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Review

Novel regulation and function of Src tyrosine kinase

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Abstract. Src tyrosine kinase is a critical signal transducer that modulates a wide variety of cellular functions. Misregulation of Src leads to cell transformation and cancer. Heterotrimeric guanine-nucleotide-binding proteins (G proteins) are another group of signaling molecules that transduce signals from cell-surface receptors to generate physiological responses. Recently, it was discovered that Gαs and Gαi could directly stimulate Src family tyrosine kinase activity. This novel regulation of Src tyrosine kinase by G proteins provides insights into the adenylyl cyclase-independent signaling mechanisms involved in ligand-induced receptor desensitization, internalization and other physiological processes.

Key words. Src tyrosine kinase; G protein; adenylyl cyclase; receptor desensitization; receptor internalization.

Introduction

Remarkable progress has been made in the past two decades in understanding the diverse functions of Src tyrosine kinase. It has been well established that Src family tyrosine kinases are involved in signal transduction pathways regulating a broad spectrum of physiological responses, including cell cycle control, cell proliferation, differentiation, adhesion, migration and survival [1]. Recently, Src family tyrosine kinases emerged to play a role in heterotrimetric G protein signaling [2]. This novel G protein-Src link is implicated in the regulation of apoptosis and ligand-induced receptor endocytosis [3, 4]. In this review, we focus on the novel regulation and function of Src tyrosine kinase.

Src Function in Cancer

Src was initially identified as the transforming protein (v-Src) of the oncogenic retrovirus Rous sarcoma virus [5]. A ubiquitously expressed and highly conserved cellular homolog of v-Src was subsequently discovered and proved to possess protein tyrosine kinase activity [5]. As the first cloned proto-oncogene and first identified nonreceptor tyrosine kinase, Src has been implicated in the development of human colon cancers. The first genetic evidence suggesting that active Src mutations may have a role in the malignant progression of human colon cancer came from the observation that a truncating mutation in Src at codon 531 (equivalent to codon 528 in chicken Src) in the C-terminal regulatory region was found in 12% of the cases of advanced human colon cancer tested. The mutation is demonstrated to be activating, transforming, tumorigenic and metastatic [6]. Biochemical evidence demonstrated that the tyrosine-specific protein kinase activity of c-Src obtained from human colon carcinoma, skeletal muscle tumor and breast cancer tumor tissues is elevated five to seven-fold over that from normal tissues. The elevated c-Src kinase activity does not appear to result solely from an increase in the abundance of c-Src protein, suggesting that specific kinase activity is increased in the tumor cells [7–9]. The progression of colon primary tumors to liver metastases correlates with increased c-Src

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kinase activity and protein expression [10]. In addition, receptor tyrosine kinase epidermal growth factor receptor (EGFR) was found to participate in the ligand activation of c-Src. Specifically, EGFR preferentially activates c-Src in highly metastatic human colon cancer cells. EFGR was found to associate with c-Src in colon cancer cells, and specific inhibitors of the EGFR resulted in a reduction of c-Src activity to basal levels [11].

Regulation of c-Src activity by phosphorylation and conformational change

Studies over the past 20 years on Src family tyrosine kinases culminated in the resolution of the c-Src crystallographic structure [12], which provides unprecedented insights into the regulation of Src kinase activity. Based on the structure, the kinase activity of c-Src is maintained at a low basal level by two intramolecular interactions. One is between the SH3 domain and the linker (between the SH2 domain and the kinase domain). The other is between the SH2 domain and the phosphorylated tyrosine residue 527 (Tyr527) in the carboxyl-terminal tail. A tyrosine kinase, Csk (for C-terminal Src kinase), specifically phosphorylates Tyr527, repressing the kinase activity of Src to generate a downregulated state.

In addition, it has become clear that autophosphrylation of Tyr416 at the activation loop is a critical step leading to full activation of Src tyrosine kinase activity [1, 13]. In the suppressed form of c-Src, this activation loop forms an α helix that packs between the upper and lower lobes of the catalytic domain, thus blocking the peptide substrate binding site [13, 14]. In the active state, the activation loop swings away from the entrance of the catalytic cleft, allowing for access of the substrate to the active site [15]. Phosphorylation of Tyr416 has been proposed to stabilize this extended conformation and activate kinase activity [13].

Consequently, Src kinase activity could be modulated by tyrosine phosphorylation and conformational changes that affect the intramolecular interactions. For example, Csk can inhibit c-Src activity by phosphorylating Tyr527 to generate intramolecular interaction between the SH2 domain and the phosphorylated regulatory tail [12]. This negative regulation has been confirmed genetically. Src family tyrosine kinases are constitutively active in mouse embryos that lack Csk [16]. Truncations of the C-terminal tail including Tyr527, which subsequently lead to elevated Src kinase activity, are found in tumors [17]. In addition, high affinity ligands for the SH2 or SH3 domain could disrupt these intramolecular interactions, cause conformational changes and activate c-Src [18]. Src kinase activity is precisely controlled under the delicate balance between phosphorylation and dephosphorylation in cells.

Src regulation by protein tyrosine phosphatase

Besides tyrosine kinases, c-Src can also be regulated by dephosphorylation on the regulatory tail by protein tyrosine phosphatases. The tyrosine phosphatase SHP2 binds to the platelet-derived growth factor receptor (PDGFR) and activates c-Src by dephosphorylating the C-terminal negative regulatory tyrosine 527 [19]. Thrombin treatment of platelets induces activation of phosphatase SHP1 and dephosphorylation of Src, which precedes the increase in catalytic activity [20]. In Swiss 3T3 cells, receptor-induced activation of Src can be blocked by vanadate, a tyrosine phosphatase inhibitor [21]. In addition, Src is activated by engagement of integrins following cell attachment to a fibronectin matrix [22]. This activation is preceded by stimulation of protein tyrosine phosphatase 1B (PTP1B) and dephosphorylation of pY527 on c-Src.

In T cell receptor (TCR) signaling, the protein tyrosine phosphatase CD45 also plays a role in regulating Src family kinases Lck and Fyn through dephosphorylation. CD45 is able to dephosphorylate the C-terminal negative regulatory Tyr527 [23]. The phosphorylation status of Lck Tyr527 is balanced by the activities of Csk and CD45. These regulatory enzymes play an important role in TCR signaling. T cells from CD45 knockout mice are impaired in TCR induction of tyrosine phosphorylation, calcium mobilization and anti-CD4 stimulation of tyrosine phosphorylation [24–26]. The defects in T cell signaling correlate with decreased catalytic activity of Lck and Fyn and increased phosphorylation on Tyr527 [23, 27–30]. Along with these well-established mechanisms, increasing evidence suggests that heterotrimetric guanine nucleotide binding proteins (G proteins) are modulators of Src tyrosine kinase.

Src regulation by heterotrimeric G proteins

G proteins transduce signals from G-protein-coupled receptors (GPCRs) to regulate a wide variety of intracellular effectors. All GPCRs have seven stretches of hydrophobic amino acids representing transmembrane domains. Each of these receptors is coupled to a heterotrimeric G protein that consists of α, β and γ subunits. So far, the identified direct effectors of G proteins include adenylyl cyclases (ACs), phospholipase Cβ, cyclic GMP (cGMP) phosphodiesterase, some guanine-nucleotide exchange factors for Rho and ion channels [31–33]. Studies from our lab established that nonreceptor tyrosine kinase Btk can be stimulated by Gαq, Gα12 and G$\beta\gamma$ [34, 35].

The classical model of Gαs and Gαi signaling is insufficient to account for many phenomena (table 1). Therefore, Gαs and Gαi might signal through transducers other

Table 1. Observations that could not be explained by Gαs and Gαi regulation of adenylyl cyclase.

Phenomena	Reference
1. cAMP-independent but Gαs-dependent inhibition of magnesium uptake in S49 cells induced by isoproterenol or prostaglandin E_1.	57
2. AC-independent induction of fibroblast transformation by Gαi.	58
3. AC-independent regulation of 3T3 L1 cells adipogenesis by Gαs.	59
4. AC-independent Gαi regulation of F 9 teratocarcinoma stem cells differentiation into primitive endoderm.	60
5. PKA-independent Gαs induced formation of wing blisters in differentiating wing epithelial cells of *Drosophila*.	61
6. PKA-independent Gαs-induced apoptosis in S49 cells.	3

than adenylyl cyclases. Indeed, several lines of evidence indicate that Src family tyrosine kinases are involved in GPCR-initiated signal transduction. For example, G-protein-coupled thrombin receptor stimulated Src activity in growth-responsive fibroblasts [36]. Also, Src mediates the phosphorylation and transactivation of epidermal growth factor receptor initiated by GPCRs [37, 38]. Similarly, phosphorylation and activation of TrkA receptors in PC12 cells and TrkB in hippocampal neurons after treatment with adenosine that acts through GPCRs could be inhibited by PP1 [39]. Furthermore, Src tyrosine kinase specific inhibitor PP1 completely blocked α1A-adrenergic receptor mediated Erk activation and PC12 cell differentiation [40]. In addition, G-protein-coupled β-adrenergic receptor (β-AR)-induced vascular endothelial growth factor gene expression and extracellular signal-regulated kinase (Erk) activation in brown adipocytes have been shown to be Src and protein kinase A (PKA) dependent [41, 42]. Activated Src was also found directly associated with the β3-adrenergic receptor and essential for Erk activation [43]. Moreover, early analysis of the phosphorylation sites on avian sarcoma virus-transforming protein suggests cyclic AMP (cAMP) dependent phosphorylation may involve the functional regulation of Src activity [44]. cAMP treatment of Rous sarcoma virus-transformed Chinese hamster ovary cells increases phosphorylation of Src and its kinase activity [45]. Besides this possible PKA-mediated Src activation, Src can also be activated by GPCRs without the participation of PKA. In S49 mouse T lymphocytes, β-AR-induced apoptosis is severely impaired upon deletion of Src family tyrosine kinase Lck. Based on the fact that recombinant Gαs could directly stimulate purified Lck activity, it was proposed that Src family tyrosine kinase plays a critical role in this signaling pathway [3]. Although all this evidence suggested the importance of Src in GPCR-induced signal transduction pathways, the biochemical mechanisms used by G proteins to activate c-Src remained largely elusive.

Direct activation of Src-family tyrosine kinases by Gαs and Gαi

To study the direct regulation of Src-family tyrosine kinases by G proteins, we used an in vitro reconstitution assay with purified G proteins and c-Src [2]. When the Csk-phosporylated Src with lower basal activity was examined, we demonstrated that Gαs and Gαi, but not Gαq, Gα12 or Gβγ, directly stimulate the kinase activity of the downregulated c-Src [2]. Gαs and Gαi similarly modulate Hck, another member of Src-family tyrosine kinases, suggesting that direct modulation of Src-family tyrosine kinases by G proteins is likely to be a general phenomenon [2].

To assess the physiological relevance of this direct link between c-Src and G proteins, we tested the effect of deletion of Src-family kinases on the overall protein tyrosine phosphorylation induced by Gαs in Src-family kinase knockout cells [2]. Embryonic fibroblast cells (SYF cells) from c-Src, Yes and Fyn triple knockout mice are devoid of any known Src-family tyrosine kinase activity [46]. In SYF cells, tyrosine phosphorylation of cellular proteins induced by Gαs was severely reduced compared with that in NIH3T3 cells. This defect could be remedied by reintroduction of c-Src into SYF cells [2]. This demonstrates that Src-family kinases play a prominent role in mediating Gαs-induced protein tyrosine phosphorylation events in vivo.

Next, we established that Gαs mutants, defective in stimulating c-Src in vitro, failed to increase c-Src kinase activity in cells [2]. To identify the residues on Gαs involved in stimulation of c-Src, we generated mutants of some residues in the switch II region of Gαs, which has been shown to directly interact with adenylyl cyclase [47]. The data showed that some of these mutants could still stimulate c-Src, although they failed to stimulate adenylyl cyclase, indicating that stimulation of adenylyl cyclase is not essential for stimulation of c-Src in cells. One mutant (I235A) failed to increase c-Src activity both in vitro and in vivo, suggesting I235 in the switch II region of Gαs is required for Gαs stimulation of c-Src [2].

To gain more insights into the mechanism used by Gαs to regulate c-Src, we mapped the binding site of Gαs on c-Src and tested the effect of Gαs on the kinetic parameters of c-Src [2]. By using glutathione *S*-transferase (GST) fusion proteins of the individual domains of c-Src, we found that activated G proteins interact with the kinase domain of c-Src. Moreover, in enzyme kinetics analysis, the major effect of Gαs on c-Src was to decrease the K_m

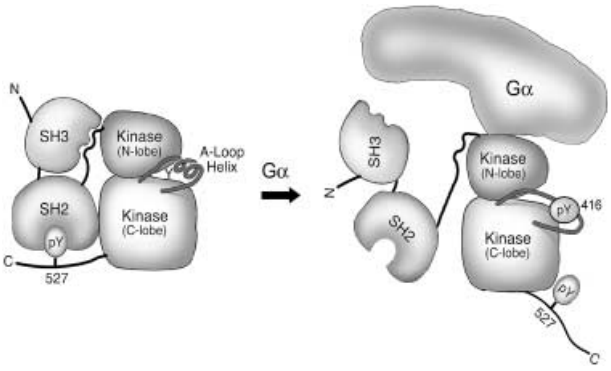

Figure 1. Proposed model for G protein activation of c-Src. Gαs binding changes the conformation of the activation loop of c-Src. This allows the substrate easier access to the active site and thus increases kinase activity.

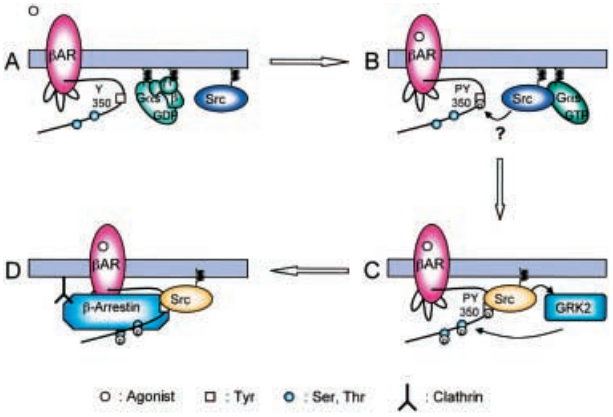

Figure 2. Role of Src in β-AR desensitization. (*A*) Agonist binding to β-AR catalyzes activation of Src by Gαs. (*B*) Activtaed Src could possibly phosphorylate Tyr-350 on the C-terminal tail of β-AR, creating a docking site for Src SH2 domains, leading to further Src activation. (*C*) Src-catalyzed GRK2 phosphorylation and activation lead to Ser and Thr phosphorylation of β-AR. (*D*) the Ser/Thr phosphorylation recruits β-arrestin to the complex to start clathrin-mediated internalization.

(Michaelis constant) for the peptide substrate about five-fold without changing V_{max} (maximum velocity) or K_{cat} (maximum turnover rate) [2]. Because K_m reflects the affinity of the substrate for Src kinase, we propose that G protein binding to the catalytic domain modulates the position and conformation of the activation loop, as well as other elements in the catalytic domain. This could lead to relief of steric hindrance at the entrance to the catalytic cleft, increased accessibility of the activate site to substrates, exposure of the side chain of Tyr416, making it a better substrate for autophosphorylation (the Tyr416 hydroxyl group is buried in the catalytic cleft in the down-regulated form), and thus increased kinase activity (fig. 1) [2]. These observations clearly indicate that Gαs and Gαi can directly signal through Src family tyrosine kinases in addition to the classically defined adnylyl cyclase system. The relative contribution of these different effectors to the physiology of G proteins in organisms remains to be addressed.

New role of Src in β-AR desensitization

β-AR and other GPCRs undergo four sequential steps in response to stimulation by agonists: activation, desensitization, internalization and resensitization [48]. Most GPCRs have phosphorylation sites in the putative cytoplasmic domains, especially the C-terminal segments. Phosphorylation is important for the desensitization of the receptors, a process that makes the receptors refractory to continuous stimulation after an initial response.

Src tyrosine kinase activation and phosphorylation play important roles in the desensitization of β-ARs (fig. 2). When challenged by agonists, β-ARs are phosphorylated on the C-terminal Tyr-350 through an unknown mechanism, creating a binding site for the SH2 domain of Src [4]. Following recruitment to the phosphorylated receptor, activated Src phosphorylates and activates GPCR kinase 2 (GRK2). GRK2, in turn, phosphorylates the β-AR on Ser/Thr residues, creating a docking site for β-arrestin. β-arrestin then initiates internalization of the β-AR complex through clathrin-coated pits, a process necessary for resensitization and redistribution of the receptors. Both expression of dominant-negative Src and treatment with specific Src inhibitor impair tyrosine phosphorylation of GRK2 and agonist-induced desensitization [4]. Phosphorylation of Tyr-350 by a tyrosine kinase initiates the whole desensitization process. Whether the activation of Src by Gαs upon β-AR stimulation plays an important role in Tyr-350 phosphorylation requires further investigation.

The desensitization and internalization process has been postulated to initiate another round of signaling waves that activate the Ras dependent mitogen-activated protein (MAP) kinase pathway [49]. The interaction between Src and the N-terminal of β-arrestin, when both are overexpressed at high levels, may reflect an additional role of Src in β-AR internalization and signaling.

New role of Src in EGFR trafficking

Similar to G-protein-coupled β-AR, receptor tyrosine kinase EGFR utilizes downstream effector signaling to regulate its internalization [50] (fig. 3). This process is initiated by recruitment of the receptor into a clathrin-coated pit at the plasma membrane. EGF binding to its receptor causes rapid phosphorylation of the clathrin heavy chain at tyrosine 1477 in the assembly control domain. In cells lacking Src kinase or treated with Src kinase inhibitor

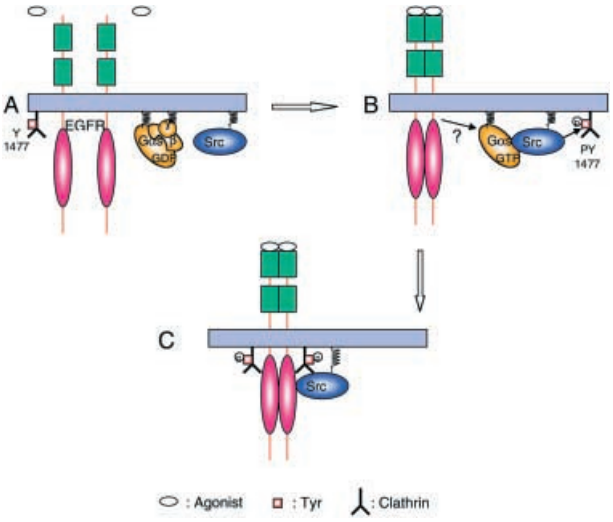

Figure 3. Role of Src in EGFR internalization. Activation of EGFR by EGF catalyzes phosphorylation of Tyr 1477 in the heavy chain of clathrin by Src tyrosine kinase. Phosphorylated clathrin mediates the internalization of EGFR. The stimulation of Src kinase activity could possibly work through Gαs.

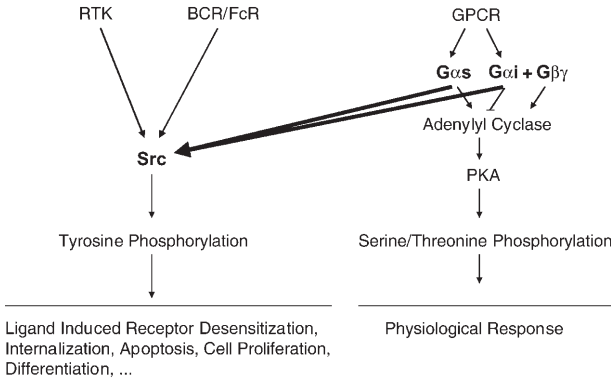

Figure 4. Direct regulation of Src tyrosine kinases by G proteins extends G protein signaling to a broad range of physiological processes. RTK, receptor tyrosine kinase; BCR, B cell receptor; FcR, Fc receptor; GPCR, G-protein-coupled receptor; PKA, protein kinase A.

PP1, EGF stimulation of clathrin phosphorylation and redistribution are impaired, and EGFR endocytosis is delayed [50]. EGFR kinase activity is necessary but not sufficient for clathrin heavy-chain phosphorylation. Clathrin heavy chain is not a direct target for EGFR kinase, but a substrate for c-Src both in vitro and in vivo [50].

Although Src has been shown to phosphorylate and bind to the EGFR [51–54], the nature of this interaction and the role of EGFR in the activation of Src kinase activity are not clear. The initial activation of Src by EGF has been postulated to be mediated by binding of the phosphorylated tyrosine residue 891 of EGFR to Src. However, since Tyr891 has been proposed to be phosphorylated by Src, the question of how Src is initially activated still exists [54, 55]. It has been reported that the juxtamembrane region of EGFR could activate Gαs in vitro [56]. Whether Gαs activation of Src tyrosine kinases is involved in clathrin heavy-chain phosphorylation and EGFR internalization is a question for further investigation

Conclusion

These observations clearly indicate that Src family tyrosine kinases are direct effectors of Gαs and Gαi. This direct G protein-Src link not only extends GPCR signaling to a broad spectrum of Src-regulated physiological responses, but also provides new insights into the understanding of GPCR-elicited protein tyrosine phosphorylation, and ligand-induced receptor desensitization and internalization (fig. 4). The novel regulatory mechanism breaks the traditional artificial linear pathways of Src tyrosine kinase and GPCR signaling and turns them into a more integrated and dynamic network.

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