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The interplay between Src family kinases and receptor tyrosine kinases

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Src family tyrosine kinases (SFKs) are involved in a diverse array of physiological processes, as highlighted in this review. An overview of how SFKs interact with, and participate in signaling from, receptor tyrosine kinases (RTKs) is discussed. And also, how SFKs are activated by RTKs, and how SFKs, in turn, can activate RTKs, as well as how SFKs can promote signaling from growth factor receptors in a number of ways including participation in signaling pathways required for DNA synthesis, control of receptor turnover, actin cytoskeleton rearrangements and motility, and survival are discussed.
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Introduction

In this chapter, we will provide an overview of how Src family tyrosine kinases (SFKs) interact with, and participate in signaling from, receptor tyrosine kinases (RTKs). Much of our understanding of SFK function in RTK signaling comes from analyses of cells in culture expressing the three ubiquitously expressed members of the Src family, Src, Fyn, and Yes. Since the roles of the individual members of the family have for the most part not been addressed, and indeed these enzymes may have redundant functions in the signaling pathways we will be describing (Roche *et al*., 1995a, b), we will not distinguish between them here, but rather use the generic term SFK. We will first give examples of RTKs that couple to SFKs, and then go on to discuss the many ways in which SFKs participate in RTK signaling. We do not intend this review to be comprehensive, rather we will use selected examples to illustrate SFK signaling, as well as point out poorly understood or ambiguous areas.

SFKs are downstream of several RTKs

The first evidence for the involvement of SFKs in RTK signaling pathways was the demonstration that Src is activated by stimulation of quiescent fibroblasts with platelet-derived growth factor (PDGF) (Ralston and Bishop, 1985). Subsequent studies have established that SFKs are involved in signaling from many RTKs, including PDGF receptor (PDGF-R), epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), insulin-like growth factor-1 receptor (IGF-1R), hepatocyte growth factor/scatter factor receptor (HGF-R), colony-stimulating factor-1 receptor (CSF-1R), stem cell factor receptor (SCF-R), muscle specific kinase (MuSK), and others (Belsches *et al*., 1997; Parsons and Parsons, 1997; Krystal *et al*., 1998; Biscardi *et al*., 1999b; Abram and Courtneidge, 2000; Dey *et al*., 2000; Hong *et al*., 2004; Mohamed *et al*., 2001; Maejima *et al*., 2003). SFKs can promote mitogenic signaling from growth factor receptors in a number of ways, including initiation of signaling pathways required for DNA synthesis, control of receptor turnover (Ware *et al*., 1997; Wilde *et al*., 1999), actin cytoskeleton rearrangements and motility (Chang *et al*., 1995; Weernink and Rijksen, 1995), and survival (Karni *et al*., 1999).

How are SFKs activated by RTKs? SFKs associate with PDGF-R (Kypta *et al*., 1990) via an interaction of their Src homology (SH)2 domains with Tyr^{579} of the activated receptor (Mori *et al*., 1993). This association is presumed to initiate SFK activation, by releasing the intramolecular interaction between the SH2 domain and the tail, thus allowing the molecule to adopt the catalytically active conformation. This model is supported by the observation that a phosphopeptide modeled on the PDGF-R binding site activates Src *in vitro* (Alonso *et al*., 1995). However, more recent studies suggest that the activation of SFKs by RTKs may be more complex than simple recruitment. For example, the activation of Src by EGF-R in transfected cells requires the small GTPases Ras and Ral (Goi *et al*., 2000). There is also a role for the tyrosine phosphatase Shp2 in promoting SFK activation in response to PDGF, EGF, and FGF. Shp2, which is recruited to activated RTKs, dephosphorylates the Csk-binding protein PAG, thereby preventing the access of Csk (the negative regulator of SFKs) to SFKs (Zhang *et al*., 2004).

The requirement for SFKs in RTK signal transduction pathways has been probed primarily in four different ways, each with its own strengths and weaknesses. Firstly, RTK-binding sites for SFKs have been identified and mutated, and then signaling from the mutated receptors measured. While this approach can

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unambiguously determine whether stable association of SFKs with an RTK is required, it does not preclude the possibility that SFKs participate in RTK signaling without association. Secondly, interfering mutants of SFKs have been introduced into cells, usually by microinjection or transfection. These 'dominant-negative' mutants usually bear a point mutation in the ATP-binding site that prevents catalysis, although sometimes a second mutation in the tail tyrosine is introduced, that has the effect of opening up the conformation of Src. It is usually considered that these dominant negatives work by competing with endogenous SFKs for binding to RTKs. However, it is equally possible that the dominant negatives compete for downstream effectors, and it can be hard to determine whether they have the appropriate specificity in cells. Thirdly, pharmacological inhibitors of SFKs have been used. Here care must be taken that the inhibitor has sufficient selectivity. For example, the related SFK inhibitors PP1 and PP2 are potent inhibitors of PDGF-Rs, as well as a number of other kinases (Blake *et al*., 2000). Fourthly, fibroblasts derived from mice lacking the three ubiquitously expressed SFKs, Src, Fyn, and Yes (SYF cells), have been a valuable tool (Klinghoffer *et al*., 1999). But these cells may not be ideal for mitogenesis studies, since the presence of the large T antigen (LT) of SV40 in these cells is thought to over-ride requirements for both SFKs and Ras (Broome and Courtneidge, 2000; Furstoss *et al*., 2002).

In the sections that follow, we will review what is known about how SFKs participate in RTK signaling.

SFKs promote RTK-initiated DNA synthesis

Whether SFKs are required for mitogenesis in response to PDGF has been the subject of intense study and debate. In favor of a role for SFKs in PDGF-induced mitogenesis are the observations that dominant-negative forms of SFKs, SFK-neutralizing antibodies, and the SFK inhibitor SU6656 all inhibit PDGF-stimulated DNA synthesis (Twamley-Stein *et al*., 1993; Broome and Hunter, 1996; Blake *et al*., 2000). However, other studies have suggested that SFKs are not required for PDGF-induced mitogenesis. For example, a mutant PDGF-R that fails to associate with and activate SFKs can still induce DNA synthesis (DeMali and Kazlauskas, 1998). But these studies leave open the possibility that the basal activity of SFKs is sufficient for proliferation. PDGF-stimulated mitogenesis is only partially inhibited in SYF cells (Klinghoffer *et al*., 1999; Kilkenny *et al*., 2003), providing seemingly compelling evidence that SFKs are dispensable for PDGF-induced mitogenesis. However, as discussed above, these conclusions are complicated by the presence of LT in the SYF cells (Broome and Courtneidge, 2000; Furstoss *et al*., 2002). A definitive answer to this debate will perhaps come when SYF cells are generated without LT.

SFKs have also been implicated in mitogenesis induced by members of the EGF family of receptors. Murine fibroblasts overexpressing Src show an increased level of EGF-stimulated DNA synthesis (Luttrell *et al*., 1988), whereas fibroblasts overexpressing dominant-negative Src fail to synthesize DNA in response to EGF (Wilson *et al*., 1989). Injection of a neutralizing SFK antibody, as well as overexpression of dominant-negative SFKs, also inhibits EGF-stimulated DNA synthesis (Roche *et al*., 1995b). Taken together, these data suggest that SFKs are indispensable for EGF-R-induced mitogenesis in fibroblasts.

The interaction between SFKs and EGF-R may be of particular relevance clinically, since both kinases are often overexpressed in breast tumors (Luttrell *et al*., 1994; Abram and Courtneidge, 2000). Src has been found to be tightly associated with overexpressed, activated EGF-R, probably via the autophosphorylation site in EGF-R. In addition, murine fibroblasts that overexpress both Src and EGF-R show not only increased DNA synthesis but also increased colony formation in soft-agar, and large tumor formation in nude mice (Maa *et al*., 1995). The mechanism for this synergy between SFKs and EGF-R is not clear, but it may involve the direct modification of EGF-R by SFKs (Parsons and Parsons, 1997 and see below). Another clinically relevant member of the EGF receptor family, Her2, also associates with SFKs in tumor cells (Muthuswamy *et al*., 1994; Muthuswamy and Muller, 1995).

In myeloid cells, and in fibroblasts that overexpress CSF-1R, stimulation by CSF-1 activates SFKs (Courtneidge *et al*., 1993), and results in ligand-dependent association of SFKs with CSF-1R. Autophosphorylation of CSF-1R at Tyr^{809} is required for SFK activation and receptor association, but it appears that the SFK-binding site is Tyr^{559} in the juxtamembrane region of the receptor (Courtneidge *et al*., 1993; Dey *et al*., 2000). CSF-1R-mediated activation of SFKs appears to be required for mitogenesis. Expression of an SFK-neutralizing antibody or a dominant-negative Src mutant blocks CSF-1-induced DNA synthesis in fibroblasts engineered to express CSF-1R (Roche *et al*., 1995b). Also, CSF-1-stimulated DNA synthesis is completely inhibited in chicken B cells lacking the SFK Lyn (Corey *et al*., 1998).

A role for SFKs in the regulation of FGF-1R signaling has also been suggested (Klint and Claesson-Welsh, 1999). Fibroblasts stimulated with FGF-1 showed SFK-dependent tyrosine phosphorylation of cortactin (Zhan *et al*., 1993, 1994). Further, the SH2 domain of Src mediates its association with the activated FGF-1R, and stimulation of fibroblasts with FGF-1 results in enhanced SFK activity (LaVallee *et al*., 1998). Another study also reported SFK activation in response to FGF treatment of some cell types, but not all (Landgren *et al*., 1995). SFKs may play a role in regulating FGF-1-stimulated mitogenesis. One study showed that mouse embryo fibroblasts (MEFs) obtained from $Src^{-/-}$ mice proliferated normally in response to FGF-1, suggesting that SFK activity is dispensable for growth factor-induced mitogenesis (Liu *et al*., 1999). However, $Src^{-/-}$ MEFs express Fyn and Yes, and these two SFKs could potentially compensate for the loss of

Src (Lowell and Soriano, 1996). Indeed, recent work demonstrates that SYF cells show an almost complete block in FGF-1-induced DNA synthesis (Kilkenny *et al*., 2003). Further, recovery of FGF-1-induced DNA synthesis was accomplished by reintroducing Src into these cells, demonstrating a role for SFKs in FGF-1-induced mitogenesis in fibroblasts.

Stimulation of IGF-1R in murine 3T3-L1 cells results in enhanced Src and Fyn kinase activity and increased DNA synthesis (Boney *et al*., 2001). The kinase inhibitor PP1 prevented both the IGF-1-stimulated increase in DNA synthesis and the tyrosine phosphorylation of Shc and ERK1/2. Further, overexpression of dominant-negative Src inhibited the phosphorylation of ERK1/2 in response to IGF-1 stimulation. In sum, these data suggest that SFKs mediate IGF-1R-induced mitogenesis via regulation of mitogen-activated protein kinase (MAPK) signaling. Additional components of this signaling pathway remain to be identified.

SCF stimulation of small cell lung carcinoma cells (H526) results in activation of the SFKs Lck and Yes (Krystal *et al*., 1998), concomitant with an interaction of SFKs with the juxtamembrane domain of SCF-R. Further, the SCF-induced activation of SFKs may regulate mitogenesis in H526 cells, since PP1 was shown to inhibit the SCF-induced increase in cell growth and DNA synthesis. Further evidence comes from studies using chimeric CSF-1R mutants that have the ligand-binding domain of CSF-1R fused to the cytoplasmic domain of an SCF-R that lacks binding sites for all signaling pathways except SFKs (Hong *et al*., 2004). This chimeric receptor is able to mediate cell proliferation.

Several other RTK families appear to signal through SFKs, including those of the HGF, NGF, Ret, Eph, and Axl families. In these cases, less is known about the mechanism of SFK activation, and how SFKs participate in signaling from each receptor class. We know that activation of the HGF receptor Met results in the association of a number of signaling molecules, including Src, and there is some evidence that HGF stimulated mitogenesis may require SFKs (Weidner *et al*., 1993; Chen *et al*., 1998; Maejima *et al*., 2003). Stimulation of RTKs of the Axl family, including Axl and Sky, also results in SFK activation (Toshima *et al*., 1995; Braunger *et al*., 1997). Furthermore, mitogenesis of fibroblasts stimulated with the Axl ligand Gas6 is inhibited by dominant-negative Src (Goruppi *et al*., 1997).

To summarize, SFKs are activated by a number of RTKs in a variety of cell types. While an absolute requirement for SFKs may depend on the cell type and the cellular context under study, the available data suggest that SFKs promote mitogenesis from a variety of RTKs.

Loss of p53 releases the SFK requirement for RTK mitogenesis

In NIH3T3 cells overexpressing LT, neither SFKs nor Ras are required for mitogenesis in response to PDGF, apparently because LT expression over-rides mitogenic control points normally regulated by these signaling molecules (Broome and Courtneidge, 2000). One mechanism by which LT exerts its effects is through association with, and inactivation of, p53, which appears to be the key regulatory molecule that is targeted by SFK signaling. For example, overexpression of kinase-dead Src or injection of a neutralizing SFK antibody fails to block PDGF-induced DNA synthesis in cells overexpressing dominant-negative p53 or in p53 null MEFs (Broome and Courtneidge, 2000; Furstoss *et al*., 2002). Further, overexpression of E1B-55 K (an adenovirus protein that inhibits p53 transactivation) overcomes the block in PDGF-stimulated DNA synthesis caused by dominant-negative Src (Furstoss *et al*., 2002). Together, these data suggest that one role of SFKs in PDGF signaling is to oppose the negative growth effects of p53 (Figure 1).

A pathway between SFKs and Myc

Myc is a transcription factor that is required for mitogenesis in response to several growth factors (Bouchard *et al*., 1998). Its production is controlled on at least three levels, transcriptionally, post-transcriptionally, and post-translationally. In fibroblasts, early reports suggested that the basal transcription of the *myc* gene was detectable in unstimulated cells, and unaffected by mitogen treatment, rather mitogen stimulation acted to stabilize *myc* mRNAs once made (Blanchard *et al*., 1985; Dean *et al*., 1986). We have recently confirmed that this mechanism operates in NIH3T3 cells stimulated with PDGF (unpublished observations). The control of the stability of short-lived mRNAs such as *myc* is under intense scrutiny, but how mitogenic signals impact this control is not yet fully understood (Ross, 1995; Guhaniyogi and Brewer, 2001). Once translated, Myc protein turnover is also tightly controlled. For example, it has recently been shown that both MAPK and phosphatidylinositol 3-kinase (PI 3-K) signaling pathways act to stabilize the Myc protein via phosphorylation at specific sites (Sears *et al*., 1999, 2000).

The observation that the block to PDGF-, EGF-, and CSF-1-stimulated DNA synthesis caused by dominant-negative SFKs can be overcome by introducing Myc on an expression plasmid (Barone and Courtneidge, 1995) led to the suggestion that SFKs initiate a signal transduction pathway (probably distinct from the classical Ras–MAPK pathway), which culminates in Myc production. The microinjection approach used for these studies precluded a more detailed analysis of how SFKs affected Myc production. But the subsequent use of SU6656, an SFK inhibitor, suggested that it is the PDGF-stimulated increase in *myc* mRNA levels that is in large part under SFK control (Blake *et al*., 2000). Exactly how SFKs participate in the production of Myc is still the subject of active investigation, and some debate. Additional complexity arises because some studies have used *myc* transcription assays, some have measured mRNA levels, and some have measured protein levels. We will describe some of these studies below, pointing out the methods used.

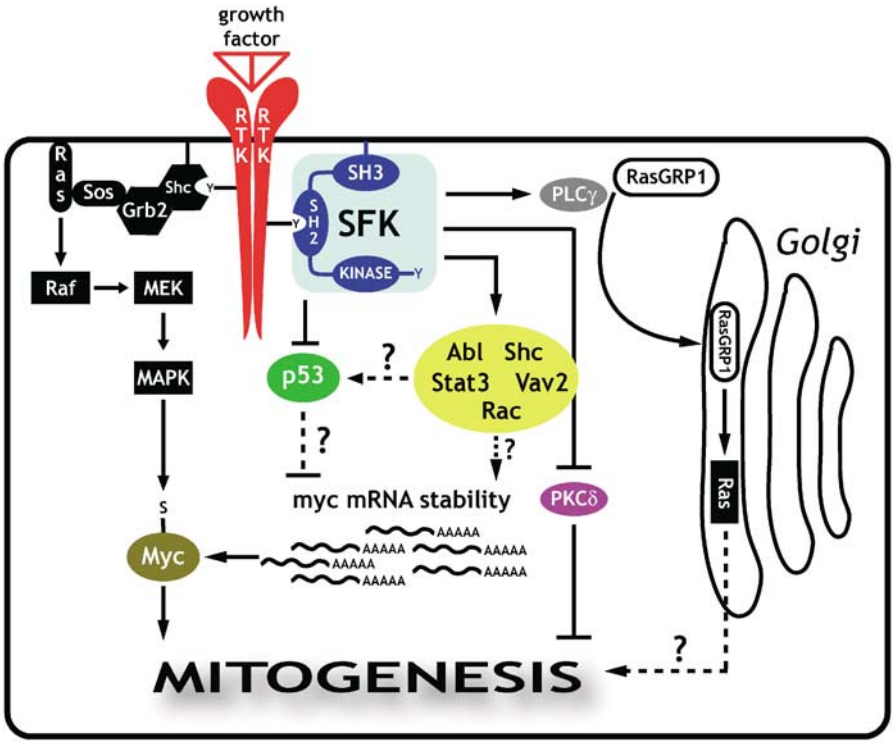

Figure 1 A model for how SFKs mediate mitogenesis in response to RTK activation. RTK-activated SFKs positively regulate DNA synthesis via stabilization of *myc* mRNA and thus increase the production of Myc, a transcription factor required for mitogenesis. Although perhaps dispensable for stabilization of *myc* mRNA, RTK-dependent activation of the traditional Ras/MAPK pathway stabilizes Myc protein in response to growth factor stimulation. The mechanism of SFK-induced *myc* mRNA stabilization is unknown, but might involve the activation of a number of effectors including Abl, Shc, Stat3, Vav2, and Rac, as these are required for SFK-mediated DNA synthesis in response to mitogens (see text, for details). SFKs also stimulate DNA synthesis by opposing the negative growth effects of p53 and PKCδ, although whether either of these effectors is involved in SFK-mediated *myc* mRNA stabilization is unknown. Finally, SFK activation results in translocation of RasGRP1 to the Golgi network and activation of Ras signaling there. Whether Ras signaling within the Golgi participates in mitogenesis is not clear

A number of possible signaling components have been identified using the microinjection assay. For example, dominant-negative forms of Abl, Shc, and Stat3 all inhibit DNA synthesis in response to PDGF, and all can be overcome by enforced expression of Myc, suggesting that they may lie in an RTK→SFK→Myc pathway (Blake *et al*., 2000; Bowman *et al*., 2001; Furstoss *et al*., 2002) (Figure 1). We will discuss each of these proteins in turn.

In fibroblasts, PDGF and EGF stimulation results in increased Abl enzymatic activity (Plattner *et al*., 1999). The PDGF-stimulated increase in Abl activity is mediated by SFKs, as it is blocked in SYF cells and by expression of dominant-negative Src. Abl activation is required for PDGF-stimulated DNA synthesis (Furstoss *et al*., 2002). Overexpression of Myc overcomes the block in PDGF-stimulated DNA synthesis mediated by dominant-negative Abl. Furthermore, serum-stimulated increases in *myc* mRNA levels are reduced in the presence of an Abl inhibitor (STI571). These data place Abl downstream of SFKs in the pathway leading to activation of Myc. However, there may be other signaling effectors that also participate in this pathway, since recent data show that the PDGF-mediated activation of Abl requires activation of both SFKs and phospholipase Cγ (PLCγ) (Plattner *et al*., 2003).

Transcription factors of the Stat family appear to be central players in SFK signaling downstream of RTKs. Stats are activated by a variety of peptide mitogens (Bromberg and Darnell, 2000). Several different studies have used both pharmacological inhibitors and dominant-negative forms of SFKs, and concluded that SFKs are required for both PDGF (Blake *et al*., 2000; Wang *et al*., 2000; Bowman *et al*., 2001; Simon *et al*., 2002) and EGF (Olayioye *et al*., 1999; Kazansky and Rosen, 2001; Guren *et al*., 2003; Kloth *et al*., 2003) stimulation of Stat tyrosine phosphorylation and activation. The nonreceptor tyrosine kinase Pyk2 is also involved in the SFK-mediated activation of Stats in response to growth factors (Shi and Kehrl, 2004), although the mechanism by which this occurs is not clear. The block to PDGF-stimulated DNA synthesis induced by a naturally occurring dominant-negative form of Stat3 (Stat3β) can be overcome by expressing Myc, potentially placing Stats in the PDGF→SFK→Myc pathway (Bowman *et al*., 2001). Because Stats are transcription factors, the simplest model would be that Stats increase transcription from the *myc* gene. However, as we have already pointed out, *myc* mRNA is constitutively transcribed in quiescent fibroblasts. A more likely mechanism then is that Stats control the transcription of other genes that participate in the stabilization of the *myc* mRNA.

A number of studies have implicated the Ras/MAPK pathway in the mitogenic stimulation of Myc production. For example, CSF-1- and PDGF-induced expression of Myc protein is inhibited by both the MEK inhibitor PD98059 and by dominant-negative Ras (Aziz *et al*., 1999; Cheng *et al*., 1999; Sears *et al*., 1999). In one case, the level of *myc* mRNA stimulated by CSF-1 was also reduced by pretreatment with PD98059 (Cheng *et al*., 1999). However, in other studies, the level of PDGF-stimulated expression of *myc* mRNA was only marginally affected by PD98059 (Sears *et al*., 1999, 2000; Chiariello *et al*., 2001). Recent work has demonstrated that the primary role of the Ras/MAPK signaling pathway is in the stabilization of the Myc protein in response to mitogenic signaling (Sears *et al*., 1999, 2000) (Figure 1). However, there remains the possibility that there is also a cell type- or context-dependent requirement for Ras signaling for the stabilization of *myc* mRNA. A further understanding of this important issue will have to await a fuller description of the mechanism by which mitogens stabilize mRNAs.

The guanine nucleotide exchange factor Vav2 and the small GTPase Rac have also been implicated in the SFK-dependent stimulation of Myc (Chiariello *et al*., 2001) (Figure 1). The data suggested a model in which Vav2 is phosphorylated by SFKs in response to mitogen stimulation, thus initiating the activation of a Rac-dependent pathway that controls the expression of the *myc* gene. Neither Ras nor MEK was involved in this pathway. These studies clearly defined that Vav2 phosphorylation in response to PDGF is dependent on SFKs, in keeping with the fact that Vav2 is required for v-Src-mediated NIH3T3 transformation (Servitja *et al*., 2003). However, caution must be used in assigning roles for Vav2 and Rac in mitogen stimulation of *myc*, since the authors employed *myc* promoter/reporter assays, even though the basal level of transcription from the endogenous *myc* gene is unaffected by mitogens. It will be important to determine whether these effectors are also involved in the regulation of *myc* mRNA stability in response to PDGF stimulation, and in what way p53 might fit into this pathway.

The adaptor protein Shc is phosphorylated on tyrosine in response to many mitogens. Two sites of tyrosine phosphorylation have been identified, Tyr^{317} and $Tyr^{239,240}$. It has been shown that phosphorylation of Tyr^{317} leads to the recruitment of Grb2 and subsequent activation of the Ras/MAPK pathway (Ravichandran, 2001). The $Tyr^{239,240}$ site is also a canonical site for Grb2 binding. However, even though overexpression of both Shc^{Y317F} and $Shc^{Y239/240F}$ mutants inhibits EGF-stimulated DNA synthesis and cell proliferation, only cells expressing Shc^{Y317F} showed inhibition of EGF-stimulated ERK activation (Gotoh *et al*., 1997). In contrast, $Shc^{Y239/240F}$, but not Shc^{Y317F}, blocked EGF-stimulated Myc expression (Gotoh *et al*., 1997). PDGF stimulation also results in the phosphorylation of Shc at both Tyr^{317} and $Tyr^{239,240}$. Here, SU6656 pretreatment blocked the phosphorylation of $Tyr^{239,240}$, but had no effect on the phosphorylation of Tyr^{317}, suggesting that $Tyr^{239,240}$ is a substrate of SFKs, while Tyr^{317} is probably phosphorylated by PDGF-R itself (Blake *et al*., 2000). In light of these data, it is more likely that the SFK-dependent activation of Shc specifically enhances mitogenesis by regulating the Myc pathway, and not the Ras/MAPK pathway. In keeping with this model, expression of a form of Shc mutated at both $Tyr^{239,240}$ and the PTB domain inhibited PDGF-stimulated DNA synthesis, and Myc but not Fos rescued this block (Blake *et al*., 2000). Thus, Shc appears to be another component of the SFK $\rightarrow$ Myc pathway (Figure 1). It will be of great interest to determine what signaling effectors are recruited to $Tyr^{239,240}$, and how they are regulated by SFK activity.

A pathway between SFKs and Ras

The signaling pathway that leads from activated RTKs to Ras and MAPKs has been described in great detail. Signaling is initiated by the recruitment of proteins such as Shc and Shp2 to the activated receptor. These adaptor proteins in turn recruit the adaptor Grb2 and its associated protein Sos (a guanine nucleotide exchange factor for Ras). Once Ras is activated in this way, it goes on to activate, in a cascade, the serine/threonine kinases Raf, Mek, and MAPKs (Medema and Bos, 1993). However, recent work has suggested that there is an alternative mechanism that activates Ras proteins that reside in the Golgi (Figure 1). In this case, EGF stimulation of cells leads to the Src-dependent phosphorylation of PLCγ, which then causes the translocation of the Ras guanine nucleotide exchange factor RasGRP1 to the Golgi where it activates Ras. The requirement for SFKs for Golgi but not plasma membrane activation of Ras was demonstrated through the use of the SFK inhibitors PP2 and SU6656, and by probing SYF cells (Chiu *et al*., 2002; Bivona *et al*., 2003). It is yet to be determined whether Ras signaling from the Golgi is required for mitogenesis in response to peptide ligands. However, it has been shown that the Golgi form of Ras is required for NGF-stimulated neurite outgrowth of PC12 cells (Rhodes *et al*., 1989). In addition, farnesylation of the *CAAX* motif in Ras targets Ras proteins to endoplasmic reticulum (ER) and Golgi membranes (Choy *et al*., 1999). It has been shown that Ras *CAAX* peptidomimetic, FTI-277, selectively blocks oncogenic Ras signaling (Lerner *et al*., 1995), suggesting that Golgi and ER localization is important in oncogenic transformation of Ras.

Other SFK mitogenic targets

A member of the protein kinase C (PKC) family, PKCδ, has been implicated in mitogenic SFK signaling downstream of RTKs. PKCδ is thought to play an inhibitory role in cell growth (Figure 1). When overexpressed, it can slow the growth of fibroblasts (Mischak *et al*., 1993), and inhibit morphological transformation in a variety of systems (Li *et al*., 1996; Perletti *et al*., 1999). PKCδ is phosphorylated on tyrosine residues in response to several different stimuli, usually as a result of SFK

activity (Li *et al*., 1994; Denning *et al*., 1996; Blake *et al*., 1999; Kronfeld *et al*., 2000; Joseloff *et al*., 2002). SFKs processively phosphorylate several tyrosines on PKCδ (with Tyr^{311} being the first residue phosphorylated), and this causes the subsequent degradation of the protein. Overexpression of a PKCδ mutant that cannot be phosphorylated by SFKs ($PKC\delta^{Y311F}$) inhibits PDGF-induced DNA synthesis (Blake *et al*., 1999). Whether PKCδ is a negative regulator of the Src→Myc or Src→Ras signaling pathways remains to be established.

SFKs modulate RTKs too

The synergy between SFKs and RTKs is complex and bidirectional. For example, SFKs have also been shown to modulate the activity and signaling of RTKs, particularly EGF-R, PDGF-R, and IGF-R (Thomas and Brugge, 1997) (Figure 2). Interestingly, this regulation seems to be most crucial to the function of EGF-R. We will discuss briefly here the consequences of SFK phosphorylation of RTKs. We will not discuss in any detail the physiological contexts in which this SFK engagement occurs: this will be covered in more detail in other papers in this issue.

Ligand-induced dimerization of the EGF-R results in the rapid phosphorylation of various EGF-R tyrosine residues, including positions 992, 1068, 1086, 1148, and 1173 (Wright *et al*., 1996; Haskell *et al*., 2001), and activation of intrinsic kinase activity. These sites then further participate in signaling by serving as recruitment points for various signaling and docking proteins. In addition to these autophosphorylation sites, however, SFK activation can lead to additional tyrosine phosphorylation of EGF-R and to activation of EGF-dependent signaling. For example, coexpression of EGF-R and of a temperature-sensitive mutant of v-Src in Rat-1 fibroblasts induces rapid tyrosine phosphorylation of two non-autophosphorylation sites in EGF-R (Wasilenko *et al*., 1991). Further studies using purified wild-type or kinase-dead EGF-R and c-Src identified three potential sites responsive to c-Src phosphorylation *in vitro*: Tyr^{1148}, Tyr^{1173}, and possibly Tyr^{703} (Wright *et al*., 1996). Tyr^{703} but not Tyr^{1173} is also phosphorylated by c-Src *in vivo*, suggesting that the conformation of the receptor may be crucial in determining its sensitivity to c-Src activity. Finally, recent work has also shown that Tyr^{845} and Tyr^{1101} are both phosphorylated by c-Src *in vitro* and *in vivo* (Tice *et al*., 1999; Biscardi *et al*., 2000).

Tyr^{845} is particularly noteworthy, since in other RTKs (including CSF-1-R, HGF-R, and FGF-R) the equivalent site is an autophosphorylation site. This might suggest that SFKs play a unique role in the regulation of EGF-R by phosphorylating this site (Haskell *et al*.,

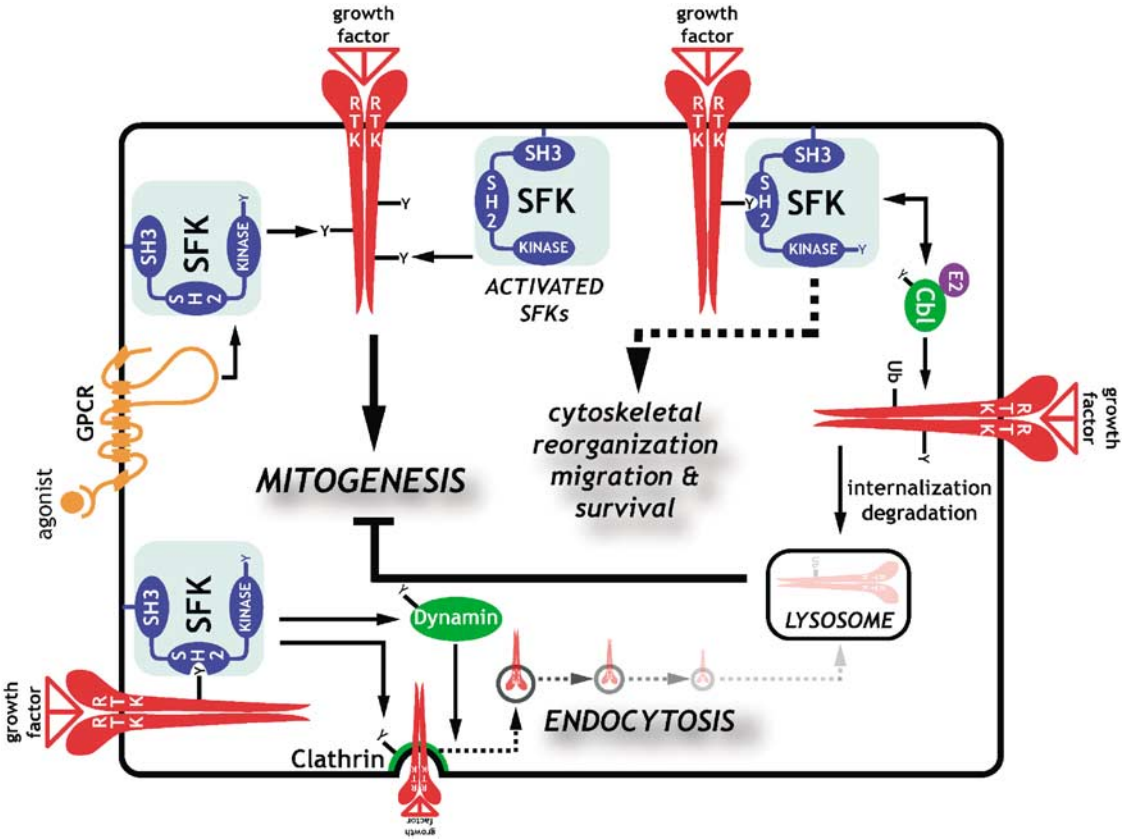

Figure 2 A model for how SFKs participate in RTK signaling. Oncogenic forms of SFKs phosphorylate RTKs. SFKs activated by cellular signaling also phosphorylate RTKs (in the example shown, SFKs are activated by a G-protein-coupled receptor (GPCR)). Once activated by RTKs, SFKs regulate receptor turnover both at the level of endocytosis and ubiquitination. SFKs also participate in RTK-stimulated cytoskeletal reorganization, migration, and survival

2001). Indeed, it has been shown recently that integrin engagement induces phosphorylation of EGF-R on a number of residues including Tyr^{845} (as well as Tyr^{1068}, Tyr^{1086}, and Tyr^{1173}) (Moro *et al*., 2002). Further, pretreatment of cells with the SFK inhibitor PP1 or the expression of dominant-negative Src blocks this integrin-mediated phosphorylation of EGF-R. These data suggest that, in response to cell adhesion, c-Src is recruited to an integrin-containing complex, where it then phosphorylates EGF-R at Tyr^{845}.

What is the functional consequence of the direct phosphorylation of EGF-R by SFKs? In most cases, Src-dependent phosphorylation of EGF-R can positively regulate EGF-R signaling and cellular proliferation, with Tyr^{845} playing a particularly important role. In cells transfected with a Y845F mutant version of EGF-R, EGF-stimulated DNA synthesis is severely impaired when compared to wild-type EGF-R-transfected cells (Biscardi *et al*., 1999a; Tice *et al*., 1999). Taken together, these data demonstrate that c-Src-mediated phosphorylation of Tyr^{845} is required for EGF-R signaling and mitogenesis.

SFKs also regulate PDGF-R by phosphorylating critical tyrosines. For example, Src phosphorylates Tyr^{934} of PDGFβ-R *in vitro* (Hansen *et al*., 1996). Phosphorylation of this site is also observed in fibroblasts stimulated with PDGF, suggesting that PDGF-R is a Src substrate *in vivo*. Fibroblasts expressing a mutant PDGFβ-R with a tyrosine to phenylalanine amino-acid substitution at position 934 (PDGFβ-R^{Y934F}) show reduced levels of PDGF-BB-stimulated DNA synthesis, in keeping with a role for SFK-dependent phosphorylation of Tyr^{934} in proliferative responses (Hansen *et al*., 1996). Interestingly, fibroblasts overexpressing PDGFβ-R^{Y934F} show increased chemotaxis and actin remodeling in response to PDGF stimulation, suggesting a negative role for Tyr^{934} in these responses.

SCF-R is closely related to PDGF-βR. Recent work shows that SFKs can directly and selectively phosphorylate Tyr^{900} of SCF-R (Lennartsson *et al*., 2003). This tyrosine, which is not phosphorylated by the intrinsic kinase activity of SCF-R, serves as a binding site for CrkII (Lennartsson *et al*., 2003). Further, cells overexpressing SCF-R^{Y900F} show decreased proliferation in response to SCF, even though levels of ERK activation are similar (Lennartsson *et al*., 2003). Interestingly, Tyr^{900} corresponds to Tyr^{934} in PDGFβ-R, suggesting that these two receptors may share some common SFK-dependent receptor phosphorylation signaling components.

Finally, EGF-R can be transactivated by PDGF-R (Countaway *et al*., 1989; Decker and Harris, 1989), and the signaling activity of PDGF-R can depend on the coexpression of EGF-R (Li *et al*., 2000), suggesting a mutual codependence of these two receptors. PDGF-R-dependent transactivation of the EGF-R appears to be SFK-dependent, since PP2 will inhibit it (Saito *et al*., 2001). While these results are complicated by the fact that PP2 is known to inhibit PDGF-R (Blake *et al*., 2000), the dose of the inhibitor used may have been selective for SFKs (Saito *et al*., 2001).

IGF-1R is also subject to regulation by SFKs (Thomas and Brugge, 1997). The β subunit of IGF-1R is constitutively phosphorylated in cells transformed by v-Src, and the level of IGF-1-stimulated receptor tyrosine phosphorylation in these cells is much greater than levels seen with IGF-1R from nontransformed cells (Kozma and Weber, 1990). Enhanced tyrosine phosphorylation is observed even in cells expressing kinase-dead IGF-1R mutants, suggesting that receptor kinase activity is dispensable (Peterson *et al*., 1994, 1996). Further, Src-dependent tyrosine phosphorylation of IGF-1R increases the levels of phosphorylation of poly(Glu,Tyr) as well as IRS-1 *in vitro*, suggesting that Src-dependent regulation of IGF-1R enhances its catalytic activity (Peterson *et al*., 1994). In human pancreatic carcinoma cells (PANC-1), overexpression of constitutively active Src increases levels of IGF-1-induced as well as IGF-1-independent proliferation, probably in part by enhancing signaling from the receptor, and also by increasing receptor number (Flossmann-Kast *et al*., 1998).

One interesting difference between the SFK regulation of IGF-1R, and the EGF and PDGF receptors is particularly noteworthy. For both EGF-R and PDGF-R, the Src-dependent tyrosine phosphorylation sites are distinct from those sites phosphorylated in response to growth factor stimulation. In the case of IGF-1R, these sites are identical both *in vitro* and *in vivo* (Peterson *et al*., 1996), suggesting that in the case of IGF-1, SFKs positively regulate the receptor in a manner analogous to ligand stimulation.

SFKs regulate the turnover of RTKs

Endocytosis

It is well established that many RTKs are endocytosed after engagement with their ligands. Endocytosis is primarily thought to be a mechanism for signal attenuation, although there are also suggestions of specific signaling pathways activated by endocytosis (Marmor and Yarden, 2004).

Many of the studies on RTK endocytosis have been carried out using EGF-R. Stimulation of cells with EGF results in rapid clustering of EGF-R complexes in clathrin-coated pits and their translocation into clathrin-coated endocytic vesicles (Gorden *et al*., 1978; Carpentier *et al*., 1982; Jiang and Sorkin, 2003). Cells overexpressing Src show an increased rate of EGF-R internalization (Ware *et al*., 1997). In addition, internalization of EGF-R is impaired in SYF cells, and in cells treated with PP1 (Wilde *et al*., 1999). The authors propose that EGF treatment of cells resulted in the SFK-mediated phosphorylation of clathrin, which drives its subsequent redistribution to the cell periphery (Wilde *et al*., 1999).

Other studies have implicated the SFK-mediated tyrosine phosphorylation of dynamin in the control of endocytosis (Ahn *et al*., 2002) (Figure 2). Dynamin, a large GTPase, was first identified as a microtubule-

binding protein (Shpetner and Vallee, 1989). Subsequent studies revealed that dynamin plays a crucial role in receptor-mediated endocytosis of EGF-R and transferrin (Kosaka and Ikeda, 1983; Kessell *et al*., 1989; Damke *et al*., 1995). Endocytosis of ligand-induced RTKs requires dynamin-mediated GTP hydrolysis, which is regulated by dynamin self-assembly. Phosphorylation of dynamin at Tyr^{597} plays a critical role in its self-assembly and GTPase activity, and it is phosphorylation at this site that is dependent on SFKs (Ahn *et al*., 2002). A strong association between Src and dynamin has also been reported and is mediated by the SH3 domain of Src and the PxxP motif of dynamin (Okamoto *et al*., 1997).

Others have failed to find a role for SFKs in EGF-R internalization, and suggested instead that kinases other than SFKs were involved (Sorkina *et al*., 2002). The reconciliation of these differences will clearly require further study. Nevertheless, a requirement for SFKs has also been reported for the efficient endocytosis of SCF-R (Broudy *et al*., 1999; Jahn *et al*., 2002), and for PDGF-Rs, both α and β forms (Mori *et al*., 1994; Avrov and Kazlauskas, 2003).

Ubiquitination

The E3 ubiquitin ligase Cbl is a negative regulator of activated SFKs (Broome *et al*., 1999; Harris *et al*., 1999), and overexpression of Cbl can inhibit Src-driven DNA synthesis (Broome *et al*., 1999) and transformation (Kim *et al*., 2004). However, under some circumstances, SFKs can negatively regulate Cbl. For example, Cbl is a substrate of SFKs, and once phosphorylated, its conformation is changed (Kassenbrock and Anderson, 2004), and its interaction with the ubiquitin-conjugating enzyme UbcH7 is destabilized (Yokouchi *et al*., 2001) (Figure 2).

The mechanism of Cbl-mediated RTK regulation has been best characterized for the EGF-R (Dikic, 2003). EGF-R activation results in recruitment of Cbl to the EGF-R via association with the autophosphorylation site Tyr^{1045} (Levkowitz *et al*., 1999) and subsequent Cbl-mediated monoubiquitination of receptor (Joazeiro *et al*., 1999; Zheng *et al*., 2000). Although previous data suggested that this Cbl-mediated monoubiquitination stimulated EGF-R endocytosis (Soubeyran *et al*., 2002; Jiang *et al*., 2003), recent studies using $Cbl^{-/-}$ MEFs suggest that Cbl regulates the sorting of EGF-R to lysosomes (and subsequent degradation there) and not EGF-R endocytosis *per se* (Duan *et al*., 2003). Regardless of the exact mechanism, Cbl-mediated EGF-R and PDGF-R ubiquitination clearly results in the increased internalization and degradation of both RTKs (Haglund *et al*., 2003; Mosesson *et al*., 2003).

Overexpression of Cbl blocks PDGF- and EGF-stimulated DNA synthesis in fibroblasts, whereas introduction of Cbl-neutralizing antibodies potentiates growth factor-stimulated DNA synthesis (Broome *et al*., 1999), suggesting that Cbl plays a negative role in growth factor-induced mitogenesis. Indeed, the EGF-stimulated ubiquitination and subsequent proteolysis of EGF-R are mediated by Cbl (Joazeiro *et al*., 1999; Levkowitz *et al*., 1999; Waterman *et al*., 1999), and in some circumstances, SFKs regulate Cbl-mediated receptor ubiquitination (Kassenbrock *et al*., 2002; Bao *et al*., 2003). Thus, activation of the PDGF-R, EGF-R, and EphA receptors results in the SFK-dependent tyrosine phosphorylation of Cbl (Blake *et al*., 2000; Rosenkranz *et al*., 2000; Kassenbrock *et al*., 2002; Bao *et al*., 2003; Sharfe *et al*., 2003). Inhibition of SFKs not only blocks the EGF-stimulated phosphorylation of Cbl in T47D cells and fibroblasts but also inhibits EGF-stimulated ubiquitination of EGF-R (Kassenbrock *et al*., 2002; Bao *et al*., 2003). Thus, the SFK-mediated negative regulation of Cbl upregulates levels of EGF-R.

For PDGF-Rα, SFKs seem to enhance Cbl-mediated degradation of the receptor (Rosenkranz *et al*., 2000). Thus, in SYF cells, or in cells expressing a mutant PDGFα-R that cannot bind SFKs, the half-life of the receptor is prolonged. Furthermore, overexpression of Cbl is only able to promote the degradation of receptors containing SFK-binding sites. How these findings can be reconciled with those demonstrating delayed internalization of PDGFα-R receptors (Avrov and Kazlauskas, 2003) is unclear.

SFKs may participate in RTK-initiated cytoskeletal reorganization, migration, and survival

It is well established that both the introduction of activated Src into cells and the stimulation of cells with peptide mitogens such as PDGF lead to rapid and profound changes in the cytoskeleton. Indeed, studies in Src-transformed cells have led to the identification of a number of Src substrates with roles in the regulation of the actin cytoskeleton (Parsons and Parsons, 1997; Frame *et al*., 2002). But surprisingly, few studies have addressed how SFKs activated by, and associated with, RTKs might be involved in the regulation of actin dynamics. It is known that PDGF stimulation of fibroblasts results in translocation of Src to the cell periphery, in a process that requires small GTPases and an intact cytoskeleton (Fincham *et al*., 1996). Acting through the tyrosine kinase Abl, SFKs are required for the generation of membrane ruffles in response to PDGF (Plattner *et al*., 1999). However, PDGF-induced chemotaxis occurs normally in SYF cells (Klinghoffer *et al*., 1999). Following EGF stimulation of cells, the p190RhoGAP protein is phosphorylated on tyrosine in an SFK-dependent fashion (Roof *et al*., 1998). This phosphorylation acts to relieve an autoinhibitory conformation of p190, and is required, but not fully sufficient, for p190 to exert its effects on actin stress fiber disassembly. It will be interesting to determine which other actin regulatory proteins that are phosphorylated following growth factor stimulation are substrates for SFKs, and how phosphorylation by SFKs might affect their function. Finally, SFKs mediate cytoskeletal anchoring and stability of acetylcholine receptor clusters in response to MuSK activation (Mittaud *et al*., 2001; Mohamed *et al*., 2001; Smith *et al*., 2001).

Two recently characterized GTPase-binding proteins, the mouse diaphanous-related formins (mDia)1 and mDia2, associate with Src and play a role in cytoskeletal reorganization through Rho GTPases. Activated mDia1 and mDia2 induce signaling downstream of Rho GTPases along with Src (Tominaga *et al*., 2000). More recently, it was shown that mDia-interacting protein (DIP) is phosphorylated by Src following EGF stimulation (Meng *et al*., 2004). Phosphorylated DIP in turn associates with p190RhoGAP and Vav2. As a result, DIP negatively regulates Rho and positively regulates Rac, in an SFK-dependent manner.

There have also been reports suggesting the involvement of SFKs in RTK-stimulated migration. For example, SFK association with SCF-R is required for migration in response to ligand (Ueda *et al*., 2002; Hong *et al*., 2004), and PP2 inhibits VEGF-induced chemotaxis and migration (Abu-Ghazaleh *et al*., 2001). The adhesive and motile responses promoted by members of the family of Eph receptors are also mediated by SFKs (Bruckner and Klein, 1998; Palmer *et al*., 2002; Vindis *et al*., 2003). But the molecular pathways by which SFKs promote migration in this context have not been elucidated.

A similar situation exists for the involvement of SFKs in RTK-promoted survival (Schlessinger, 2000; Belsches-Jablonski *et al*., 2001). The use of SFK inhibitors and receptor mutants has suggested that SFKs are involved in survival in response to GDNF (a ligand for Ret) (Melillo *et al*., 1999; Encinas *et al*., 2001, 2004), SCF (Hong *et al*., 2004), and VEGF (Abu-Ghazaleh *et al*., 2001), via a mechanism that likely involves the activation of PI 3-K and Akt. In the case of EGF-R and Her2, SFKs appear to promote survival via a Stat-3 dependent increase in Bcl-xL expression (Karni *et al*., 1999; Song *et al*., 2003). Ultraviolet irradiation of cells can induce ERK activation, which serves as a survival signal: in this case, ERK activation occurs via SFK-dependent phosphorylation of EGF-R and subsequent downstream signaling (Kitagawa *et al*., 2002). Similarly, cisplatin-induced activation of EGF-R occurs via SFKs and results in protection (Benhar *et al*., 2002). These data suggest that DNA-damaging agents can generate survival signals that may be dependent on the regulation of RTKs by SFKs.

In summary, we have learned much in recent years about how SFKs are regulated by, regulate, and participate in signaling from, RTKs. In the coming years, we anticipate that more molecular details will be added to these pathways. The involvement of SFKs in RTK signaling may also be exploited clinically, for example by testing the effects of SFK inhibitors on the growth, metastasis, and survival of cancers driven by members of the EGF-R family.

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