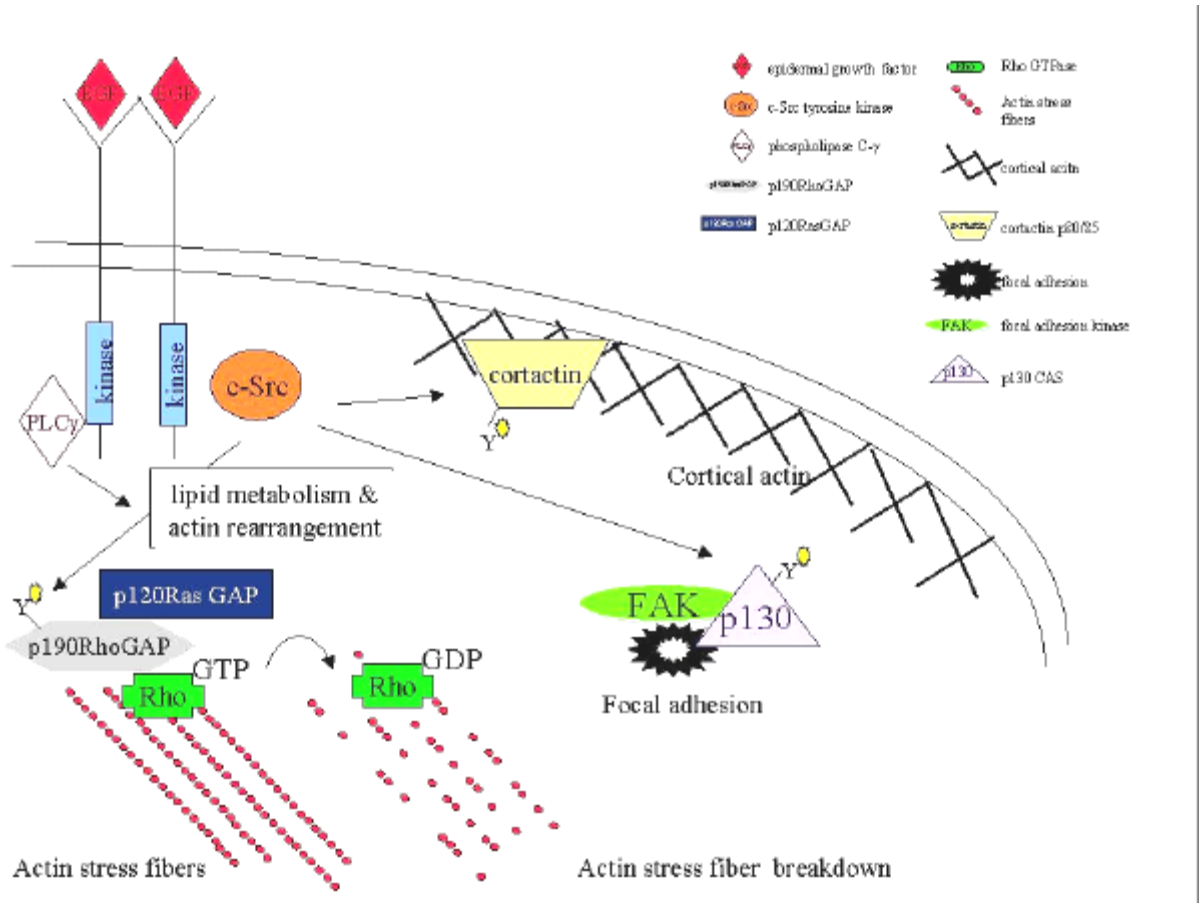

Figure 3. c-Src phosphorylates proteins that are involved in cytoskeletal organization. c-Src phosphorylates cortactin, p190RhoGAP, and p130CAS. Cortactin is found associated with cortical actin at peripheral sites within the cell. p130CAS is found associated with focal adhesion kinase in focal adhesions. p190RhoGAP stimulates hydrolysis of Rho-GTP to Rho-GDP leading to actin stress fiber breakdown. Stimulation of C3H10T1/2 cells with EGF stimulates lipid metabolism and actin rearrangement involving all three of these substrates, but the effect of tyrosine phosphorylation is uncertain at this time

4.2 p130CAS

P130CAS was first identified as a highly tyrosyl-phosphorylated protein in cells transformed by a variety of oncogenes (112-114) and in normal cells following integrin-engagement (115-117) and stimulation with mitogenic neuropeptides, such as bombesin, vasopressin, and endothelin (118, 119). In the latter two processes, an intact actin cytoskeleton is required for phosphorylation of CAS. c-Src has also been implicated in integrin-mediated events and mitogenesis, and in fact, tyrosine phosphorylation of p130CAS in response to adhesion is largely dependent

upon c-Src (117, 120). That p130CAS may be an integral part of the action of c-Src in EGF-induced mitogenesis is suggested by our finding that p130CAS co-precipitates with c-Src in 10T½ cells overexpressing c-Src and that its tyrosine phosphorylation is elevated in these cells as compared to control cells (N. Sevilir-Williams, J.-H. Chang, and S. Parsons, unpublished). Tyrosine phosphorylation of c-Src-associated p130CAS is not altered by EGF, confirming that it is a preferred substrate of c-Src. How p130CAS participates in c-Src-mediated EGF signaling, however, is not clear. Recent evidence indicates that p130CAS functions as an adapter molecule, binding a number of signaling molecules that participate in cell adhesion, such as p125FAK (121) and PTP-PEST (122). Thus, the link between cell adhesion, the actin cytoskeleton, and mitogenesis is repeated, and the common involvement of c-Src in both processes suggests that c-Src may be a critical factor that links the two.

4.3 p190RhoGAP

P190RhoGAP was first identified as a tyrosine phosphorylated protein that co-precipitated with p120RasGAP from v-Src transformed Rat-2 cells (90). The p190RhoGAP molecule has an amino-terminal GTPase domain that binds GTP (123, 124) and a carboxyl-terminal GTPase-activating domain (GAP) that is specific for small GTP-binding proteins of the Rho family (125). Rho in the GTP-bound form stimulates stress fiber formation while Rho in the GDP-bound form

permits actin disassembly (108), a fact that functionally links p190RhoGAP to the actin cytoskeleton. The two functional domains of p190RhoGAP are separated by an 801 amino acid middle region that contains tyrosine residues 1087 and 1105. Both these residues are found in a motif (YXXPXD) thought to mediate *in vitro* binding to the SH2 domains of p120RasGAP (126). Results from transfection experiments suggest that p190 has tumor suppressor characteristics, in that it can inhibit Ras transformation of NIH3T3 cells. The GTP binding domain is thought to mediate this suppressive activity (127).

Overexpression of c-Src in 10T½ cells results in an increase in the basal level of tyrosine phosphorylation of p190RhoGAP that is not further increased by EGF stimulation, which indicates that p190RhoGAP is a preferred substrate of c-Src and not the EGF receptor. Upon stimulation of normal murine fibroblasts by EGF, p190RhoGAP undergoes a rapid and transient redistribution from a diffuse cytoplasmic localization into concentric arcs that radiate away from the nucleus with a time course that mimics EGF-stimulated actin dissolution (128). Overexpression of *wt* c-Src expands the window of time in which EGF-induced p190RhoGAP arc formation and actin dissolution occurs, and overexpression of kinase defective c-Src contracts that window (128). These results implicate a role for tyrosine phosphorylated p190RhoGAP in regulating cytoskeletal reorganization, possibly by inactivating Rho. Several questions are raised by these experiments. Since the results are correlative, one question is whether tyrosine phosphorylation of p190 is required for the effects of c-Src overexpression on EGF-induced rearrangement of both p190 and actin. The second is related and asks whether or not the changes observed in the actin cytoskeleton that occur within 30 min after EGF stimulation are necessary for the two-five fold increase in EGF-induced DNA synthesis that is observed at 21 hours after addition of EGF in fibroblasts that overexpress c-Src (30). These questions are currently being addressed experimentally.

Recently, our laboratory has characterized the complexity and relative levels of tyrosine phosphorylation on p190 from different c-Src overexpressors (Roof *et al*., 1997, in preparation). Phosphotryptic peptide analysis of endogenous p190 has uncovered only two tyrosine phosphorylated polypeptides in the molecule, one peptide containing Y1105 (located in the middle domain), and another as yet unidentified minor peptide. This pattern of phosphorylation holds true for p190 from control cells and from *wt* c-Src overexpressors. That c-Src is directly responsible for phosphorylating Y1105 *in vivo* is suggested by an increase in the level of phosphorylated Y1105 upon c-Src overexpression, a decrease in phosphorylation of Y1105 in cells overexpressing a kinase-deficient c-Src and phosphorylation of Y1105 by purified c-Src in an *in vitro* kinase assay (Roof *et al*. 1997, in preparation).

One presumed function of tyrosine phosphorylation of p190RhoGAP is to enhance its interaction with p120RasGAP (84, 129, 130). Using deletion and site directed mutagenesis, Hu and Settleman (130) have proposed a model in which phosphorylation of both Y1087 and Y1105 are necessary for the interaction of p190RhoGAP with the two SH2 domains of p120RasGAP. In this model, the engagement of the two SH2 domains of p120RasGAP with Y1087 and Y1105 in p190RhoGAP induces a conformational change in the p120RasGAP molecule which exposes the RasGAP SH3 domain for interaction with its binding partners. This model represents a mechanism whereby tyrosine phosphorylation of p190 can regulate SH3 domain interactions of p120RasGAP.

The data from our laboratory support a role for tyrosine phosphorylation of p190RhoGAP in enhancing its interaction with p120RasGAP but suggest that interactions other than the p120RasGAP SH2 domain/p190RhoGAP pTyr interaction may also be important. Phosphotryptic peptide mapping and phosphoamino acid analyses have been used to compare the level of tyrosine phosphorylation of p190 in control, *wt* c-Src overexpressors, and kinase deficient c-Src overexpressors at both Y1105 and the unidentified site. These comparisons have revealed that the level of phosphorylation of Y1105 in the *wt* c-Src overexpressors is increased approximately seven fold above that of control cells and decreased approximately six fold in the kinase deficient c-Src overexpressors relative to control cells. In contrast, the level of tyrosine phosphorylation at the unidentified site remains constant and below the level of Y1105 phosphorylation in all cell lines (approximately 25% of the level of Y1105 phosphorylation in control cells), irrespective of either c-Src overexpression or EGF addition (Roof et al. in preparation).

The fact that we have been unable to identify two, equally phosphorylated tyrosines in p190 brings into question the dual pTyr/SH2 model for complex formation between p190 and p120RasGAP. Further questions are raised by the finding that the amount of p120RasGAP that is immunoprecipitated with p190RhoGAP from the cells overexpressing *wt* c-Src or kinase inactive c-Src is not consistent with the overall level of p190 tyrosine phosphorylation observed in these two cell lines. For instance, the amount of p120RasGAP that co-immunoprecipitates with p190RhoGAP from c-Src overexpressors is less than expected, while the amount of p120 in complex with p190 from kinase deficient c-Src overexpressors is greater than expected, given the striking differences in the extent of p190 tyrosine phosphorylation in the two cell lines. These data suggest that factors in addition to tyrosine phosphorylation may contribute to p190RhoGAP/p120RasGAP interactions and that tyrosine phosphorylation of p190RhoGAP may regulate another function of p190RhoGAP independently of its interaction with p120RasGAP. In fact, Koch *et al*. (131) have indicated that SH2 domains can interact with phosphoserine-containing peptides, which implies that

there may be a role for phosphorylated serines in p190RhoGAP/p120RasGAP interactions. Another possibility is that tyrosine phosphorylation of p190RhoGAP regulates the two known functional activities ascribed to p190RhoGAP, namely, the GTP-binding and GAP activities. Preliminary data from our lab has shown that tyrosine phosphorylation of p190RhoGAP inhibits the *in vitro* binding of GTP to the N-terminal domain of p190 (Roof, R. and S. Parsons, unpublished observation).

In conclusion, it appears that the mechanism of interaction between p190RhoGAP and p120RasGAP is more complicated than previously anticipated and that the consequences of the interaction await further experimentation before the exact function of the interaction between these two proteins is delineated.

4.4 Cortactin

The v-Src and c-Src substrate p75/p80/85 cortactin is an actin-binding protein that consists of five tandem repeats in the N-terminus and a SH3 domain in the extreme C-terminus of the molecule (85-87). Both structural features of cortactin are found in a number of cytoskeletal proteins, and indeed the N-terminal repeats of cortactin mediate binding of the molecule to actin (87). Phosphoamino acid analysis of cortactin from normal chick embryo cells indicates that the protein is phosphorylated on serine and threonine residues, whereas in chick embryo cells transformed with activated c-Src (527F), cortactin is also phosphorylated on tyrosine (86). In normal murine 10T½ fibroblasts, cortactin has a low level of tyrosine phosphorylation that is increased upon overexpression of c-Src. (85). In addition, EGF stimulates a time-dependent increase in the level of tyrosine phosphorylation of cortactin that is further enhanced by overexpression of c-Src (66, 85). Thus, cortactin may be a substrate of the EGF receptor as well as of c-Src, or c-Src may mediate EGF-induced phosphorylation of cortactin. The observation that increased tyrosine phosphorylation of cortactin is seen in Csk-deficient cells favors the notion that c-Src and/or its family members are responsible for phosphorylating cortactin (132, 133). Interestingly, two phases of EGF-induced cortactin tyrosine phosphorylation can be observed in 10T½ cells, one occuring within 2-10 min following stimulation and another occurring later in G1, with the maximum level being reached approximately nine hours post-treatment. The level of cortactin tyrosine phosphorylation in both phases is increased by c-Src overexpression.

Indirect immunofluorescence microscopy of 10T½ cells reveals that cortactin is localized within the cytoplasm to punctate sites that are concentrated around the nucleus (85). A significant amount of cortactin is also co-localized with actin in the plasma membrane and at peripheral adhesion sites (85). This pattern of subcellular distribution is not altered upon EGF treatment or c-Src overexpression. However, in cells transformed with activated c-Src (527F), cortactin collapses into rosettes or podosomes, which are sites of membrane/substratum interactions (86). These studies are suggestive of a role for peripheral cortactin in maintaining cortical actin integrity and membrane association, but the role of intracellular cortactin is unknown. Furthermore, the role that tyrosine phosphorylation plays in regulating cortactin function is not clear. Recent studies have shown that *in vitro* tyrosine phosphorylation of cortactin results in a dramatic decrease in F actin cross-linking activity which indicates a role for cortactin in regulating actin cytoskeletal reorganization (134).

4.5 Sam68

Although Sam68 is not a known cytoskeleton-associated protein, it is included in this section, because it is a well-documented substrate of c-Src. The activity of c-Src increases when cells enter mitosis, suggesting the existence of mitosis-specific substrates (135). Sam68 (Src-associated in mitosis) is a 68 kDa RNA-binding protein that is both a c-Src binding protein and a c-Src substrate (figure 1). Sam68 was discovered simultaneously by two laboratories that were comparing pTyr-containing proteins from cells arrested in mitosis with those found in asynchronous cells (136, 137). These two groups found that in mitotic cells but not in asynchronous cells a heavily tyrosine phosphorylated protein of 68 kDa can be co-immunoprecipitated with c-Src. The apparently high affinity interaction of Sam68 with c-Src requires both the SH2 and SH3 domains of Src, although it appears that the association is mediated mainly through the Src SH2 domain (136, 137). The functional consequence of phosphorylation of Sam68 is currently unknown, although it has been reported that phosphorylation of Sam68 decreases its RNA binding ability (138).

Recent work by Barlat *et al*. (139) has shown that a naturally occurring isoform of Sam68 exists in NIH3T3 cells. This isoform has a portion of the RNA-binding domain deleted (amino acid residues 170-208) and is called Sam68deltaKH. Sam68deltaKH is expressed at growth arrest in confluent cells but is absent from cells that have been transformed by activated c-Src (Y527F) (139). Interestingly, transfection of NIH3T3 cells with a myc-tagged Sam68deltaKH leads to a 50% decrease in BrdU incorporation in response to serum stimulation, while transfection of either myc-tagged Sam68 or vector alone had no effect on BrdU incorporation (139). Furthermore, co-expression of myc-tagged Sam68 with myc-tagged Sam68deltaKH reversed the myc-tagged, Sam68deltaKH-induced inhibition of BrdU incorporation. These results indicate that the RNA-binding domain of Sam68 is important for progression through the cell cycle. However, it is unclear what effect tyrosine phosphorylation of Sam68 has on cell cycle progression. Whether the deletion of the KH domain affects the ability of SAM68 to become tyrosine phosphorylated is not known, although the portion of the KH domain that is deleted does not contain any tyrosine residues.

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