Src-Related Protein Tyrosine Kinases in Hematopoiesis

By Seth J. Corey and Steven M. Anderson

HEMATOPOIESIS DEPENDS ON GROWTH FACTOR RECEPTOR-MEDIATED TYROSINE KINASE SIGNALING

The proliferation and differentiation of blood cell progenitors and precursors are tightly regulated by approximately a dozen different growth factors, which act primarily on hematopoietic cells. In addition, growth factors that stimulate a wider variety of cell types, such as insulin-like growth factor 1, are also able to stimulate the proliferation of hematopoietic progenitor cells. Both types of growth factors activate receptor-mediated tyrosine kinase signaling pathways. Acting as extracellular signals, hematopoietic growth factors bind to cell-surface receptors and induce a rapid increase in tyrosine phosphorylated cellular proteins. Tyrosine phosphorylation of growth factor receptors and cytoplasmic signaling molecules then serves to amplify the intracellular signal and activate a diverse number of intracellular signaling pathways. These signaling pathways suppress apoptosis and induce the transcription of genes required for mitogenesis. Many of the same signaling molecules are also activated by growth factors that stimulate terminal differentiation, rather than proliferation, of precursor cells.

The receptors for three well-established hematopoietic growth factors, macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), and the Flt-3 ligand, are transmembrane tyrosine kinases in which the cytoplasmic domain of these receptors encodes a tyrosine kinase. Ligand binding leads to receptor dimerization and immediate activation of the receptor tyrosine kinase domain. In contrast, the receptors for the majority of hematopoietic growth factors, including granulocyte CSF (G-CSF), GM-CSF, erythropoietin (Epo), thrombopoietin (Tpo), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, and IL-15, do not encode a tyrosine kinase catalytic domain. This latter group of receptors are all members of the cytokine receptor superfamily. Cytokine receptors share several structural motifs in both the extracellular ligand binding domain and their cytoplasmic tails. A series of four conserved cysteine residues and the WSXWS motif (Trp-Ser-X-Trp-Ser, where X is any amino acid) are both present in the extracellular ligand binding domain of all cytokine receptors. Other highly conserved motifs found in the extracellular region of the cytoplasmic tail of cytokine receptors are referred to as the box 1 and box 2 motifs. Excellent reviews describing the structural features of cytokine receptor family members have recently been presented.

The molecular cloning of multiple cytokine receptors a decade ago showed that none of these receptors encoded a protein tyrosine kinase, despite the fact that stimulation of cells with various cytokines resulted in a rapid increase in tyrosine-phosphorylated proteins. This observation provided the stimulus to identify the tyrosine kinase(s) activated by these receptors. The structural features of cytokine receptors, however, did not provide any clues as which tyrosine kinases might be activated or to the mechanism(s) by which these kinases might be activated. Studies conducted with a variety of cytokine receptors have provided evidence that the binding of ligands to cytokine receptors is able to activate multiple tyrosine kinases including members of the Janus family kinases, the Src family of kinases, and, more recently, members of the Tec family of tyrosine kinases. For the last 5 years the majority of research on cytokine receptor signaling has focused on the Janus family of tyrosine kinases, because one or more Janus kinase family members have been observed to be activated by every cytokine receptor identified to date. Furthermore, genetic evidence supports a role for Janus kinases in promoting cytokine-induced differentiation of hematopoietic cells. A group of patients with severe molecular defects in Jak3 have pronounced B- and T-cell deficiencies, and Myeloid cells derived from fetal liver cells of Jak2-deficient mice failed to differentiate in response to IL-3, Epo, or TPO. However, these cells differentiated normally in response to G-CSF or M-CSF. Myeloid cells derived from Jak1-deficient mice responded to GM-CSF, G-CSF, IL-5, and IL-3. These data suggest unique roles for Jak1, Jak2, and Jak3.
Regardless of receptor type, the activation of kinases and the phosphorylation of substrates drive proliferation and differentiation. A common set of downstream targets is phosphorylated by receptors that induce proliferation or differentiation (Fig 1). We hypothesize that cytokine-stimulated proliferation is dependent on the activation of Jak and Src-family of tyrosine kinases. This review will discuss the evidence that Src-like kinases are required for myeloid hematopoietic cell signaling, highlight those substrates that are activated/phosphorylated in an Src-like dependent manner, and describe potential mechanism(s) by which these kinases are regulated by cytokine receptors. The critical role of Src-related kinases in the development of T-cell and B-cell lymphocytes and activation signaling of the multimeric immune receptors (T-cell receptor, B-cell receptor, and the high-affinity IgE receptor) have been well reviewed.

THE Src FAMILY OF TYROSINE KINASES

The v-Src oncogene is derived from the Src cellular proto-oncogene (frequently identified as c-Src). Seven additional related genes have been identified either by cloning of related cDNAs or by identification of related viral oncogenes. The members of the Src kinase family include: Src, Yes, Fgr, Fyn, Lck, Lyn, Blk, and Hck (Table 1). Some Src family members are expressed in a wide variety of tissues, while others show a more restricted pattern of expression. For example, Blk, Hck, Fgr, Lck, and Lyn are each expressed primarily in a narrow range of hematopoietic cells (see below). In addition, several Src family members exist in multiple isoforms formed by either alternative splicing or, in the case of Hck, by the use of an alternative start codon. Adding to the complexity of Src kinase expression is the fact that these different isoforms can also show differences in tissue expression; that is, different forms of Fyn are expressed in T lymphocytes and the brain. Although three Src kinases, Src, Yes, and Fgr, were discovered in naturally occurring oncogenic retroviruses, the introduction of specific point mutations can lead to the oncogenic activation of all members of the Src kinase family. The ability of specific point mutations to induce constitutive activation of the kinase activity of Src family kinases can readily be understood based on the three-dimensional crystal structure of downregulated Src and Hck (see below).

As noted above, the pattern of tissue expression of Src family members varies greatly. Src is expressed in different tissues of the body with the highest proteins levels detected in neurons and in platelets. Yes is expressed primarily in neural tissues.

### Table 1. Members of the Src Family of PTK

<table>
<thead>
<tr>
<th>PTK</th>
<th>Molecular Weight</th>
<th>Tissue Expression</th>
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<tr>
<td>Src</td>
<td>60</td>
<td>Platelets, neural, fibroblasts, mammary</td>
<td>PDGF, M-CSF, EGF</td>
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<tr>
<td>Yes</td>
<td>62</td>
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<td>Lck</td>
<td>56</td>
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</tr>
<tr>
<td>Lyn</td>
<td>53, 56</td>
<td>B cells, myeloid cells, natural killer cells</td>
<td>IL-3, GM-CSF, IL-6</td>
</tr>
<tr>
<td>Hck</td>
<td>55</td>
<td>Myeloid</td>
<td>BcR</td>
</tr>
<tr>
<td>Blk</td>
<td>55</td>
<td>B cells</td>
<td></td>
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</tbody>
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[Fig 1. Similarities in signaling between a hematopoietic receptor tyrosine kinase and a hematopoietic cytokine receptor. The receptors for M-CSF (CSF-1) and G-CSF serve as models for a hematopoietic receptor tyrosine kinase and a hematopoietic receptor without intrinsic tyrosine kinase activity. In both cases, upon receptor engagement, the receptor dimerizes and becomes tyrosine phosphorylated. The phosphotyrosine residues serve as specific docking sites for SH2 containing signal transduction molecules, such as Src, PI 3-kinase, Grb2, and STAT. The same pathways involving Cbl-PI 3-Kinase-Akt kinase-Akt or Src-Grb2-Sos-Raf-MapKK (Map Kinase Kinase)-MapK may be activated by both types of receptors via the receptor and Src. Mutagenesis studies show that the loss of Y559 in the M-CSF receptor and loss of box 1 and box 2 in the G-CSF receptor lead to the loss of cytokine-induced mitogenesis.]
Hematopoietic lymphocyte src kinase are present in B lymphocytes. As kinase is cell kinase. Csk inhibition of c-Src requires functional B receptor. The N-terminal seven residues comprise the SH4 immunoreceptor tyrosine activation motif (ITAM) in the B-cell N-terminus of Lyn and Fyn binds to the nonphosphorylated specific motifs common to all known protein kinases. The kinase domain was the first region of homology domain. The kinase domain includes approximately 75 amino acids that are not conserved among different Src-like kinases. The SH2 domain permits its association with CXCXXXXC motif found in CD4, and the N-terminus of Lyn and Fyn binds to the nonphosphorylated immunoreceptor tyrosine activation motif (ITAM) in the B-cell receptor. The N-terminal seven residues comprise the SH4 domain of the molecule, which contains potential fatty acylation sites required for association of Src kinases with the plasma membrane. The glycine residue at position 2 serves as an acceptor site for myristate, and the cysteine residue at position 3, found in all family members except Src and Blk, serves as an acceptor site for palmitate. Palmitoylation, which is reversible, may serve several purposes. It may serve to strengthen the association of the kinase with the membrane. Alternatively, palmitoylation could serve to concentrate Src family kinases in caveolae and thereby allow them to function as effectors for glycanphosphatidylinositol-linked surface proteins.

Another critical structural feature of all Src-like kinases is the conserved C-terminal tyrosine residue which plays a critical role in regulating their catalytic activity. Dephosphorylation of this tyrosine, or the binding of antibodies to the C-terminus, results in the catalytic activation of the Src-related kinases. Phosphorylation of this C-terminal tyrosine is mediated by csk (c-src kinase). Csk inhibition of c-Src requires functional SH2 and SH3 domains of c-Src, suggesting that the SH2 domain binds to the C-terminal phosphotyrosine in a manner that is stabilized by the SH3 domain. Thus on the simplest level, it could be argued that the activation of Src-related kinases merely requires dephosphorylation of the C-terminal tyrosine by an unidentified protein tyrosine phosphatase (PTPase). Indeed, it has been suggested that the requirement for transmembrane PTPase CD45 in signal transduction by the T-cell receptor reflects the need for dephosphorylation of this regulatory tyrosine in the activation of Src-like kinases.

The crystal structures of the downregulated forms of Src and Hck have been recently determined (Fig 2). The region of the protein characterized included the SH3 domain, the SH2 domain, the kinase domain, and the SH4 domain. The kinase domain was the first region of homology identified among different Src family members, and it contains specific motifs common to all known protein kinases. The SH2 domain is approximately 100 amino acids long and binds to phosphorylated tyrosine residues in a sequence-specific context. The SH3 domain contains approximately 75 amino acids and binds to proline-containing sequences. The unique region of Src-like kinase includes approximately 75 amino acids that are not conserved among different Src-like kinases. Although the precise function of the unique domain is not currently clear, there are two examples of specific functions that correlate with specific amino acid sequences present in the unique domain. The unique region of Lck permits its association with CXCXXXXC motif found in CD4, and the N-terminus of Lyn and Fyn binds to the nonphosphorylated immunoreceptor tyrosine activation motif (ITAM) in the B-cell receptor. The N-terminal seven residues comprise the SH4 domain of the molecule, which contains potential fatty acylation sites required for association of Src kinases with the plasma membrane. The glycine residue at position 2 serves as an acceptor site for myristate, and the cysteine residue at position 3, found in all family members except Src and Blk, serves as an acceptor site for palmitate. Palmitoylation, which is reversible, may serve several purposes. It may serve to strengthen the association of the kinase with the membrane. Alternatively, palmitoylation could serve to concentrate Src family kinases in caveolae and thereby allow them to function as effectors for glycanphosphatidylinositol-linked surface proteins.

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The crystal structures of the downregulated forms of Src and Hck have been recently determined (Fig 2). The region of the protein characterized included the SH3 domain, the SH2 domain, the kinase domain, and the C-terminal tail with the regulatory tyrosine residue phosphorylated. These studies showed that the C-terminal phosphotyrosine residue bound to the SH2 domain in an intramolecular fashion. The kinase domain was found to be composed of two lobes in a fashion similar to that observed with cyclic adenosine monophosphate (AMP)-dependent protein kinase and cyclin-dependent protein kinase 2. The adenosine triphosphate (ATP)-binding site is present in the smaller N-terminal lobe while the active site is present in the larger more C-terminal lobe. Contrary to the expectation that the binding of the SH2 domain to the C-terminal tyrosine would block access to the active site, the kinase domain and the SH2 domain were on opposite faces of the molecule. The SH3 domain was observed to bind to the region that links the kinase domain to the SH2 domain, even though this region of the protein does not contain a predicted SH3 domain binding site. The binding of the SH3 domain to this linker peptide disrupts the structure of the kinase domain rendering it inactive. This is largely accomplished through the displacement of "helix C" which forces Glu to a critical residue in catalysis, out of the catalytic pocket. The structure of downregulated Src and Hck clearly indicates that both the SH2 and SH3 domains play important roles in the regulation of Src-like kinases. This is consistent with data showing that point mutants in the SH2 and SH3 domains can lead to the oncogenic activation of c-Src. Furthermore, structural changes that prevent the interaction of either the SH3 or the SH2 domains with other regions of the kinase molecules can alter the regulation of the kinase. This is most easily demonstrated by mutation of the C-terminal tyrosine to phenylalanine which results in the constitutive activation of the kinase. Also consistent with this concept, it has been shown that binding of the SH3 domain of Hck to the HIV Nef protein results in the catalytic activation of Hck. This clearly indicated that binding of the SH3 domain to a ligand can activate Src-like kinases. The dephosphorylation of the C-terminal tyrosine residue, displacement of the SH2 domain from the C-terminal phosphotyrosine of the kinase, or binding of the SH3 domain of the enzyme to another protein represent molecular events that can lead to activation of the enzyme by altering the three-dimensional structure of the molecule and allowing the enzyme to assume an active conformation.
This figure not available online; please see print version
Src-LIKE KINASES REGULATE CELL-CYCLE PROGRESSION

Although it has been known for many years that the constitutive activation of Src-like kinases leads to the oncogenic transformation of cells, the role played by these kinases in normal cellular physiology is just recently becoming clearer. The contribution of Src kinases to cell-cycle progression has been established from reports of Src’s structural and functional interaction with receptor tyrosine kinases in nonhematopoietic cells. Recent studies have shown the activation of Src-like kinases in the G1 phase after stimulation of cells with platelet-derived growth factor (PDGF), epidermal growth factor (EGF), colony-stimulating factor-1, and fibroblast growth factor. Microinjection of antibodies directed against Src or Fyn, or of dominant negative mutants of Src or Fyn, blocked mitogenesis induced by PDGF. Both Src and Fyn bind to a specific phosphorylated tyrosine residue present in the cytoplasmic tail of the PDGF receptor, and phosphorylated peptides based on this sequence were capable of activating Src and Fyn. In hematopoietic cells, the best-studied interaction of receptor and nonreceptor tyrosine kinase has been that between c-Kit and Lyn. A member of the same subfamily of receptors as that for PDGFR and CSF-1R, c-Kit, when stimulated, leads to the activation of Lyn and their association. Targeting of Lyn by either lyn antisense or specific Src kinase inhibitor resulted in a dramatic decrease of SCF-induced proliferation. We have recently reported that the presence or absence of Lyn correlated with G-CSF-induced proliferation in hematopoietic cells (see below).

The inhibition of PDGF-induced mitogenesis mediated by dominant negative mutants of Src or Fyn could be rescued by the overexpression of c-myc, suggesting that Src-like kinases may be required for induction of the immediate early gene c-myc and progression through G1 to S phase. Activation of Src, Fyn, and Yes has been noted in G2 phase and may be required for the G2 to M phase transition. The activated form of cyclin-dependent kinase Cdc2, which functions at the G2/M checkpoint, associates with Fyn, Lck, and Lyn. When cells have been subjected to ionizing radiation, Lyn’s association with Cdc2 correlated with inhibition of the Cdc2 kinase.

DEVELOPMENTAL IMPORTANCE OF Src: INSIGHTS GAINED FROM KNOCKOUT MICE AND CELL LINES

The generation of mice bearing homologous disruption of different Src family member genes has resulted in mice with specific developmental defects and/or diseases (Table 2). To date the disruption of both alleles of a single Src family member has not proved lethal, although the disruption of csk has been lethal. In a few cases (fgr−/−, yes−/−, and hck−/−), the knockout mice have very subtle defects. When these mice were crossed-bred to create double knockouts, more pronounced defects were unmasked. Thus, while gene targeting may show important clues about the function of a specific Src family member, conclusions about specific function must be tempered by the recognition that there appears to be redundancy in the function of different family members, as well as developmental variation in levels or tissue expression of specific Src kinases. In addition, it is possible that some of the phenotypes observed in knockout mice may be species specific.

Mice deficient in Src develop severe osteopetrosis. The defect in bone development is caused by a nonredundant contribution of Src to proper osteoclast function. Src-deficient osteoclasts cannot resorb bone, which may be due to a defect in their failure to form a ruffled border. Expression of kinase-defective alleles of Src in Src-deficient mice ameliorated the osteopetrosis. These findings suggest that regions of Src other than the kinase domain play a critical role in cytoskeletal rearrangement and cell shape (see below). Other nonredundant contributions by Src may not be observed because of the increased mortality of Src−/− mice. Because osteoclasts are derived from monocyte lineage, it may not be surprising that CSF-1−/− mice (op/op) also develop osteopetrosis. No patients with osteopetrosis have yet been identified with defects in either the Src protein tyrosine kinase or in the production of CSF-1. This highlights the problem that phenotypes observed in genetically altered mice may not directly correspond to genetic changes that contribute to disease in humans.

The critical importance of several different Src family members in lymphocyte development is shown in three differ-
ent knockout mice. Mice deficient in Lck have reduced number of thymocytes, whereas mice deficient in Fyn have reduced T-cell receptor responsiveness. Mice deficient in Lyn have reduced number of mature B cells and aberrant B-cell receptor signaling. The failure to detect more profound defects in myeloid-derived cells with single or combinational disruptions of the genes for hck, fgr, and lyn is most likely due to redundancy of Src kinases. Cytokine receptor signal transduction is defective, however, in cell lines that lack expression of Src family members through posttranslational addition of myristate. 10.106

Signal transduction in myeloid cells is also defective in mice bearing disruption of Src-family kinases. Neutrophils from hck−/−fgr−/− mice display incompetent integrin receptor signaling. 10.106 Erythrocytes from these mice have higher mean corpuscular hemoglobin due to enhanced K/Cl cotransport activity. 10.108 The proline-rich motif RPLPXLP is a generic poly-proline II helix conformation. 118

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As shown in Fig 2, the structure of a Src-like kinase consists of catalytic and noncatalytic domains. Both parts of the molecule contribute to generating and diversifying signaling cascades by trapping specific substrates and through the formation of multi-protein complexes. Although an Src family tyrosine kinase might be expected to have a diversity of substrates, multiple factors appear to be important in the selection of the proteins that are actually phosphorylated in a particular cell. Factors include membrane localization of Src family members through posttranslational addition of myristate ± palmitate, the binding of the SH2 and/or the SH3 domains of Src family members to potential substrates or adapter molecules which themselves are bound to substrates, and, finally, the presence of preferred phosphorylation sites. The ability to predict proteins that may be substrates for specific Src family members is complicated by the contribution of each of these different factors. Degenerate peptide library screening has been used to identify the preferred phosphorylation sequence for Src as EEEYG/EFD. 112 However, none of the proteins identified as likely Src substrates (Table 3) contain this phosphorylation sequence. Although this approach has not been useful in predicting preferred protein substrates for Src family members, it has suggested that there are significant differences in the preferred phosphorylation sequences for different Src-like kinases. For example, the preferred phosphorylation site for Lck is XEXYGΦΦ (with Φ = L, V, F, or I and X is indistinguishable) while that for Src is EEEYG/EFD. The logical explanation for our inability to predict specific substrates based on amino acid sequence analysis is that binding of the SH3 and/or SH2 of the kinases to potential substrates are major determinants in substrate selection and result in the phosphorylation of “suboptimal” phosphorylation sites. SH3 domains bind to proline-rich motifs with moderate affinity (micromolar), however, SH2 domains can bind with nanomolar affinity to phosphotyrosine residues in a sequence-specific context. Thus, the interactions of SH3 and SH2 domains with proteins have a major effect on substrate selection.

SH2 and SH3 domains are found in a wide range of proteins including non–Src-related protein tyrosine kinases, cytoskeletal proteins, transcription factors, and adaptor molecules. Peptide library screening has been used to identify the preferred binding sites for the SH2 domains from many different proteins. SH2 domains have been determined to recognize phosphorylated tyrosine residues in a sequence specific context with the three amino acids that lie C-terminal the tyrosine residue being critical in determining which specific SH2 domain will bind to a specific phosphotyrosine. This information has proved to be very useful in predicting whether the SH2 domain of a specific protein will bind to the phosphorylation site in a second molecule. Peptide library screening and screening of phage display libraries have been used to develop consensus sequences to which different SH3 domains might bind. These sequences all adopt a poly-proline type II helix conformation. The poly-proline-rich motif RPLPXL is a generic poly-proline II helix motif which has been defined as a SH3 binding site by phage display methods, but significant differences exist between the sites predicted to be favored by different Src family SH3 domains. It is clear that SH3 domains may recognize other unrecognized motifs because the analysis of downregulated Src (described above) showed that the SH3 domain of Src can recognize a motif within itself which structurally resembles a poly-proline type II helix but does not contain the minimal Pro-X-X-Pro sequence. One of the most accurate approaches to identifying substrates for Src family members has been to express the oncogenic

Table 3. Substrates of Src-Related PTK

<table>
<thead>
<tr>
<th>Adaptor Molecules</th>
<th>Structural Proteins</th>
<th>Enzymes</th>
<th>Transcription Factors</th>
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forms of these kinases in the cell of interest and characterize the phosphotyrosine-containing proteins present in these cells. One consideration inherent in this approach is that the identified phosphorylated proteins may represent those that are present in the highest concentration and thus may not reflect the critical substrates. Another important consideration is that the proteins phosphorylated in different cell types may vary considerably. This was pointed out by a study in which the tyrosine phosphorylated proteins present in v-Src-transformed NIH3T3 cells were compared with those present in the murine myeloid cell line 32Dcl3 expressing v-Src.115 This study concluded that either there were different substrates in the proteins phosphorylated or that the proteins phosphorylated by v-Src in fibroblasts were missing from myeloid cells. The phosphorylation of p125Fak, p120, and p85 observed in v-Src transformed 32Dcl3 cells using antibodies directed against these proteins. A second approach that has been used is to employ cells that lack the expression of either all known Src family members, or cells that lack expression of a specific Src kinase. The latter cell lines include fibroblast cell lines derived from knockout mice, or cells derived from embryonic stem cell lines lacking both alleles of specific Src kinases. An alternative, but less accurate, method is to look for in vitro phosphorylation using recombinant Src and candidate substrates.

By initiating signaling cascades, Src kinases affect cell shape, migration, adhesion, cell-cycle progression, secretion, and differentiation. These responses occur through the recruitment of a second level of signaling molecules. As a result, pathways emanating from Ras, PI 3-Kinase, and focal adhesion kinase (Fak) are activated. The role of Src in these signaling pathways is to either phosphorylate the signal transduction molecule itself (eg, Fak) or to phosphorylate an adaptor molecule (eg, Cbl and Shc) that links Src to specific signaling molecules (PI 3-kinase and Ras, respectively). Activation of Ras, PI 3-kinase, and nonreceptor protein tyrosine kinases such as Zap70/Syk, Tec, and Fak cascade leads to an amplification of signals along different signaling pathways.

The Ras and PI 3-kinase cascades recruit serine/threonine kinases, which control proliferation, differentiation, and apoptosis. Src activates Ras via a domino effect mediated by SH2 and SH3 interactions: Src → Shc → Grb2 → Sos → Ras. Loss of Shc phosphorylation in lyn-deficient cells and the coprecipitation of Lyn and Shc show the critical role played by Src kinases in initiating this cascade.120,122 Overexpression of Fyn increases Sos activity, which is associated with complex formations of Fyn-Shc, Shc-Grb2, and Grb2-Sos.122 Ras may also be activated by Src kinases via PI 3-kinase and Fak (see below). In turn, Ras triggers a serine/threonine kinase cascades consisting of Raf → Map Kinase Kinase → Map Kinase → S6 Kinase. PI 3-kinase is a heterodimeric complex, consisting of a p110 catalytic subunit and a p85 regulatory subunit. The latter contains two SH2 and one SH3 domains. PI 3-kinase becomes activated via adaptor molecules, such as Cbl, and produces 3'-phosphatidylinositides. This lipid species, which is resistant to phosphodiesterase, serves as a docking site for proteins containing a pleckstrin domain. One such protein is the proto-oncogene product Akt, also a serine/threonine kinase. Recent studies suggest that Akt negatively regulates apoptosis through its phosphorylation of Bad, a Bcl-2 binding partner.123

Src kinases activate and/or associate directly with other nonreceptor protein tyrosine kinases, such as Zap70/Syk, Tec/Btk, and Fak/Raf/ltk. As a result of Src-mediated phosphorylation of immunoreceptor tyrosine activation motifs, found in components of T-cell receptor, B-cell receptor, and Fc receptor complexes, Zap70 or Syk become activated. The Src kinases phosphorylate the two tyrosine residues found in the ITAM, which then serves as a docking site for the two SH2 domains found in Zap70/Syk. As a result of this dual phosphotyrosine-SH2 association, the kinases become activated. Major substrates of these kinases are phospholipase Cγ and Vav, which recruit additional signaling cascades. Tec, Btk, and Ikh comprise another family of nonreceptor protein tyrosine kinases. In addition to containing an SH2 and an SH3 domain, the Tec family of kinases has two unique features: a pleckstrin domain and a Tec homology domain, which mediates association of Tec with Lyn.124 Stimulation by Epo, G-CSF, IL-3, and SCF leads to tyrosine phosphorylation of Tec. Cytokine-induced association of Tec and p85 provides means by which PI3-kinase may be activated.125 Src kinases regulate the cytoskeleton via multiple signal transduction molecules. Src kinases also regulate the cytoskeleton, mostly through its association with paxillin and the nonreceptor protein tyrosine kinase Fak was first identified in cells transformed with v-Src.126,127 Whereas Zap70/Syk and Tec family of kinases are predominantly found in hematopoietic tissues, Fak is more ubiquitously expressed. Engagement of integrins results in activation of Fak.128-130 In activated Fak, the phosphorylated tyrosine residue (Tyr 493) becomes a docking site for an Src SH2 domain,130,131 and for the binding of the p85 subunit of PI3 kinase.119 The recruitment of Src is critical for the role of Fak in promoting the formation of focal adhesions, as evidenced by defective adhesion properties in fibroblasts derived from Src-deficient mice.131 Activation of the Ras pathway appears to occur via binding of the SH2 domain of Grb2 to Tyr925 of Fak.132,133

**Src-RELATED KINASES IN HEMATOPOIESIS AND CYTOKINE-STIMULATED PROLIFERATION OF HEMATOPOIETIC CELLS**

Several lines of evidence suggest a critical role for Src-related kinases in blood cell function: (1) Half of the eight known mammalian Src kinases (Blk, Hck, and Lck) are found exclusively, and two others (Fyn and Lyn) are predominantly found in blood cells. (2) Knock-out mice bearing disruption of several of the Src-related genes (Fyn, Lck, Lyn, and Hck/Fgr) display prominent hematologic abnormalities.98,105-109 (3) Defects in Src-related kinases have been observed in patients with hematologic disease. A patient with T-cell acute lymphoblastic leukemia has been described with a chromosomal translocation t(1;7)(p34;q34) that resulted in the fusion of the lck gene with the gene encoding the β subunit of the T-cell receptor.134 Also, defects in the closely related protein tyrosine kinases, ZAP-70 and Btk, play a critical role in either severe combined immunodeficiency or X-linked agammaglobulinemia.135-138 (4) Src tyrosine kinase inhibitors block blood cell function or leukemic cell growth.139 (5) Several different Src family kinases have been observed to co-precipitate with hematopoietin/cytokine receptors21-25,140 or transmembrane receptor tyrosine kinases.95,96,87,90
As noted above, a wide range of hematopoietic growth factors also stimulate the activation of Janus family kinases. The importance of Janus family kinases is underscored by the association of defects in Jak3 with some forms of severe combined immunodeficiency, the generation of a novel fusion protein involving Jak2 in some forms of T-cell leukemia.150 Jak3 deficiency in hematopoietic/cytokine receptors,12,14,15,144,145 In the absence of ligand binding, Janus kinases are not tyrosine-phosphorylated or catalytically active. After cytokine stimulation, Janus kinases are rapidly phosphorylated and activated, suggesting that they are critical in cytokine receptor mediated signaling events. Expression of a dominant negative form of Jak2 inhibits Epo-sensitive apoptosis in FDCP-1 cells or suppresses GM-CSF-induced c-Fos and c-Myc promoter activities in Ba/F3 cells.146,147 Studies with cells lines lacking specific Janus kinase family members clearly indicate that they play a critical role in cytokine receptor signaling.30,32,146,149 Genetic deficiency of Jak2 by gene targeting profoundly inhibits Epo or IL-3-mediated hematopoiesis, but leaves G-CSF-induced myeloid colony formation undisturbed.30,31

The biological basis of the difference between Epo/IL-3 and G-CSF signaling rests on receptor-activated signaling components caused by differences in receptor structure and the precise role that a Jak or Src kinase plays in cellular physiology. It is our view is that although activation of Janus kinases is important in cytokine-mediated signaling events, the main mitogenic response to cytokines is mediated by activation of Src family members.

One of the approaches we have used to examine the role of Src family members in cytokine receptor signaling is to utilize the DT40 cell line. DT40 is a chicken B-lymphocyte cell line that is notable because it undergoes homologous recombination at a high rate, thereby facilitating the disruption of genes in this cell line. In addition, the only known Src kinase family member expressed in these cells is Lyn. Expression of Lyn has been disrupted by homologous recombination to yield the D33-3 cell line. Likewise, variants of DT40 cells have been generated which lack Syk, or Lyn and Syk expression. Ectopic expression of the receptors for either G-CSF or GM-CSF has been achieved in both the DT40 and the lyn-deficient D33-3 cell line91 (and unpublished data, S.M.A., September 1998). Both G-CSF or GM-CSF can stimulate DNA synthesis in DT40 cells expressing the cognate cytokine receptor. Conversely, neither cytokine was able to stimulate the proliferation of the lyn-deficient cells above background levels91 (and unpublished data, S.M.A., September 1998). Cytokine stimulation of lyn-deficient cells expressing either receptor resulted in activation of Jak2 and STAT5, indicating that cytokine stimulation of these cells activated these molecules, but this was not sufficient to induce cytokine-dependent proliferation91 (and unpublished data, S.M.A.). Transfection of Lyn-deficient cells with a Lyn-encoding expression vector restored cytokine-induced DNA synthesis in these cells. These data clearly provide significant evidence that cytokine-induced proliferation requires activation of Src-like kinases and that activation of Jak2 and STAT5 is not sufficient to support mitogenesis. This also suggests that the activation of Src family members occurs downstream of Jak2, or that it occurs independently of Jak2 in these two receptor systems. Analysis of the substrates activated in Lyn-deficient cells indicates that the cytokine-induced phosphorylation of Cbl and the activation of PI3-kinase are negligible in G-CSF–stimulated Lyn-deficient cells. Ras activation, as determined by an increase in GTP-bound Ras, was not suppressed in the Lyn-deficient cells (unpublished data, S.J.C., September 1998).

Additional groups have found that either Jak activation is neither sufficient nor necessary for cytokine-induced proliferation or differentiation. A Tpo receptor mutant has been made that does not activate either Jak2 or Stat family members, but is able to induce mitogenesis.151 Expression of a dominant negative Jak2 did not affect IL-3–induced proliferation of 32D cells, although it did accelerate apoptosis upon IL-3 withdrawal.152 An essential role for Lyn has been established with the discovery that the mutant J2E line, which failed to differentiate in response to Epo, is deficient in lyn.153 At the same time that Jak activation is not needed for all hematopoietic signaling responses, evidence is accumulating that Src kinases can activate Stats in hematopoietic and nonhematopoietic cells.154-157 Altogether, these reports suggest that either Jak activation precedes that of Src in a hierarchical series or Jak and Src kinases are activated independently.

A MODEL FOR HOW SRC-LIKE KINASES MIGHT BE REGULATED IN RESPONSE TO CYTOKINE STIMULATION

Perhaps the most critical issue in understanding signal transduction by cytokine receptors is how the receptor induces activation of Jak and Src family. The mechanism(s) whereby these kinases interact or bind to the receptor must be elucidated. Although we do not fully understand this process, clues are starting to accumulate. It is clear that Jaks physically associate with and can be coprecipitated with multiple cytokine receptors.12,14,15,144,145 In those cases where physical association has not been observed, it is likely to be caused by experimental artifact. One or more members of the Janus family are activated by all members of the cytokine receptor family. Activation of Janus family members appears to require the phosphorylation of one of two tyrosine residues present in the “activation loop” of the kinase; in the case of Jak2, phosphorylation of Tyr1007 appears to be both necessary and sufficient for catalytic activation.158 Phosphorylation of the adjacent tyrosine in the activation loop, Tyr1006, is not required. Ligand-induced dimerization of cytokine receptors appears to be all that is required to bring two different molecules of Jak2 into immediate proximity such that they are able to transphosphorylate the critical tyrosine residue in the activation loop. Association of Janus kinases with cytokine receptors appears to require the membrane proximal box 1 sequence present in the cytoplasmic tail of all cytokine receptors.159 No other kinase appears to be required for activation of Jak2.143,144,146 This model would be consistent with the mechanisms by which other tyrosine kinases, such as the receptors for fibroblast growth factor and insulin, become activated.161,162 Dimerization is critical for function, and if it occurs inappropriately, it turns the kinase into an oncopogene. Ligand-independent dimerization and activation contribute critically to leukemogenesis, as in the cases of Tel-Jak2, Tel-Abl, and Tel-PDGFβR. In these examples, the oligomerization motif of Tel provides the mechanical basis for dimerization.163 Coprecipitation of several Src-like kinases, including Fyn,
Hck, and Lyn, with cytokine receptors has been described; this includes the receptors for G-CSF, GM-CSF/IL-3/IL-5, Epo, IL-6, and prolactin. This association has generally been described as ligand-dependent or independent; these differences may be a reflection of the lysis conditions and the antibodies used in these studies. The constitutive association of Hck with the murine IL-3 receptor has been observed in studies using a monoclonal antibody directed against the cytoplasmic tail of the β subunit. Bacterial fusion proteins containing different regions of Hck coupled to glutathione S-transferase (GST) have been used to demonstrate both phosphotyrosine-dependent and phosphotyrosine-independent binding of different regions of Hck to the β subunit of the IL-3 receptor. The Hck SH2 domain was observed to bind to the β subunit in a phosphotyrosine-dependent manner, while the Hck SH3 domain bound in a phosphotyrosine-independent manner. A GST fusion protein containing a 236-amino acid region of the cytoplasmic tail of the murine β subunit was used to localize the region(s) of phosphotyrosine-dependent and phosphotyrosine-independent binding of Hck and the β subunit. This region of the cytoplasmic tail of the murine β subunit has four tyrosine residues; however, none of them would be predicted to be binding sites for the SH2 domain of Src family kinases. Three of these four tyrosine residues are conserved in the human βc subunit, although these sequences are not conserved among other cytokine receptors. There are several poly-proline se-

Fig 3. Models for the activation of src-like kinases by the hematopoietic growth factor (HGF) receptor. In (A), Src and Janus kinases act in parallel. Src kinases are weakly associated with the receptor, perhaps via the SH3 domain of the kinase binding to proline-rich sequences in the cytoplasmic tail of the receptor and/or by the binding of the unique domain to unidentified peptide sequences. Ligand-induced receptor dimerization brings the kinase domains of two different Src-like kinases into close proximity, allowing the transphosphorylation of tyrosine residues in the activation loops of the kinases. This may result in a change in the structure of Src-like kinases such that the C-terminal regulatory tyrosine residue is released from the SH2 domain, dephosphorylated by a protein tyrosine phosphatase such as CD45, and the kinase is then fully activated. After HGF stimulation, activation of Jak2 occurs independently of Src and is mediated by phosphorylation of Tyr1007 in Jak2. This phosphorylation results when two molecules of Jak2 are brought into close proximity after receptor dimerization. In (B), Src and Janus kinases act in series, with Src being downstream of Jak. The Src-like kinases are weakly associated with the cytokine receptor, as suggested above. The SH2 domain of the Src-like kinase is bound to the C-terminal tyrosine residue holding the kinase in an inactive conformation. Ligand-induced dimerization leads to the activation of nonreceptor protein tyrosine kinases, such as Jak2, which phosphorylate different tyrosine residues in the cytoplasmic tail of the receptor. The SH2 domain of the Src kinase binds to one of the newly phosphorylated tyrosine residues in the C-terminal tail of the receptor, thereby displacing the C-terminal tyrosine residue. This results in a conformational change of the kinase such that it is now enzymatically active. The displaced C-terminal tyrosine residue is eventually dephosphorylated by a protein tyrosine phosphatase such as CD45, thus helping to maintain Src in an activated conformation. The activation of Src kinases would require prior activation of a Jak kinase in this model.
quences that could represent binding sites for the SH3 domains of Fyn or Lyn. A more direct approach to determining receptor-kinase interaction lies in using yeast two-hybrid in vivo screening. Tilbrook et al. reported that the EpoR interacted with Lyn using this approach. However, our own studies have not detected an interaction between Lyn or Jak2 and either the G-CSF receptor or the β subunit of the GM-CSF receptor (unpublished data, S.J.C., September 1998).

Based on these data we propose two models whereby Src and Janus kinases transduce hematopoietin cytokine receptor signaling. In the first model (Fig 3A), Src and Janus kinases are in parallel. Src family tyrosine kinases are constitutively associated with cytokine receptors in a weak interaction based on the binding of the SH3 domain, and perhaps also the unique domain, of the kinase to specific sequences present in the cytoplasmic tail of the receptor. After ligand binding the receptors undergo ligand-induced dimerization. Activation of Src-like kinases might proceed in the same manner described above for the activation of Janus family kinases, i.e., receptor dimerization brings two different Src kinases into close proximity so they can phosphorylate the critical tyrosine residues present in the activation loop of the Src kinase. In this model, activation of Src is totally independent of Jak2. In the second model (Fig 3B), Src lies downstream of Jak. Receptor dimerization leads to the activation of Jak2, which results in the phosphorylation of multiple sites in the cytoplasmic tail of the receptor. The phosphorylation of one or more specific tyrosine residues in the cytoplasmic tail of the receptor now presents an alternative binding site for the SH2 domain which displaces the unique domain, of the kinase to specific sequences present in the catalytic activation loop of the Src kinase. Although the precise details of the binding of Hck to the β subunit of the IL-3 receptor are not known, how cytokine receptors interact with which protein tyrosine kinase(s) remains one of the most important issues in hematopoietic cell signaling.

Finally, understanding the molecular mechanisms that regulate activation of Src-like kinases and the roles of these kinases in mitogenesis will provide insights into novel therapeutic approaches that can be used to block proliferation of malignant or auto-reactive cells. Alternatively, this information can be used to develop approaches to stimulate hematopoietic cell proliferation in the absence of cytokines.

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