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Jak1 Has a Dominant Role over Jak3 in Signal Transduction through γc-Containing Cytokine Receptors

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SUMMARY

Genetic deficiency of Jak3 leads to abrogation of signal transduction through the common gamma chain (γc) and thus to immunodeficiency suggesting that specific inhibition of Jak3 kinase may result in immunosuppression. Jak1 cooperates with Jak3 in signaling through γc-containing receptors. Unexpectedly, a Jak3-selective inhibitor was less efficient in abolishing STAT5 phosphorylation than pan-Jak inhibitors. We therefore explored the roles of Jak1 and Jak3 kinase functionality in signaling using a reconstituted system. The presence of kinase-inactive Jak1 but not kinase-inactive Jak3 resulted in complete abolishment of STAT5 phosphorylation. Specific inhibition of the "analog-sensitive" mutant AS-Jak1 but not AS-Jak3 by the ATP-competitive analog 1NM-PP1 abrogated IL-2 signaling, corroborating the data with the selective Jak3 inhibitor. Jak1 thus plays a dominant role over Jak3 and these data challenge the notion that selective ATP-competitive Jak3 kinase inhibitors will be effective.

INTRODUCTION

The physiological effects of interleukins (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 are initiated by binding of these cytokines to their cognate receptor which triggers a complex signal transduction cascade. All these cytokines utilize receptors containing the common gamma chain (γc, CD132) which is paired with a corresponding α chain (for IL-4, -7, -9, -21) or β chain (for IL-2 and IL-15) which determine ligand specificity. IL-2 and IL-15 receptors also contain a nonsignaling α chain. Work performed in the last two decades established that Janus kinases (Jak) 1 and 3 are essential tyrosine kinases associated with the intracellular domains of the above-mentioned cytokine receptors with Jak3 binding to γc and Jak1 to the other chain (Benczik and Gaffen, 2004; Gaffen, 2001; Imada and Leonard, 2000; Johnston et al., 1994, 1996; Kawamura et al., 1994; Oakes et al., 1996). While Jak1 is widely expressed and is part of the signaling apparatus of several cytokine receptors, Jak3 expression is restricted to cells of the hematopoietic lineage and is coexpressed and exclusively associated with γc. Loss-of-function mutations in either γc or Jak3 in humans or experimental deletion of either one of these in mice result in severe combined immunodeficiency (Cao et al., 1995; DiSanto et al., 1995; Notarangelo et al., 2000; Rodig et al., 1998; Thomis et al., 1995) indicating the importance of this pathway in the establishment and maintenance of a functionally competent immune system (O'Shea et al., 2004a). Thus, it has been assumed that inhibitors of signal transduction through γc/Jak3, in particular, selective inhibitors of the Jak3 kinase activity, might be effective immunosuppressive drugs with a potentially improved safety profile compared with drugs targeting other mechanisms (Borie et al., 2004; O'Shea et al., 2004b, 2005; Pesu et al., 2005, 2008).

Signaling through γc cytokines, however, also requires Jak1. Cells lacking either Jak3 or Jak1 are unable to respond to γc cytokines (Rodig et al., 1998; Thomis et al., 1995). Although differences in the biochemical steps associated with cytokine receptor-triggered signal transduction have been described, it has been assumed that both Jak kinases are equally important for signal transduction. (Chen et al., 2000; Cheng et al., 2008; Fujii, 2008; Kirken et al., 1995; Liu et al., 1997; Zhou et al., 1997). Several low molecular weight Jak inhibitors have been reported recently which potently inhibit kinase activity in vitro and cellular responses to cytokines (reviewed in Haan et al., 2010; Pesu et al., 2008). One such inhibitor, CP-690,550, has been described to be effective in models of autoimmune diseases, transplant rejection, and inflammation, suggesting the importance of this signaling pathway and its therapeutic potential (Kudlacz et al., 2004, 2008). Clinical trials with Jak inhibitors are underway with CP-690,550 being currently assessed in multiple indications (http://clinicaltrials.gov). Although originally described as being selective for Jak3 (Changelian et al., 2003), CP-609,550, also potently inhibits Jak1 (Jiang et al., 2008; Karaman et al., 2008; Williams et al., 2009) and thus efficacy could be determined by the combined inhibition of both kinases. Therefore, it is not elucidated whether inhibition

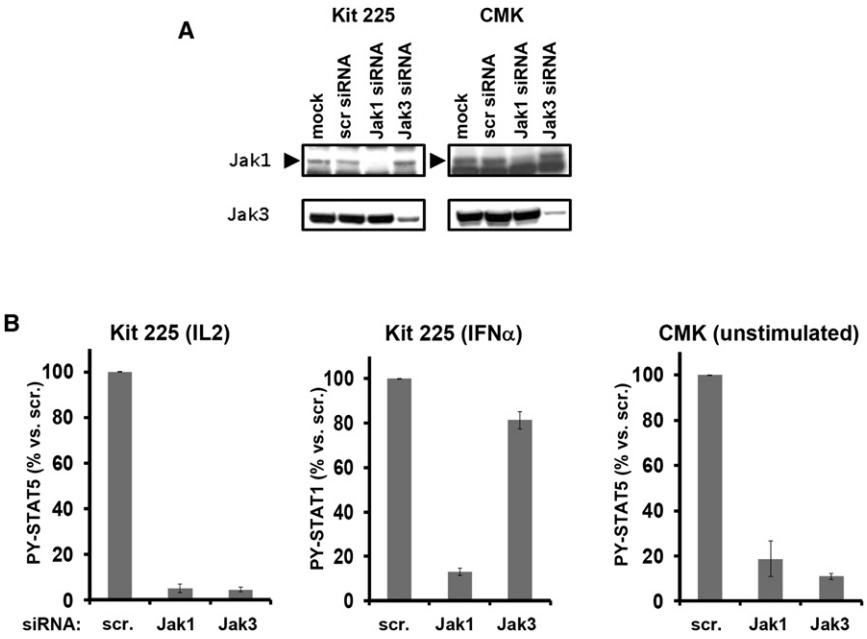


Figure 1. si-RNA Knockdown of Jak1 or Jak3 Abrogates IL-2-Induced and Jak3A572V-Dependent Constitutive STAT5 Phosphorylation

(A) Kit225 and CMK cells were transfected with si-RNA specific for Jak1 or Jak3 or a scrambled control si-RNA. Western blots of lysates were probed with Jak1 and Jak3 antibodies.

(B) STAT5 and STAT1 phosphorylation was assessed by flow cytometry in Kit225 cells stimulated with IL-2 (left panel) or IFN-α (middle panel) or in unstimulated CMK cells (right panel). The cells were treated with si-RNAs as indicated and the graph shows the standard deviations for three to four experiments.

of Jak3 kinase activity is sufficient to efficiently suppress γc cytokine signaling as inferred from the phenotype of the γc and Jak3 deficiencies.

Given the importance of this pathway and intrigued by observations that selective inhibitors of Jak3 kinase showed unexpectedly poor cellular potency in cellular assays involving signaling through γc (Thoma et al., 2011), we set out to further explore the relative contributions of Jak1 and Jak3 in γc cytokine signaling. For this, we generated a reconstituted IL-2 signaling system in which we studied combinations of different mutations in Jak1 and Jak3. We demonstrate that, surprisingly, the two Jak kinases are not of equal importance in IL-2 signal transduction. Our data indicate that the contribution of Jak1 kinase functionality is dominant while Jak3 kinase activity is subordinate and its activity merely enhances the effect of Jak1.

RESULTS

Knockdown of Jak1 or Jak3 with si-RNA Abrogates IL-2-Dependent and Jak3A572V-Mediated Constitutive STAT5 Phosphorylation

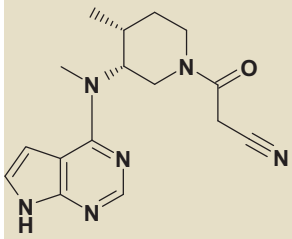
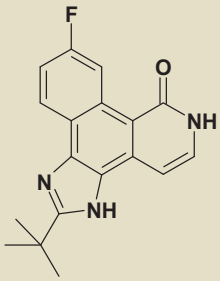
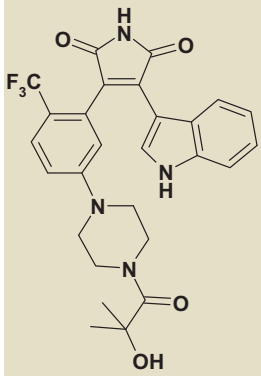
To interrogate whether Jak1 is required for signal transduction initiated by IL-2 or through constitutively active Jak3, we selectively depleted either Jak1 or Jak3 by specific si-RNAs in the human T lymphoma line Kit225 (expressing Jak1WT and Jak3WT) and in the human acute megakaryocytic leukemia cell line CMK which expresses a constitutively active mutant of Jak3 (Jak3A572V) (Walters et al., 2006) and does not require IL-2 for STAT5 phosphorylation. Each si-RNA downregulated its specific target efficiently in both cell lines as assessed by western blot analysis (Figure 1A). The functional consequence of the specific downregulation of either Jak was probed by monitoring IL-2-dependent (in Kit225 cells) or constitutive (in CMK cells) phospho-STAT5 (P-STAT5) by flow cytometry. In both cell lines, either Jak1 or Jak3 si-RNA markedly reduced STAT5 phosphorylation (Figure 1B). As a control, IFN-α-induced STAT1 phosphorylation (which is known to be dependent on Jak1 and Tyk2 but does not involve Jak3) was only reduced by Jak1 si-RNA but not by Jak3 si-RNA in Kit225 cells. Thus, in IL-2-dependent signaling as well as in signaling through the constitutively active mutant Jak3A572V, both Jak1 and Jak3 proteins are required. Constitutively catalytically active Jak3 is insufficient to lead to STAT5 phosphorylation in the absence of Jak1.

Specific Inhibition of Jak3 Kinase Activity Is Less Effective in Blocking IL-2-Dependent Phosphorylation of STAT5 and Other Signaling Molecules than pan-Jak Inhibition

Three kinase inhibitors (structures shown in Table 1) with activity against Jak family members were tested in enzyme assays with kinase domains of the four Jaks. In agreement with literature data (Changelian et al., 2003; Karaman et al., 2008; Thompson et al., 2002; Williams et al., 2009), CP-690,550 and Jak inhibitor I showed no significant selectivity for Jak3 over Jak1. NIBR3049 (Thoma et al., 2011) selectively inhibited Jak3 at low nanomolar concentrations and showed greater than 150-fold selectivity over the other Janus kinases (Table 1).

The effect of the three compounds was investigated on IL-2 or IFN-α-dependent stimulation of STAT phosphorylation in CMK cells and the human T lymphoma line Kit225. CMK cells were investigated for basal P-STAT5 and for IFN-α-induced P-STAT1. STAT5 and STAT1 phosphorylation were measured by flow cytometry with phosphospecific antibodies (Table 2A). In agreement with the enzymatic data, CP-690,550 and Jak inhibitor 1 efficiently inhibited both cytokine-induced and constitutive STAT phosphorylation. NIBR3049 showed poor activity on signaling induced by IFN-α, in agreement with its poor potency toward Jak1, Jak2, and Tyk2. Surprisingly, the concentrations of the Jak3-specific compound NIBR3049 needed to suppress IL-2-dependent (Kit225) or constitutive (CMK) STAT5 phosphorylation were 20 to 50 fold higher compared with the pan-Jak inhibitors despite similar potencies in the Jak3 kinase assay. NIBR3049 also less efficiently inhibited other signaling pathways activated by IL-2 in Kit225 cells (see Figure S1 and Table S1

Table 1. Characterization of the Jak Inhibitors Used in This Study

Inhibitor		IC_{50} in Enzymatic Assay (nM)			
Structure	Denomination	Tyk2	Jak1	Jak2	Jak3
	**CP-690,550**	176 ± 25	6.1 ± 2	12 ± 2	8.0 ± 2
	**Jak inhibitor 1**	7 ± 0.5	5.2 ± 0.4	4.5 ± 0.5	41 ± 4.1
	**NIBR3049**	8055 ± 589	1017 ± 117	2550 ± 326	8.0 ± 2

IC_{50} values of the Jak inhibitors in enzyme assays are reported in nM as the average ± SEM of at least three experiments. Kinase activities were assessed against synthetic peptide substrates using the Caliper technology and were carried out as described in Experimental Procedures.

available online). The phosphorylation of STAT1 and STAT3 (which is also induced upon IL-2 albeit at much lower levels than STAT5) as well as of Erk1/2 and Akt, was inhibited at lower concentrations by the pan Jak inhibitor CP-690,550 compared with the selective Jak3 inhibitor NIBR3049.

CP-690,550 and NIBR3049 showed similar passive permeability, a measure indicating cell penetration (Wohnsland and Faller, 2001), and determinations of the intracellular concentrations of the two compounds indicated similar levels in CTLL cells (Thoma et al., 2011). Thus, the differences observed when comparing the Jak3-specific inhibitor to the pan-Jak inhibitors are not due to a lower intracellular availability of the Jak3-specific compound.

We next explored whether this pattern of activity applies to other cytokines utilizing γc-containing receptors and used primary human lymphocytes activated with anti-CD3 and anti-CD28 and expanded in the presence of IL-2. As shown in Table 2B, the pan–Jak inhibitors potently inhibit STAT5 phosphorylation induced in these cells by IL-2 and IL-15 as well as STAT6 phosphorylation induced by IL-4 (all three cytokines using receptors which utilize γc) while the Jak3-specific inhibitor NIBR3049 was less active. The response to IFN-α in these cells was similar to that seen in the cell lines shown in Table 2A. The striking difference in potency of pan-Jak inhibitors compared with a Jak3-selective inhibitor in preventing STAT5 phosphorylation was also seen in the mouse T cell line CTLL-2 upon IL-2 stimulation (Thoma et al., 2011) and the human acute megakaryoblastic leukemia line M-07e stimulated with IL-15 (see also Figure S1 and Table S1).

Jak1 Kinase Activity Is Required for STAT5 Phosphorylation by IL-2 or Constitutively Active Jak3

The si-RNA experiments are not informative whether presence of Jak3 protein as such is needed (to act as a scaffold) or whether its catalytic activity is mandatory to "support" Jak1 activity. The data obtained with small molecule inhibitors suggested that a selective Jak3 antagonist is much less potent at inhibiting

Table 2. Inhibition of STATPhosphorylation by Jak Inhibitors in (A) CMK and Kit225 Cells and (B) Human PBL

A

Cell Line	CMK		Kit225	
Stimulation	None	IFN-α	IL-2	IFN-α
Readout	*P-STAT5*	*P-STAT1*	*P-STAT5*	*P-STAT1*
CP-690,550	**90.5** ± 15	**199.0** ± 34	**26.6** ± 2	**32.3** ± 8
Jak inhibitor 1	**63.4** ± 8	**101.7** ± 12	**34.8** ± 12	**33.8** ± 1
NIBR3049	**2126.8** ± 282	**>20,000**	**738.1** ± 43	**4933.3** ± 97

B

Stimulation	IL-2	IL-4	IL-15	IFN-α
Readout	*P-STAT5*	*P-STAT6*	*P-STAT5*	*P-STAT1*
CP-690,550	**53.0** ± 7	**92.8** ± 27	**93.7** ± 29	**60.2** ± 9
Jak inhibitor 1	**55.7** ± 8	**57.0** ± 12	**62.3** ± 16	**24.7** ± 5
NIBR3049	**1232.7** ± 50	**1067.0** ± 131	**1460.7** ± 445	**5070.3**[a]

IC_{50} values in nM are indicated (three to eight independent experiments). See also Figure S1 and Table S1.
[a] Average of two measurements.

STAT5 phosphorylation compared with inhibitors which are about equipotent on Jak1 and Jak3, but they do not unambiguously prove that NIBR3049 inhibited Jak3 kinase function in a cellular context. To further investigate the mechanisms underlying these observations, we reconstituted IL-2 signaling in U4C cells (Kohlhuber et al., 1997) which are devoid of IL-2 receptor chains as well as Jak1 and Jak3. First, we generated stable transfectants expressing IL-2Rβ and IL-2Rγ. Then, all nine combinations of wild-type (WT), kinase-deficient (KD), and constitutively active mutants (CA) of Jak1 and Jak3 were stably expressed from the bidirectional pBOF-vector in U4C-IL-2Rβγ-TetOn-FlpIn cells (see Table 3 for constructs used). The transfected vector inserts into a Flp recombinase target (FRT) site and consequently, the different mutants are expressed from the same site in the genome. Induction by doxycycline (Dox) enabled the expression of controlled and equivalent levels of both Jaks, dependent on the Dox concentration used. At the Dox concentration of 5 μg/ml the expression of Jak1 was about 2-fold the endogenous levels seen in 2C4 cells, the parent cells from which the U4C cells were derived (see Figure S2 in the supplemental material). All nine cell lines were investigated with respect to their ability to induce STAT5 phosphorylation upon IL-2 stimulation as indicated. Figure 2A shows a representative western blot (see Figure S3) and Figure 2B the quantitation of four western blots.

In Jak1WT/Jak3KD-cells the IL-2-induced STAT5 phosphorylation was reduced by 50% compared with Jak1WT/Jak3WT-cells, while STAT5 was not phosphorylated at all in Jak1KD/Jak3WT-cells (lanes a-c in Figures 2A and 2B). The combination Jak1CA/Jak3CA led to constitutive IL-2-independent STAT5 phosphorylation (lane h in Figures 2A and 2B). Expression of Jak3CA translated into constitutive phosphorylation of STAT5 only when combined with Jak1WT, but not when combined with Jak1KD (lanes e and f in Figures 2A and 2B). The phospho-STAT5 signal was reduced to about 50% in Jak1CA/Jak3KD cells compared with Jak1CA/Jak3WT cells (lanes g and i in Figures 2A and 2B).

Thus, we conclude that for both IL-2-dependent and constitutive Jak signaling, Jak1 catalytic activity plays an essential role while Jak3 activity is not necessary although a decrease in intensity was associated with the loss of Jak3 activity (Figures 2A and 2B). It is noteworthy that constitutively active Jak1 could phosphorylate STAT5 independently of Jak3 kinase activity while the inverse was not the case (lanes i and e in Figures 2A and 2B).

Jak3 Phosphorylates Jak1 but Does Not Induce STAT5 Phosphorylation

Lysates of the stable transfectants treated as described above were investigated for Jak1 and Jak3 phosphorylation (Figure 2; Figure S4). Upon expression of Jak1WT/Jak3KD or the "inverse" pair Jak1KD/Jak3WT, only the kinase-deficient Jak was phosphorylated (a, b, and c in Figure 2C), i.e., in these combinations Jak1 phosphorylated Jak3 and vice versa. Note that only Jak1WT/Jak3KD could mediate STAT5 phosphorylation. No Jak or STAT5 phosphorylation was observed in Jak1KD/Jak3KD cells (d in Figure 2C).

When the constitutively active Jak1CA or Jak3CA were combined with each other or with their corresponding WT counterpart, constitutive Jak1 and Jak3 phosphorylation was observed which, however, could further be enhanced by IL-2 stimulation (a, g, f, and h in the right panel of Figure 2C).

In contrast, when Jak1CA and Jak3CA were paired with the corresponding inactive counterpart, no constitutive phosphorylation (Jak1KD/Jak3CA) or a very weak phosphorylation (Jak1CA/Jak3KD) was observed (e and i in the left panel of

Table 3. The Jak Constructs Used

Construct	Abbreviation	Function	Description
Jak1WT	WT	Wild-type	
Jak1K908A	***KD***	Kinase deficient	Mutation of the ATP coordinating lysine.
Jak1V658F	**CA**	Constitutively active	Activating mutation found in T and B cell lymphomas (Jeong et al., 2008; Mullighan et al., 2009; Staerk et al., 2005).
Jak1M956G	AS	Analog sensitive	Gatekeeper residue Met 956 mutated to Gly.
Jak3WT	WT	Wild-type	
Jak3K855A	*KD*	Kinase deficient	Mutation of the ATP coordinating lysine.
Jak3A572V	**CA**	Constitutively active	Activating mutation found in AMKL (Malinge et al., 2008; Walters et al., 2006).
Jak3M902G	AS	Analog sensitive	Gatekeeper residue Met 902 mutated to Gly.

See also Figure S2.

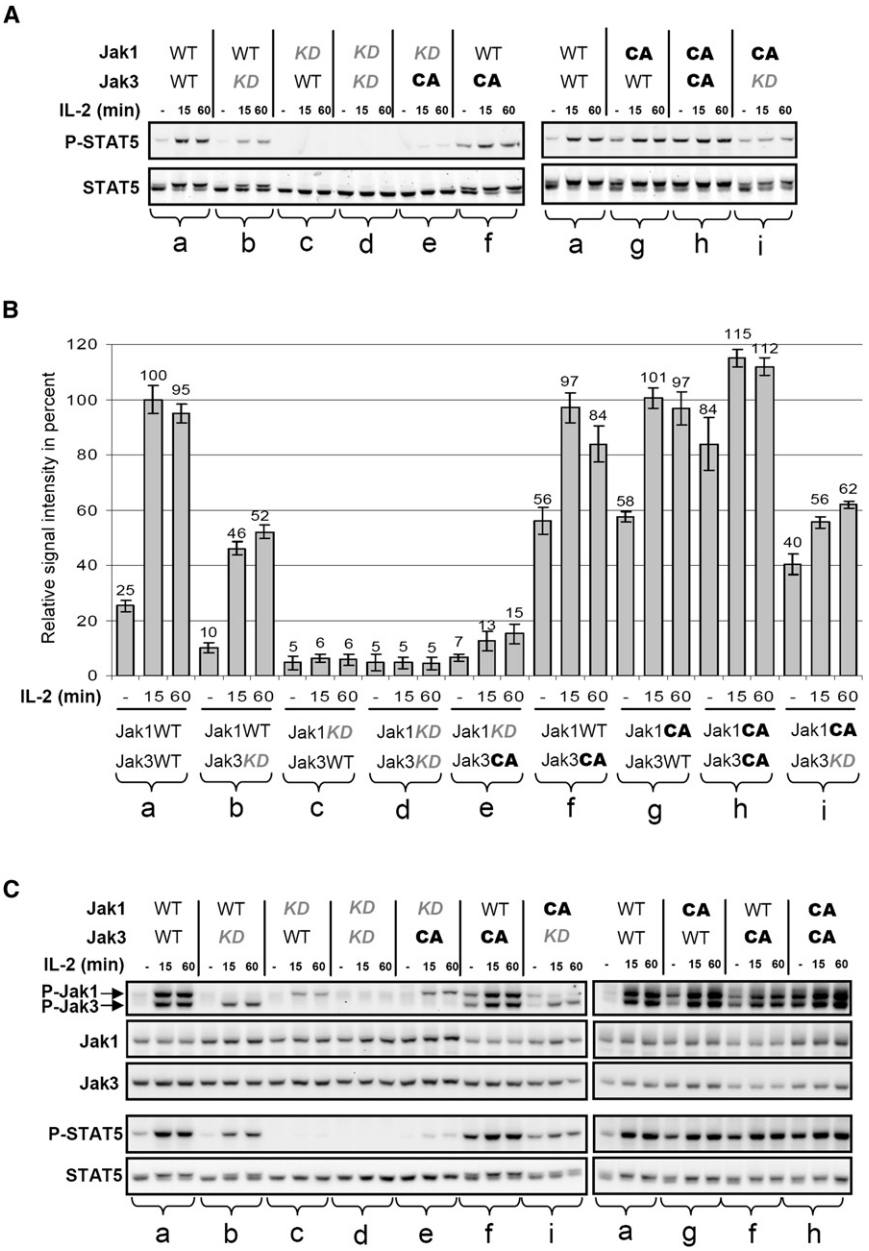


Figure 2. Investigation of the Contributions of Jak1 and Jak3 in a Reconstituted Cell System Expressing Different Combinations of Jak1/Jak3 Mutants

(A) U4C-IL-2Rβ/γ-FlpIn-TetOn cells expressing different combinations of Jak1 and Jak3 mutants were induced with 5 μg/ml Dox for 48 hr and stimulated with 1000 U/ml of IL-2 for 15 or 60 min as indicated. Western blots were probed with antibodies against P-STAT5 and STAT5. Fluorescently labeled secondary antibodies were used and the signals were quantified as described in Experimental Procedures.

(B) Results of the quantitation of four experiments performed as in (A). The P-STAT5 signals were represented as percent of the 15 min IL-2-stimulated P-STAT5 signal obtained for cells expressing Jak1WT/Jak3WT. The abbreviations are as shown in Table 3. Standard deviations are shown.

(C) Phosphorylation of the Janus kinases in the context of different combinations of Jak1/Jak3 mutants. U4C-IL-2Rβ/γ-FlpIn-TetOn cells expressing different combinations of Jak1and Jak3 mutants were induced with 5 μg/ml Dox for 48 hr and stimulated with 1000 U/ml of IL-2 as indicated. Western blots were probed with antibodies against P-Jak1. The blots were stripped and consecutively reprobed with anti P-STAT5, STAT5, Jak1 and Jak3 antibodies. The phosphospecific Jak1 antibody is cross-reactive and recognizes Jak1 and Jak3 phosphorylation equipotently.

See also Figure S3.

Figure 2C). Again only Jak1CA/Jak3KD led to a significant constitutive STAT5 phosphorylation, despite the low phosphorylation level of Jak1. However, upon IL-2 stimulation, both Jak1CA and Jak3CA phosphorylated their corresponding counterparts Jak3KD or Jak1KD. Interestingly, again the Jak1CA/Jak3KD but not the Jak1KD/Jak3CA situation led to significant STAT5 phosphorylation even in the absence of Jak1 phosphorylation (see e and i in Figure 2C).

Taken together, these data lead to the following model: Engagement of the IL-2 receptor activates Jak1 and Jak3 kinase activity independently of their phosphorylation state. Subsequently Jak1 and Jak3 phosphorylate each other. The role of Jak3 seems to be restricted to the phosphorylation of Jak1, while Jak1 phosphorylates Jak3 as well as STAT5. The constitutively active Jak3 and Jak1 mutants retain the same functions with the difference that for these hyperactive kinases IL-2 stimulation is not required for induction of STAT5 phosphorylation.

Specific Inhibition of Analog-Sensitive Mutants of Jak1 and Jak3 Confirms that Jak3 Catalytic Activity Is Dispensable for IL-2-Induced STAT5 Phosphorylation and that Jak1 and Jak3 Transphosphorylate

The contribution of the catalytic activity of a given kinase can be investigated by a chemical genetics approach. The ATP binding pocket of the kinase is enlarged by mutation of the gatekeeper residue and becomes thereby accessible to a bulky kinase inhibitor (1NM-PP1) which does neither fit into the ATP binding pocket of the wild-type kinase nor into other kinases present in the cell (Bishop et al., 2000, 2001). In both, Jak1 and Jak3, the gatekeeper amino acid methionine was replaced by site-directed mutagenesis with glycine, yielding "analog-sensitive" kinases AS-Jak1 and AS-Jak3 (see Table 3 for constructs used). The resulting constructs AS-Jak1/Jak3WT and Jak1WT/AS-Jak3 were stably and inducibly expressed in the U4C-IL-2Rβγ-TetOn-FlpIn cells as described above. Stimulation of these cells with IL-2 resulted in phosphorylation of both STAT5 and the Jaks, confirming that the analog-sensitive mutants were functional (lanes 2 and 5 in Figure 3).

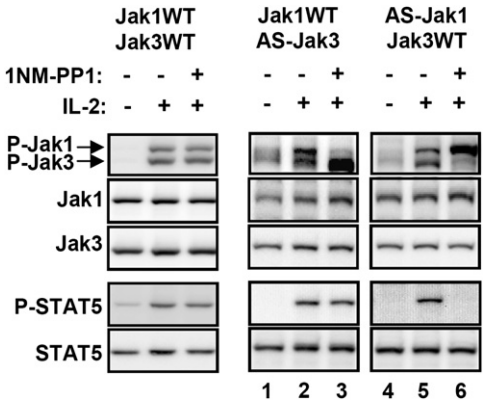


Figure 3. Specific Inhibition of the AS-Jak3- and AS-Jak1 Mutants
U4C-IL-2Rβ/γ-FlpIn-TetOn cells expressing different combinations of Jak1 and Jak3 wild-type and analog-sensitive mutants were induced with 5 μg/ml Dox for 48 hr and stimulated with IL-2 for 15 min as indicated. The mutants were pretreated for 30 min prior to IL-2 stimulation with 1NM-PP1 (40 μM) as indicated. Western blots were probed with antibodies against P-Jak1, P-STAT5, STAT5, Jak1, and Jak3.

Treatment of cells expressing Jak1WT/Jak3WT with IL-2 and 1NM-PP1, a prototype analog-sensitive kinase inhibitor, did not lead to reduced STAT5 phosphorylation indicating that the inhibitor did not affect Jak1WT or Jak3WT activity (left panel in Figure 3). Interestingly, treatment of cells expressing either AS-Jak1/Jak3WT or Jak1WT/AS-Jak3 with IL-2 and 1NM-PP1 led to a hyperphosphorylation of the respective analog sensitive Jak (lanes 3 and 6 in Figure 3). In addition, the trans-phosphorylation of Jak3 by AS-Jak1 and of Jak1 by AS-Jak3 was reduced by 1NM-PP1. Importantly, STAT5 phosphorylation was effectively inhibited by 1NM-PP1 in cells expressing AS-Jak1/Jak3WT but not in cells expressing Jak1WT/AS-Jak3 (lanes 3 and 6 in Figure 3) again indicating that specific inhibition of Jak3 kinase activity does not result in efficient abrogation of signal transduction initiated by the IL-2 receptor.

DISCUSSION

Based on the critical role Janus kinase 3 plays in cytokine signal transduction and its restricted expression in immune cells, it is believed that selective inhibitors of this enzyme would represent a novel immunosuppressive principle with an unprecedented safety profile. However, this hypothesis still remains to be proven. The lack of Jak3 expression leading to a SCID phenotype in patients as well as knockdown experiments in cells causing abrogation of signaling do not allow to distinguish a potential structural role of the Jak3 protein from the catalytic activity of the Jak3 enzyme. As Jak3 is always paired with Jak1, the efficacy of recent pan-Jak inhibitors (equally potent on Jak3 and Jak1), in particular, CP-690,550 (Changelian et al., 2003; Jiang et al., 2008; Karaman et al., 2008; Kudlacz et al., 2004, 2008, 2004; Williams et al., 2009), does not necessarily prove that the enzymatic function of Jak3 plays a pivotal role in signal transduction. Although a number of reports addressed the biochemistry of Jak1 or Jak3 cytokine receptor interaction, STAT phosphorylation or kinase activation (Cacalano et al., 1999; Chen et al., 2000; Fujii, 2008; Gaffen, 2001; Goldsmith et al., 1994; Haan et al., 2001, 2006, 2008; Kirken et al., 1995; Liu et al., 1997; Macchi et al., 1995; Zhou et al., 2001; Zhu et al., 1998), the cross-talk between the two Jak kinases and their individual contributions to signal transduction has not been fully understood.

The knockdown experiments suggested that the structural integrity of the receptor/Jak1/Jak3/STAT-signaling-complex is essential and a constitutively active mutant of Jak3 is not sufficient to trigger STAT5 phosphorylation (see Figure 1). Specific pharmacological blockade of Jak3 with the novel highly potent and selective Jak3 inhibitor NIBR3049 did not result in potent blockade of STAT5 phosphorylation as anticipated from the kinase inhibition profile assuming equal contribution of the two Jak kinases in IL-2 signal transduction. To confirm these findings, we undertook a systematic analysis of the contributions of Jak1 and Jak3 kinase functionalities to signal transduction in a reconstituted system ensuring equivalent and about physiological levels of expression of the two kinases together with the IL-2 receptor complex. The data obtained from the stable cell lines expressing all possible combinations of wild-type, kinase-inactive, and constitutively active mutants of Jak1 and Jak3 pointed out a key role for Jak1 and showed that the kinase functionality of Jak3 was not essential for STAT phosphorylation. In an IL-2 receptor dependent context Jak3 activation loop tyrosine phosphorylation was not necessary for STAT5 activation (Liu et al., 1997), which seems to support our data. The selective pharmacological inhibition of either Jak1 or Jak3 using analog-sensitive (AS) mutants confirmed our observation with the novel synthetic small molecule Jak3 inhibitor NIBR3049.

Collectively all data are consistent with a model in which Jak1 is the kinase responsible for Jak3 as well as STAT5 phosphorylation upon cytokine receptor activation. Since the level of STAT5 phosphorylation observed in the presence of catalytically inactive Jak3 but active Jak1 is reduced to about half the levels achieved with catalytically active Jak3, we conclude that the role of Jak3 is to phosphorylate Jak1 (but not STAT5) thereby enhancing its activity toward STAT5. Interestingly, earlier work with in vitro kinase assays with immunoprecipitated Jak1 and Jak3 had already suggested that Jak1 has an intrinsically higher activity toward STAT5 than Jak3 (Liu et al., 1997).

Similar to most other kinases, Janus kinases are known to become phosphorylated upon activation, and full activation is associated with phosphorylation of residues within the activation loop of the kinase domain. In our experiments with the kinase-inactive and analog-sensitive-Jak mutants, we find evidence for Jak1 and Jak3 transphosphorylation (Figures 2C and 3) as has also been proposed before (Fujii, 2008; Liu et al., 1997; Wang et al., 2003).

Importantly, our experiments show that Jak1 is able to phosphorylate STAT5 upon IL-2 stimulation even without prior phosphorylation by Jak3 indicating the existence of a phosphorylation independent conformational activation of Jak1 and Jak3 upon cytokine stimulation. Whether an unphosphorylated Jak1 kinase domain is catalytically active is not known to date since the isolated kinase domain has only been described in the phosphorylated state. In the published X-ray structures of both Jak1 and Jak3 kinase domains, the Tyr are phosphorylated (Boggon et al., 2005; Lucet et al., 2006; Williams et al., 2009) but the

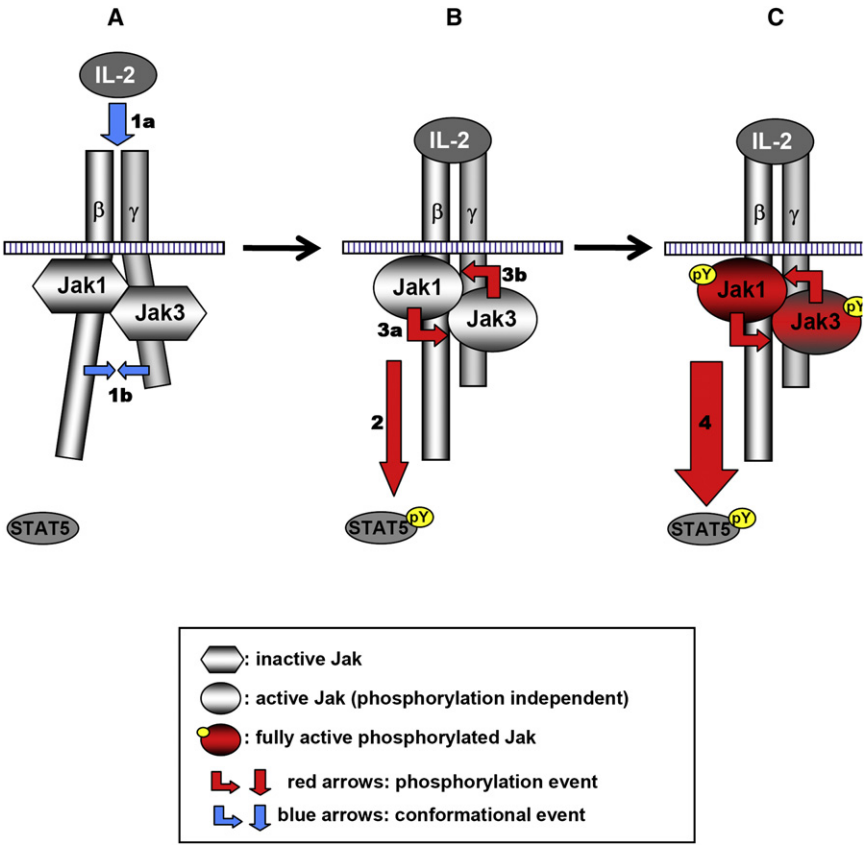


Figure 4. Schematic Representation of the Proposed Sequence Of Events Leading to STAT5 Phosphorylation through the IL-2R Complex upon IL-2 Stimulation

Receptor engagement by IL-2 binding leads to receptor chain rearrangement and activation of kinase activities of Jak1 and Jak3. This step is phosphorylation-independent (step 1 in A). Upon this conformational activation, Jak1 is able to phosphorylate STAT5 although it is not yet fully activated (step 2 in B). The active but unphosphorylated Jaks now transphosphorylate (step 3 in B) and are stabilized in their active state (see phosphorylated Jaks in C). The fully active Jak1 now leads to increased phosphorylation of STAT5 (4 in C). The IL-2 receptor α chain has been omitted in our reconstituted system and in the figure as it is not essential for signal transduction.

Jak3 kinase domain appears to be catalytically active even in the unphosphorylated state (P.D., H.-G.Z., unpublished data). The data shown here suggest that in a cytokine receptor-dependent system the kinase activities of both Jaks become activated independently of phosphorylation, presumably by the release of inhibitory constraints upon cytokine-induced receptor complex aggregation. Trans-phosphorylation of the two Jaks then induces a stabilization of their activated states, as described for many other kinases, and promotes full signal transduction. Jak1 herein plays the dominant role, activating both STAT5 and Jak3, while Jak3 supports signal transduction by phosphorylating Jak1. A schematic illustration of the model is shown in Figure 4. Even in the presence of constitutively activated Jak1 and Jak3, IL-2 stimulation and the associated conformational rearrangements lead to an increase in Jak- and STAT-phosphorylation (see f and g in Figures 2B and 2C) which is reminiscent of the conformational activation of Jaks by receptor rearrangements upon cytokine binding to the extracellular part of cytokine receptors (Figure 4).

As has been reported for several kinases (Cameron et al., 2009; Frye and Johnson, 2009; Okuzumi et al., 2009), the analog-sensitive Jak kinases become hyper-phosphorylated in the presence of an ATP-competitive specific inhibitor. Comparable "inhibitor priming" of Jaks has been described in the context of the oncogenic Jak2V617F mutant for other Jak inhibitors (Jak inhibitor 1 and Gö6976) (Grandage et al., 2006; Haan et al., 2009). Our data suggest that the kinase responsible for the hyper-phosphorylation of AS-Jak1 is Jak3 and vice versa (see Figure 2). ATP-competitive kinase inhibitors in general often target and block the kinase domain in the active conformation. Since we have shown that both Jak1 and Jak3 can phosphorylate their Jak counterpart even though they are not phosphorylated themselves, it is conceivable that the inhibited analog-sensitive Jak (presented in the active conformation induced by the inhibitor) is phosphorylated by its Jak counterpart because the inhibitor stabilizes the active form of the cytokine/receptor/Jak complex which is particularly prone to efficient Jak phosphorylation. The inhibited analog-sensitive Jak cannot phosphorylate its Jak counterpart efficiently.

The finding that Jak1 and not Jak3 kinase activity is essential in mediating cytokine-dependent signal transduction was unexpected and has important consequences for drug discovery. Based on the data shown here the concept that a highly selective ATP-competitive Jak3 kinase inhibitor will result in the desired safe and effective immunosuppression is questionable. Concomitant inhibition of Jak1 kinase activity seems to be required for suppression of γc cytokine signaling. However, the involvement of Jak1 (in combination with Jak2 or Tyk2) in the majority of cytokine receptor signaling via the Jak-STAT pathway raises concerns about target-related side effects. Unfortunately, neither specific Jak1-blocking agents nor dual Jak1/Jak3 inhibitors (devoid of Jak2 and Tyk2 activity) are available to date which would allow the exploration of their pharmacological potential. In any case, clinical data from several studies with CP-690,550 and other pan-Jak kinase inhibitors will soon show whether this approach is safe and effective in humans.

SIGNIFICANCE

Immunosuppressive drugs are of crucial importance in disease treatment today and more specific drugs with minimal side effects are sought. Janus kinase 3 (Jak3) is essential for signal transduction via cytokines utilizing the

common gamma chain (CD132/γc) and its absence results in immunodeficiency. Jak1, however, also participates in signaling through γc containing receptors. It is generally assumed that specific inhibition of Jak3 kinase activity will result in immunosuppression and because of the restricted expression of Jak3 this will have therapeutic potential as a selective and potentially safe immunosuppressive principle.

Surprisingly, we find that a highly potent small molecule Jak3 kinase inhibitor showing >150-fold selectivity over the other Janus kinases, is >20-fold less potent in abolishing γc-cytokine (IL-2, IL-4, or IL-15)-induced STAT phosphorylation compared with pan-Jak inhibitors in several cell lines and human lymphocytes.

To further explore this unexpected observation we studied IL-2 -dependent signaling in a reconstituted system using transfectants in which all combinations of wild-type, constitutively active and kinase-inactive mutants of Jak1 and Jak3 were stably coexpressed. The presence of kinase-inactive Jak1 resulted in complete abolishment of STAT5 phosphorylation regardless of the Jak3 status, whereas it was only reduced partially in the presence of kinase-inactive Jak3. We next generated analog-sensitive (AS) mutants of Jak1 and Jak3 which were coexpressed in the reconstituted cell system. Again specific inhibition of AS-Jak1 but not of AS-Jak3 by the ATP-competitive analog 1NM-PP1 abrogated IL-2 signaling: These results corroborated the finding with the Jak3-specific small molecule inhibitor and taken together our results demonstrate that the two Jak kinases involved in signaling through γc utilizing cytokine receptors are not equally important and that Jak1 plays a dominant role. These data challenge the concept that ATP-competitive kinase inhibitors selectively targeting Jak3 will be sufficient to achieve immunosuppression.

EXPERIMENTAL PROCEDURES

Materials

The cytokines used were IL-2 (Peprotech or Chiron [Proleukin]), IFN-α (Immunotools), and IFN-γ (Roche). Doxycycline (Sigma) was usually used at a concentration of 5 μg/ml. Jak inhibitor 1 and 1NM-PP1 were purchased from Calbiochem. CP-690,550 was purchased from Selleck Chemicals LLC and NIBR3049 was synthesized at Novartis.

Jak Enzyme Assays

For enzyme assays affinity-purified GST fusions of the active kinase domains (GST-Jak1(866-1154), GST-Jak2(808-1132), GST-Jak3(811-1124), and GST-Tyk2(888-1187) were expressed in insect cells or purchased from Invitrogen (Carlsbad, CA). All assays were performed in 384-well microtiter plates with 8 point serial dilutions of compounds. The kinase reactions were started by stepwise addition of 4.5 μl per well of a 2 × peptide/ATP-solution and 4.5 μl per well of a 2 × enzyme solution. The constitution of the assay buffer was: 50 mM HEPES (pH 7.5), 1 mM DTT, 0.02% Tween20, 0.02% BSA, 0.6% DMSO, 10 mM beta-glycerophosphate, and 10 μM sodium orthovanadate. The other components were adjusted specifically for the respective kinase assay: Jak1: 16 nM enzyme, 70 μM ATP, 2 μM peptide substrate, 12 mM $MgCl_2$. Jak2: 1.8 nM enzyme, 20 μM ATP, 2 μM peptide substrate, 9 mM $MgCl_2$. Jak3: 13 nM enzyme, 18 μM ATP, 2 μM peptide substrate, 1.5 mM $MgCl_2$. Tyk2: 3.5 nM enzyme, 35 μM ATP, 2 μM peptide substrate, 9 mM MgCl2. The stop solution was 100 mM HEPES (pH 7.5_, 5% DMSO, 0.1% Caliper coating reagent, 10 mM EDTA, and 0.015% Brij35. The peptide substrate used in the Jak1 and Tyk2 assays was FITC-Ahx-KKSRGDYMTMQIG-NH_2 and Carboxyfluorescein-Ahx-GGEEEEYFELVKKKK for the Jak2 and Jak3 assays. Kinase reactions were incubated at 30°C for 60 min and terminated by addition of 16 μl per well of stop solution. Phosphorylated and unphosphorylated peptides were separated using the Caliper microfluidic mobility shift technology on a Caliper LC3000 workstation and kinase activities were calculated from the amounts of formed phosphopeptide.

Knockdown of Jak1 and Jak3 with si-RNA

The Cell Line Nucleofector kit V and the Amaxa Nucleofector electroporation apparatus (Lonza) were used and the cells were treated according to the manufacturer's instruction. Human Jak1 or Jak3 validated stealth oligo duplex 1 + duplex 2, or si-RNA negative (medium GC duplex) or fluorescein-labeled si-RNA positive control (all from Invitrogen) were used. The efficiency of the transfection and the response to cytokines (IL-2, IFN-α) were assessed at 24 hr or 48 hr after transfection.

Assessment of STAT Phosphorylation By Flow Cytometry

Cells treated with inhibitor for 30 min at 37°C or untreated cells were stimulated with 20 U/ml IL-2 for 15 min at 37°C, fixed with paraformaldehyde (final concentration 2%), permeabilized with 90% methanol on ice, washed, and stained with Alexa647-labeled anti-STAT5-pY694 (BD Biosciences) for 45 min followed by analysis on a FACS Calibur flow cytometer (BD Biosciences). IC_{50} values for inhibitors were calculated based on an 8 point concentration-response curve. Recombinant human IFN-α or IFN-γ (Immunotools, Berlin), were used to stimulate STAT1 phosphorylation which was assessed following the same protocol but using PE-labeled anti-STAT1-pY701 antibody (BD Biosciences).

Expression Plasmids

Standard cloning procedures were performed throughout. The generation of pBOF is described in the supplemental information (Supplemental Experimental Procedures and Figure S2B). Starting from pBOF-Jak1WT/Jak3WT, the mutants Jak1V658F/Jak3WT (CA/WT), Jak1K908A/Jak3WT (KD/WT), Jak1V658F/Jak3A572V (CA/CA), Jak1WT/Jak3A572V (WT/CA), Jak1K908A/Jak3A572V (KD/CA), Jak1WT/Jak3K855A (WT/KD), Jak1K908A/Jak3K855A (KD/KD), Jak1V658F/Jak3K855A (CA/KD), Jak1M956G/Jak3WT (AS/WT), and Jak1WT/Jak3M902G (WT/AS) were generated by point mutagenesis using the QuikChange XL site-directed mutagenesis kit (Agilent Technologies). The constructs pEF6-V5-His-hIL2Rβ and pTetOn-hIL2Rγ-$IRES_{puro}$ are described in the Supplemental Experimental Procedures.

Cell Lysis, Immunoprecipitation, and Western Blot Analysis

All steps of cell lysis, immunoprecipitation, and western blot analysis were performed as described (Radtke et al., 2005; Vollmer et al., 2009). Anti-phospho-STAT5 (BD Biosciences), anti-phospho-Jak1 (Tyr1022/Tyr1023, Santa Cruz Biotech.), anti-Jak1 (BD Biosciences), anti-Jak3 (Santa Cruz Biotech.) and anti-STAT5 (Santa Cruz Biotech.) were used for western blot detections. For a further characterization of the anti-phospho-Jak1 (Tyr1022/Tyr1023) antibody used to detect phosphorylated Jak1 and Jak3, see Figure S4. ECL detection of western blots was performed as described (Haan and Behrmann, 2007) with horseradish peroxidase-conjugated secondary antibodies from Dako or Cell Signaling. For western blot quantitation IRDye 680 anti-mouse IgG (LI-COR) and Dylight 800 anti-rabbit IgG (Pierce) were used for detection on the Odyssey instrument (LI-COR). For quantification the average signals of four blots were determined. Anti-phospho-STAT5 (BD Biosciences) and anti-STAT5 (Santa Cruz Biotechnology) were used as primary antibodies. The STAT5 signals served for normalization of phospho-STAT5 levels. Quantitation was performed on single channels with the analysis software V3.0 provided by the manufacturer (Li-COR Biosciences). The signals to be quantitated were normalized with respect to the loading control for each lane and the signal intensity was then represented as fold induction or as percentage of the strongest signal.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.01.012.

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