Minireview

Modulation of Cellular Signaling Pathways: Prospects for Targeted Therapy in Hematological Malignancies

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Abstract

The high remission rates observed in patients with chronic myelogenous leukemia who receive Imatinib mesylate (Gleevec) indicate that targeted therapy for hematological malignancies is achievable. At the same time, progress in cellular biology over the past decade has resulted in a better understanding of the process of malignant transformation, a better classification of subtypes of each disease on the basis of molecular markers, and a better characterization of the molecular targets for drug development. These advances have already spawned the development of such effective agents as monoclonal antibodies and specific enzyme inhibitors. This review attempts to provide a practical introduction to the complex and growing field of targeted therapy in hematological malignancies.

Introduction

Cellular proliferation, differentiation, and death are regulated by a number of extracellular molecules such as cytokines and hormones, as well as intercellular interactions mediated by neighboring cell surface antigens. These effectors mediate gene transcription either directly or indirectly by activating intracellular signaling pathways, which in turn activate appropriate cellular machinery (1). Cell-surface receptors that convert external stimuli into intracellular signals are pivotal in this signaling process. They activate intracellular pathways either through their inherent enzymatic function or as a result of their association with other catalytic proteins. Indeed, most growth factors and cytokines bind these receptors and exert their function through their activation, commonly by phosphorylation (1).

Normal hematopoiesis is dependent on intricately regulated signaling cascades that are mediated by cytokines and their receptors. Orderly function of these pathways leads to the generation of appropriate constellation of hematopoietic cells, and their abnormal activation results in neoplastic transformation, impaired apoptosis, and uncontrolled proliferation. Cytokines function in a redundant and pleiotropic manner; different cytokines can exert similar effects on the same cell type, and any particular cytokine can have several differing biological functions (2). This complexity of function is a result of shared receptor subunits as well as overlapping downstream pathways culminating in activation of common transcription factors (3).

The signal transduction cascades involve three major classes of proteins: kinases, adaptor or docking proteins, and transcription factors. Early insights into the cellular signaling pathways came from studies of IFN function (4–6). These experiments led to the identification of a family of nonreceptor TKs[2] called Jaks and their target proteins, Stats, which mediate gene transcription (4, 7). The Jak-Stat pathways are commonly activated during cytokine signaling through phosphorylation of specific tyrosine residues (3). The interaction of a cytokine with its receptor induces its tyrosine phosphorylation and leads to activation of downstream protein TKs including Jaks and Stats. Apart from their catalytic domain, protein TKs contain several other characteristic motifs including the SH2, SH3, and pleckstrin homology domain, which enable them to interact with other signaling molecules and propagate the message (8, 9).

The phosphorylation of serine and threonine residues is integral to the activation of numerous other intracellular proteins that mediate a number of other signaling pathways (10). Cytokine receptors without intrinsic kinase activity transmit their signals primarily through activation of Jak kinases. These receptors as well as those with intrinsic kinase activity, the RTKs, were previously thought to transmit their signals independently of the serine/threonine kinase cascades. More recently, it has been established that both of these pathways interact with serine/threonine kinase cascades such as the Ras/Raf/MEK/ERK (MAPK; Ref. 10). For example, after ligand binding, the β-subunit of IL-3 and GM-CSF receptors are phosphorylated and, through recruitment of adaptor proteins such as Shc, Grb2, and

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[2] The abbreviations used are: TK, tyrosine kinase; Jak, janus kinase; Stat, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinase; PKB, protein kinase B; PI3K, phosphatidylinositol 3′-kinase; PDGFR, platelet-derived growth factor receptor; TNF, tumor necrosis factor; SHP-1, SH-2 domain containing protein tyrosine phosphatase-1; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitor of activated stat; RTK, receptor tyrosine kinase; FLT3R, FMS-like tyrosine kinase 3 receptor; Grb2, growth factor receptor binding protein 2; RSK, ribosomal S6 kinase; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ALK, anaplastic lymphoma kinase; NPM, nucleophosmin; ATRA, all-

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Sos, activate the Ras signaling pathway (11). This in turn activates Raf followed by downstream activation of ERK1 and ERK2, and increased expression of transcription factors c-*fos* and c-*jun* (12–14). Other members of the MAPK family such as p38, and JNK/SAPK are also activated after phosphorylation of their serine/threonine residues as a result of cytokine/receptor interaction (15–18). Similarly, PI3K associates with the β chain of IL-3 receptor, recruits PKB/AKT by phosphorylation of its serine residues, and transmits cellular survival signals (19–21). Another downstream protein to IL-3 activation is the p70S6 kinase, which also interacts with the β chain and mediates appropriates signals (22, 23). Ultimately, these pathways influence gene transcription through their ability to recruit transcription factors, regulate apoptosis through the phosphorylation of apoptotic proteins, and cause the cell to progress through cell-cycle checkpoints by activation of specific kinases.

Of considerable interest is the description of a number of oncogenes with constitutive kinase activity. These molecules are derived from genes including *c-ABL*, *c-FMS*, *FLT3*, *c-KIT*, and *PDGFR*β, which are normally involved in the regulation of hematopoiesis (24). The kinase activity of the oncogene is constitutively activated by mutations that remove inhibitory domains of the molecule or induce the kinase domain to adopt an activated configuration (24). As a result of such constitutive activation a number of signaling cascades such as the Jak-Stat pathway, the Ras/Raf/MAPK pathway, and the PI3K pathway are activated.

With better characterization of aberrant signaling through the cell surface receptors and their downstream pathways in neoplastic cells, current research is exploring ways to reverse such dysregulated stimuli (25). Here, we will briefly review the role of cellular signaling pathways in normal cellular processes, neoplastic transformation, and development of hematological malignancies. We then explore the possible ways that their modulation can lead to clinically meaningful benefits.

Jak-Stat Signaling Pathways

Hematopoietic cell proliferation and differentiation is regulated by a number of soluble polypeptides such as IFNs, interleukins, and colony-stimulatory factors known collectively as cytokines (26). Cytokines bind to their cognate receptors and mediate downstream effects. A cytokine receptor consists of a unique ligand binding subunit as well as a signal transducing subunit, which may be structurally similar to the other cytokine receptors (27–29). On the basis of their characteristic structural motifs in their extracellular domains a number of subfamilies of cytokine receptors have been identified (27, 29, 30). These include the gp130 family, the IL-2 receptor family, the growth hormone receptor family, the IFN family, and the gp140 family of receptors (3). A detailed description of the structure of these receptors is beyond the scope of this review, but in general they consist of two or more subunits including the α, β, and γ chains (3, 27). For example, the IL-2 receptor family includes the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 each consisting of a ligand-binding α-subunit, and signal transducing β and γ subunits. Alternatively, the gp140 family including the receptors for IL-3, IL-5, and GM-CSF have a unique ligand-binding α-subunit and a common β (gp140) signal-transducing unit (3).

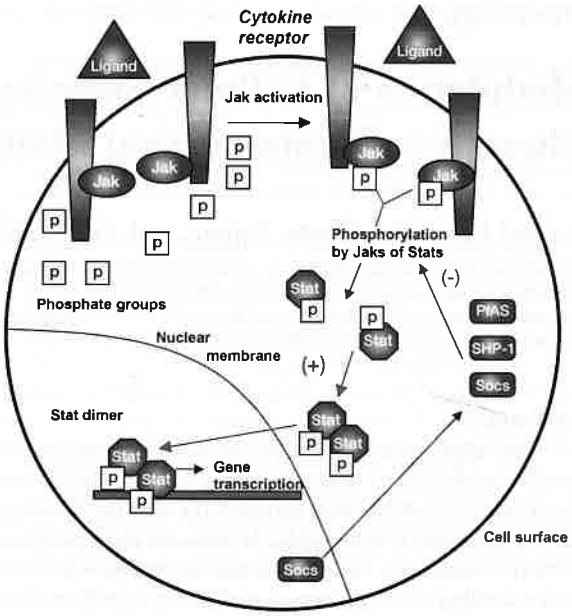

Fig. 1 The Jak-Stat pathway and its regulation.

Unlike growth factor receptors (RTKs), cytokine receptors do not possess a cytoplasmic kinase domain, and most cytokines transmit their signal by recruiting other TKs (3). Dimerization of the cytoplasmic component of the cytokine receptor as a result of ligand binding is the initial step in the initiation of cellular signaling (31). The dimerized subunits then associate with intracellular TKs such as members of the *Src* or Jak families of kinases, and the signal is propagated (Fig. 1). Different Src family members are associated with different receptors and phosphorylate distinct but overlapping sets of downstream target molecules. For example, *Lck*, *Lyn*, and *Fyn* can be activated by IL-2 (29, 32).

The Jak family of kinases comprises four known relatively large proteins (Jak1, Jak2, Jak3, and Tyk2) that can bind cytokine receptor subunits, phosphorylate them, and in doing so creat docking sites on the receptors for binding of SH2-containing proteins (33, 34). In general, Jaks consist of several domains (JH1-JH7) of which the functional significance has been characterized by mutational analysis and include a TK domain (JH1; Refs. 3, 33, 35, 36). The precise functions of JH2-JH7 domains are under current investigation (3). Jaks are able to associate with the cytokine receptors as well as with each other (37, 38). Dimerization/oligomerization of cytokine receptor subunits as a result of ligand binding leads to juxtaposition of Jaks (3). This results in transphosphorylation and activation of their kinase activity and the phosphorylation of downstream signaling proteins such as Stats, *Src*-kinases, and adaptors such as Shc, Grb2, and Cbl (Fig. 1; Refs. 39, 40).

Abnormalities of Jak function have been associated with a number of disorders (34, 41). For example, chromosomal translocations resulting in TEL-JAK2 constructs lead to the constitutive activation of Stat5, IL-3-independent cellular proliferation, and leukemogenesis (42, 43). The translocation t(9;12)(p24;p13) results in the fusion of the kinase catalytic

region of JAK2 with the transcription factor TEL generating the constitutively active TEL-JAK2. Similarly, infection with oncogenic viruses such as human T-cell lymphotrophic virus, type I, and Abelson murine leukemia viruses results in enhanced TK activity of Jaks, possibly accounting for their leukemogenic potential (44, 45).

The Stat transcription factors are coded by six known mammalian genes and include 10 different Stat proteins including different isomers of Stats 1, 3, 4, and 5 (3, 46). Like other transcription factors Stats have a well-defined structure including a DNA-binding domain, a conserved NH_2-terminal domain, a COOH-terminal transactivation domain, and SH2 and SH3 domains (3). Their activation through tyrosine phosphorylation results in their dimerization and translocation into the nucleus where they activate specific genes (6, 47–49).

Jak proteins activate a number of intracellular signaling proteins, among which Stats are the best defined (46, 50). Binding of a cytokine to its receptor rapidly induces tyrosine phosphorylation of the cytoplasmic domains of the receptor by activated Jak kinases, thus providing a docking site for Stat proteins, which are then phosphorylated. This phosphorylation of Stats leads to their homo- or heterodimerization and translocation to the nucleus, followed by DNA binding and gene activation (Fig. 1; Refs. 51, 52). The specificity for Stat phosphorylation is determined by the receptor docking sites and not the Jak kinases (53, 54). Also, different Stat proteins have different DNA-binding affinities, resulting in activation of specific genes. Stats also interact with other transcription factors such as the p300/cyclic AMP-responsive element binding protein family of coactivators to activate genes (55, 56). The transcriptional activity of Stats may also be regulated by the phosphorylation of their serine and threonine residues, although the implications of such regulation are not known (7, 57).

Stats mediate diverse and sometimes opposite cellular events affecting growth, differentiation, and apoptosis (58, 59). For example, Stats can mediate both growth arrest and cellular proliferation. Specifically, Stat1 mediates the growth-inhibitory effects of IFN-γ, through the induction of the CDKI $p21^{waf1}$, whereas Stat5 mediates proliferative effects of IL-3 and GM-CSF (60, 61). Similarly, phosphorylation of Stat3 can result both in IL-6- and IL-10-induced growth arrest, and in GM-CSF- and IL-3-induced proliferation (61–63). Stats also modulate cellular differentiation and apoptosis. Reconstitution of Stat1 in Stat1-null U3A cells (which do not respond to TNF-α) restores basal caspase expression and renders them sensitive to TNF-induced apoptosis (64). Conversely, Stat3 and Stat5 mediate the anti-apoptotic effects of IL-6 and IL-2, respectively (65, 66). Stat1 activates the caspase cascade through up-regulation of Fas and FasL expression in response to IFN-γ (67). The exact mechanisms underlying these diverse effects are being elucidated.

An important property of cellular signaling pathways is that their activation is both rapid and transient. This is because of effective mechanisms of inactivation. In the Jak-Stat system, proteasome-mediated degradation, tyrosine dephosphorylation, and inhibition by various inhibitory proteins are responsible for this process (4). The ubiquitin-proteasome pathway governs the degradation of many intracellular proteins including activated Stats, and effective inhibitors of this system have shown promising early results in clinical trials (68, 69). The cytokine-activated Jak-Stat pathways are also inhibited by tyrosine dephosphorylation mediated by cytoplasmic phosphatases such as SHP-1 (70, 71). SHP-1-deficient mice demonstrate multiple hematopoietic abnormalities, including hyperproliferation and abnormal activation of granulocytes and macrophages in the lungs and in the skin (72). SOCS and PIAS are other important inhibitors of the activated Jaks and Stats (70, 73). Recent studies have established that SOCS are negative regulators of cytokines, and there is ample evidence suggesting the importance of Stats in the induction of SOCS expression, thereby constituting a negative feedback mechanism (74–76). The role of these inhibitory proteins in the pathogenesis of neoplastic transformation is also becoming clearer (77).

RTK and Serine/Threonine Signaling Pathways

RTKs are membrane-bound enzymes with an extracellular ligand-binding domain, a transmembrane domain, and a highly conserved intracellular domain that mediates the activation, through tyrosine phosphorylation, of a number of downstream signaling proteins (78–80). These enzymes are activated by ligand binding, by cell-cell interactions via cell adhesion molecules, and by stimulation of G-protein coupled receptors (81). Phosphorylated tyrosine residues in specific domains of these receptors serve as high-affinity docking sites for SH2-containing adaptor and effector proteins (82). RTKs include diverse molecules, which are considered as members of several distinct classes: class I including epidermal growth factor receptor; class II including insulin-like growth factor-1 receptor; class III including PDGFR, macrophage colony-stimulating factor (FMS-R or CSF-1R), stem cell factor receptor (KIT), and FLT3R; and class IV including FGFR (79, 83, 84). The importance of these receptors in malignant transformation and the possibility of modulating them as therapeutic targets are subjects of intense research. The recent reports of constitutive activation of FLT3R resulting in stimulation of multiple signaling pathways and leading to malignant transformation has been of significant interest in leukemia research (85, 86). Such constitutive activation of this receptor has been reported in >30% of patients with AML and results from two well-described molecular events. Internal tandem duplication mutations of *FLT3R* gene occur at exons 11 and 12 of the gene that code for the juxtamembrane domain of receptor (87–90). More recently, point mutations of codon 835 of *FLT3R* receptor gene, located in the activation loop of its TK domain, have been reported in 7% of patients with AML (87, 91). Inhibitors of such aberrant activation are undergoing clinical evaluation (92, 93).

Many intracellular signaling proteins bind the phosphotyrosine on the activated RTKs. These proteins include GTPase activating protein, PI3K, Grb2, and *Src*-like tyrosine-kinases (1, 10, 78). The activation of these proteins by serine/threonine phosphorylation in turn activates a number of downstream signaling cascades that lead to gene transcription (10).

Although knowledge of the Jak-Stat pathway has been instrumental in understanding cytokine signaling (94), the importance of signaling cascades that involve the activation of serine/threonine kinases is increasingly apparent (10). The serine/threonine MAPKs, which include the Ras-Raf-MEK-ERK pathway, the p38 family of kinases, and the JNK (SAPK)

family, are activated by upstream signals and mediate effects on inflammation, cell growth, cell cycle progression, cell differentiation, and apoptosis (95). The Ras family of proteins belongs to the large superfamily of GTPases that localize to the inner surface of the plasma membrane (1, 96). Ras proteins play a pivotal role in a number of signaling pathways mediated by RTKs and other receptors. Ligand binding to these receptors initiates the autophosphorylation of specific tyrosine residues in their cytoplasmic domain and creates phosphotyrosyl-binding sites for adapter proteins such as Shc and Grb2, which in turn recruit guanine nucleotide exchange factors and thereby initiate Ras activation (97, 98).

Once induced, Ras activates Raf serine/threonine kinase, which then phosphorylates MAPK kinases (otherwise known as MEKs; Refs. 10, 97, 99). These in turn activate MAPKs (or ERKs; Refs. 100, 101), which in turn move to the nucleus where they phosphorylate and activate nuclear transcription factors such as Elk-1 (102). ERKs were initially described as a novel family of protein kinases that, when activated, produced proliferative stimuli (103). ERKs can also activate other kinases such as RSKs (also known as MAPK-activated protein kinases), which are involved in cell-cycle regulation and apoptosis (104). ERK-activated RSK kinase catalyzes the proapoptotic protein Bad and suppresses Bad-mediated apoptosis (105). Similarly, the Ras-Raf-MEK-ERK cascade modulates cellular proliferation by regulating the activity of several proteins, including cell-cycle regulators (*e.g.*, cyclin D1, $p21^{waf1/cip1}$, $p27^{kip1}$, and cdc25A) and transcription factors (*e.g.*, c-Myc; Ref. 106).

The G_1/S cell cycle checkpoint is a critical point determining the commitment of cells to growth arrest or proliferation. During this stage cells are responsive to cytokines (107). Regulatory proteins $p21^{waf1/cip1}$ and $p27^{kip1}$ are of particular importance in this transition, which is controlled by both positive and negative regulators. Distinct Rb-E2F repressor complexes suppress the transcription of genes required for progression of various phases of cell cycle. For example, Rb-E2F1 complex suppresses the progression through G_1 (108). During this progression from G_1 to S-phase cyclin/CDKs are sequentially activated, which then inactivate suppressor complexes such as Rb-E2F1 (109). Cyclin/CDK activity results in Rb phosphorylation and its dissociation from E2F1 leading to activation of genes necessary for S phase (110). Activity of a number of these cyclin/CDKs as well their inhibitors such as $p21^{waf1/cip1}$ is modulated by cytokine-mediated signals through their phosphorylation.

The p38 family of MAPKs is involved in various cellular processes such as inflammation, cell cycle progression, and cell death (111, 112). The four different p38 isoforms (α, β, γ, and δ) are activated by two MEK isoforms (113). Originally, the p38 kinase pathway was reported to have a critical role in the generation of signals in response to stress stimuli. However, its role in cytokine signaling and regulation of the Jak-Stat pathway has been elucidated recently (114). In particular, from the standpoint of leukemogenesis, it modulates the growth-inhibitory effect of type I IFNs in BCR-ABL-expressing cells as well as normal hematopoietic progenitors (115, 116).

The third group of MAPKs includes the JNK (otherwise known as SAPK; Ref. 95). The four different JNK kinases have a similar role to p38 kinases in cellular function and are activated by specific MAPK kinases (MEKKs) in response to inflammatory cytokines such as TNF-α, and other stress stimuli such as reactive oxygen species, heat, and withdrawal of growth factors (117). The MEKK1/JNK signaling increases p53 stability and transcriptional activation, and MEKK1/JNK potentiates the ability of p53 to initiate apoptosis (118).

Normal functioning of MAPK-mediated signaling necessitates its efficient inactivation (95). A number of dual-specificity MAPK phosphatases serve to dephosphorylate and, hence, inactivate MAPKs (119–121). Similarly, protein phosphatases PP1 and PP2 dephosphorylate and inactivate a number of phosphoproteins including components of the MAPK pathway (122, 123).

Other signaling pathways such as those mediated by PI3K, AKT (also known as PKB), and protein kinase C are also controlled by serine/threonine phosphorylation (Fig. 2; Ref. 10). PI3K consists of two subunits, the p85 regulatory subunit and the p110 catalytic subunit (124, 125). The p85 subunit binds to the cytokine receptor as a consequence of ligand-receptor interaction and receptor autophosphorylation (126). As a result, phosphatidylinositol-dependent kinases and their downstream substrate AKT/PKB are recruited to the membrane (127). PI3K-AKT pathway activates several downstream targets including p70 RSK, forkhead transcription factors, and NFκB (128–130). The serine/threonine kinase AKT is an important component of the cell survival machinery (10, 131–133). Its activation via the PI3K pathway leads to a number of events (10, 131, 134, 135). For example, the phosphorylation of the cytosolic protein IκB by AKT releases NFκB from its association with IκB. NFκB then moves into the nucleus, where it induces a number of genes involved in cell survival (131). Meanwhile, the inhibitory protein IκB is degraded by the proteasome (136). AKT also phosphorylates the proapoptotic protein Bad, which leads to higher levels of free antiapoptotic Bcl-x_L and thereby inhibits the cell-death protease caspase-9 (134). The tumor suppressor gene *PTEN* codes for a phosphatase that acts by removing a phosphate group from the 3 position of the inositol ring of the $PIP_{3,4,5}$ phospholipids located at the cellular membrane. This prevents the proximation of AKT and phosphatidylinositol-dependent kinases, and prevents AKT activation (137–140). Several lines of evidence including studies of PTEN knockout mice support the role of *PTEN* as a tumor suppressor gene (141). Serine/threonine kinases, in general, also influence the activity of other antiapoptotic proteins of the Bcl-2 family (10, 135, 142). In the normal cell cycle, Bcl-2 is phosphorylated on its serine/threonine residues at several points during the G_2 to M phase transition (10, 143).

PKC, another important signaling enzyme, phosphorylates specific serine or threonine residues on target proteins in different ways (Fig. 2). For example, PKC is a potent activator of Raf-1, which activates the MAPK cascade. This leads to phosphorylation of IκB, release of NFκB, translocation of NFκB into the nucleus, and gene transcription as described above (144–146). PKC also regulates cytokine signals through its effects on the Jak-Stat pathway in some myeloid progenitor cell lines (147). The significant role of PKC in phosphorylation and activation of Raf has led to its targeting for inhibition of the Raf-Ras-MEK-ERK pathway (95, 148). For example, stauros-

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