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This information is current as of August 4, 2022.

J Immunol 2007; 178:2623-2629; ;
doi: 10.4049/jimmunol.178.5.2623
<http://www.jimmunol.org/content/178/5/2623>

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BRIEF REVIEWS

The JAK-STAT Signaling Pathway: Input and Output Integration¹

Peter J. Murray

Universal and essential to cytokine receptor signaling, the JAK-STAT pathway is one of the best understood signal transduction cascades. Almost 40 cytokine receptors signal through combinations of four JAK and seven STAT family members, suggesting commonality across the JAK-STAT signaling system. Despite intense study, there remain substantial gaps in understanding how the cascades are activated and regulated. Using the examples of the IL-6 and IL-10 receptors, I will discuss how diverse outcomes in gene expression result from regulatory events that effect the JAK1-STAT3 pathway, common to both receptors. I also consider receptor preferences by different STATs and interpretive problems in the use of STAT-deficient cells and mice. Finally, I consider how the suppressor of cytokine signaling (SOCS) proteins regulate the quality and quantity of STAT signals from cytokine receptors. New data suggests that SOCS proteins introduce additional diversity into the JAK-STAT pathway by adjusting the output of activated STATs that alters downstream gene activation. The Journal of Immunology, 2007, 178: 2623–2629.

The mammalian JAK and STAT family members have been extensively, and seemingly exhaustively, analyzed in the mouse and human systems. All four JAK and seven STAT family members have been deleted in the mouse, in addition to the creation of conditional alleles for genes whose loss of function leads to embryonic or perinatal lethality (*Stat3*, combined deficiency of *Stat5a* and *Stat5b*, and *Jak2*). In humans, detailed genetic studies have been performed in people bearing mutant *Jak* or *Stat* genes. Specific Abs to phosphoforms of each protein are used to study how the JAK-STAT cascade is activated by cytokine receptors. Crystallographic studies have illuminated structural information for multiple STAT family members in different forms. Pharmacological inhibitors have been developed for clinical use where JAK-STAT signaling is implicated in disease pathology and progression. Finally, in most cases, a specific JAK-STAT combination has been

paired with each cytokine receptor, and this information translated into cell-type specific patterns of cytokine responsiveness and gene expression.

Major questions remain concerning how the JAK-STAT cascade functions to control specific gene expression patterns, and how the cascades are regulated. I will describe three elements of JAK-STAT signaling that require experimental investigation. First, I will address an unexpected experimental complication that arises from the analysis of mice and cells that lack one or more STAT family member. Second, I will use JAK1-STAT3 signaling from the IL-10R and IL-6R systems to illustrate that we lack detailed understanding of how specificity in gene expression is generated by receptors that use identical JAK-STAT members. Third, we have yet to explain how STAT activation is negatively regulated. Although the suppressor of cytokine signaling (SOCS)³ proteins are the best understood negative regulators of the JAK-STAT pathway, the biochemical mechanism of SOCS-mediated inhibition is unexplained. Moreover, additional inhibitory pathways have also been proposed to block the production of activated STATs. Collectively, I will argue that our understanding of the pathway from cytokine receptor to gene expression profile is in its infancy, but remains one of the best opportunities to dissect signal transduction.

Overview of the proximal JAK-STAT activation mechanism

The current model of JAK-STAT signaling holds that cytokine receptor engagement activates the associated JAK combination, which in turn phosphorylates the receptor cytoplasmic domain to allow recruitment of a STAT, which in turn is phosphorylated, dimerizes and moves to the nucleus to bind specific sequences in the genome and activate gene expression. Cytoplasmic domains of cytokine receptors associate with JAKs via JAK binding sites located close to the membrane (1). The postulated role of JAKs in trafficking or chaperoning the receptors to the cell surface is debated (2–6). Regardless of the when and where cytokine receptors and JAKs associate, their close apposition at the membrane is required to stimulate the kinase activity of the JAK following cytokine binding. At this stage in the activation

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Received for publication January 8, 2007. Accepted for publication January 12, 2007.

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¹ This work was supported by grants from the National Institutes of Health, Sandler Program for Asthma Research, Cancer Center Support CORE Grant P30 CA 21765, and the American Lebanese Syrian Associated Charities.

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³ Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; EpoR, erythropoietin receptor.

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of the pathway, we understand next to nothing about the structural basis of the JAK-receptor interaction, how receptor intracellular domains reorient upon cytokine binding and physically contact the JAK to receive the phosphorylation modification.

JAK-mediated phosphorylation of the receptor creates binding sites for the Src homology 2 (SH2) domains of the STATs. STAT recruitment is followed by tyrosine, and in some cases, serine phosphorylation on key residues (by the JAKs and other closely associated kinases) that leads to transit into the nucleus. This brief summary of the activation of the JAK-STAT pathway omits numerous unresolved details: the STAT monomer to dimer transition has been questioned, as has the role of phosphorylation in dimerization and nuclear transit (7). Furthermore, it is unclear how many configurations of STAT homo- and heterocomplexes are present in cells before, during, and after cytokine stimulation (8–10). We do not understand the detailed structural basis for the preference of one SH2 domain for a given receptor, and we have little knowledge of how other non-JAK kinases are recruited to the receptors and phosphorylate the STATs.

Many receptors signal through a small number of JAKs

Cytokine receptors signal through two types of pathways: the JAK-STAT pathway and other pathways that usually involve the activation of the MAP kinase cascade. Although the latter will not be discussed here, it is worth noting that elegant genetic studies have demonstrated the importance of these pathways in various pathological systems (11–14). There are now ~36 cytokine receptor combinations that respond to ~38 cytokines (counting the type I IFNs as one because they all signal through the IFN- $\alpha\beta$ R). Different cells and tissues express distinct receptor combinations that respond to cytokine combinations unique to the microenvironment or systemic response of the organism. Hence, at any given time, a single cell may integrate signals from multiple cytokine receptors. Genetic studies have established that the cytokine receptor system is restrictive in that different classes of receptors preferentially use one JAK or JAK combination (7): receptors required for hemopoietic cell development and proliferation use JAK2, common γ -chain receptors use JAK1 and JAK3 whereas other receptors use only JAK1 (Fig. 1). Unexplained is the selective use of these combinations: why the IFN- γ R rigidly uses the JAK1, JAK2 combi-

nation is unknown as is the restricted use of TYK2. Compared with JAK1–3, TYK2 is unusual in that loss of function mutations in the mouse have shown obligate, but not absolute, requirements in IFN- $\alpha\beta$ R and IL-12R signaling (15, 16). In contrast, human TYK2 seems to be essential for signaling through a broader range of cytokine receptors (17).

The preferential association of JAKs to certain receptor classes raises several issues. First, how did the JAK-receptor combinations evolve? Because the number of receptors is relatively large, why has the number of JAKs remained small? Why have the combinations of JAK pairs also remained small given that there are 10 possible combinations that can be used (Fig. 1)? Second, how flexible is the cytokine receptor-JAK pair? That is, can receptors be engineered for interchangeable JAK use, or is a given JAK combination fixed for a specific receptor class? For example, can JAK1, JAK3, or TYK2 activate erythropoietin receptor (EpoR) signaling (if so engineered) or is JAK2 obligatory for signaling? These questions allude to a fundamental issue that concerns the function of the JAK in cytokine receptor activation: if the only function of the JAKs is to phosphorylate tyrosine residues on the cytoplasmic domain of the receptors, then it should be possible to trade JAK-receptor pairs. If these receptors retain identical downstream gene expression profiles, then the signal generated by the JAK is generic and functions primarily to activate the receptor (6). Conversely, it is also possible that each receptor-JAK combination retains crucial specificity functions and swapping, for example, JAK1 for JAK2 on the EpoR will modify or destroy a specific function in erythropoiesis. These questions can be addressed experimentally by replacing one preferred JAK binding site for another in genes encoding different receptors. The EpoR is a good test example because the activity of the receptor and its signaling pathway is essential for life and erythropoiesis is readily assayed.

Core versus cell-type specific STAT signaling

Microarray experiments designed to monitor changes in gene expression induced by JAK-STAT signaling have revealed that both cell-type specific transcription and core, or stereotypic, mRNA profiles are induced by activated cytokine receptors in different cell types (Fig. 2). For example, IFN- γ , via STAT1, induces the expression of a similar cohort of genes regardless of the cell type tested (18). These genes are often termed the “IFN

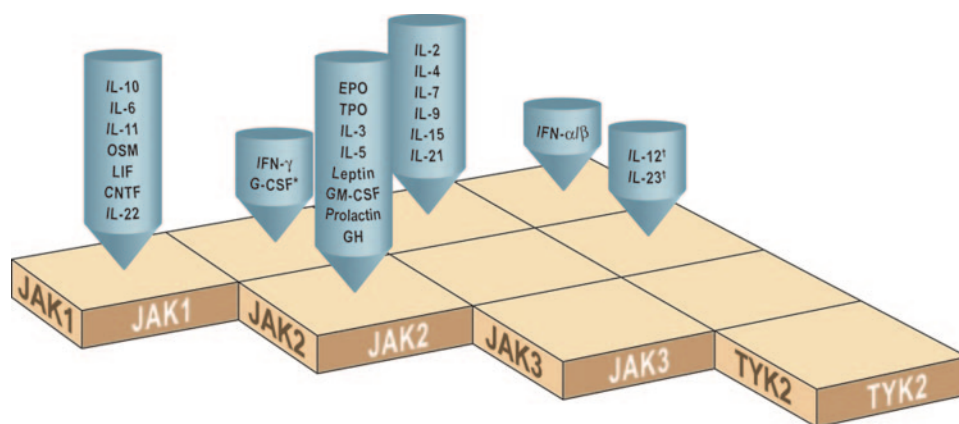


FIGURE 1. The majority of cytokine receptors use three JAK combinations. Shown are well-studied cases where JAK usage by each cytokine receptor has been established by genetic and biochemical studies. Exceptions shown are the G-CSFR (*) where it is currently unclear whether both JAK1 and JAK2 are required together. Additionally, the IL-12R (†) and IL-23R (†) require TYK2 but the requirement for JAK2 has not been definitively determined. Receptors that use JAK2 and JAK3, JAK3 alone, TYK2 alone, or JAK3 and TYK2 have not been described.

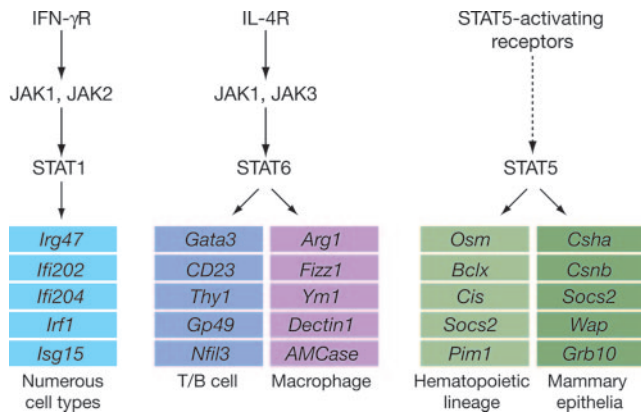


FIGURE 2. Core signaling by STATs. Representative examples of gene expression induced by STAT signaling in different tissues. The examples were extracted and edited from numerous microarray and empirical studies.

signature” and overlap with the gene expression pattern induced by IFN- $\alpha\beta$ signaling that also involves STAT1, in cooperation with STAT2 and IRF9. The IFN signature is readily observed in microarray experiments and is indicative of STAT1 activity. The STAT6 pathway activated by IL-4 or IL-13 provides an example of a cell-type specific response. IL-4-regulated genes in T cells have a distinct signature compared with IL-4/IL-13 signaling in macrophages or other non-lymphocytes (19–22). In the latter, genes such as *Arg1* (encoding arginase 1) are often induced >100-fold but are silent in T cells (23–27). Collectively these data argue that STATs activate defined gene sets, depending on their genomic accessibility, and possibly on cofactors that further refine gene expression profiles. STAT3 signaling illustrates a more complex system and will be discussed below to illustrate the distinctions between IL-6 and IL-10 signaling.

Interpreting experiments using STAT loss-of-function systems

Experiments with the different STAT knockout mice, and cells derived from these animals, have been critical for understanding specific requirements of individual STATs in gene expression following cytokine receptor signaling. The interpretation of these experiments is generally straightforward. For example, STAT5a and STAT5b are essential for the expression of genes that promote hemopoietic survival (28–30) whereas STAT1 is required for the expression of IFN-regulated genes that are involved in the protection against pathogens (18). However, by EMSA and immunoblotting experiments, most cytokines have been shown to activate multiple STATs, prompting experiments to determine transcriptional responses that can be activated in the absence of a given STAT. An initial example of this type of approach was performed by Schreiber and colleagues who interrogated gene expression profiles induced by IFN- γ signaling in the absence of STAT1 (31, 32). In these experiments, IFN- γ was used to stimulate STAT1-deficient bone marrow-derived macrophages and fibroblasts. Numerous genes were induced by IFN- γ in the absence of STAT1, leading to the conclusion that the IFN- γ R activates a STAT1-independent gene expression program. However, inspection of the genes induced by IFN- γ signaling in STAT1-deficient cells shows many to be STAT3-regulated genes such as *Socs3*, *Gadd45*, and *Cebpb*. STAT3 phosphorylation is normally induced by IFN- γ in wild-type cells but in the absence of STAT1, STAT3 signal-

ing is dominant. What is the mechanism of this effect? We now know from experiments using STAT-deficient cells that receptor occupancy, or lack of occupancy by the dominant STAT that binds the receptor, causes a switch from one activated STAT to another (33). A converse example is the conversion of IL-6R signaling to a dominant STAT1 activation in STAT3-deficient cells (34). This switch causes the downstream induction of the IFN gene expression pathway just as IFN- γ would cause in wild-type cells.

A related example is observed when IL-6 signaling is tested in the absence of SOCS3. SOCS3 is induced by STAT signaling from different cytokine receptors and functions as a feedback inhibitor of the IL-6R (and the G-CSFR, LIFR, and leptinR) by binding to phosphorylated Y757 on the gp130 cytoplasmic domain (see below). However in the absence of SOCS3, STAT3 phosphorylation is greatly increased (35–37). At the same time however, STAT1 phosphorylation is also induced, leading to a dominant IFN-like gene expression signature (35, 36). Thus SOCS3 regulates both the quantity and type of STAT signal generated from the IL-6R. Although the mechanism of the SOCS3 effect is unclear, the promiscuity of different receptors for different STATs argues that loss-of-function experiments must be carefully examined for the activation of other STAT molecules that fill the “hole” created by the loss of one STAT. These data also suggest that different cytokine receptors have evolved selectivity for different classes of STATs. Although STAT1 and STAT3 can apparently interchangeably bind the IL-6R or IFN- γ R when either molecule is missing, signaling in wild-type cells shows a strong preference for one STAT over the other. Likewise, other receptors may have evolved to bind only one STAT, and in the absence of the key STAT, the other STATs cannot bind and/or be activated by the receptor.

The above examples primarily describe experiments using STAT1–STAT3-activating receptors but these are not isolated cases. In T cells stimulated by IL-12, STAT4 is activated and drives IFN- γ production. This pathway is a central regulatory event in the development of the Th1 type T cell responses. IFN- $\alpha\beta$, via the IFN- $\alpha\beta$ R, also activates STAT4 (in addition to STAT1 and STAT2 that forms a complex with IRF-9 to mediate anti-viral gene expression) but cannot activate strong IFN- γ production and therefore cannot drive Th1 development (38). However, in the absence of STAT1, IFN- $\alpha\beta$ causes a large increase in IFN- γ production, especially in vivo during viral infection (39, 40). These data were originally interpreted to mean that STAT1 normally suppressed IFN- γ production. However, the data can just as easily be resolved when we consider that STAT4 activation from the IFN- $\alpha\beta$ R is increased in the absence of STAT1. Recent data confirm this interpretation but also show that STAT4 activation by the IFN- $\alpha\beta$ R, although increased, cannot sustain IFN- γ production from T cells when compared with IL-12 (38). This is probably because of the stronger differential activity of SOCS1 on the IFN- $\alpha\beta$ R versus the IL-12R (discussed below). I would predict that an IFN- $\alpha\beta$ R that is refractory to SOCS1 (or active in a *Socs1*^{-/-} background) would behave identically to the IL-12R in the absence of STAT1.

Although loss of gene expression may be observed in a given STAT knockout, a corresponding increase in the ectopic activation of another STAT pathway may confound the interpretation of results in both in vitro and in vivo systems. Because specific Abs are available for each tyrosine-phosphorylated

STAT molecule, a simple solution is to first measure which other STATs are activated by a given receptor in the absence of the STAT of interest. Experiments using STAT knockout systems should also be supported by additional data that uses complementary mutations in the receptor that ablate STAT recruitment, or complete loss of the receptor. Finally, it is worth noting that the loss of a STAT pathway from a receptor signaling system can cause additional loss of key negative regulatory systems including feedback loops such as SOCS induction as presently debated for G-CSFR signaling and receptor systems discussed below (41–45).

Negative regulation of the JAK-STAT signal

Many biochemical processes conspire to regulate the JAK-STAT pathway, including phosphorylation-mediated autoinactivation of the FERM domain of the JAKs, loss of receptor numbers at the surface, dissociation of JAKs from a receptor, heterodimer competition and transport of STATs out of the nucleus. I will discuss two mechanisms of JAK-STAT regulation whose biochemical processes are mechanistically unclear but likely play central roles in negative regulation of the STAT activation process, and therefore translate into large effects at the level of gene regulation.

SOCS proteins are induced by cytokines and other stimuli and function as negative feedback inhibitors of cytokine receptor signaling. The prevailing hypothesis for SOCS function has focused on an initial receptor binding step, mediated by the SH2 domain of the SOCS protein, followed by a second step involving the activity of the SOCS box, which forms a complex with proteins involved in ubiquitin E3 ligase activity (46). SOCS1, SOCS2, and SOCS3 have been found to have surprisingly selective essential functions in regulating cytokine signaling (47). The genetic studies raise a complex issue in that loss-of-function studies can only identify the non-redundant functions of each SOCS protein. Other targets of the SOCS proteins that are not cytokine receptors have been proposed. The functions of these interactions are not readily determined by genetics and their roles normal cellular physiology have yet to be firmly established.

The functions of the SH2 domain and the SOCS box can be separated and, at least for SOCS1, the key inhibitor of the IFN- γ R (and also the IFN- $\alpha\beta$ R (48, 49), IL-12R (50), IL-4R (51) and γ_c receptor (52, 53)). Mice lacking SOCS1 die rapidly from excessive IFN- γ signaling: a phenotype that can be rescued in multiple ways that reduce the amounts of IFN- γ or IFN- γ signaling (47). However, mice lacking the SOCS box of SOCS1 but retaining the SH2 domain that binds to the IFN- γ R are partially protected from the toxic effects of unregulated IFN- γ signaling (54). This suggests that both SH2 and SOCS box domains have inhibitory activity toward cytokine signaling. How the two inhibitory signals delivered by a SOCS protein are integrated with other negative signals to block cytokine receptor signaling are unknown and require further study.

Although impressive inroads into understanding the functions and specificities of the SOCS proteins have been made, one major question is outstanding: what are the substrates of the ubiquitin E3 ligase activity of each SOCS protein? A simple model for SOCS function is that binding of the SOCS SH2 domain anchors the protein complex close to the receptor, increasing the effective concentration of the complex to where it is needed. But what then are the targets? Although we suspected

that the receptor chains themselves could be the target for ubiquitination and degradation, we could not find compelling evidence to support this idea in the case of the SOCS3-gp130 interaction (36). Similarly, it is difficult to imagine how SOCS proteins could selectively and specifically block JAK signaling at one receptor class while leaving other receptors using the same JAK unaffected, although many investigators favor this hypothesis as a partial solution to the problem of identifying SOCS substrates (55–57).

Since the overall biochemical activity of SOCS proteins is to reduce the output of activated cytokine signaling, maybe the SOCS proteins direct the ubiquitination of the phosphorylated STATs, and thereby their degradation, reducing the amplitude of the STAT signal. Such a mechanism would occur in a dynamic way, because STATs are continuously recruited to activated receptors during signaling, while selected SOCS proteins are continuously made in response to STAT activation. Early work, preceding the discovery of the SOCS proteins, noted that proteasome inhibition increased cytokine receptor signaling, implicating ubiquitin-mediated degradation in blocking the JAK-STAT pathway (58, 59). Validation of this idea requires sensitive tests to measure percentages of STAT proteins that are modified by ubiquitination. However, the identity of the relevant SOCS substrates is an intrinsically complex biochemical problem that will not be solved easily.

Several investigators have observed that STAT DNA binding activity can rapidly (preceding the induction of the SOCS proteins) be inhibited by other receptor signaling systems. For example, STAT3 DNA binding activity is blocked by cosignaling through the IL-1R or TLR4, which does not use the JAK-STAT system (60–62), suggesting rapid inhibitory effects on STAT activity seems to occur in a membrane proximal way. These data argue that kinases, phosphatases, and other enzymes can be recruited in the vicinity of cytokine receptors and influence their function. These poorly understood pathways likely have significant effects during inflammatory responses where coincident TLR, TNF- α R and IL-1R signaling are acting. For example, in the example of IL-10 versus IL-6 signaling that follows, LPS seems to affect early signaling from the IL-6R but not the IL-10R.

Is there functional equivalence in signaling from receptors using the same JAK-STAT combination in the same cell?

In macrophages stimulated with either IL-10 or IL-6, the JAK1-STAT3 pathway is activated. Even though both the IL-10R and IL-6R activate a seemingly identical process, the downstream readouts of signaling from these receptors are remarkably distinct. The major function of IL-10 is to negatively regulate inflammatory responses from activated macrophages and dendritic cells (63). The inhibitory effects of IL-10 are mediated solely by STAT3 and are indirect (63–66). That is, IL-10-mediated activation of STAT3 targets one or more STAT3-regulated genes whose products are responsible for inhibiting inflammatory gene expression at the level of transcription.

The obligate activity of STAT3 in the IL-10 pathway raises a key issue. Do other STAT3-activating receptors also activate the same anti-inflammatory response, and if not, why not? The answer to these questions have been partly resolved through the use of modified receptors that activate STAT3 but are either refractory to the effects of SOCS3 or regulated by SOCS3 (37, 67). Cytokine receptors that are unrelated to the IL-10R but

activate STAT3 in a SOCS3-independent way activate the anti-inflammatory response. By contrast, receptors that are regulated by SOCS3 cannot activate the anti-inflammatory response, such as the IL-6R (37, 67). However, the IL-6R robustly activates STAT3, even in wild-type cells where SOCS3 is active (67). In reconstituted primary macrophages expressing these different types of receptors, STAT3 tyrosine and serine phosphorylation are activated, but the kinetics of the decay of STAT3 phosphorylation are largely controlled by SOCS3 (67).

The interpretation of these data is not straightforward, because the use of immunoblotting as a readout for STAT3 activation does not correlate well with the response at the level of gene expression. Instead, I would argue that two conclusions can be drawn from these studies that allude to an unknown aspect of JAK-STAT signaling: that receptors generate different pools of the same activated STAT but have overlapping yet distinct activities (Fig. 3). Consider the examples described above. In the case of the IL-6R versus the IL-10R, both activate STAT3 and both can activate some STAT3-dependent genes in common, such as *Sox3*. However, only the IL-10R activates the genes that control the anti-inflammatory response, whereas the IL-6R is incapable of this activity. The activation of STAT3 by the IL-10R is not unique, because an EpoR engineered to activate STAT3 and not STAT5 activates an anti-inflammatory response identical with the IL-10R (67). Therefore, the STAT3 activated by the IL-10R differs from STAT3 activated by the IL-6R. The only obvious distinction between receptor types is that the IL-6R is regulated by SOCS3 whereas the other “anti-inflammatory” STAT3-activating receptors are not. Yoshimura has argued that the STAT3 signal from the IL-6R becomes “prolonged” in the absence of SOCS3 and thereby mimics STAT3 activation by the IL-10R (37). This view postulates that the strength of the STAT3 signal determines the output in terms of gene regulation. This concept cannot be formally excluded but is, however, potentially flawed for three reasons. First, the anti-inflammatory effects of IL-10, and the lack of the anti-inflammatory effects induced by IL-6, begin before the ro-

bust induction of *Sox3* expression (64). Second, IL-6 is a stronger inducer of STAT3 activation, correlated by immunoblotting or accumulation of tyrosine-phosphorylated STAT3 in the nucleus, than IL-10 on a weight or molar basis (67). Third, the strength of signal hypothesis cannot readily accommodate common gene expression induced by both IL-6 and IL-10, such as *Sox3*.

An alternative way to account for the differences between STAT3-activating receptors is to consider that the IL-10R (or receptors like the IL-10R or engineered to behave so) activates a “generic” type of STAT3 activation in activated macrophages. That is, STAT3 generated by the IL-10R is unencumbered by additional regulatory steps that connect to SOCS3-mediated inhibition. In contrast, the IL-6R has evolved to respond to regulatory steps that restrain the ability of the receptor to activate the expression of the genes that execute the anti-inflammatory response. SOCS3 is the central, but probably not the only, regulatory molecule in this process. Thus, in the absence of SOCS3, the IL-6R can now generate an anti-inflammatory response because a greater fraction of STAT3 activated by the IL-6R falls into the pool that behaves like IL-10R-activated STAT3 (37, 67). How can this idea be tested? An obvious experiment would be to engineer a SOCS3-binding site into the IL-10R and determine whether the anti-inflammatory effect of IL-10 is lost. This experiment is a “straw-man” and not informative because the effects of SOCS3 would be likely strong enough to block receptor activity leaving the question of the mechanism unresolved. Another approach would be to ask whether the STAT3 activated by the IL-6R at any given time is homogeneous with respect to phosphorylation or other post-translational modifications. An appealing hypothesis is that multiple kinases and phosphatases and other enzymes linked to the IL-6R modify pools of STAT3 recruited to the receptor. In wild-type cells there is not enough “IL-10-like” STAT3 activated by the IL-6R. However in the absence of SOCS3, the pool of “IL-10-like” STAT3 increases enough to where the anti-inflammatory effects are observed. This problem can be addressed by mass spectrometry of total STAT3 activated by different receptors, as well as chromatin-immunoprecipitation (ChIP) experiments to determine where the pools of STAT3 localize at the genomic level.

Future directions

The biochemical, cell biological, and genomic analyses of the JAK-STAT signaling pathway are in their infancy. Restraining progress is a lack of structural information on cytoplasmic domains of cytokine receptors, particularly in complex with JAKs and STATs. In addition, mass spectrometric measurements of posttranslational alterations made to both JAKs and STATs before, during, and after cytokine receptor activation are essential for understanding how one STAT can generate multiple outcomes at a gene expression level. Related to the preceding point, genome-wide ChIP analysis of STAT target genes is now available: using this technique, it should be possible to determine all the gene targets of any STATs in any cell at any time. This data needs to be then translated into functional information because a ChIP assay only gives the genomic address of a specific factor at a given time. Together, these approaches can be applied to any cytokine receptor signaling system.



FIGURE 3. Proposed differential STAT activation by IL-10 or IL-6. Shown are three classes of genes activated by STAT3 where *Sox3* is a representative “common” gene induced by both receptors. In the absence of SOCS3, the IL-6R can activate the anti-inflammatory genes in the same way as the IL-10R. The mechanism of this effect remains to be established.

Acknowledgments

I apologize to the many researchers whose primary publications could not be cited due to space constraints. I thank Doug Hilton and Stephanie Watowich for discussion and critique, and Josh Stokes for help with Fig. 1.

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