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Src kinases in G-CSF Receptor Signaling

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1. ABSTRACT

The Granulocyte Colony-Stimulating Factor (G-CSF) Receptor, a member of the hematopoietin cytokine receptor superfamily, functions as a homodimer and requires the recruitment of cytosolic protein tyrosine kinases (PTKs) to transduce its signal. At least two cytosolic PTKs are primarily involved: Jak2, a member of the Janus family, and Lyn, a member of the Src family. Through poorly understood mechanisms, these kinases functionally interact with the G-CSF Receptor. Jak2 primarily enlists members of the signal transducer and activator of transcription (STAT) family and Lyn phosphorylates a number of adaptor molecules, which link the G-CSF Receptor to phosphatidylinositol (PI) 3'-kinase and extracellular signal-regulated kinases (Erk) pathways. This review presents evidence that the Src kinases play a major role in the pathways of G-CSF-mediated proliferation, survival, and differentiation. Identification of Src-dependent pathways provides drug targets useful in the treatment of myeloid leukemias.

2. INTRODUCTION

A small number of polypeptide growth factors, e.g. stem cell factor (SCF), Interleukin (IL)-3, IL-11, and granulocyte-macrophage colony stimulating factor (GM-CSF), drive hematopoietic stem cell expansion. A few more polypeptide growth factors, e.g. erythropoietin (Epo), G-CSF, monocyte-colony stimulating factor (M-CSF), thrombopoietin (Tpo), and IL-5, drive the differentiation of progenitors cells into lineage-restricted, differentiated myeloid blood cells (1). A few of these growth factors (e.g. SCF and M-CSF) have receptors that are tyrosine kinases. Almost all of the other hematopoietic growth factors have cognate receptors, which are members of the hematopoietin cytokine receptor (HCR) superfamily. The HCRs lack intrinsic enzymatic function and, instead, recruit cytosolic PTKs to transduce their signals (2). Common to both classes of hematopoietic growth factor receptors is rapid changes in cellular phosphotyrosine content. In turn, these tyrosine phosphorylated proteins lead to the activation of serine/threonine kinases. Both tyrosine and serine/threonine

Table 1. Basic Features of Src and Jak kinases

	Src	Jaks
Family members	Src, Fyn, Yes, Lyn, Fgr, Hck, Lck, Blk	Jak1, Jak2, Jak3, Tyk2
Structure domains	Unique	JH7 JH6 JH5 JH4 JH3
	SH3	JH2 (pseudokinase domain
	SH2	JH1(kinase domain)
	SH1(kinase` C-terminal regulatory	
Autophosphorylation site	Y416 (Src)	Y1007(Jak2)
Negative regulatory site	Y527 (Src)	JH2 domain(Jak2)
Mechanism of G-CSFR binding	?Via SH2 to pY764	via JH7, JH6 to Box 1
	?Via SH3 to Box 1	Phosphorylation dependent
Kinase activity	Phosphorylation dependent	Phosphatase (?CD45, TCPTP)
	Phosphatase (?CD45, Shp-2)	
Signal effects	Proliferation	?Proliferation
	?Differentiation	?Differentiation
	Survival	?Survival
	Cytoskeletal	
Oncogenic	Yes (mammals, chickens)	Yes (JAK2V617)

kinases ultimately affect transcription factors, causing their migration to the nucleus and activation. For some transcription factors, serine/threonine phosphorylation promotes their degradation or prevents their migration into the nucleus. Different sets of genes are either expressed or silenced, affecting cell cycle progression, survival, cytoskeletal organization, differentiation, and senescence.

G-CSF is an essential growth factor for the optimal production of neutrophils and their precursors. When either G-CSF or its cognate receptor is genetically ablated, the resulting mice are severely neutropenic and susceptible to opportunistic infections (3-6). Loss of G-CSF signaling for proliferation, differentiation, and survival at the progenitor, precursor, or terminally differentiated neutrophil stages accounts for these defects. The characterization of the specific intracellular signaling pathways to distinct cell responses elicited by G-CSF is a major objective of current studies. This review focuses on the role of Src kinases in transducing a component of G-CSF's intracellular signaling and in contributing to leukemogenic growth. Understanding how Src kinases contribute to G-CSF-mediated growth can lead to targeted therapies in some forms of leukemia.

3. THE G-CSF RECEPTOR

Almost all of the receptors for the hematopoietic growth factors belong to the HCR. These receptors share structural properties and features. G-CSF binds to its high-affinity receptor, which functions as a homodimer (7). The receptor is a member of the class I cytokine receptor family, most closely related to gp130 which is a component of the IL-6 Receptor complex. As a member of the HCR superfamily (8), it possesses the characteristic features of a conserved WSXWS motif, a single transmembrane domain, and two regions ("Box 1" and "Box 2") of homology within the proximal intracellular domain (9). G-CSF binds to the external domain in a pocket formed by the N-terminal immunoglobulin domain and the cytokine receptor homology domain (10, 11). Three fibronectin domains make up the rest of the external domain.

Mutation of both proline residues in the PDP sequence within Box 1 or the W650 residue in the intervening sequence between Box 1 and Box 2 results in the loss of G-CSF-induced mitogenesis (12, 13). The receptor's intracellular region does not contain any intrinsic enzymatic function. It does have four tyrosine residues (Y704, Y729, Y744, and Y764), all of which appear to be phosphorylatable(14-20).

Current models of cytokine receptor signaling, including that for the G-CSF Receptor, assign the critical signal transduction role to the Janus kinases. Unlike the Src PTKs, the Janus kinases (i.e., Jak1, Jak2, Jak3, and Tyk2) do not contain well-established functional signaling domains (21). Notably absent are SH3 and SH2 domains (Table 1). In the non-catalytic region of the Jaks are conserved Janus homology (JH) domains. The C-terminal kinase domain is JH1 (see below for further discussion). In distinction to Jak2-deficient mice that display major defects in IL-3, GM-CSF, Epo, and TPO signaling, mice deficient in either Jak1 or Jak2 have intact G-CSF Receptor signaling (22-24). As discussed below, Src kinases have a non-redundant function in transducing G-CSF-induced cell cycle progression. Src kinases have a wider range of physiological substrates than do the Jaks, which primarily affect the STAT proteins (Figure 1). Src kinases may also play a role in G-CSF-mediated survival and metabolism. These pathways that involve the Erk1/2 or PI 3-kinase may contribute to G-CSF-induced differential and cytoskeletal reorganization.

4. THE SRC FAMILY OF PROTEIN TYROSINE KINASES

Src is the cellular homolog of the retroviral oncogene (v-Src), first appreciated a century ago by Peyton Rous (25). Rous identified a filterable substance that produced sarcomas in chickens. Eventually, v-Src was identified as the genetic cause of those sarcomas. Due to a mutation, the negative regulatory site of cellular Src (c-Src) is lost, and its enzymatic activity is constitutive. Tyrosine phosphorylation itself was first identified in protein lysates from v-Src transformed cells (26, 27). Eight other members of the Src family of PTKs are found in different types of mammalian cells (28). Retroviral oncogenic forms of Src kinases have been isolated: Src, Fgr, and Yes. These three kinases are also the most widely expressed in mammalian tissues. The other members of the Src family have a more limited distribution: Blk in B lymphocytes, Lck in T lymphocytes and Natural Killer cells, Hck and Fgr in myeloid cells, and Lyn in B lymphocytes and myeloid cells (2). Members of the Src family contain an N-terminal unique domain with unknown function, an SH3 domain that recognizes poly-proline residues in a helical pattern, an SH2 domain that recognizes phosphotyrosine motifs, a linker that binds to the SH3 domain (29, 30), and the C-terminal kinase domain (Figure 2). The kinase domain

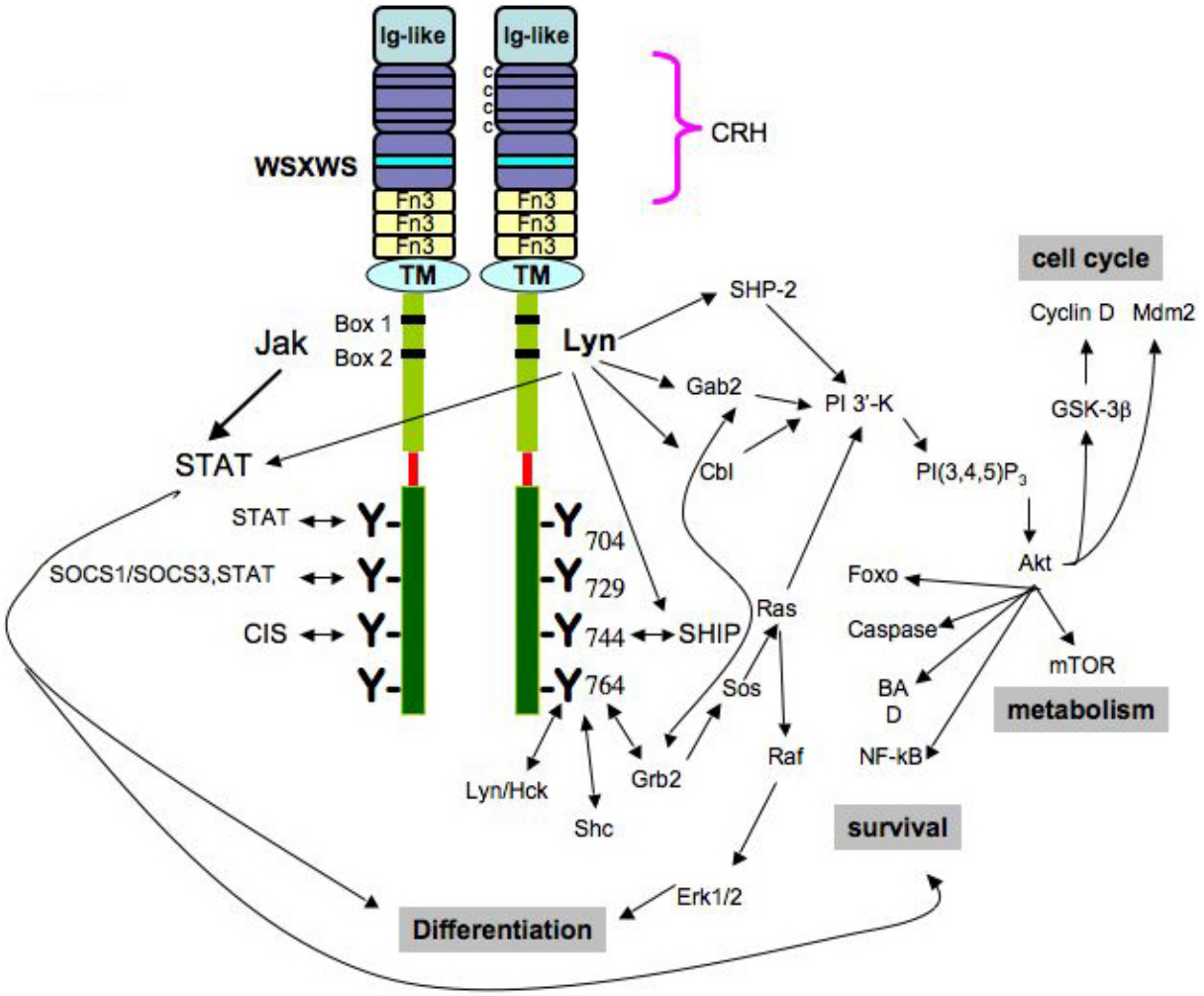

Figure 1. Intracellular signaling pathways of the G-CSF Receptor. The G-CSF Receptor is a member of the hematopoietin/cytokine receptor family. The external domain is composed of an immunoglobulin domain (Ig), the Cytokine Receptor Homology domain (CRH) that contains the WSXWS motif and conserved cysteine residues (C) that provide disulfide linkage necessary for homodimerization, and three fibronectin (fn) domains. The cytoplasmic domain of the receptor contains Box 1 and Box 2 and Tyr704 in the proximal domain, which is necessary for mitogenesis. The distal domain drives differentiation and contains three tyrosine residues (Tyr729, Tyr744, and Tyr764). When phosphorylated, the four tyrosine residues serve as docking sites for SH2-containing proteins. Additional recruitment of receptor-associated molecules provides signal diversification. Neither the structural basis of how cytosolic PTKs, such as Jak and Lyn, associate with the receptor, nor the the specificity or redundancy of each tyrosine residue's kinase are known.

contains a positive autophosphorylation tyrosine residue (corresponding to Src Y416) that is correlated with Src kinase activity and a negative phosphorylation tyrosine residue (corresponding to Src Y527) that is phosphorylated by CSK (C-terminal Src kinase). In the resting state, Src is phosphorylated at Y527, which binds to its intrinsic SH2 domain and stabilizes the inactive confirmation of Src. Following activating signals, that Y527 is dephosphorylated and Src undergoes an activating conformational change. The most likely tyrosine phosphatases to activated Src are CD45 or Shp-2 (31-34). Src then autophosphorylates itself, augmenting the kinase activity (35).

Another important feature of Src regulation is its enrichment in lipid rafts(36), a cholesterol-rich region in the plasma membrane. Recruited to lipid rafts in the plasma membrane by a transmembrane protein (Cbp), CSK phosphorylates that C-terminal tyrosine (37). Although there are no reports to date of G-CSF Receptor localizing to the lipid raft, IL-2 and IL-15 receptors have been recovered in these membrane microdomains (38).

Src PTKs interact with different classes of receptors. The receptor tyrosine kinases (RTK), such as SCF Receptor (c-kit), Flt-3, and M-CSF Receptor, undergo autophosphorylation, resulting in the tyrosine

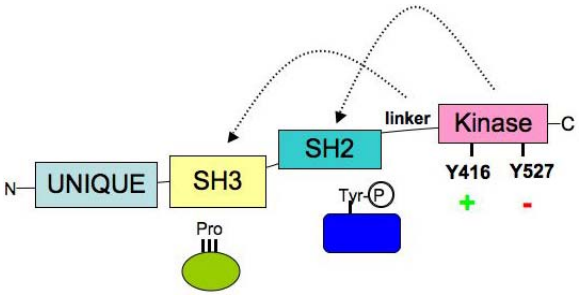

Figure 2. The structural and functional features of a typical Src family tyrosine kinase. Src PTK have an N-terminal unique domain, an SH3 domain that recognizes proline-rich sequences, an SH2 domain that recognizes phosphotyrosine motifs, a linker region, and the kinase domain. The kinase domain contains both a positive regulatory tyrosine (for Src, Tyr416) and a negative regulatory tyrosine site (for Src, Tyr527). The unique domain also contains sites for post-translational addition of myristate. All of the Src PTK, except for Blk and c-Src, also undergo acylation with palmitate at the N-terminus. Post-translational acylation permits membrane localization and enrichment in lipid rafts. To become activated, Src must undergo a conformational change that follows displacement of the SH3-linker interaction and/or dephosphorylation of the C-terminal phosphotyrosine. Substrate recognition may occur through an SH3-proline-rich motif interaction or by a substrate's trafficking to the membrane.

phosphorylation of residues. Specific phosphotyrosine residues in the cytoplasmic domain of the receptor serve as docking sites for the SH2 domain of Src kinases. Src PTKs are thus recruited to the active receptor, where it enlists additional signaling pathways (discussed below). Based on experiments whereby anti-Src antibodies were injected into cells expressing RTK (39), Src PTKs contribute to RTK-induced mitogenesis. The HCR, integrins (e.g. β_3) (40), G-protein coupled receptors (e.g. the chemokine receptor CXCR4) (41,42), membrane channels (43), and even steroid hormone (e.g. estrogen) receptors (44), functionally interact with Src PTK.

Src PTKs contribute to the wide range of cellular responses: cytoskeletal reorganization, cell cycle progression, survival, differentiation, and DNA repair (28, 45). These diverse responses are due to Src's tyrosine kinase activity or ability to form specific protein-protein complexes. The Src SH3 domain binds to proline-rich sequences with low affinity (uM) and low specificity. A minimal four-residue motif, PXXP, forms a polyproline helix that fits into a groove within the SH3 domain (46). The Src SH2 domain binds to phosphotyrosine residues with high affinity (nM) and high specificity due to amino acid residues C-terminal to the phosphotyrosine (47). Proteins that interact directly with Src kinases are: adaptors to Ras or PI 3-kinase (e.g. Grb2, Gab2, Shc, and Cbl), cytoskeletal proteins (e.g. paxillin, CAS, WASp, and HS-1), nuclear proteins such as Sam68 and STAT3, and other enzymes (e.g. RTK, Abl, FAK, SHIP, and Shp-2). From this incomplete list of Src interactors (for a more complete listing, refer to http://bind.ca), one can appreciate Src's manifold functions. Thus, Src kinases contribute to proliferation, differentiation, survival, and cytoskeletal reorganization.

5. INTERACTION BETWEEN G-CSF RECEPTOR AND PROTEIN TYROSINE KINASES

The physical basis by which PTK interact with any of the HCR is not well understood. First, the PTK must physically be close to the receptor. Signaling molecules can be concentrated into the lipid rafts or membrane micro-domains, however it is not known whether the G-CSF Receptor is found in lipid rafts. Another mechanism by which PTK can associate with the receptor is through membrane localization. Such motifs occur in the N-terminus of both Jak and Src molecules. The Jaks contain a band 4.1/ezrin/radixin/moesin (FERM) homology domain (48), and the Src PTK undergo post-translational acylation that permits binding to the plasma membrane (49). Reports suggest that the Jak and Src PTKs associate with the proximal domain of the HCR. Through an incompletely defined physical association, HCR requires Box 1 to bind Jak. The N-terminal JH3-7 domains bind to the Epo Receptor in the endoplasmic reticulum, perhaps stabilizing it, and acts as a chaperone to promote its trafficking to the cell surface (48). Whether all HCR requires Jak for stabilization and trafficking is not known. The Epo Receptor behaves differently than the Growth Hormone Receptor in its dimerization. For the Growth Hormone Receptor, the ligand binds to one polypeptide chain on the cell surface, which then attracts a second polypeptide chain where it binds to a different site (50). On the other hand, the Epo Receptor exists on the cell surface as a pre-formed, ligand-independent dimer (51, 52). Conformation of the Epo Receptor dimer prevents spontaneous Jak2 activation, but upon ligand binding, the receptor undergoes a conformational change that facilitates Jak activation. Structural analysis of the G-CSF Receptor's external domain suggests that it behaves differently from the Epo Receptor. Instead of a 1(ligand):2(polypeptide chain) Epo:Epo Receptor complex, the functional receptor complex exists as 2(ligands):2(polypeptide chains) of G-CSF:G-CSF Receptor (11). Therefore, the model of Jak association and activation based on the Epo Receptor may not be applicable to that for the G-CSF Receptor.

Src kinases can associate with HCR. In co-precipitation studies, Lyn associates with both the proximal region of the βc subunit of the GM-CSF/IL-3/IL-5 receptor complex and the βc subunit's tyrosine phosphorylated residues (53). Lyn interacts with both the proximal domain and the tyrosine phosphorylated residues in the Epo Receptor (54). Both co-precipitation and yeast two-hybrid screening showed Lyn's association with Epo Receptor (55). Lyn's association with the HCR is due to its SH2 domain binding to phosphotyrosine residues approximating the Src SH2 consensus site pYEEI/L (56). In the G-CSF Receptor, phospho-Tyr764 and the adjacent ENL resides resembles that sequence. How does a Src kinase, such as Lyn, interact with the non-phosphorylated receptor? One report showed that Lck's tyrosine kinase domain interacted

with the acidic serine region in the cytoplasmic domain of IL-2Rβ (57). Another possibility is that even though the proximal domain does not contain a proline-rich (PXXP) sequence, there is a highly conserved PXP sequence (X = Asp for the G-CSF Receptor). This motif is critical for mitogenesis. A PXXP motif is also found at residues 634-637 in the juxtamembrane region of the G-CSF Receptor. Thus, it is possible that Src kinases interact with the G-CSF Receptor via an SH3-proximal domain interaction.

6. TYROSINE PHOSPHORYLATION OF THE G-CSF RECEPTOR

Structural functional analysis of the G-CSF Receptor attributes proliferative signaling to the proximal domain (~60 amino acids proximal to the plasma membrane) and differentiation to the distal domain (~100 amino acids at the C-terminus) (Figure 1). Mutation of PDP or Trp650 in the proximal domains abolishes the proliferative response (12, 13). The four tyrosine residues are, however, dispensable for proliferation. Of the four tyrosine residues, only Tyr704 resides in the proximal domain, but mutation of this residue does not impair proliferation. However, the tyrosine residues modify the proliferative response. Results from growth factor-dependent cell lines transfected with different G-CSF receptor forms show enhanced growth with deletion of the distal domain or tyrosine to phenylalanine mutants. Confusingly, retroviral transduction of primary hematopoietic cells derived from G-CSF Receptor null mice reveals enhanced growth requires Tyr764 and differentiation Tyr729 (15). A tyrosine-null Epo Receptor transmits a growth signal, albeit attenuated (58, 59). Thus, when phosphorylated, tyrosine residues likely "fine-tune" the signal generated by the G-CSF Receptor.

Multiple PTK (e.g. Jak2 and Src) probably phosphorylate the tyrosine residues (Tyr704, Tyr729, Tyr744, and Tyr764) of the G-CSF Receptor (16). While the SH2 domains of distinct signaling molecules recognize specific phosphotyrosine motifs, no rules exists to predict which PTK phosphorylates specific tyrosine residues. Typically, the potential phosphotyrosine site does reside in a hydrophilic region.

When phosphorylated, the phosphotyrosine residues serve as docking sites for signaling proteins containing a phosphotyrosine binding domains (e.g. SH2 or PTB). Recruitment of these signaling proteins serves to diversify and inactivate G-CSF Receptor's signal. Diversification involves recruitment of the STAT transcription factors and Ras/Erk1/2 and PI 3'kinase pathways. Y^{704}VLQ fits the YXXQ motif, which can be phosphorylated by the Jaks and then serve as a docking site for the SH2 domains of STAT proteins (17). Resembling that site, when phosphorylated, Y^{729}GQL may also serve as a docking site for the SH2 domain of STAT. Y^{764}ENL best approximates the YEEI/L motif favored by both the Src kinase and the Src SH2 domain (56, 60). This site is also the preferred binding site (i.e. YpEN) for the SH2 domain of Grb2 and is functionally coupled to Shc and the SH2-containing tyrosine phosphatase-2 (Shp-2)(18). Grb2 also interacts with Gab2, which leads to PI 3'-kinase activity (61). Tyr764 is also functionally coupled to Ras activation and Jun kinase (62). Thus, phospho-Tyr764 can transduce several different signals with both positive and negative effects on growth. Substrate availability and sustained activation may determine functional outcome. In their phosphorylated states, Y^{744}LRC and Y^{729}GQL may serve as docking sites for cytokine inducible SH2 protein (CIS)/suppressor of cytokine signaling (SOCS) and SH2-containing inositol phosphatase (SHIP) (63-66). Both molecules are negative regulators of Jak-STAT and PI 3'-kinase, respectively. The C-terminal domain also recruits SH2-containing tyrosine phosphatase-1 (Shp-1), which desphosphorylates positive signaling molecules such as Lyn and STAT (67).

7. INTRACELLULAR SIGNALING IN MYELOID CELLS

Well characterized pathways occur in myeloid cells, although how these form circuits and networks that drive cell cycle progression, survival, repair and aging, differentiation, cytoskeletal reorganization, and inflammatory responses is unknown. Genetic ablation of G-CSF, G-CSF Receptor, and the transcription factors C/EBPα, C/EBPε and Gfi-1 result in severe neutropenia (68-70). However, granulopoiesis appears to be intact in both Jak and Src-deficient mice (23, 71). Jak2-null mice die during embryogenesis, but hematopoietic stem cells respond to G-CSF with formation of granulocyte colonies (22, 24). Thus, murine models provide few insights into the role of specific PTK in G-CSF signaling for myeloid progenitor and precursor proliferation and lineage commitment/differentiation.

G-CSF primes neutrophils for pro-inflammatory responses (72, 73), and Src PTKs play a major role. Phagocytic cells from Src-deficient ($Hck^{-/-}Fgr^{-/-}$ or $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$) mice are defective in superoxide production, degranulation, or migration (74-77). Defects are most profound for integrin-coupled responses, less so in Fc or cytokine receptor signaling. Integrins signal through activation of Focal Adhesion Kinase (FAK) and Src PTK, although the relationship of these two and the presence of FAK in phagocytic cells have not been fully elucidated. However, Src clearly phosphorylates the adaptor protein Cbl. Cbl, which also contains E3 ubiquitin ligase activity in its RING domain, couples Src PTK to PI 3'-kinase via the p85 regulatory subunit (78-81). In turn, PI 3'-kinase affects cytoskeletal reorganization through the activation of RhoGTPases.

The identification of a somatic mutation (Jak2V617F) in the majority of patients with Polycythemia Vera, Essential Thrombocythemia, and Myeloid Metaplasia highlights the importance of the pseudokinase domain (JH2), adjacent to the C-terminal catalytic domain (JH1), in regulating Jak activity (82-85). It also provides an important clue to the role of Jak in granulopoiesis. The absence of neutrophilia in these diseases suggests that a gain-of-function Jak2 has less impact than Bcr-Abl, which causes chronic myeloid or chronic neutrophilic leukemia. Signaling pathways for Bcr-Abl more closely approximate

those of Src, not Jak2 (Figure 1). While there may be cross-talk between Src and Jak signaling pathways, each kinase can trigger a stereotyped response, e.g. Jak-STAT and Src-Ras/PI 3-kinase. This reviews focuses on the contribution of Src to myeloid signaling due to G-CSF.

8. ROLE OF SRC KINASES IN G-CSF RECEPTOR SIGNALING

Because Lyn is the predominant Src PTK expressed in myeloid cells throughout their lineage, it has received the most attention. G-CSF treatment of neutrophils and myeloid leukemia cell lines rapidly increases Lyn kinase activity (86). G-CSF also activates Hck (87), which is most phylogenetically related to Lyn. The precise role of Lyn vis-à-vis Jak2 (the predominant Jak in myeloid tissues) in G-CSF-induced cell signaling is not known. This problem arises because biochemical analysis shows basal Src activity, as determined by either an in vitro kinase assay or blotting with anti-phospho-Src Tyr416 antibody. Blotting for phosphotyrosine content of Jak1 or Jak2 demonstrates complete absence in myeloid cells until G-CSF is added (14), which supports a ligand-dependent response for Jak activation. To determine the specific role of Src in G-CSF Receptor signaling, we have adopted the use of the avian DT40 B lymphocyte cell line, which expresses only one Src kinase, Lyn. Through gene targeting, Lyn-deficient DT40 B cells have been established. In cells that stably express the human G-CSF Receptor, wild-type, Lyn-deficient, and Syk-deficient DT40 cells respond to G-CSF with rapid changes in phosphotyrosine content, and wild-type and Syk-deficient, not Lyn-deficient, cells will respond with cell cycle progression (16). Jak1 and Jak2 activation occurs in the Lyn-deficient cells. Thus, Lyn plays a role in promoting cell cycle progression in these blood-derived cells. Enhancement of Jak-STAT activation in these cells led to decreased cell proliferation (88). Additional studies suggested that PI 3'-kinase, not Ras-Erk1/2, promotes G-CSF-induced cell proliferation (78). The contribution of Lyn and PI 3'-kinase to cell growth and differentiation occurs in the murine Ba/F3 blood cell line that expresses the human G-CSF Receptor (61).

Two models for how cytosolic PTKs transduce downstream signals of the G-CSF Receptor may be proposed: Jak and Src act in series or in parallel. The use of Src PTK and Jak for HCR signaling fits the theme of tandem tyrosine kinases to effect signal transduction for other receptor systems: RTK and Src for RTK (39), Src and Fak for integrins (89, 90), and Src and ZAP-70/Syk for multimeric immune receptors (91, 92).

As a Src kinase, Lyn affects both positive and negative signaling pathways in B lymphocytes (93) and thrombopoietin-stimulated cells (94, 95). The functional outcome 2 depends on nature of the stimulus, duration in exposure to the stimulus, developmental state of the responsive cell, and the relative abundance of known substrates.

Additional circumstantial evidence points to the role of Src PTK. G-CSF induces the tyrosine phosphorylation of adaptor proteins, such as Gab2 (61), Cbl (78), and Shc (78), which are Lyn substrates and effect PI 3'-kinase and Ras pathways. Other hematopoietic growth factors, such as Epo and GM-CSF, induce the phosphorylation of other Src substrates such as CrkL (79) and the GTP exchange factor Vav (81), which affect Rho GTPases. The Jaks phosphorylate members of the STAT, CIS/SOCS, and cytokine receptor families. Although there are many reports of Src PTK phosphorylating STAT proteins (96), there is little evidence that the Jaks affect Ras and PI 3'-kinase pathways.

The Ras-Erk1/2 pathway was one of the first mitogenic pathways to be established (97). G-CSF treatment leads to activation of Erk1/2 as well as other related kinases such as Erk5 and stress activated protein kinases (98-102). Maximal activation of Ras and expression of c-Myc in G-CSF treated cells require Tyr764 (103). Since Src participates in both Ras and Rho GTPases pathways, Src indirectly affects the Erks and, possibly, influences cell proliferation. To confuse matters, prolonged activation of Erk1/2 has also been associated with thrombopoietin-induced differentiation (104).

Another major signaling pathway which is Src-associated is that involving PI 3'-kinase (105). G-CSF stimulates the activity of Akt (Protein Kinase B, PKB), which is Src dependent (106). Truncation of the G-CSF Receptor, as found in patients with Severe Congenital Neutropenia (SCN, also known as Kostmann Syndrome) who develop myelodysplasia or acute myeloid leukemia (AML) (107), results in prolonged Akt activity (61, 106). Akt phosphorylates a number of substrates, such as Bad, Caspase-9, Forkhead transcription factors, mdm2, and glycogen synthase kinase (GSK)-3β (108-111). These molecules critically regulate a variety of responses: survival, metabolism, and differentiation. Besides cell survival, G-CSF-induced Akt activity promotes differentiation and generation of reactive oxygen species (61, 112).

G-CSF induces the tyrosine phosphorylation of Tec, also a cytosolic PTK that is immediately downstream of Lyn (113, 114). One of Tec's chief roles in cell signaling is its association with and activation of Vav. Tec also contribute to G-CSF-induced differentiation (114).

While Jak is only associated with the STATs as downstream effectors, the downstream effectors of Src are diverse and include the Erks, PI 3-kinase, and Tec. Of unknown physiologic significance, the Src kinases can also activate the STATs (115, 116). There is little evidence that the Jaks can directly affect the Src targets. Furthermore, G-CSF signaling is intact in Jak-deficient mice but disturbed in Lyn-deficient blood cells. Altogether, these studies suggest a primary role for Src kinases in transducing the G-CSF signal.

9. IMPLICATION OF SRC KINASES IN G-CSF RECEPTOR SIGNALING

Alterations in the G-CSF Receptor contribute to myeloid diseases. Germ-line mutations in the external

domain of the G-CSF Receptor have been found in patients with SCN, and these do not respond to filgrastim. In two patients, the receptor has a nonsense mutation, so it behaves as a dominant negative (117, 118). A third patient had a point mutation that affected Jak and STAT3 activation (119). Truncation of the G-CSF Receptor due to a nonsense mutation is almost always found in patients with SCN. The loss of the C-terminal 80-90 amino acids results in prolonged STAT and Akt activation, probably through the failure to recruit negative regulators such as CIS/SOCS, SHIP, and the Shp-1. Similarly, expression of a differentiation-defective isoform of the G-CSF Receptor is increased in AML or myelodysplastic syndromes (MDS) (120). Rare cases of AML display *de novo* mutation of the G-CSF Receptor. A functional polymorphism has been recently reported in ~6% of patients with high risk MDS (121). How these diverse mutations contribute to leukemia pathogenesis is not known, but deregulated Src-related pathways, e.g. PI 3'-kinase/Akt, are likely to be involved.

Since Src kinases contribute to cell cycle progression, survival, and motility, they provide an excellent target in cancer therapeutics. Small molecule inhibitors, antisense oligonucleotides, and RNAi have blocked Src PTK and induced growth arrest and apoptosis in myeloid leukemia cell lines and primary cells (122). Therapeutic implications go beyond cancer. Excessive neutrophil production and function play a role in a variety of inflammatory diseases. Targeting Src PTK may ameliorate these conditions. A dual Src/Abl inhibitor, dasatanib, has progresses to phase II studies for patients with either Bcr-Abl+ leukemia and solid tumors. Dasatanib inhibits Src and Abl with an *in vitro* IC_{50} ~ 1 nM (123). It affects other kinases, such as the platelet-derived growth factor receptor and c- kit, with an IC_{50} ~ 50 nM. The drug has been well tolerated at doses in excess of Src inhibition. Toxicity has been mild and includes pericardial myeloid suppression. Because Grade III/IV myelosuppression has been uncommon, it is possible that the Jaks provide sufficient signaling or other salvage pathways exist for near normal granulopoesis. Alternatively, the Src kinases may play both positive and negative roles in hematopoiesis (124).

10. CONCLUSIONS

The G-CSF Receptor is a member of the hematopoietin cytokine receptor superfamily, which recruits cytosolic PTK to transduce its signal. The signals generated by the G-CSF Receptor drive the proliferation, survival, differentiation of neutrophil precursors and the function of the terminally differentiated neutrophil. At least two classes of PTK affect these responses: members of the Janus (Jak2) and Src (Lyn) families. The physiological significance of each class of PTK and how the G-CFS Receptor functionally interacts with them are incompletely understood. Jak primarily phosphorylates and activates the STAT family of transcription factors. Lyn phosphorylates a variety of adaptor molecules, such as Gab2, Shc, and Cbl, and enzymes, such as Shp-1, Shp-2, and SHIP-1. Secondary signaling pathways include PI 3-kinase and Ras-Raf-Erk1/2. The role for Src kinases in G-CSF-induced cell cycle progression and survival is based on several lines of evidence. Granulopoiesis is intact in mice with genetic ablation of Jak1 or Jak2. Cells expressing the G-CSF Receptor but completely lacking Src PTK do not undergo DNA synthesis, although mice with loss of Lyn, Hck, and Fgr show normal myelopoiesis. Src kinases contribute to activation of both PI 3'-kinase and Erk1/2. These pathways are associated with cell proliferation, survival, and differentiation. Treatment of myeloid cells with either Src or PI 3-kinase inhibitors results in their death. One implication for Src PTK's role in growth factor-induced myelopoiesis is that forms of myeloid leukemia can be treated with a Src inhibitor.

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