Biochemical approaches to discovering modulators of the JAK–STAT pathway

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Regulation of cytokine activity has application in the treatment of numerous diseases, as illustrated by the successful clinical use of recombinant cytokines. The discovery of two key families of signaling proteins – the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs) – has greatly increased our understanding of cytokine signal transduction. This review describes some of the key interactions between JAKs, STATs and other components of the signal transduction cascade, and the potential for developing high-throughput biochemical screens for the detection of small molecules that target these interactions. Novel compounds of this type have potential as agonists or antagonists of cytokine action.

Cytokines and growth factors form an extensive family of secreted proteins that regulate the growth and differentiation of numerous cell types. In the hematopoietic and immune systems, cytokines act to expand populations of progenitor cells, promote differentiation along particular pathways and regulate the function of terminally differentiated cells[1]. These activities provide an organism with a mechanism for meeting various environmental challenges by adjusting both the number and activity of multiple cell types in the blood.

Altering cytokine activity to treat disease

Modulation of cytokine activity provides a powerful tool for the treatment of a variety of diseases. Several cytokines already have clinical application, exemplified by the interferons (IFNs) and colony-stimulating factors (CSFs)[2–7].

The biological activities of the IFNs include antiviral, antitumor and immunomodulatory effects. The stimulatory effects of IFN-α on the immune system have been exploited to treat chronic hepatitis B and C viral infection. IFN-α is also used to treat a variety of cancers, including hairy cell leukemia, chronic myeloid leukemia, cutaneous T-cell lymphoma, multiple myeloma, Kaposi's sarcoma and malignant melanoma. IFN-β is approved for treatment of multiple sclerosis, although the mechanism through which it exerts a beneficial effect is unknown. The current estimated world-wide interferon sales (IFN-α and IFN-β) are more than $1.8 billion.

The CSFs are involved in the proliferation, differentiation and activation of hematopoietic cells, and include three important and profitable cytokines: erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF). EPO is responsible for the maturation of red blood cells and is approved for treatment of a variety of anemias, including anemia associated with chronic renal failure[3,4]. The estimated world-wide sales of EPO are in excess of $2.5 billion. G-CSF and GM-CSF are used to treat neutropenia associated with chemotherapy and myeloid recovery following bone-marrow transplantation[5–7]. These CSFs are also used to mobilize stem cells into the circulation, where they can be harvested and used for transplantation as an alternative to

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bone marrow. Finally, CSFs are used to treat diseases associated with bone-marrow failure. Current world-wide sales of G-CSF and GM-CSF are estimated at almost $2 billion annually.

Development of small-molecule mimics of the IFNs and CSFs would be advantageous since they could be administered orally rather than by injection, as required for protein therapeutics. Furthermore, the costs of producing small molecules may be significantly less than the costs of producing protein cytokines.

Antagonists of specific cytokines are likely to be useful in treating numerous diseases of immune dysregulation, particularly chronic inflammatory disease, asthma, allergy and graft rejection[8–10]. A number of protein cytokine antagonists (antibodies, soluble receptors and receptor antagonists) are in clinical development for these conditions[11]. Small-molecule cytokine antagonists are likely to be even more useful clinically. However, there are few examples of these currently in use. Nevertheless, small molecule antagonists of interleukin 2 (IL-2) signal transduction, such as rapamycin, have proved a significant aid to transplant surgery because of their ability to prevent graft rejection through down modulation of the cellular immune response[12].

IL-2, IFN-γ and IL-12 are involved in the maturation of the T helper 1 (Th1)-cell subset[13]. Th1 cells are characterized by production of tumor necrosis factors α (TNF-α), TNF-β, IFN-γ and IL-2. Th1 cells are associated with cell-mediated immunity, and are thus involved in chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, and in graft rejection[14,15]. Small molecules capable of inhibiting the activity of IFN-γ or IL-12 could selectively abrogate the Th1 response and the diseases associated with Th1-mediated action. Evidence that specific cytokine blockades can blunt Th1-mediated disease states has been provided by animal models in which antibodies are used to neutralize IFN-γ (Ref. 9).

Similarly, antagonists of the cytokines that cause maturation of T cells to the Th2 subset are also likely to be medically useful in the treatment of diseases that are related to the humoral or antibody immune response, such as allergy and asthma[14–16]. IL-4 plays a key role in Th2 maturation, and these cells are thought to activate B cells to produce immunoglobulins such as IgE (Ref. 17). In addition, IL-4 acts directly on B cells to promote Ig class switching from IgM to IgG or IgE (Ref. 17). Thus, IL-4 controls the two critical steps in the production of IgE, the mediator of allergy, and IL-4 antagonists are therefore good candidates for the treatment of allergy.

As described below, the similarity in the mechanisms through which many cytokines activate signal transduction makes it possible to envisage approaches towards the development of small molecule modulators of cytokine signal transduction that are broadly applicable. On the basis of the currently approved uses of recombinant cytokines, and the many possible uses of cytokine antagonists, the potential market for such small-molecule cytokine modulators is enormous.

Cytokine signaling via JAKs and STATs

Cytokines and growth factors exert their effects by binding to specific cell-surface receptors that belong to several families depending on conserved sequence and functional motifs[18,19]. These include the intrinsic tyrosine kinase receptors, such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and stem cell factor (SCF) receptors, the TNF receptor family, which includes receptors for TNF and Fas ligand, and the type I and type II cytokine receptors such as the EPO, G-CSF, growth hormone (GH) and IFN receptors (Figure 1). Although some members of

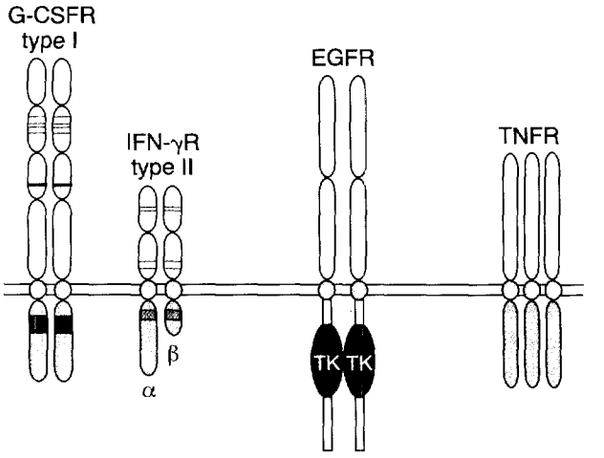

Figure 1. *Domain structure of three families of cytokine/growth factor receptors. Shown are type I and type II cytokine receptors (G-CSFR and IFN-γR, respectively), an intrinsic tyrosine kinase receptor (EGFR) and TNFR. Thin horizontal red lines in the type I and II cytokine receptors represent conserved cysteines; the thick horizontal black line is the WSXWS motif. EGFR, epidermal growth factor receptor; G-CSFR, granulocyte colony-stimulating factor receptor; IFN-γR, interferon γ receptor; TK, tyrosine kinase domain; TNFR, tumor necrosis factor receptor.*

the intrinsic tyrosine kinase receptor family can activate signal transducers and activators of transcription (STATs), this review will focus on type I and type II cytokine receptors, because it is largely in this context that signaling via Janus kinases (JAKs) and STATs has assumed its current prominence.

Type I cytokine receptors are characterized by the presence of one or more copies of a WSXWS motif in the extracellular segment of the receptor, together with a characteristic pattern of cysteine residues[19,20]. Type II receptors lack the WSXWS motif, but also have a distinctive pattern of cysteine residues in the extracellular domain (Figure 1). In the past few years, our understanding of the processes by which these classes of receptors mediate signal transduction has increased substantially. Most significant was the discovery the JAKs and STATs, which provide a key information conduit from the receptor at the membrane to genes in the nucleus[21]. Although initially discovered in the context of interferon regulation of gene expression, it rapidly became apparent that numerous cytokines signal via activation of both JAKs and STATs. JAKs, a novel class of cytoplasmic tyrosine kinases, are thought to mediate many of the rapid tyrosine phosphorylation events that occur after binding of a cytokine to its receptor, thereby triggering multiple downstream signal transduction pathways. STATs, a family of latent transcription factors presumed to be substrates for the JAKs, acquire the ability to bind DNA and regulate gene expression after phosphorylation on tyrosine.

The fundamental steps in this signal transduction pathway are outlined in Figure 2. Binding of cytokine to its receptor triggers oligomerization of receptor chains, which brings JAKs associated with the cytoplasmic face of the receptor chains into close proximity. JAK activation then occurs, probably by *trans*-phosphorylation on tyrosine residues. A specific tyrosine in the kinase domain of JAK2 has been implicated in regulation of its catalytic activity[22]. Activated JAKs are then thought to phosphorylate multiple tyrosines on the receptor chains. These phosphotyrosines serve as the binding sites for a variety of signal transduction molecules, such as Shc, the regulatory p85 subunit of phosphoinositide 3-kinase, phosphatases and STATs. These proteins often become phosphorylated on tyrosine after association with the receptor, possibly by the direct action of JAKs. Phosphorylation of STATs causes them to form dimers, translocate to the nucleus and bind to specific DNA sequences present in the promoters of cytokine-responsive genes. Changes in the transcription of these genes alters the complement of proteins present in a cell, resulting in changes in cellular phenotype.

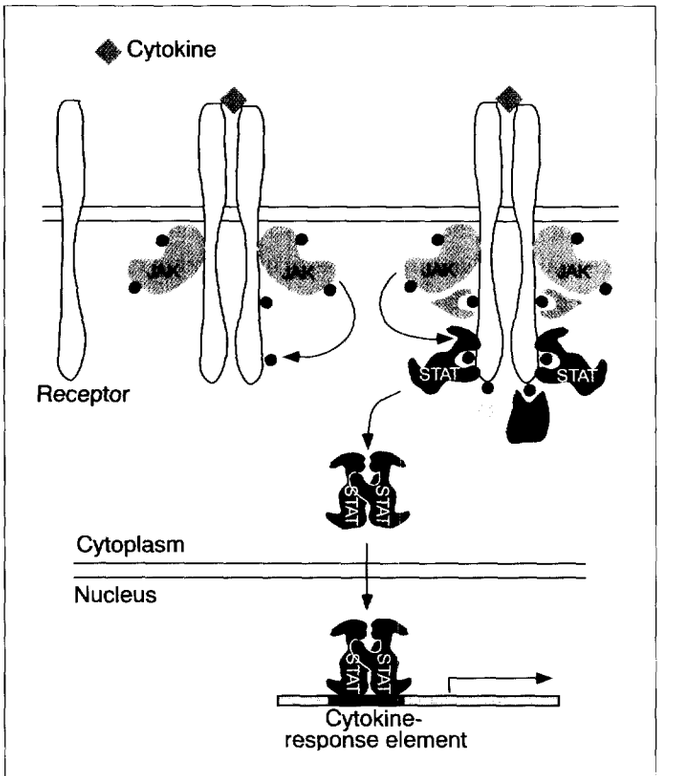

Figure 2. *Activation of the JAK–STAT pathway by cytokines. Cytokine binding to its receptor triggers receptor oligomerization, activation of Janus kinases (JAKs) and tyrosine phosphorylation of receptor chains. Multiple signaling proteins, including the signal transducers and activators of transcription (STATs), bind to the phosphorylated receptor chains and become activated. Activated STATs dimerize, translocate to the nucleus and alter the expression of cytokine-regulated genes. Red circles represent phosphotyrosine residues.*

Structure and function of JAKs and STATs

A considerable amount of work has been directed at defining the functional domains of both JAKs and STATs. Several activities have now been mapped to distinct domains of each family of proteins. The results of these studies are summarized in Figure 3.

Four members of the JAK family have been described: JAK1, JAK2, JAK3 and Tyk2 (Refs 23–28). Alignment of their amino acid sequences reveals the presence of a series of conserved JAK homology (JH) domains, JH1–JH7. The most

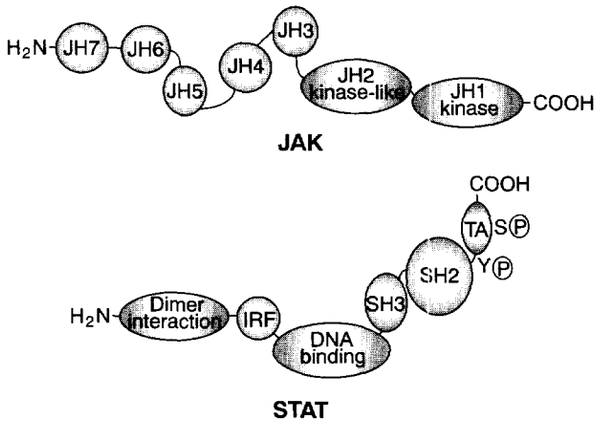

Figure 3. *Domain structure of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). IRF, interferon-regulatory factor; JH, JAK homology domain; P, phosphate; SH, src-homology domain; TA, transcriptional activation region.*

C-terminal domain, JH1, is responsible for the tyrosine kinase activity of the protein[23]. Adjacent to JH1 is a related domain, JH2, which has some of the features of a kinase domain but does not have enzymatic activity, and its function is not understood. The remainder of the protein contains five blocks of homology (JH3–JH7) for which distinct activities have not been assigned. They may be involved in interactions between JAKs and cytokine receptors or other signaling proteins.

The STAT family consists of seven members: STAT1, 2, 3, 4, 5a, 5b and 6, encoded by distinct genes[29–37]. Alternative splicing of several of the STATs leads to the production of additional isoforms (Refs 29,38,39). Comparison of the amino acid sequences of the STAT proteins reveals a series of conserved domains. A region of homology, 100 amino acids in length, starting close to the N-terminus has recently been shown to mediate interactions between dimers of STATs (Refs 40,41). As discussed below, this interaction stabilizes the binding of STAT dimers to pairs of weak STAT-binding elements found in the promoters of certain cytokine-responsive genes. A region adjacent to this has been identified that mediates the interaction of STAT1 with p48, a protein that is a member of the interferon-regulatory factor (IRF) family[42]. Together with STAT1 and STAT2, p48 forms the IFN-α-induced DNA-binding complex ISGF3. The conservation of this region in other STATs, and the existence of multiple members of the IRF family, raises the intriguing possibility that additional STAT–IRF interactions remain to be discovered.

A novel domain of 110 amino acids lies in the center of the protein. This domain confers DNA-binding specificity on the STATs and is presumed to be the region that contacts DNA (Refs 43,44). Exchanging this region of STAT1 for the equivalent region of STAT3 results in a chimeric protein with the DNA-binding specificity of STAT3. The primary sequence and predicted secondary structure of this domain are unlike DNA-binding domains of any previously studied DNA-binding protein.

A region with some similarity to *src*-homology 3 (SH3) domains lies C-terminal to the DNA-binding domain, but it has not been assigned any function to date. Downstream of this region is an SH2 domain[45]. Several studies have shown that this phosphotyrosine-binding domain is required for dimerization of STAT proteins (and hence is important for DNA binding) and for interaction with the appropriate cytokine receptors (described in more detail below)[46–48]. The critical tyrosine residue required for STAT activation, and the presumed target of JAK kinases, is located immediately following the SH2 domain.

Finally, although there is limited primary sequence homology in the C-terminal section of several STATs, this region is involved in transcriptional activation[49–51]. A serine residue present in this region of some of the STATs is thought to be the target of phosphorylation by mitogen-activated protein (MAP) kinases[52,53]. Phosphorylation at this site increases transcriptional activation by STATs (Ref. 52). STATs lacking this region, either through artificial mutation or alternate splice site selection, are still able to bind DNA upon tyrosine phosphorylation, but no longer induce gene expression[39,54,55].

STAT function as revealed by inactivating mutations in mice

Inactivating mutations in selected *Stat* genes produced in mice by homologous recombination ('knockout mice') have illustrated the important role that STATs play in cytokine signaling, and have also revealed a surprising degree of selectivity in the actions of different STATs. Inactivation of the *Stat1* gene results in mice with a remarkably specific loss of responsivity to IFN action, even though STAT1 is activated by several cytokines in addition to the IFNs (Refs 56,57). Inactivation of the *Stat4* gene results in loss of responsiveness to IL-12 (Refs 58,59). In *Stat4*-deficient mice,

Th1-cell differentiation is markedly inhibited, consistent with a central role for STAT4 in IL-12 signaling, and therefore in T-cell development. *Stat6*-knockout mice lose many of their responses to IL-4, and do not produce IgE antibodies[60–62]. The phenotypes of the *Stat1*-, *Stat4*- and *Stat6*-knockout mice demonstrate that these STATs play a key role in mediating the effects of these cytokines, and are therefore likely to be excellent targets for compounds useful in the modulation of inflammatory processes. *Stat5a*- and *Stat5b*-knockout mice also have highly selective phenotypes. *Stat5a*-deficient mice develop normally, but fail to lactate, perhaps as a result of defects in prolactin signaling[63]. *Stat5b*-deficient mice are defective in male-characteristic body growth rates and male-specific liver gene-expression patterns, consistent with defects in growth hormone signaling[64]. For reasons that are not understood, *Stat3*-deficient mice die early in embryogenesis[65]. *Stat2*-knockout mice have not yet been reported.

Exploiting key protein–protein interactions

The JAKs and STATs normally function as part of a large multiprotein complex that assembles at the cytoplasmic face of cytokine receptors shortly after a cytokine binds to the extracellular domain[66]. The best characterized of these protein–protein interactions provides the opportunity to develop highly efficient high-throughput biochemical screens to detect compounds that modulate the assembly of these complexes, and therefore the activity of JAKs and STATs. In this section, we focus on a few well-characterized interactions that lend themselves to the development of biochemical assays that can detect either antagonists or agonists of specific cytokine signal transduction pathways. Table 1 summarizes these, as well as other potential high-throughput biochemical assays that could be contemplated on the basis of our current knowledge of JAK–STAT signaling. Assays of this type have the advantage of being very efficient in terms of throughput, they do not require compounds to cross the cell membrane and they yield compounds with known mechanisms of action. However, they do require detailed knowledge of the target and the ready availability of recombinant proteins.

Table 1. Potential biochemical assays that could be developed to discover modulators of the JAK–STAT signaling pathway

Assay	Type	Detects
STAT receptor	SH2 domain–phosphopeptide	Antagonists
STAT dimerization	SH2 domain–phosphopeptide	Antagonists
STAT dimer interaction	Protein–protein	Antagonists (partial)
STAT DNA	Protein–DNA	Antagonists
JAK receptor	Protein–protein	Antagonists
JAK activity	Enzymatic	Antagonists
Cytokine receptor	Protein–protein	Antagonists
Phosphatase receptor	Protein–protein	Agonists
Phosphatase activity	Enzymatic	Agonists
Receptor receptor	Protein–protein	Agonists/antagonists

JAK, Janus kinase; SH2, *src*-homology 2; STAT, signal transducer and activator of transcription.

Interactions involving STATs

The interaction of STATs with several cytokine receptors has been studied in some detail. One general theme that has emerged is that STATs can interact with one or more phosphotyrosine residues on the cytoplasmic face of the receptor via their SH2 domains. For example, single phosphotyrosine residues on the IFN-γ or IFN-α receptor chains have been identified that interact with STATs 1 and 2, respectively (Refs 67,68). Mutation of these tyrosines to phenylalanines, preventing phosphorylation at these sites, blocks activation of the requisite STATs and inhibits other downstream actions of the IFNs. Multiple phosphotyrosines, embedded in the consensus sequence YXXQ, on the gp130 component of the receptors for IL-6, leukemia inhibitory factor and oncostatin M are responsible for interacting with STAT3 (Refs 66,69). A short peptide containing this motif is sufficient to direct STAT3 activation when appended to a receptor that does not normally activate STAT3. The receptor sequences known to mediate STAT docking to receptors are summarized in Table 2. The STAT SH2 domains can also provide specificity with respect to targeting STATs to particular receptors[47,48]. Exchanging the SH2 domain of STAT1 for that of STAT2 yields a chimeric protein that is targeted to the IFN-α receptor instead of the IFN-γ receptor, demonstrating the key role that the SH2 domain plays in receptor selectivity[47].

In several of these studies it was shown that short phosphotyrosine-containing peptides of the appropriate sequence can inhibit association of the STAT with its cognate receptor, suggesting that it might be possible to find small molecules capable of disrupting the interaction between STATs and receptors. Such compounds would act as novel antagonists of cytokine signaling. Most

Table 2. Peptide sequences that mediate STAT interactions with cytokine receptors

Receptor	Peptide sequence	STAT	Refs
IFN-γ,-α	GY*DKPH	1	67
IFN-α R1	NY*VFFP	2	68
hgp130	SY*LPQT	3	
	GY*RHQV		66,69
	QY*FKQN		
	GY*MPQ		
LIFR	MY*QPQ	3	66
hG-CSF	TY*VLQG	3	66
hIL-9	AY*LPQE	1, 3, 5	70
hIL-2R β-chain	AY*LSLQ	5	71
hIL-7	AY*VTMS	5	71
hEPO	TY*LVLD	5	
	LY*LVVS	5, 1	72–74
	GY*VACS		
hIL-4	GY*KAFS	6	36
	GY*KPFQ		

EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; h, human; IFN, interferon; IL, interleukin; LIF, leukemia inhibiting factor; R, receptor; STAT, signal transducer and activator of transcription; Y*, phosphorylated tyrosine.

attractive as targets are the STATs that have a relevant interaction with only one receptor, as this restriction provides inherent specificity to their antagonists. This applies to STAT1 (IFN-γ receptor), STAT4 (IL-12 receptor) and STAT6 (IL-4 receptor). The data using *Stat*-knockout mice described earlier strongly support the notion that antagonists of these STATs would show selectivity for their cytokine signaling pathways. The peptide competition and mutagenesis data discussed above also indicate that the STAT–receptor interaction requires only a short segment of the receptor and just the SH2 domain of the STAT. This greatly facilitates the configuration of a biochemical assay that mimics this interaction, and suggests that it is reasonable to expect to find small molecules able to disrupt it. The receptor component can conveniently be provided by using a synthetic phosphotyrosine-containing peptide corresponding to the STAT docking site of interest. The STAT can be full-length or a fragment that includes the SH2 domain, and can be overexpressed and purified from a variety of available high-expression systems in bacteria, yeast or insect cells. The assay could be configured in many different ways to allow detection of the STAT interacting with phosphopeptide[75,76]. One configuration is described below in the context of a phosphatase–receptor interaction. Antagonists would then be scored on the basis of their ability to block the signal resulting from STAT–phosphopeptide interaction.

After tyrosine phosphorylation of STATs at the critical residues in the C-terminal region, they assemble into homo- or heterodimeric complexes, which in some cases may include additional non-STAT proteins. Dimerization of STATs also requires an intact SH2 domain as this process is mediated through a pair of SH2–phosphotyrosine interactions that can be disrupted by short phosphotyrosine-containing peptides derived from the STATs themselves[46]. With regard to assay development, this is very similar to the STAT–receptor interaction described above, and a similar assay could be set up to detect antagonists of STAT–STAT dimerization. Indeed, compounds that prevent STAT–receptor interaction by binding to the SH2 domain of a STAT may also inhibit STAT dimerization.

Recently, a second mode of STAT–STAT interaction was identified, involving the N-terminal 100 amino acid region of the STATs – a region of conservation among the STAT family members[40,41]. DNA footprinting and gel retardation of wild-type or N-terminally truncated STATs to DNA probes containing adjacent weak STAT-binding sites showed that the N-terminal region is required for occupation of these sites by STAT dimers. However, this domain is not required for binding to DNA probes containing strong STAT-binding sites. This suggests that the N-terminal region mediates cooperative binding to weak sites by stabilizing interactions between STAT dimers. This view is further supported by an experiment in which addition of a purified 100 amino acid fragment from the N-terminal region of a STAT to a DNA-binding reaction disrupted the binding of STAT dimers to a DNA fragment containing two adjacent weak sites, but not to a DNA fragment containing strong sites[40]. These observations suggest that it may be possible to find inhibitors of this interaction that would selectively inhibit binding of STATs to a subset of its normal target sites in the control regions of genes. Compounds of this type would be partial antagonists of STAT action. The potential biological effects of such compounds are at present unknown.

Interactions involving phosphatases

The pivotal role of tyrosine phosphorylation in the activation of cytokine signaling, and its transient nature, indicates that tyrosine phosphatases are important regulatory components serving to terminate and dampen the signals initiating at cytokine receptors through the removal of key phosphate groups on signaling proteins. Experiments with vanadate, a

general tyrosine phosphatase inhibitor, indicate that inhibition of tyrosine phosphatase activity can both trigger and prolong activation of JAKs and STATs (Refs 77,78). A careful study examining the turnover of STAT protein and tyrosine phosphate on STATs has also concluded that phosphatases are involved in the inactivation of STATs (Ref. 79). Therefore, small molecules that target the activity of specific phosphatases or interfere with their localization may trigger the selective activation of JAKs in the context of a specific receptor, and thus act as cytokine agonists or potentiators.

The nature of the phosphatases involved in negative regulation of cytokine signaling is still the subject of investigation. The SH2-domain-containing phosphotyrosine phosphatase 1 (SHP-1) is currently one of the best understood[80]. This protein contains a tyrosine phosphatase catalytic domain linked to tandem SH2 domains with different substrate specificities[80,81], which allows it to dock with a variety of tyrosine-phosphorylated substrates, including EPO receptor, IL-3 receptor β-chain, CD22, FcγR1, Ly-49A and ZAP-70.

The role of SHP-1 in cytokine signaling is most graphically demonstrated by a series of studies using motheaten mice, which have mutations in their *hcph* gene that result in a defect in SHP-1 activity[82,83]. These mice have multiple hematopoietic abnormalities, and cells from these animals have altered responses to a number of cytokines, including EPO, G-CSF and IFN-α (Refs 84–86). Monocytes from motheaten animals show a greatly increased activation of STAT1 in response to IFN-α compared with monocytes from normal animals, suggesting that SHP-1 normally plays a role in downregulating IFN-α activation of STAT1. Receptor mutagenesis experiments also support a negative regulatory role for SHP-1 in cytokine signaling. Cells expressing mutated versions of the EPO receptor that lack the SHP-1 interaction site are hypersensitive to the effects of EPO, and show prolonged JAK2 phosphorylation after EPO stimulation[87]. A model of the role of SHP-1 in EPO signaling is shown in Figure 4. These studies suggest that inhibitors of SHP-1 catalytic activity or localization to the EPO receptor may act as agonists or potentiators of EPO signal transduction, respectively. The latter mechanism is particularly attractive because it provides an opportunity to inhibit some of the actions of SHP-1 selectively. Because the two SH2 domains of SHP-1 are involved in localization to different substrates, targeting one domain rather than the other will result in inhibiting only a subset of the actions of SHP-1. Mutagenesis and peptide competition experiments show that short phosphotyrosine-containing peptides constitute the binding sites for SHP-1, allowing the use of such peptides as receptor surrogates in high-throughput biochemical assays. An example of an assay of this type is shown in Figure 5. In this assay, a phosphotyrosine-containing peptide derived from the EPO receptor is immobilized in the wells of a 96-well plate, then incubated in the presence of test compounds with a recombinant fusion protein that includes the SH2 domains of SHP-1. After washing to remove unbound fusion protein, bound protein is detected using a two-step ELISA. Phosphopeptides that interact with the SH2 domain but do not bind to the plate serve as model inhibitors in the assay and, as expected, prevent binding of fusion protein to the plate. Likewise, small molecules that prevent the interaction of the fusion protein with the phosphopeptide are easily identified (Figure 5). Such compounds are then tested in secondary assays that allow detection of compounds with non-specific effects on protein structure. After elimination of these 'nuisance' compounds, the remaining leads can be tested to determine their selectivity with respect to other SH2 domain–phosphopeptide interactions, and for their effects on EPO signal transduction.

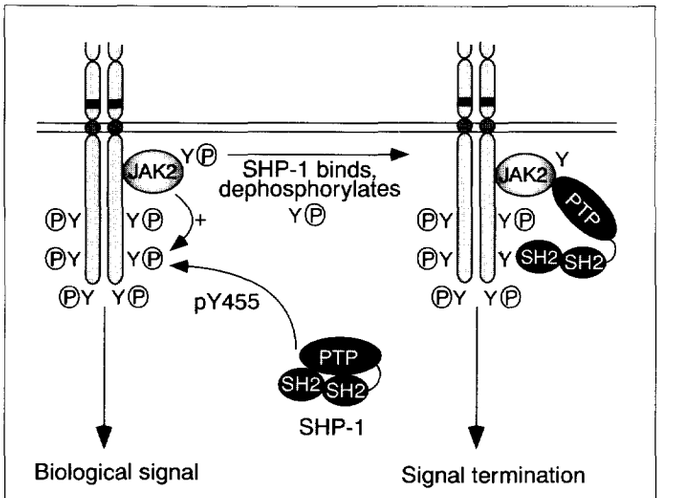

Figure 4. *A model for the role of the SH2-domain-containing phosphotyrosine phosphatase 1 (SHP-1) in the regulation of erythropoietin (EPO) signaling. Tyrosine phosphorylation of the EPO receptor by activated Janus kinase JAK2 results in recruitment of SHP-1. SHP-1 then dephosphorylates critical tyrosine(s) on JAK2, causing a loss of kinase activity and a reduction in signal transduction from the EPO receptor. P, phosphate; PTP, phosphotyrosine-containing peptide; SH2, src-homology 2 domain.*

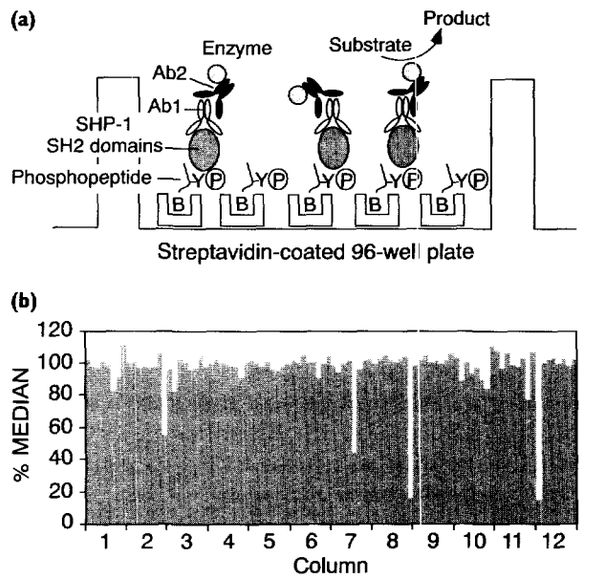

Figure 5. *A high-throughput biochemical assay for small molecules capable of inhibiting the interaction of SH2-domain-containing phosphotyrosine phosphatase 1 (SHP-1) with the erythropoietin (EPO) receptor. (a) Schematic diagram of the assay. A phosphopeptide is immobilized in the wells of a 96-well plate, and incubated with recombinant SHP-1 src-homology 2 (SH2) domains. After washing, the bound SHP-1 is detected using a two-stage ELISA. Ab, antibody; B, biotin; P, phosphate. (b) Example of the use of this assay to screen compounds. SHP-1 SH2 domain protein was incubated with bound peptide in the presence of 30 μM compounds. Compounds inhibiting the interaction are easily scored.*

Conclusions

Cytokine signal transduction via JAKs and STATs involves a series of ordered protein–protein interactions that are modulated by enzymatic activities that control the degree of tyrosine phosphorylation on key signaling molecules. As such, this pathway lends itself to the development of high-throughput biochemical assays that target individual protein–protein interactions or enzymatic activities. We have used as examples, protein–protein interactions that appear to be attractive targets either for antagonists or agonists of cytokine signal transduction. However, assays for compounds that inhibit JAK enzymatic activity are also attractive, particularly in the context of JAK3. On the basis of results with knockout mice, and the phenotype of individuals with JAK3 mutations, JAK3 inhibitors would be expected to be novel immunosuppressive agents[88–90]. As our detailed understanding of the JAK–STAT pathway increases, and the role of JAKs and STATs in various disease states becomes clearer, additional opportunities for assay development and drug discovery should become apparent.

Although we have focused on biochemical approaches in this review, small-molecule modulators of JAK–STAT signaling can also be discovered by employing cell-based screens[91,92]. The discovery of STATs as cytokine-triggered transcriptional activators, and the definition of DNA sequence elements to which they bind, has provided the tools for the development of such screens[93]. These assays consist of cytokine-responsive cells expressing the requisite receptors, JAKs and STATs, stably transfected with a reporter construct that incorporates STAT-binding elements into its promoter. If a test compound is a cytokine agonist, it will activate the appropriate STATs, which will bind to the introduced promoter and trigger an increase in reporter activity. Alternatively, if antagonists are sought, a test compound is added to cells in the presence of the cytokine, and a reduction in reporter activity (reflecting the ability of the compound to block STAT activation or function) is indicative of a potential inhibitor of cytokine signaling. Assays of this type could yield compounds that act on novel targets that could ultimately be incorporated into new generations of biochemical assays. Thus, cell-based screens and the biochemical approaches detailed above constitute complementary approaches to drug discovery using JAKs and STATs.

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