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The role of Stat5a and Stat5b in signaling by IL-2 family cytokines

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The activation of Stat5 proteins (Stat5a and Stat5b) is one of the earliest signaling events mediated by IL-2 family cytokines, allowing the rapid delivery of signals from the membrane to the nucleus. Among STAT family proteins, Stat5a and Stat5b are the two most closely related STAT proteins. Together with other transcription factors and co-factors, they regulate the expression of the target genes in a cytokine-specific fashion. In addition to their activation by cytokines, activities of Stat5a and Stat5b, as well as other STAT proteins, are negatively controlled by CIS/SOCS/SSI family proteins. The outcome of Stat5 activation in regulating expression of target genes varies, depending upon the complexity of the promoter region of target genes and the other signaling pathways that are activated by each cytokine as well. Here, we mainly focus on the IL2-/IL-2 receptor system, as it is one of the best-studied systems that depend on Stat5-mediated signals. We will summarize what we have learned about the molecular mechanisms of how Stat5 is activated by IL-2 family cytokines from *in vitro* biochemical studies as well as the role that is played by Stat5 in each of the cytokine signaling pathways from *in vivo* gene-targeting analyses. *Oncogene* (2000) **19,** 2566–2576.

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An overview of IL-2 family cytokines

Interleukin-2 (IL-2) family cytokines play critical roles in the development of the immune system and modulation of lymphocyte activities during immune responses (Leonard, 1999). This family of cytokines includes IL-2, IL-4, IL-7, IL-9 and IL-15, each of which shares the IL-2 receptor γ chain (IL-2Rγ) as a receptor component (Giri *et al*., 1994; Kondo *et al*., 1993, 1994; Noguchi *et al*., 1993a; Russell *et al*., 1993, 1994; Figure 1). Because of its shared nature, IL-2Rγ is now known as the common cytokine receptor γ chain (γ_c) (Leonard, 1996). The gene for γ_c was localized to human chromosome Xq13 (Noguchi *et al*., 1993b) in the region previously determined to be the locus for X-linked severe combined immunodeficiency (XSCID) (de Saint Basile *et al*., 1987), and mutation of this gene was shown to cause XSCID (Noguchi *et al*., 1993b). XSCID is a disease characterized by profoundly decreased numbers of T-cells and natural killer (NK) cells and by normal numbers of non-functional B-cells (Fischer *et al*., 1997; Leonard, 1996). The critical roles played by γ_c in mediating the signals for IL-2 family cytokines is underscored by the profound phenotype in XSCID. In addition to γ_c, the receptor for each of the IL-2 family cytokines contains at least one other component, such as IL-2Rβ, IL-4Rα, IL-7Rα and IL-9Rα (Figure 1), that contributes both to binding and to transduction of specific signals (Leonard, 1999). Because the receptors for IL-2 and IL-15 additionally share IL-2Rβ (Figure 1), IL-2 and IL-15 have the most overlapping biological activities of the five cytokines. In contrast to IL-4, IL-7 and IL-9, the receptors for IL-2 and IL-15 also have third components, IL-2Rα and IL-15Rα (Lin and Leonard, 1997; Waldmann *et al*., 1998; Figure 1). IL-2Rα only contributes to IL-2 binding affinity but not to the recruitment of signaling molecules. However, its importance in the development of a normal immune response is emphasized by the finding that a truncation mutant of IL-2Rα results in an immunodeficiency in humans characterized by an increased susceptibility to viral, bacterial and fungal infections (Sharfe *et al*., 1997). In addition, gene targeting analysis also reveals that IL-2Rα-deficient mice exhibit autoimmunity and premature death (Willerford *et al*., 1995).

Each member of the IL-2 family of cytokines not only acts on T cells, but can act on other lineages as well. These cytokines collectively exhibit both overlapping and distinctive biological actions (Paul and Seder, 1994; Leonard, 1999; Waldmann *et al*., 1998). For example, IL-2 and IL-15 play vital roles in proliferation of T cells and augmentation of the cytolytic activity of NK cells (Waldmann *et al*., 1998). IL-2 also plays an important role in the elimination of autoreactive T cells (Lenardo, 1991; Refaeli *et al*., 1998; Van Parijs *et al*., 1997) and can promote immunoglobulin synthesis by B cells (Lin and Leonard, 1997). IL-15 appears to be essential for the development of NK cells, as well as proliferation of $CD8^+$ memory T cells (Lodolce *et al*., 1998; Zhang *et al*., 1998b) and lymphocyte homing (Lodolce *et al*., 1998).

IL-4 is a critical mediator that promotes differentiation of T helper precursor (Thp) cells into Th2 cells, while suppressing differentiation of Thp into Th1 cells (Nelms *et al*., 1999). It is thus important for IgE production (Corry and Kheradmand, 1999; Nelms *et al*., 1999). IL-4 also plays a role in activation of endothelial cells (Nelms *et al*., 1999). IL-7 is required for development of T cells in thymus (Baird *et al*., 1999; Hofmeister *et al*., 1999), while IL-9 stimulates late T cell growth and mast cell growth (Renauld *et al*., 1995).

How does IL-2 transduce its signals?

Analogous to other cytokines/growth factors, IL-2 activates a number of signaling pathways. As shown in Figure 1, the IL-2 receptor is composed of three subunits, IL-2Rα, IL-2Rβ, and γ_c (Lin and Leonard, 1997; Sugamura *et al*., 1996; Taniguchi, 1995). On the basis of their binding affinities to IL-2, the receptor complexes are denoted as low- (α chain only,

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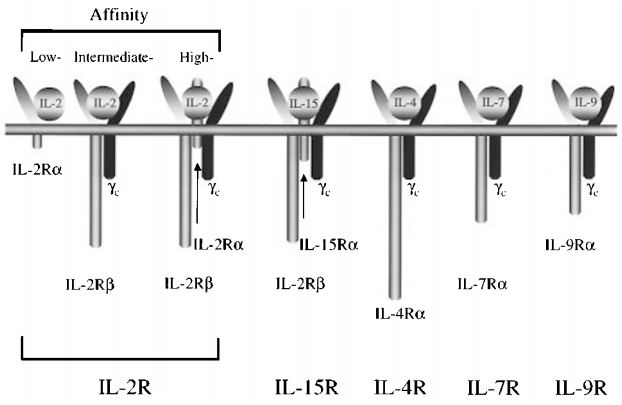

Figure 1 Schematic illustration of the receptors for IL-2 family cytokines. The common cytokine receptor γ chain (γ_c) is shown in black. The low- intermediate- and high-affinity receptors are shown for IL-2 receptor complexes. Each of the corresponding cytokines interacts with the extracellular domains of the receptor subunit(s)

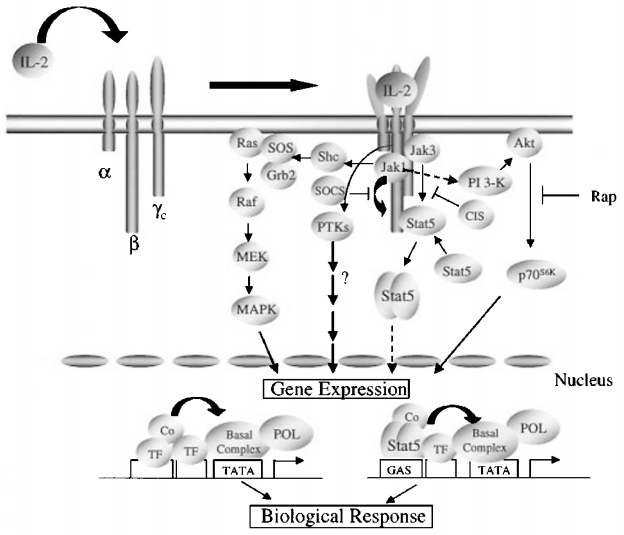

Figure 2 Schematic cartoon illustrating signaling pathways associated with IL-2Rβ and/or γ_c and known to be activated by IL-2. PTKs represent the protein tyrosine kinases Syk and Pyk2 and the Src family kinases $p56^{lck}$, $p53/p56^{lyn}$ and $p59^{fyn}$. The broken lines and question mark indicate signaling pathways whose functional roles in IL-2 signaling still require further investigation. Rapamycin is indicated as Rap. In the nucleus, transcription factors are indicated as TF, coactivators are shown as Co, and DNA polymerase II is labeled as POL

$K_d = 10^{-8}$ M), intermediate- (β and γ_c, $K_d = 10^{-9}$ M) and high-affinity (α, β and γ_c, $K_d = 10^{-11}$ M) receptors (Lin and Leonard, 1997). Only the intermediate- and high-affinity receptors are able to transduce IL-2 signals, and the cytoplasmic domains of both IL-2Rβ and γ_c are essential for this purpose. Like most type I cytokine receptors, the cytoplasmic domains of both IL-2Rβ and γ_c contain certain structural features, including 'Box1/Box2' regions for binding JAK kinases, and a number of tyrosine residues (Bazan, 1990; Leonard and O'Shea, 1998). At least in the case of IL-2Rβ, phosphorylation of some of these tyrosine residues creates docking sites for the recruitment of signaling molecules (Friedmann *et al*., 1996; Fujii *et al*., 1995; Goldsmith *et al*., 1995; Ravichandran and Burakoff, 1994). In contrast, IL-2Rα has a short cytoplasmic domain (only 13 amino acids); it mainly contributes to IL-2 binding affinity and is unlikely to contribute to signaling per se (Leonard *et al*., 1984; Nikaido *et al*., 1984).

The interaction of IL-2 with either the intermediate- or high-affinity IL-2 receptors mediates hetero-dimerization of the receptor complexes (Nakamura *et al*., 1994; Nelson *et al*., 1994). Some interaction of IL-2Rβ and γ_c can occur in the absence of IL-2 (Damjanovich *et al*., 1997; Eicher and Waldmann, 1998), but IL-2 significantly stabilizes this interaction (Takeshita *et al*., 1992). This then leads to the recruitment and activation of the signaling molecules associated with IL-2Rβ and γ_c, thereby initiating the IL-2 signaling cascade (Lin and Leonard, 1997) (Figure 2). As is typical of type I cytokine receptors, neither IL-2Rβ nor γ_c contains protein kinase activity in its cytoplasmic domain (Hatakeyama *et al*., 1989; Takeshita *et al*., 1992). However, within minutes following the interaction of IL-2 with the high- or intermediate-affinity IL-2 receptors, a number of cellular proteins, including IL-2Rβ and γ_c, are phosphorylated on tyrosine residues (Farrar and Ferris, 1989; Turner *et al*., 1991), at least in part by the receptor-associated protein tyrosine kinases, Jak1 and Jak3 (Friedmann *et al*., 1996; Johnston *et al*., 1994; Witthuhn *et al*., 1994). It has been demonstrated that in the absence of IL-2, Jak1 is associated with IL-2Rβ (Johnston *et al*., 1994; Russell *et al*., 1994; Witthuhn *et al*., 1994) and Jak3 is with γ_c (Johnston *et al*., 1994; Russell *et al*., 1994; Witthuhn *et al*., 1994; Figure 2). Moreover, in addition to its principal interaction with γ_c, Jak3 also can contact IL-2Rβ (Zhu *et al*., 1998). IL-2-mediated hetero-dimerization of its receptor triggers a rapid increase in the recruitment of Jak3 and activation of both Jak1 and Jak3 (Johnston *et al*., 1994; Witthuhn *et al*., 1994). These kinases phosphorylate the receptor as well as each other, and activate other signaling molecules associated with the receptor. The phosphorylated tyrosines on IL-2Rβ can then serve as docking sites for signaling molecules that otherwise cannot associate with IL-2Rβ, including the adaptor protein Shc, Stat5a, and Stat5b (Figure 2). These proteins are themselves then available for tyrosine phosphorylation.

An important pathway activated following stimulation by IL-2 is the Ras-Raf-MAP kinase pathway (Graves *et al*., 1992; Turner *et al*., 1991, 1993; Zmuidzinas *et al*., 1991; Figure 2). The adaptor protein Shc associates with Tyr-338 of IL-2Rβ via its PTB domain and it is tyrosine phosphorylated by Jak1 (Friedmann *et al*., 1996; Ravichandran *et al*., 1996). Another adaptor protein, Grb2, and the guanine nucleotide exchange factor mSOS can be detected in IL-2R-immune complexes (Ravichandran and Burakoff, 1994; Zhu *et al*., 1994). These findings provide a mechanism as to how IL-2 activates Ras-Raf-MAP kinase pathway. Like many other cytokines and growth factors, IL-2 also activates phosphatidyl inositol 3-kinase (PI 3-K) (Augustine *et al*., 1991; Merida *et al*., 1991; Remillard *et al*., 1991; Figure 2). Jak1 has been shown to be important for recruiting PI 3-K (Migone *et al*., 1998). Activated PI 3-K is most likely then responsible for activation of its downstream kinase Akt (protein kinase B) and p70 S6 kinase (Ahmed *et al*., 1997; Kuo *et al*., 1992), which are involved in anti-apoptotic and proliferative activities in response to IL-2 (Reif *et al*., 1997). In addition, the protein tyrosine

kinases Syk and Pyk-2 and Src-family kinases including $p56^{lck}$ $p53/p56^{lyn}$ and $p59^{fyn}$ can associate with IL-2Rβ and can be activated by IL-2 (Hatakeyama *et al*., 1991; Kobayashi *et al*., 1993; Minami *et al*., 1993, 1995; Miyazaki *et al*., 1998; Figure 2). However, the biological significance of their activation *in vivo* is not yet clear and requires further investigation.

STAT proteins and their activation

Interferons (IFNs) are the prototype cytokines that activate a unique class of transcription factors known as STAT (signal transducers and activators of transcription) proteins (Horvath and Darnell, 1997; Leonard and O'Shea, 1998; Stark *et al*., 1998). As illustrated in Figure 3 for both Stat5a and Stat5b, STAT family proteins typically contain an amino-terminal region, followed by a coiled-coil region, a DNA binding domain (except Stat2), a linker region, an SH2 domain, a conserved tyrosine residue approximately 700 amino acids from the amino-terminus, and a carboxyl-terminal transactivation region. Interestingly, STAT proteins can be subdivided into two groups, with Stat2 and Stat6 being longer proteins by approximately 50–100 amino acids than are Stat1, Stat3, Stat4, Stat5a, and Stat5b (Horvath and Darnell, 1997; Leonard and O'Shea, 1998).

In the absence of stimulation, STAT proteins are present in a latent state in the cytosol, most likely as a monomeric form. The exact location of STAT proteins in the cytosol remains unclear. Following stimulation by cytokines, JAK kinases, which are either constitutively associated with receptors or recruited to receptors, phosphorylate tyrosine residues on the receptor that serve as docking sites for STAT proteins. STAT proteins are themselves then available for phosphorylation by JAK kinases. After tyrosine phosphorylation, the activated STATs are released from their docking sites on the receptors, and dimerize. The exact mechanism(s) as to how tyrosine phosphorylation facilitates dissociation of the activated STAT protein from the docking site is not fully understood. However, after dissociation, the bivalent dimerization is favored over monovalent association with the receptor. After dimerization, STATs translocate into the nucleus, bind to their DNA binding site GAS (IFN-γ activated site) motif, and regulate transcription of the target genes. This rapid and dynamic tyrosine phosphorylation-driven association-dissociation-association cascade during STAT activation process was demonstrated *in vitro* for Stat1 using the BIAcore surface plasmon resonance technique (Greenlund *et al*., 1995). These findings seem likely to be applicable to activation of STAT proteins by other cytokines or growth factors as well. Purified Stat1 can associate with a tyrosine-phosphorylated peptide containing the docking site on the IFNγ receptor α chain in a direct, specific, and reversible manner with a moderate affinity ($K_d = 137$ nM). Activated Stat1 proteins then dissociate from the receptor, and the free, tyrosine-phosphorylated Stat1 monomers favor the formation of homodimers as they have a greater avidity for each other than for reassociating with the docking site on the receptor. Therefore, the SH2 domain on each STAT protein serves two roles (Horvath and Darnell, 1997; Leonard and O'Shea, 1998; Stark *et al*., 1998). First, it provides the ability for the STAT protein to dock on a phosphorylated tyrosine on the receptor. This docking makes the STAT protein available for phosphorylation by JAK kinases. Second, the SH2 domains mediate the dimerization of activated STAT proteins where the SH2 of each STAT monomer interacts with the phosphorylated tyrosine on each other.

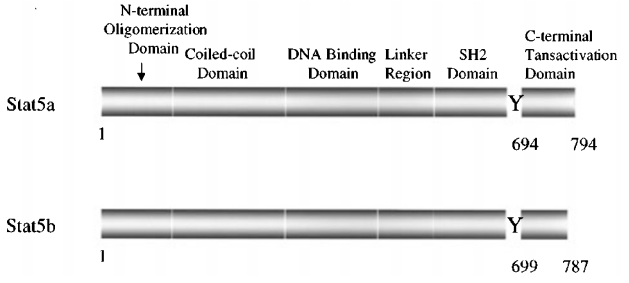

Figure 3 Schematic illustration of the known structural features of the human Stat5a and Stat5b. The conserved tyrosine residue in both proteins is indicated as Y

Stat5 proteins are encoded by two closely related genes

Stat5 was originally described as a prolactin-induced mammary gland factor (MGF) that could regulate transcription of the β casein gene (Wakao *et al*., 1994). Cloning of ovine MGF revealed that it had amino acid sequence homology to Stat1 and Stat2 proteins and that it shared the structural features of these STAT proteins, including DNA binding domain, SH2 domain and the conserved tyrosine residue (Wakao *et al*., 1994). Thus, it was later renamed as Stat5. Cloning of two closely related Stat5 cDNAs from both mouse and human cDNA libraries indicated that Stat5 proteins are encoded by two distinct but closely related genes, *STAT5A* and *STAT5B* (Hou *et al*., 1995; Lin *et al*., 1996; Liu *et al*., 1995; Mui *et al*., 1995). These genes are tandemly linked on human chromosome 17 (Lin *et al*., 1996; Imada *et al*., unpublished) and murine chromosome 11 (Copeland *et al*., 1995). Both human and mouse Stat5a and Stat5b are 91% identical at the amino acid level. While the coiled-coil region, DNA binding domain, linker region, SH2 domain and the conserved tyrosine residue are highly conserved; the amino terminal and carboxy terminal regions are divergent, with the extreme carboxyl-terminal transactivation domain showing the most divergence. The high degree of sequence conservation and tandem localization for the two Stat5 genes indicate that they are most likely derived from an ancestral gene duplication event. In addition to full-length transcripts transcribed from these two genes, alternatively spliced transcripts have also been identified from both human and mouse cDNA libraries (Lin *et al*., 1996; Wang *et al*., 1996).

How are Stat5 proteins activated by IL-2 family cytokines?

In addition to prolactin, Stat5 proteins are activated by a wide variety of cytokines and growth factors, including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, granulocyte-macro-

phage colony-stimulating factor, erythropoietin, growth hormone, thrombopoietin, epidermal growth factor and platelet-derived growth factor.

The docking sites on a particular receptor seem to determine the specificity as to which STAT is activated by a cytokine. This has been clearly demonstrated for IL-2 family cytokines by eletrophoretic mobility shift assays (EMSA) using the peptides spanning the putative docking sites as competitive blockers of STAT dimerization (Demoulin *et al*., 1996; Lin *et al*., 1995). For example, only phosphorylated (but not non-phosphorylated) peptides spanning either Tyr-392 or Tyr-510 of IL-2Rβ can efficiently compete with IL-2-induced Stat5 DNA binding to a GAS motif (Lin *et al*., 1995), but they cannot compete with the IL-4-induced Stat6 DNA binding activity. This indicates the specificity of these tyrosine-phosphorylated peptides for the Stat5 SH2 domain.

Interestingly, a phosphorylated peptide spanning Tyr-429 of the IL-7 receptor α chain can also efficiently compete with IL-2-induced Stat5 DNA binding activity (Lin *et al*., 1995). These data explain the mechanisms by which IL-2, IL-7, and IL-15 activate the same STAT proteins: the IL-2 and IL-15 receptors share IL-2Rβ as a receptor component, whereas in the case of IL-7, a similar docking site is present in IL-7Rα. Similarly, a phosphorylated, but not unphosphorylated, peptide containing Tyr-367 of the IL-9Rα chain competes with IL-9-induced Stat5 binding activity, indicating that Tyr-367 serves as the docking site for the activation of Stat5 by IL-9 (Demoulin *et al*., 1996). A comparison of the motifs of the key docking tyrosines from the IL-2Rβ, IL-7Rα, and IL-9Rα chains shows sequence similarities among them (Figure 4). However, the minimal sequence requirement for the docking sites and the critical residues and properties thereof has not been rigorously tested.

IL-4 has also been reported to activate Stat5 in lymphocytes; however, this activation appears not to depend on the phospho-tyrosine docking sites in IL-4Rα but rather it may instead depend on interaction with a JAK kinase (Friedrich *et al*., 1999) or γ_c (Lischke *et al*., 1998). It therefore likely represents additional mechanism(s) by which IL-2 family cytokines activate Stat5 proteins. It is conceivable that Stat5 activation by IL-4 could be directly mediated by Jak3, as Stat5 proteins can associate with Jak3, as demonstrated by co-immunoprecipitation in HTLV-I transformed leukemia T cell lines (Migone *et al*., 1995). Furthermore, Stat5 proteins can also be activated in

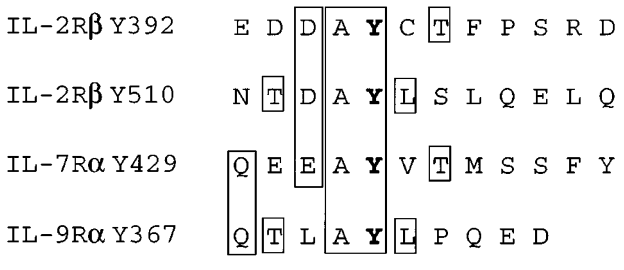

Figure 4 Sequence comparison of the peptides derived from IL-2Rβ, IL-7Rα and IL-9Rα that span Stat5 docking sites. Phosphorylation of the tyrosine is required for Stat5 docking. Open boxes indicate the conserved residues among these peptides and the phosphorylated tyrosine is shown as Y in bold

COS-7 cells when only the cDNAs encoding for Jak3 and Stat5 are co-transfected (Lin *et al*., 1996). Clearly, Stat6 is a critical signaling molecule that mediates essential actions of IL-4, as demonstrated by the greatly diminished IL-4 function in Stat6$^{-/-}$ mice (Kaplan *et al*., 1996; Shimoda *et al*., 1996). However, analyses of Stat5 genetargeted mice also indicate that at least Stat5b protein plays an important role in T cell proliferation in response to IL-4 (Imada *et al*., 1998; Moriggl *et al*., 1999a).

As noted above, a given STAT protein can only efficiently recognize certain docking sites on cytokine receptors. Similarly, SH2-phosphotyrosine mediated dimerization between STAT proteins is restricted and does not occur between all STATs (Greenlund *et al*., 1995). Therefore, the SH2 domain on each STAT protein and the tyrosine-phosphorylated docking site on each of the cytokine receptors are major features for determining the specificity as to which STAT protein will be activated by a given cytokine. However, Stat5 proteins can also be activated by their interaction with JAK kinase(s), as discussed above for Stat5 activation by IL-4.

Jak3 plays a critical role in activation of Stat5 proteins by IL-2

IL-2 activates both Stat5a and Stat5b in normal T lymphocytes (Fujii *et al*., 1995; Gaffen *et al*., 1995; Hou *et al*., 1995; Lin *et al*., 1995) and NK cells (Yu *et al*., 1996). The importance of Stat5 activation in IL-2 signaling was first suggested by transfection experiments using either deletion or other mutated forms of IL-2Rβ. Transfection of an IL-2Rβ cDNA containing a deletion of the region spanning Tyr-392 and Tyr-510, or of an IL-2Rβ mutant with substitution of these two tyrosine residues by phenylalanine residues, abolished the ability of IL-2 to activate Stat5 in 32D cells (Friedmann *et al*., 1996; Goldsmith *et al*., 1995; Lin *et al*., 1996). Furthermore, expression of these IL-2Rβ mutant constructs also diminished IL-2-induced proliferation in these cells (Friedmann *et al*., 1996; Goldsmith *et al*., 1995). In COS-7 cells which constitutively express Jak1, when an expression vector encoding Jak3 was co-transfected with expression vectors encoding IL-2Rβ, γ_c, and Stat5, IL-2-inducible activation of Stat5 was reconstituted (Lin *et al*., 1996). Transfection of Jak1 results in constitutive phosphorylation of Stat5. These data are consistent with the possibility that Jak3 is required for phosphorylation of Tyr-694 of Stat5a and Tyr-699 of Stat5b in response to IL-2, whereas Jak1 is involved mainly in phosphorylation of Tyr-392 and Tyr-510 of the IL-2Rβ chain (Friedmann *et al*., 1996). However, it remains unproven which JAK is responsible for phosphorylation of IL-2Rβ *vs* Stat5 proteins *in vivo*. What is clear, however, is that both JAKs are required for normal IL-2-mediated Stat5 activation (Nosaka *et al*., 1995; Park *et al*., 1995; Rodig *et al*., 1998; Thomis *et al*., 1995).

Stat5 proteins have also been reported to be phosphorylated on serine (Yamashita *et al*., 1998). However, in contrast to Stat1 and Stat3 where serine phosphorylation is shown to be important for their transactivation activity (Wen *et al*., 1995), a definitive mapping of serine phosphorylation sites on Stat5a and

Stat5b has not been reported. One study used an antibody specific for a phospho-serine and provided indirect evidence for its phosphorylation but this serine phosphorylation did not appear to be important for Stat5 transactivation (Yamashita *et al*., 1998).

Complexity of the DNA binding sites recognized by Stat5

Consistent with the high overall sequence similarities between Stat5a and Stat5b, including in their DNA binding domains (which only differ by six amino acids), a DNA binding site selection assay revealed that Stat5a and Stat5b dimers bind to similar core TTC(T/C)N(G/A)GAA γ-interferon activated sequence (GAS) motifs (Soldaini *et al*., 2000). Consistent with this observation, specific binding sites for Stat5a and Stat5b have not been clearly demonstrated. Interestingly, purified Stat5a appeared to have a higher DNA binding affinity than was seen with Stat5b (Soldaini *et al*., 2000). In addition to binding as a dimer, the purified Stat5a protein bound to DNA as a tetrameric form as suggested by a distinct mobility, while the purified Stat5b protein preferentially bound DNA as dimers (Soldaini *et al*., 2000).

Sequence analysis of the sites selected by the tetramers revealed that only 18 out of the 50 tetrameric binding sites contained a consensus $TTCN_3GAA$ motif (GASc), while all of the rest contained a GAS motif with single nucleotide change from the $TTCN_3GAA$ consensus (GASn). Another interesting feature is that either a TTC or GAA 'half-GAS' motif at a distance of $\geqslant 5$ bp away from the GAS motif was found in 33 of the 50 tetrameric binding sites. The majority of the TTC half-sites were located 6 bp downstream from the GASc or GASn, while most of the GAA half-sites were located 12 bp apart from the GASc or GASn. It therefore appears that the presence of a second, more divergent GAS motif spaced 6 bp from a more conserved GAS motif is sufficient for helping to recruit a Stat5 tetramer (Soldaini *et al*., 2000). The wide range of sites selected by Stat5a tetramers indicates that a large repertoire of potential physiological tetrameric Stat5 binding sites is likely to be capable of regulating target gene expression. This finding also implies that when examining promoter sequences, many important Stat5 binding sites might potentially be missed if one only scores for canonical $TTCN_3GAA$ sites. Stat5a is the only STAT protein whose binding as a tetramer has been formally analysed by a binding site selection analysis. However, preferences of Stat1, Stat3, Stat4, and Stat6 binding as dimers have been analysed. Whereas Stat5a and Stat5b dimers both preferred a TTC(T/C)N(G/A)GAA sequences, Stat1, Stat3, and Stat4 were reported to prefer somewhat more stringent TTCC(C/G)GGAA motifs (Horvath *et al*., 1995; Xu *et al*., 1996) while Stat6 prefers a TTCNTNGGAA motif (Schindler *et al*., 1995).

How do Stat5 proteins regulate transcription of target genes?

To achieve its biological effects, IL-2 regulates expression of a number of cellular genes, including IL-2Rα, IL-2Rβ, genes required for cell cycle progression, anti-apoptotic functions, and others. IL-2 regulates the expression of some of these genes mainly at the transcriptional level, in part through Stat5. So far, one of the best understood examples of transcriptional regulation by IL-2 through Stat5 proteins is from analysis of the IL-2Rα promoter (John *et al*., 1996; 1999; Lecine *et al*., 1996; Meyer *et al*., 1997; Serdobova *et al*., 1997; Sperisen *et al*., 1995). As illustrated in Figure 5, the human IL-2Rα promoter contains three well-characterized positive regulatory regions (PRRs). PRRI is located at -299 to -228 relative to the major transcription initiation site and binds to NF-κB serum response factor (SRF) and Sp1 (Ballard *et al*., 1989; Bohnlein *et al*., 1988; Cross *et al*., 1989; Leung and Nabel, 1988; Roman *et al*., 1990; Toledano *et al*., 1990). PRRII spans the region between -137 to -64 and binds to an Ets family protein, Elf-1, and the high mobility group proteins, HMG-I/Y (John *et al*., 1995; Lecine *et al*., 1996). PRRIII is located between -3780 and -3703 and binds to Stat5, Elf-1, as well as a GATA-1-like protein (John *et al*., 1996; Meyer *et al*., 1997; Serdobova *et al*., 1997). These three positive regulatory regions are conserved in both the human and murine IL-2Rα promoters, except that the binding site for SRF is not conserved in the murine IL-2Rα promoter region.

Within PRRIII, there are two tandemly-linked Stat5 binding sites, a consensus GAS motif followed by a non-consensus one. Both sites are required for efficient binding of Stat5 proteins, indicating cooperative binding of Stat5 to these sites (John *et al*., 1999). The cooperative binding of Stat5 is at least in part mediated by tetramer formation of Stat5 through protein-protein interactions (John *et al*., 1999). A key residue involved in the tetramerization of STAT proteins is Trp 37 (Vinkemeier *et al*., 1998). This residue is conserved in all STAT proteins, including Stat5. In the context of Stat5, Lys 70 in the Stat5 N-terminal domain is also important (John *et al*., 1999). Stat5 tetramer formation undoubtedly plays an important role in mediating cooperative binding of Stat5 proteins to the tandemly-linked low affinity GAS motifs. This notion is supported by the observation that a tetramerization-deficient Stat5 protein (mutated at Trp 37 alone or also mutated at Lys 70) cannot mediate PRRIII-dependent transcription (John *et al*., 1999). Interestingly, conversion of the low affinity GAS motifs in PRRIII into the high affinity GAS motifs, as predicted and confirmed by the DNA binding assay, still allows efficient activation by a mutant Stat5 protein that can no longer form tetramers (John *et al*., 1999). This demonstrates that the need for

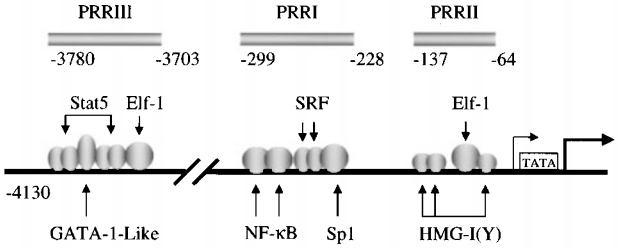

Figure 5 Summary of the regulatory elements and corresponding factors identified in the human IL-2Rα promoter region

tetramerization can be overcome by augmenting binding affinity, and suggests that a physiological role of tetramerization is to allow augmented binding affinity of the STAT proteins to low affinity GAS motifs, thus potentially expanding the repertoire of DNA binding sites that can be recognized by STAT proteins.

Interestingly, binding of Stat5 alone does not appear to be sufficient for maximal IL-2-mediated PRRIII activity; the Elf-1 binding site in PRRIII is also required (John *et al*., 1996; Lecine *et al*., 1996). This suggests that more than one element is required for IL-2-induced activation of PRRIII. Consistent with this idea, in 32D cells transfected with IL-2Rβ (so that they can respond to either IL-2 or IL-3) only IL-2 induces IL-2Rα mRNA levels even though both of IL-2 and IL-3 activate Stat5 proteins (Ascherman *et al*., 1997). All of these data emphasize the importance of the functional cooperativity of Stat5 with other transcription factors in regulating IL-2Rα expression *in vivo*.

Another important regulatory mechanism to influence the expression of STAT-mediated target genes is undoubtedly determined by the ability of a given STAT protein to interact with other transcription factor(s) and/or coactivator(s). In this regard, a number of proteins have been reported to interact with STAT family proteins. For instance, Sp1 has been reported to interact with Stat1 (Look *et al*., 1995), c-Jun with Stat3β (Schaefer *et al*., 1995), MCM5 with Stat1α (Zhang *et al*., 1998a), and the glucocorticoid receptor with Stat3, Stat5a, and Stat5b (Cella *et al*., 1998; Stocklin *et al*., 1996; Zhang *et al*., 1997). In addition, coactivators CBP/p300 have been shown to interact with Stat1, Stat2, Stat5a, Stat5b and Stat6 (Bhattacharya *et al*., 1996; Gingras *et al*., 1999; Pfitzner *et al*., 1998; Zhang *et al*., 1996; Zhu *et al*., 1999). N-*myc* interacting protein (Nmi) was shown to associate with Stat5b in a yeast two-hybrid interaction trap and by coprecipitation (Zhu *et al*., 1999). The exact mechanism(s) by which these transcription factors and coactivators integrate their effects into the signals mediated by STAT proteins require further work, but it seems that ubiquitously expressed coactivators CBP/p300 are generally important for STAT-mediated transcription.

In addition to direct activation of Stat5 proteins by JAK kinases in response to cytokines, Stat5-mediated transactivation can be augmented by other substrates of JAK kinases. In this regard, stimulation of T cells or NK cells by IL-2 also induces tyrosine phosphorylation of SH2 containing phosphatase (SHP) 2 and the scaffolding protein p97/Gab2 (Gadina *et al*., 1998, 1999; Gesbert *et al*., 1998; Gu *et al*., 1998). Tyrosine phosphorylation of p97/Gab2 is most likely mediated by Jak3 and seems to be specific for certain cytokines, since tyrosine phosphorylation of p97/Gab2 is not induced by IL-4 (Gadina *et al*., 1999). SHP-2 is constitutively associated with Jak1 and Jak3; however, it is not yet clear whether it is phosphorylated by these JAK kinases. Over-expression of SHP-2 augments STAT-mediated reporter activity (Gadina *et al*., 1998). Whereas over-expression of the dominant negative forms of either SHP-2 or p97/Gab2 diminishes IL-2-induced STAT-mediated reporter activity (Gadina *et al*., 1998; Gu *et al*., 1998). These data suggest that SHP-2 and p97/Gab2 are both involved in positively regulating Stat5-mediated transactivation in response to IL-2 stimulation. In contrast to these findings, it has recently been reported that SHP-2, but not SHP-1, can directly dephosphorylate Stat5 proteins *in vitro*, implying that it may be instead involved in the negative regulation of Stat5 activity (Yu *et al*., 2000). Therefore, the underlying mechanisms as to how SHP-2 and p97/Gab2 regulate Stat5-driven transcription in response to IL-2 clearly remain to be further elucidated.

How are IL-2-activated Stat5 signals turned off?

To maintain a balanced activation status of STAT proteins, they are also negatively regulated by a number of mechanisms. It has been demonstrated that STAT signals can be inactivated by dephosphorylation of the conserved tyrosine through tyrosine phosphatase(s) (Haspel and Darnell, 1999; Haspel *et al*., 1996; Shuai *et al*., 1996). This can also be achieved by ubiquitin/proteasome-mediated degradation of the STAT proteins (Kim and Maniatis, 1996; Yu and Burakoff, 1997), or by a direct inhibition through a more classical negative feedback loop mediated by CIS/SOCS/JAB/SSI family of proteins (Kovanen and Leonard, 1999). The specific association of PIAS1 (protein inhibitor of activated STAT) with Stat1 (Liu *et al*., 1998) and PIAS3 with Stat3 (Chung *et al*., 1997) as inhibitors of Stat1 and Stat3 DNA binding activities, respectively, provides another mechanism to negatively regulate STAT activity at DNA binding level. Competition of Stat6 binding by BCL-6 (Dent *et al*., 1997) represents an additional mechanism for this kind of negative regulation. Among these mechanisms, the one mediated by CIS/SOCS/JAB/SSI family of proteins has attracted more investigations.

The CIS (cytokine-inducible SH2-containing) protein is the prototype member of the CIS/SOCS/JAB/SSI family of proteins that are rapidly induced by a number of cytokines, including IL-2 (Yoshimura *et al*., 1995). So far, eight members of this family of proteins have been identified (Hilton *et al*., 1998; Kovanen and Leonard, 1999). Expression of CIS is regulated at least in part by cytokine-induced STAT proteins (Matsumoto *et al*., 1997; Verdier *et al*., 1998). The biological importance of this family of proteins in modulating cytokine signals was first implicated by those *in vitro* studies and then further underscored by analyses of CIS transgenic mice and mice deficient in SOCS1 and SOCS3.

An *in vitro* study shows that CIS protein associates with IL-2Rβ through the 'A' region of the IL-2Rβ chain (residues 313–382) (Aman *et al*., 1999). When over-expressed, the wild type form of CIS but not a mutant form of CIS in which the critical arginine in the SH2 domain is mutated to alanine, inhibited IL-2-induced Stat5 activation. This indicates that CIS might be involved in negative regulation of Stat5-mediated IL-2 signals and that the SH2 domain of CIS is required for this activity. It is known that CIS does not inhibit the catalytic activity of Jak1 and cannot interact with JAK kinases (Aman *et al*., 1999). However, the basis for the inhibitory effect of Stat5 activation by CIS is unclear. In CIS transgenic mice (Matsumoto *et al*., 1999), expression of IL-2Rα in T cells and proliferation of T cells in response to IL-2 are partially suppressed. This is most

likely explained by a marked inhibition of Stat5 activation by IL-2 in T cells from these mice, consistent with the *in vitro* findings. Unlike CIS, which associates with IL-2Rβ but not Jak kinases, SOCS3 primarily interacts with Jak1 and can inhibit Jak1 activation. When over-expressed in BaF3 cells, SOCS3 suppresses phosphorylation of Stat5 and proliferation in response to IL-2. However, analyses of either SOCS3 transgenic or SOCS3$^{-/-}$ gene-targeted mice indicate that SOCS3 plays a critical role in negatively regulating fetal liver erythropoiesis (Marine *et al*., 1999a). The integrity of the IL-2 signaling pathway remains to be investigated in these mice. SOCS1$^{-/-}$ mice exhibit abnormal thymocyte differentiation and spontaneous activation of peripheral T cells so that they proliferate in response to IL-2 in the absence of priming by anti-CD3. These data indicate that SOCS1 plays a vital role in directing thymocyte differentiation as well as in controlling signaling pathways of certain cytokines, including IL-2 (Marine *et al*., 1999b). Thus, more than one CIS/SOCS/SSI family protein may be involved in negative regulation of IL-2 signaling *in vivo*. As discussed above, at least *in vitro* studies show that CIS and SOCS3 can influence Stat5 signals through inhibition of the different steps of the activation cascade. The exact mechanism of how CIS/SOCS/SSI family proteins mediate their inhibitory effects as well as the proteins that are recruited to the SH2 domain of this family of proteins remain areas for further investigation.

Are Stat5a and Stat5b functionally redundant in the immune system?

The biological significance of Stat5 proteins in IL-2 signaling is further established from analyses of gene-targeted mice that lack expression of Stat5 proteins. Originally, Stat5a was discovered as a mammary gland factor and indeed Stat5a$^{-/-}$ mice exhibit a profound defect in adult mammary gland development and lactogenesis (Liu *et al*., 1997). On the other hand, Stat5b$^{-/-}$ mice develop the defects related to the growth hormone actions (Udy *et al*., 1997). However, both types of the knockout mice additionally exhibit marked defects in the immune responses. In Stat5a$^{-/-}$ mice, there is a defect in IL-2-induced IL-2Rα expression (Nakajima *et al*., 1997). Consistent with this observation, splenocytes from Stat5a$^{-/-}$ mice exhibit markedly decreased proliferation to low concentrations of IL-2, although maximal proliferation is still achieved at concentrations of IL-2 high enough to titrate intermediate affinity IL-2Rβ/γ_c receptors. This defect also correlates with defective Vβ8$^+$CD8$^+$ T-cell expansion *in vivo* in response to the superantigen, staphylococcus enterotoxin B (Nakajima *et al*., 1997). Moreover, although the number of thymocytes is normal, the number of splenocytes is modestly reduced (Nakajima *et al*., 1997). In Stat5b$^{-/-}$ mice, there is a modest decrease in the number of both thymocytes and splenocytes (Imada *et al*., 1998). There is a much greater defect in proliferation in response to IL-2 in Stat5b$^{-/-}$ mice than is seen in the Stat5a$^{-/-}$ mice. In addition, proliferation of splenocytes in response to either a combination of PMA and IL-4 (Imada *et al*., 1998) or IL-4 alone was diminished in Stat5b$^{-/-}$ mice but not in Stat5a$^{-/-}$ mice (Moriggl *et al*., 1999a,b;). Therefore, analyses of the Stat5a$^{-/-}$ and Stat5b$^{-/-}$ single knockout mice clearly reveal that the functions of these two proteins are not fully redundant or overlapping at least in terms of their ability to mediate some of the IL-2 and IL-4 actions. Both Stat5a and Stat5b contribute to normal NK cell development, with a greater defect being seen for NK function in Stat5b$^{-/-}$ mice (Imada *et al*., 1998), which appears to be explained at least in part by a marked defect in IL-2- and IL-15-induced perforin gene induction in these mice. Interestingly, expression of both IL-2Rα and IL-2Rβ is diminished in Stat5b$^{-/-}$ mice, at least in part explaining the defects in both of IL-2 and IL-15 response in these mice. Perhaps in part for this reason, unlike the situation seen in Stat5a$^{-/-}$ mice, high dose IL-2 failed to normalize the defective proliferation in splenocytes from Stat5b$^{-/-}$ mice.

Interestingly, Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice both also exhibit defects in antigen-induced airway eosinophilia, CD4$^+$ T-cell infiltration and IL-5 production in the airways (Kagami *et al*., 2000). In addition, antigen-specific IgE and IgG1 production was decreased only in Stat5a$^{-/-}$ mice but not in Stat5b$^{-/-}$ mice, whereas antigen-specific IgG2a production was increased in Stat5a$^{-/-}$ mice but not in Stat5b$^{-/-}$ mice, suggesting an increased Th1 response in Stat5a$^{-/-}$ mice (Kagami *et al*., 2000).

In Stat5a$^{-/-}$/Stat5b$^{-/-}$ double knockout mice, the phenotype is not surprisingly more severe than that found in either of the individual knockout mice (Moriggl *et al*., 1999a; Teglund *et al*., 1998). In these mice, peripheral T cells are profoundly deficient in proliferation and fail to undergo cell cycle progression. These phenotypes indicate that Stat5 proteins play a vital role in IL-2 signaling in T cells. The inability of T cells from the double knockout mice to proliferate in response to anti-CD3 activation could be potentially explained by an observation that activation of T-cell receptor by antigen can trigger a transient activation of Stat5 proteins in murine cells (Welte *et al*., 1999). However, the activation of Stat5 by TCR signals is controversial and has not been observed in human T cells (Beadling *et al*., 1994) or in another study in murine T cells (Moriggl *et al*., 1999a). One of the striking phenotypes in the double knockout mice is the absence of NK cells. Thus, Stat5a and Stat5b together are vital for NK cell development, whereas Stat5b appears to be more important for perforin gene induction and cytolytic activity.

Potential roles of Stat5 in transformation, immune surveillance and tolerance

As illustrated above, in normal lymphocytes and those cell lines dependent upon IL-2 for growth, activation of Stat5 proteins by IL-2 at least in part contributes to the proliferative response of IL-2. In contrast, in human T cell lymphotropic virus I (HTLV-1) transformed T-leukemia cell lines, Stat5 proteins were constitutively activated (Migone *et al*., 1995). In these cells, both Jak1 and Jak3 were also activated, suggesting that constitutive activation of the Jak1/Jak3-Stat5 pathway may be at least in part responsible for IL-2-independent growth of these HTLV-1 transformed cells. In this regard, acquisition of the constitutive activation of JAK-STAT

in these cells was correlated with a transition of HTLV-1 transformed cells from an IL-2-dependent to an IL-2-independent phase of growth (Migone *et al*., 1995). The HTLV-1 transactivation protein Tax did not appear to be responsible for constitutive activation of JAK-STAT pathway in these cells (Migone *et al*., 1995), although it has been shown to modulate transactivation activity of a number of transcription factors (Yodoi and Uchiyama, 1992; Yoshida and Seiki, 1987). More work is needed to clarify the molecular mechanism(s) by which constitutive activation of JAK-STAT pathway occurs in these HTLV-1 transformed leukemia cells. Interestingly, in patients with HTLV-I-induced adult T-cell leukemia, a constitutively activated Jak-STAT pathway was associated with augmented cell-cycle progression, indicating that the *in vitro* findings extend *in vivo* to an human disease (Takemoto *et al*., 1997).

Progression of tumor growth in immuno-compromised animals and in the advanced stages of tumor patients is usually associated with impaired immune functions. An analysis of immuno-compromised tumor-bearing mice suggests that Stat5 proteins may play an important role in the immune responses against tumor progression (Pericle *et al*., 1997). In both T and B cells isolated from these mice, the expression levels of Stat5a and Stat5b proteins are markedly decreased. In contrast, the expression levels of Stat1, Stat3, Stat6 and TCR are normal. These data show a correlation between the selective depression of Stat5 protein levels and progression of tumor growth in these experimental immuno-compromised tumor-bearing animals. Elucidation of the molecular mechanism(s) of the selective depression of Stat5 proteins in these mice should eventually help to clarify how progression of tumor growth can be controlled.

Two groups have recently reported the potential effects of HIV infection on STAT proteins. One group found that the levels of Stat5a, Stat5b, and Stat1 were all decreased in purified T cells isolated from HIV patients, suggesting that the reduction of these STAT proteins may contribute to the loss of T cell function over the course of HIV infection (Pericle *et al*., 1998). In contrast, an independent study showed that the C-terminal truncated Stat5 and Stat1α were constitutively activated in peripheral blood mononuclear cells isolated from HIV patients (Bovolenta *et al*., 1999). The discrepancies between these two studies remain to be clarified.

Another important role played by IL-2 is to render the activated T cells susceptible to Fas-mediated activation-induced cell death (AICD). This function is especially important for eliminating auto-reactive T cells *in vivo* and thus for terminating abnormal T cell responses and maintain tolerance. It has recently been demonstrated that activation of Stat5 proteins by IL-2 leads to an increased expression of Fas ligand (FasL) (Refaeli *et al*., 1998; Van Parijs *et al*., 1999). The evidence for the involvement of Stat5 proteins in promoting IL-2-induced AICD is provided recently (Van Parijs *et al*., 1999). Analyzing Stat5a$^{-/-}$/Stat5b$^{-/-}$ mice shows that T cells from these mice have a decreased expression of FasL following TCR cross-linking and a reduced sensitivity to AICD that can be rescued by over-expression of full-length wild type Stat5a proteins. Moreover, over-expression of dominant negative, but not the wild type, Stat5 protein reduces the sensitivity of T cells from normal mice to AICD.

Conclusion

The JAK-Stat5 pathway is one of the most rapidly activated signaling pathways following stimulation by IL-2 family cytokines. This pathway, presumably in concert with others, allows an efficient cellular reaction in response to the micro-environmental changes *in vivo*. This system plays roles in lymphoid development as well as in modulation of T-cell and NK-cell immune responses. Over the past few years, a large amount of information obtained from extensive investigations of this signaling pathway have tremendously advanced our understanding of the biological actions induced by IL-2 family cytokines.

One of the striking features of Stat5a and Stat5b proteins is that their overall amino acid sequence similarities are greater than 90%, including in the DNA binding domain. Consistent with this highly conserved structural feature, homodimers of each of these two proteins recognize very similar high affinity core GAS motifs. At least *in vitro*, purified Stat5a protein was able to efficiently bind to tandemly-linked low affinity GAS motifs through formation of homo-tetramers, whereas Stat5b was less effective in forming homo-tetramers (Soldaini *et al*., 2000). Whether hetero-tetramers can form between Stat5a and Stat5b dimers has not been reported, but studies on regulation of the IL-2Rα gene suggests that this might be able to happen based on the following two analyses. First, both Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice exhibit defective IL-2-induced IL-2Rα expression (Imada *et al*., 1998; Nakajima *et al*., 1997), indicating the importance of both of these Stat5 proteins in the regulation of this gene. Second, Stat5 tetramer formation appears to be important for IL-2-induced IL-2Rα regulation (John *et al*., 1999). Given that both Stat5a and Stat5b are required and that tetramerization is important, it is therefore reasonable to think that heterotetramers might be able to form over the tandem GAS sites in the PRRIII IL-2 response element in the IL-2Rα gene. It is clear, however, that Stat5 tetramer formation is not critical for Stat5-mediated transcription of reporter constructs containing high affinity GAS motifs (John *et al*., 1999), a finding that potentially extends to certain Stat5-dependent genes. Given the apparent difference between Stat5a and Stat5b in terms of the efficiency of tetramer formation and the different phenotypes observed in Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice, it is reasonable to postulate that these two closely related proteins regulate the expression of some distinct target genes as well as the identical ones, and that they have both overlapping and more distinctive actions. SAGE (serial analysis of gene expression) and DNA array technology may be ways to compare the target genes regulated primarily by Stat5a and Stat5b in gene-targeted mice, allowing a clarification of the molecular bases for both functional similarities and differences for these two closely related proteins.

A careful analysis of the IL-2Rα promoter also has revealed that achieving maximal IL-2-induced IL-2Rα transcription is dependent on the functional cooperativity of Stat5 proteins with other transcription factor(s). An interesting area that remains to be carefully investigated is whether Stat5a and Stat5b proteins equally interact with the same kind of transcription factor(s) and/or co-factor(s), or whether

there are differences, perhaps mediated by their divergent transactivation domains.

Activation of SHP-2 and p97/Gab2 by IL-2 may represent additional regulatory controls to modulate Stat5-dependent gene expression, although the mechanisms involved remain to be further clarified. A related area that requires attention is how different signaling pathway(s) activated by a given member of the IL-2 family cytokines, such as serine/threonine kinases and/or other tyrosine kinases, might contribute to Stat5-mediated gene expression and eventually to the biological outcome of the cytokine action. Finally, negative regulatory control mechanisms undoubtedly play important roles in determining the magnitude and duration of Stat5-mediated transcription. Such mechanisms potentially include dephosphorylation of Stat5 or potentially of phosphotyrosine docking sites on the receptor, degradation of Stat5 proteins, and the negative feedback loop mediated by CIS/SOCS/SSI family proteins. Each of these clearly warrants further investigation.

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