Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3–receptor interaction domain

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Signaling through the hematopoietic receptors requires activation of receptor-associated Janus (Jak) kinases. For example, Jak1 and Jak3 bind specifically to the IL-2 receptor beta (IL-2R β) and common gamma (γ_c) chains, respectively, and initiate biochemical signals critical in controlling immune responses. The region of Jak responsible for receptor interactions, however, is not well characterized. Here we describe a naturally occurring Jak3 mutation from a patient with autosomal severe combined immunodeficiency (SCID), where a single amino acid substitution, Y100C, in Janus homology domain 7 (JH7) prevents kinase–receptor interaction. This mutation also results in a loss of IL-2-induced signaling in a B-cell line derived from this patient. Using mutational analysis we have identified a region of Jak3, including portions of JH6 and JH7, that is sufficient for kinase–receptor contact and show that this segment interacts with the proline-rich Box1 region of the receptor. Furthermore, a Jak3–Jak1 chimera containing only the JH6 and JH7 domains of Jak3 interacts with γ_c and can reconstitute IL-2-dependent responses, including receptor phosphorylation and activation of signal transducer and activator of transcription (STAT) 5b. Our results suggest that the N-terminus of Jak kinases is critical for receptor binding, and is therefore likely to determine specificity of Jak kinase–receptor interactions.

Keywords: common gamma chain/interleukin-2/Jak3/Jak kinase/receptor

Introduction

Binding of cytokines to their receptors induces a cascade of intracellular signaling events that results in increased tyrosine phosphorylation of specific target proteins, and initiates a program of altered gene expression and proliferation. Cytokine receptors lack intrinsic tyrosine kinase activity and therefore signal via non-receptor tyrosine kinases of the Janus kinase (Jak) family that associate with the cytoplasmic domains of receptor chains (Ihle *et al.*, 1995).

The paradigm for ligand-induced signaling is that *trans*-phosphorylation and activation of receptor-associated Jak kinases occurs upon receptor aggregation. These activated kinases tyrosine phosphorylate the receptor chains, providing docking sites for the src-homology (SH2) domains of signal transducers and activators of transcription (STATs), as well as other signaling molecules, such as the adapter molecule Shc (Ihle *et al.*, 1995; Schindler and Darnell, 1995; Taniguchi, 1995; Bach *et al.*, 1997; Leonard and O'Shea, 1998). STATs are recruited to the phosphorylated receptor, where they are in turn phosphorylated. This allows them to dimerize, translocate to the nucleus, and stimulate the expression of cytokine-inducible genes. While the general model for cytokine-induced signaling has been elucidated, the molecular details of the association of Jak family members with the cytoplasmic domains of receptor chains are not well understood.

The cytokines interleukin (IL)-2, IL-4, IL-7, IL-9 and IL-15 all bind to the common gamma chain (γ_c) signaling subunit. The importance of signaling through γ_c is underscored by the fact that specific mutations in either γ_c or the Janus kinase that interacts with it, Jak3, result in phenotypically identical severe combined immunodeficiency (SCID) (Noguchi *et al.*, 1993; Macchi *et al.*, 1995; Russell *et al.*, 1995). Infants with T⁺B⁺ SCID suffer from severe infections due to reduced levels of T and natural killer (NK) cells, as well as hypogammaglobulinemia, and require heterologous bone marrow transplantation.

The Jak family of kinases consists of four known mammalian homologs, each consisting of 1100–1200 amino acids organized into seven Janus homology (JH) domains, based on sequence similarity among the family members (Wilks *et al.*, 1991; Harpur *et al.*, 1992). They do not possess classic SH2 or SH3 domains, and, except for the catalytic domains, exhibit little homology to other protein tyrosine kinases (PTKs). Although the N-terminal half of the Jak kinases containing the JH7–JH3 domains has been shown to be required for receptor interaction in several systems, the precise nature of the interaction has not been resolved. Experiments with Jak2–Jak1 chimeras have suggested that the JH7–JH6 domains of Jak2 are sufficient for physical association to interferon (IFN)- γ R2 by co-immunoprecipitation, and for IFN- γ -inducible STAT1 activation in a Jak2-deficient cell line (Kohlhuber *et al.*, 1997). Reciprocal experiments using Jak1–Jak2 chimeras suggest that the entire N-terminal half (JH7–JH3) of Jak1 is required for binding to the IFN- γ RI and for IFN- γ -inducible STAT1 activation (Kohlhuber *et al.*, 1997). Deletion analysis has shown that the N-terminal 239 amino acids (JH7–JH6) of Jak2 are indispensable for granulocyte–macrophage colony stimulating factor (GM-CSF) and growth hormone receptor (GHR) association (Frank *et al.*, 1995; Zhao *et al.*, 1995). In addition, work by Pellegrini and colleagues has demonstrated that the

N-terminal half of Tyk2 is required for IFN- α receptor stabilization in a Tyk2-deficient cell line, and for efficient IFN- α -induced phosphorylation (Gauzzi *et al.*, 1997; Richter *et al.*, 1998; Yan *et al.*, 1998). In the IL-2 receptor (IL-2R) system, a fragment of Jak3 containing JH6 and JH7 could bind γ_c , but reconstitution of IL-2-induced responses was shown only with a larger region, consisting of the JH7-JH4 domains (Chen *et al.*, 1997). Thus, although the regions of Janus kinases necessary for receptor association are clearly in the N-terminus, as yet the shortest regions or discrete domains required have not been defined.

The membrane-proximal region of the cytoplasmic tail of cytokine receptors, known as Box1/Box2 (Murakami *et al.*, 1991) has been shown to be required for signaling and is thought to interact with Jak kinases. Specifically, mutational analysis of the proline-rich Box1 region of the signaling chain of the leukemia inhibitory factor (LIF) and IL-6 receptors, gp130, has revealed a major role for this short eight amino acid motif in the binding of Jak2 (Tanner *et al.*, 1995). Although this Box1 region is loosely conserved, each receptor has a proline-rich motif within Box1 and mutations within this region can disrupt association. Interestingly, a point mutation (L271Q) in the Box1 region of γ_c disrupts Jak3- γ_c interaction, and causes X-linked combined immunodeficiency (XCID) (Russell *et al.*, 1994; Schmalstieg, 1995). Therefore, although Jaks interact with Box1/Box2 in the cytoplasmic domains of hematopoietic receptors, the region of Jaks that interact with Box1 has not been well characterized.

In this paper, we describe a point mutation of the JH7 domain of Jak3 from an autosomal SCID patient, which prevents kinase-receptor interaction and blocks downstream signaling. Using this information, we have defined a short region around this mutation that has the ability to interact with γ_c specifically at the Box1 region, and show that a Jak3-Jak1 chimera containing this region can mediate IL-2 responses. This suggests that the specificity of Jak-receptor interaction exists in a short region that overlaps JH6 and JH7.

Results

Jak3 mutation (Y100C) blocks IL-2-induced tyrosine phosphorylation

SCID can result from several distinct molecular defects (Candotti and Blaese, 1996); the X-linked form of the disease results from loss or mutation of γ_c , and a clinical phenotype indistinguishable from XSCID, which is due to loss or mutation of Jak3, and results in impaired signaling through γ_c -using cytokine receptors. While some cases of autosomal SCID result from the absence of Jak3 protein, several patients have been identified who express detectable levels of Jak3.

As shown in Figure 1A, lane 2, an Epstein-Barr virus (EBV)-transformed B-cell line from an autosomal SCID patient (GM) (Macchi *et al.*, 1995) expresses significant levels of Jak3, although in some experiments, we have observed Jak3 levels lower than in cells from normal donors expressing wild-type Jak3 (JY, lane 1). A number of patients have single point mutations (Macchi *et al.*, 1995; Russell *et al.*, 1995; Candotti *et al.*, 1998), so understanding the effects of these subtle mutations may

Fig. 1. IL-2 responses in EBV-transformed B-cell lines from SCID patients. (A) Lysates from BCL obtained from normal control (JY, lane 1), Jak3-negative SCID (MC, lane 3), or Jak3-expressing SCID (GM, lane 2) patients or a T-cell line Kit 225 (lane 4), were immunoblotted with Jak3 antiserum (top panel), then stripped and reblotted with Jak1 antiserum (lower panel). (B) EBV-transformed B-cell lines from normal donor (JY) or Jak3 Y100C-expressing patient (GM) were untreated or stimulated with 1000 U/ml IL-2 for 15 min at 37°C. Cells were lysed and lysates were immunoprecipitated with rabbit polyclonal antiserum specific for Jak3. The blot was probed with anti-phosphotyrosine (upper panel) and subsequently reblotted for JAK3 (lower panel). (C) Cells were stimulated and lysed as above and immunoprecipitated with STAT5b antibody (upper panel) and subsequently reblotted with STAT5b antibody (lower panel).

provide valuable insight into the functions of various domains of Jak3.

The patient described in Figure 1 is homozygous for a single amino acid substitution, Y100C, in the JH7 domain. We reasoned that the mutation might affect the signaling ability of Jak3, thus providing a molecular basis for the patient's autosomal SCID phenotype. To determine whether Jak3 phosphorylation could be observed in response to IL-2 in the patient's cells, we examined an EBV-transformed B-cell line derived from the SCID patient. The SCID patient cell line and a control cell line from a patient expressing wild-type endogenous Jak3 (JY) were incubated in serum-free medium for 4 h prior to challenge with IL-2. As shown in Figure 1B, lane 2, wild-type Jak3 became inducibly tyrosine phosphorylated in response to IL-2 in normal EBV-transformed B-cells (JY).

In the SCID patient's cells (GM), no inducible tyrosine phosphorylation of Jak3 was detected in response to IL-2, although Jak3 Y100C was tyrosine phosphorylated even in the absence of cytokine treatment (Figure 1B, lanes 3 and 4). The expression controls show that the levels of precipitated endogenous Jak3 Y100C are lower compared with WT Jak3 (Figure 1B, lower panel), demonstrating that the phosphorylation state of Jak3 Y100C in the absence of IL-2 does not result from Jak3 Y100C overexpression.

We further analyzed downstream signaling in the patient cell line, by examining the phosphorylation of the transcription factor STAT5b. Again, cells were incubated in serum-free medium for 4 h prior to stimulation, then were challenged with IL-2 for 15 min. STAT5b became tyrosine phosphorylated in response to IL-2 in JY cells, but no IL-2-induced STAT5b phosphorylation was detected in the GM cell line (Figure 1C). The lack of IL-2-induced responses in the SCID patient was not due to loss or downregulation of IL-2 receptor components, as the levels of IL-2R β and γ_c in the GM and JY cell lines were equal (not shown). These results suggest that although Jak3 Y100C was constitutively phosphorylated in the patient's cells, it was not inducibly phosphorylated and was unable to initiate downstream signaling events in response to IL-2.

Jak3 Y100C mutant does not interact with γ_c

The N-terminal portion of Jak kinases has been implicated in receptor association, but the precise region responsible has not been reported (Chen *et al.*, 1997). Because the severe phenotype observed in this SCID patient results from a single amino acid change in the N-terminus, we reasoned that this mutation might affect the ability of Jak3 to associate with γ_c . To study the effect of the Y100C mutation on γ_c interaction, we expressed wild-type Jak3, a kinase-inactive form of Jak3, a form of Jak3 with a Y105F mutation in a conserved tyrosine adjacent to Y100, and the Y100C SCID patient mutant, in 293T cells, and tested their ability to co-immunoprecipitate with γ_c . Wild-type or mutant Jak3 were immunoprecipitated, and blots were probed with anti- γ_c antibody. As shown in Figure 2A, lanes 2, 3 and 5, we observed co-immunoprecipitation of γ_c with wild-type Jak3, the kinase inactive mutant, K855A, and the Y105F mutant. However, we did not detect co-precipitation of γ_c with Jak3 Y100C or the Jak3 Y100C/Y105F double mutant (Figure 2A, lanes 4 and 6), although equal levels of Jak3 and γ_c were expressed in these cells (Figure 2A, lower panels), suggesting that the Y100C mutation disrupts receptor interaction. The same result was obtained when epitope-tagged γ_c was co-precipitated with wild-type or mutant Jak3 (Figure 2B). Again, wild-type, kinase-inactive and Y105F Jak3 interacted strongly with γ_c , while the Y100C mutant and the Y100C/Y105F double mutant were unable to bind the receptor, suggesting that Jak3 Y100C lacks the ability to interact with γ_c (Figure 2B). Interestingly, a naturally occurring point mutation in the cytoplasmic domain of γ_c has been identified that impairs but does not abolish interaction with Jak3. This mutation results in XCID (Schmalstieg *et al.*, 1995). Our data clearly suggest that a Jak3 point mutation in this autosomal SCID patient can also disrupt receptor association.

Following IL-2 stimulation, Jak3 becomes rapidly tyrosine phosphorylated and recruited to the receptor complex

(Leonard and O'Shea, 1998). Therefore, we investigated the ability of Jak3 Y100C to become tyrosine phosphorylated and recruited to the receptor in response to IL-2. Fibroblasts that stably express the IL-2 receptor chains (3T3 $\alpha\beta\gamma$) (Chen *et al.*, 1997) were infected with retrovirus expressing epitope-tagged wild-type Jak3, a kinase-inactive form of Jak3 or the Y100C mutant. The parental cell line expresses all receptor components and Jak1, but lacks Jak3, and thus no IL-2-inducible Jak3 phosphorylation was detected (Figure 2C, lanes 1 and 2). In contrast, in cells expressing wild-type Jak3, Jak3 became rapidly tyrosine phosphorylated and associated with the IL-2 receptor complex following IL-2 stimulation (Figure 2C, lanes 3 and 4). Interestingly, we observed tyrosine phosphorylation of the kinase-inactive Jak3 protein, although at much reduced levels compared with wild-type, presumably via *trans*-phosphorylation by receptor-associated Jak1 (Figure 2C, lanes 5 and 6). However, no IL-2-induced phosphorylation of Jak3 Y100C was observed, although we did detect high basal tyrosine phosphorylation, in agreement with our results with the GM patient's cells (Figure 2C, lanes 7 and 8). The expression controls again showed that the high basal phosphorylation state of Y100C was not due to overexpression of the mutant Jak3, as Y100C was expressed at levels comparable with wild-type and kinase-inactive Jak3 (Figure 2C, middle panel).

It has been shown previously that unlike other receptor systems, where Jak kinase–receptor association is constitutive, Jak3 is recruited to γ_c in response to cytokine (Johnston *et al.*, 1994; Russell *et al.*, 1994). In cells expressing wild-type Jak3, or kinase-inactive Jak3, γ_c co-precipitated with Jak3, and an enhanced interaction was observed after IL-2 stimulation (Figure 2C, bottom panel, lanes 4 and 6). This result suggested that Jak3 kinase activity was not necessary for receptor recruitment in response to IL-2. However, the cytokine-induced recruitment of Jak3 to the receptor complex was not observed with Jak3 Y100C (Figure 2C, bottom panel, lanes 7 and 8). These data confirm that the Y100C mutation results in defective interaction with γ_c , providing an explanation for the SCID phenotype observed in this patient.

Other Jak3 mutations that affect receptor interaction

As the Y100C mutation clearly blocks Jak3–receptor association, we investigated whether other mutations in this region of Jak3 could disrupt receptor interaction. Figure 2D shows that both wild-type and a kinase-inactive mutant of Jak3 interacted strongly with γ_c (lanes 1 and 2). A kinase-inactive form of the patient mutant, Jak3 Y100C/K855A, also did not interact with γ_c , and anti-phosphotyrosine Western blotting confirmed that Jak3 Y100C/K855A was not phosphorylated in 293T cells (Figure 2D, second panel, lane 4), suggesting that the defect in receptor binding is not due to increased or aberrant phosphorylation of the mutant kinase, and that neither kinase activity nor phosphorylation of Y100 is necessary for γ_c interaction. In addition, a Jak3 Y100A mutant abrogates γ_c binding while a conservative Jak3 Y100F mutant still interacted with γ_c (Figure 2D, lanes 5 and 6), presumably by preserving secondary structure required for receptor binding.

Fig. 2. Jak3 mutations can abrogate binding to γ_c . **(A)** Lysates from 293T cells transfected with plasmids encoding FLAG-tagged wild-type (WT) (lane 2), kinase-inactive mutant Jak3 K855A (lane 3), Y100C (lane 4), Y105F (lane 5), and Y100C/Y105F double mutant (lane 6) and with γ_c were immunoprecipitated with the FLAG antibody M2. Blots were probed with a rabbit polyclonal antibody specific for γ_c (upper panel). Expression of Jak3 and γ_c were checked on lysates from these cells (lower panels). **(B)** Lysates from 293T cells transfected with FLAG-tagged γ_c and non-tagged Jak3 constructs were immunoprecipitated with M2. Blots were probed with a rabbit polyclonal antibody specific for Jak3 (upper panel). Again, expression of Jak3 and γ_c were checked on lysates from these cells (lower panels). **(C)** NIH 3T3 fibroblasts expressing IL-2 receptor were stably infected with vector (lanes 1 and 2), FLAG-tagged WT Jak3 (lanes 3 and 4), kinase-inactive Jak3 K855A (lanes 5 and 6), or Jak3 Y100C (lanes 7 and 8), and were untreated or stimulated for 15 min with 1000 U/ml IL-2. Cells were lysed and immunoprecipitated with M2. Blots were probed with anti-phosphotyrosine (top panel) and stripped and reprobed for Jak3 and γ_c (lower panels). **(D)** 293T cells transfected with plasmids encoding γ_c and FLAG-tagged WT Jak3 (lane 1), or FLAG-tagged Jak3 constructs with mutations K855A (lane 2), Y100C (lane 3), Y100C/K855A double mutant (lane 4), Y100A (lane 5), Y100F (lane 6), L98A/L99A/I102A triple mutant (lane 7), L98A (lane 8), L99A (lane 9), I102A (lane 10) were lysed and immunoprecipitated with M2. Blots were probed with antisera to γ_c (top panel), stripped and reprobed with antisera specific for anti-phosphotyrosine and Jak3 (middle panels). Expression of γ_c was checked on lysates from these cells (lower panel).

Because this region of Jak kinases contains several highly conserved residues, we examined mutations of these amino acids to determine if this conserved region represents a domain that mediates binding to receptor chains. Mutations in highly conserved aromatic amino acids Y105A and W109A of Jak3 had no significant effect on γ_c binding in 293T co-immunoprecipitation experiments (not shown). However, several aliphatic amino acids, L98, L99 and I102, within this region of Jak3 that are highly conserved among mammalian Jak kinases, may also contribute to receptor interaction. Consistent with this idea are

recent findings of leucine-rich domains within transcription factors that mediate protein–protein interactions. Mutation of these residues abrogates functional association (Heery *et al.*, 1997; Darimont *et al.*, 1998). We generated a triple mutant (L98A, L99A, I102A), and compared γ_c association in 293T cells with wild-type Jak3. As shown in Figure 2D, lane 7, we observed a marked reduction of γ_c association by co-immunoprecipitation.

Single amino acid substitutions within this conserved region also affected the γ_c –Jak3 interaction. L98A and I102A mutations disrupted the binding of Jak3 to γ_c by

Fig. 3. Jak3 N-terminus interacts with γ_c . **(A)** Structure of wild-type and Jak3 mutants. The JH7 domain of Jak3 is shaded. **(B)** Lysates from 293T cells transfected with γ_c and myc-tagged wild-type Jak3 (lane 1), or the Jak3 fragments 1–130 (lane 2), 1–193 (lane 3), 72–130 (lane 4), or 1–109 (lane 5) were immunoprecipitated with goat polyclonal anti- γ_c . Blots were probed with mouse monoclonal anti-myc antibody 9E10. Expression of γ_c and the Jak3 fragments were analysed in these cells (lower panels).

co-immunoprecipitation (Figure 2D, lanes 8 and 10), while the L99A mutation did not have a significant effect on the interaction (Figure 2D, lane 9), suggesting that very subtle changes in this region of Jak3 can affect its ability to interact with its cognate receptor.

Defining a minimal receptor interaction domain on Jak3

The results above indicated that the Y100C mutation might be located in a receptor interaction domain situated in the N-terminus of Jak3. We have used computational prediction of the N-terminal region of Jak kinases as described by Shultz *et al.* (1998). The analysis predicts that this region is particularly rich in β -strands, and specifically that the region around tyrosine 100, amino acids 72–130, contains four β -strands and may form a core region for a receptor binding domain. To test the idea that a region of Jak3 that includes tyrosine 100 may encode a receptor interaction domain, we made truncations of Jak3 and Jak3–Jak1 chimeras, and tested γ_c interactions in both 293T cells and in IL-2R-expressing fibroblasts. The N-terminal region of Jak3 encoding Janus homology domains JH6 and JH7 encodes 256 amino acids. To narrow down the receptor interaction domain, we generated a panel of staggered deletion mutants shown schematically in Figure 3A. J3 1–193 encodes all of the JH7 domain and 73 amino acids of the JH6 domain, J3 1–130 encodes all of the JH7 domain and five residues of the JH6 domain, J3 72–130 encodes 38 amino acids of the JH7 domain and five amino acids of JH6, and J3 1–109 deletes 11 amino acids of the JH7 domain. Epitope-tagged fragments of Jak3 were co-expressed in 293T cells with γ_c . The receptor was immunoprecipitated, and blots were probed with antibody recognizing the Jak3 fragments. As shown in Figure 3B, lane 4, the shortest region of Jak3 sufficient

to interact with γ_c was amino acids 72–130. In addition, longer fragments, containing amino acids 1–130 and 1–193 also interacted strongly with γ_c (Figure 3B, lanes 2 and 3). However, a region of Jak3 further truncated from the C-terminus, containing amino acids 1–109 was unable to bind γ_c in this experiment (Figure 3B, lane 5), suggesting that the γ_c binding region of Jak3 can be mapped to a 58 amino acid region consisting of portions of JH6 and JH7.

Mutations in γ_c that affect Jak3 binding

The membrane-proximal region of the cytoplasmic tail of hematopoietic receptors, specifically, the proline-rich Box1 motif, has been implicated as an important point of contact with Jak kinases. As mentioned previously, a naturally occurring mutation of γ_c (L271Q) from a patient with XCID decreases the association of γ_c with Jak3. This single amino acid substitution occurs within the Box1 region, just C-terminal to proline-rich sequences in γ_c (RTMPRIIP) from amino acids 263–269 (Puck, 1996).

We introduced P266,269A or P266,269G mutations into the membrane-proximal Box1 region of the γ_c cytoplasmic tail, and analysed its interaction with Jak3 in 293T cells. As shown in Figure 4A, mutation of these two proline residues reduced the interaction of γ_c with full-length Jak3 to barely detectable levels (Figure 4A, lanes 3 and 4), although equal levels of Jak3 and γ_c were expressed in each cell (Figure 4A, lower panels). Furthermore, these γ_c Box1 proline mutations also reduced the interaction of γ_c with a myc-tagged N-terminal fragment of Jak3 encoding amino acids 1–193. As shown in Figure 4B, the fragment interacted strongly with wild-type γ_c but only very weakly with the P266,269A or P266,269G mutants, demonstrating a direct interaction between the N-terminus of Jak3 and

Fig. 4. Box1 mutations of γ_c inhibit Jak3 association. (A) Lysates from 293T cells co-transfected with wild-type (WT) or mutant Jak3 and γ_c in the following combinations: WT Jak3 and WT γ_c (lane 1), Jak3 Y100C and WT γ_c (lane 2), WT Jak3 and P266,269A γ_c (lane 3), or WT Jak3 and P266,269G γ_c (lane 4) were immunoprecipitated with anti-FLAG M2, and blots were probed with Jak3 antibody (upper panel). Lower panels show γ_c and Jak3 expression. (B) Myc-tagged Jak3 fragment 1–193 was co-expressed in 293T cells with WT γ_c or the Box1 mutants of γ_c as indicated. Lysates were immunoprecipitated with goat polyclonal anti- γ_c antibody, and blots were probed with anti-myc monoclonal antibody 9E10. Again, lower panels show γ_c and myc-tagged 1–193 fragment expression.

the proline-rich Box1 region of γ_c , and confirming the results obtained with full-length Jak3.

Binding of γ_c and functional responses of Jak3–Jak1 chimeras

To establish which region of the N-terminus of Jak3 was required for specific functional receptor association, we generated a series of six staggered chimeras between Jak3 and Jak1 as shown in Figure 5A. The γ_c receptor chain and the chimeras were transfected into 293T cells and their ability to associate was examined by immunoprecipitating the epitope-tagged chimeric Jak proteins, and blotting for γ_c . When compared with wild-type Jak3, the chimera containing a C-terminal truncation of the receptor interaction domain (J3/1 1–109) did not bind γ_c (Figure 5B, lanes 2 and 3). This finding is in agreement with Jak3 truncation experiments and suggests that this region is not

sufficient to mediate γ_c binding. The chimera J3/1 1–130 retained the ability to interact with γ_c , although weakly (Figure 5B, lane 4), while the J3/1 chimeras J3/1 1–193, J3/1 1–256, J3/1 1–300 and J3/1 1–519 all interacted with γ_c to an extent similar to wild-type Jak3 (Figure 5B, lanes 5–8). These results indicate that the N-terminal region containing all of JH7 and the first five amino acids of JH6 is sufficient to allow γ_c binding, but that the interaction is enhanced when a larger portion of the N-terminus is included.

The interaction of Jak3 with the receptor is known to be required for IL-2 responses. We therefore next tested the ability of the chimeras to signal in response to IL-2. The J3/1 chimeras were introduced by retroviral transduction into fibroblasts that express the IL-2 receptor but not Jak3, and cells stably expressing the chimeras were stimulated with IL-2. Epitope-tagged wild-type Jak3 or J3/1 chimeras were precipitated, and blots were probed with anti-phosphotyrosine. The J3/1 chimera containing the minimal receptor interaction domain was not tyrosine-phosphorylated in response to IL-2 (Figure 5C, lanes 5 and 6). In addition, a longer chimera containing 60 additional amino acids at the C-terminus was also not inducibly tyrosine phosphorylated (not shown). As shown in Figure 5C, lanes 7 and 8, the smallest region of Jak3 that could reconstitute IL-2-inducible tyrosine phosphorylation contains the entire JH7 and JH6 domains, considerably longer than the minimal receptor interaction domain, as defined in co-expression studies. The ability of the JH7–JH6 chimera to reconstitute IL-2 responses was further demonstrated by the IL-2-inducible phosphorylation of IL-2R β that is co-precipitated with the chimera (Figure 5C, lanes 4 and 8). This inducible phosphorylation of the IL-2R β chain, which is a hallmark of a functional IL-2 response, is not seen in cell lines expressing J3/1 chimeras that do not contain the entire JH7 and JH6 domains.

We further confirmed the ability of the chimeras to reconstitute IL-2 responses by analyzing the phosphorylation of STAT5b transiently expressed in the 3T3 cell lines that stably express wild-type Jak3 and the chimeric Jak3–Jak1 proteins. Cells that did not express Jak3 showed no significant IL-2-inducible STAT5b phosphorylation (Figure 5D, lanes 1 and 2), while in cells expressing wild-type Jak3, tyrosine phosphorylation of STAT5b was detected (Figure 5D, lanes 3 and 4). Moreover, no STAT5b tyrosine phosphorylation above background levels was detected in cells expressing the J3/1–130 chimera (Figure 5D, lanes 5 and 6), suggesting that this portion of Jak3 is not sufficient to elicit downstream responses following IL-2 stimulation. However, consistent with the J3/1 chimera phosphorylation studies, the JH7–JH6 chimera (J3/1–256) was able to mediate STAT5b phosphorylation (Figure 5D, lanes 7 and 8). These data are consistent with a model in which the JH7 and JH6 domains of Jak3 are sufficient for functional reconstitution of IL-2-induced responses.

Discussion

Mutational analysis of the cytoplasmic domains of cytokine receptors has shown that the membrane-proximal regions containing Box1 and Box2 motifs (Murakami, 1991; O'Neal and Yu-Lee, 1993) are essential for signal

Fig. 5. Jak3-Jak1 chimeras physically associate with γ_c and reconstitute cytokine responses. (A) Jak3-Jak1 chimeras, shown schematically, are described in Materials and methods. (B) Lysates from 293T cells co-expressing γ_c and the indicated Jak3-Jak1 chimeras were immunoprecipitated with Sepharose-conjugated M2 anti-FLAG monoclonal antibody, and blots were probed with rabbit polyclonal anti- γ_c antibody. (C) IL-2R+ fibroblasts lacking Jak3 (lanes 1 and 2), or stably expressing wild-type (WT) Jak3 (lanes 3 and 4) or Jak3-Jak1 chimeras J3/1 1-130 (lanes 5 and 6), or J3/1 1-256 (lanes 7 and 8) were stimulated with 1000 U/ml IL-2 for 15 min. Cells were lysed as described in Materials and methods. Lysates were immunoprecipitated with rabbit polyclonal anti-FLAG antibody and blots were probed with anti-phosphotyrosine monoclonal antibody 4G10. (D) IL-2R+ fibroblasts transiently expressing STAT5b and lacking Jak3 (lanes 1 and 2) or expressing WT Jak3 (lanes 3 and 4) or Jak3-Jak1 chimeras J3/1 1-130 (lanes 5 and 6), or J3/1 1-256 (lanes 7 and 8) were stimulated with IL-2 and lysed as in (B). Lysates were immunoprecipitated with rabbit polyclonal anti-STAT5b antibody and blots were probed with anti-phosphotyrosine monoclonal antibody 4G10.

transduction and Jak activation. The Jak kinases have been shown to associate directly with these membrane-proximal regions, but the regions of Jak kinases responsible for this interaction have not been well defined. In this study we show that a point mutation, found in an autosomal SCID patient, in the JH7 domain of Jak3 disrupts the interaction of Jak3 with its cognate receptor γ_c . We further show that other point mutations in this region can also block this interaction. We have used this information to define a region within JH6-JH7 of Jak3 that interacts with Box1 of the γ_c receptor chain and also show that a Jak3-Jak1 chimera containing this region can interact with γ_c , and can restore IL-2 signaling. These studies show that the N-terminal region of Jak3 is necessary for γ_c -mediated signaling responses and suggest that this region carries the domain responsible for binding specificity in Jak-receptor interactions.

The most common form of human SCID is typically characterized by an absence of T cells and NK cells, but the immunological phenotype can vary depending upon

the mutation. One SCID patient who did develop some mature T cells had a nonsense mutation which truncated most of the cytoplasmic tail of γ_c . T cells from this patient exhibited proliferation and no Jak3 activation in response to IL-2 (Morelon *et al.*, 1996). Another naturally occurring point mutation in the cytoplasmic tail of γ_c (L271Q) within Box1/Box2 partially disrupts binding to Jak3 and leads to a less severe phenotype (Russell *et al.*, 1995; Schmalstieg *et al.*, 1995). Therefore, these intracellular truncations and point mutations can result in immunodeficiency by disrupting proper interaction with Jak3. Interestingly, we show that a single point mutation in Jak3 (Y100C) can also lead to a severe phenotype by blocking Jak3 interaction with γ_c and hence downstream signaling responses to IL-2. Additionally we show that other mutations in this region of Jak3 (Y100A, L98A, I102A) block Jak3- γ_c interaction, suggesting that this region of Jak3 probably encodes a domain that is essential for receptor interaction. The mutation Y100A, like the SCID patient mutation, Y100C, markedly reduces Jak3 receptor binding. Since a

conservative change (Y100F) has no significant effect on Jak3- γ_c interaction, this suggests that tyrosine 100 in Jak3 forms part of the structure that directly contacts γ_c , and that ablation of the aromatic residue by an alanine or cysteine substitution disrupts the domain fold.

A number of reports have suggested that the N-termini of Jak kinases are required for receptor interaction. Several studies have shown that deletion of the JH6–JH7 domains of Jak2 abrogates its association with the growth hormone receptor and GM-CSF receptor chain β_c (Frank *et al.*, 1995; Tanner *et al.*, 1995; Zhao *et al.*, 1995). This region has also been shown to be necessary for Tyk2 interaction with IFN- α R1 (Gauzzi *et al.*, 1997; Richter *et al.*, 1998; Yan *et al.*, 1998). The JH7 and JH6 domains were shown to be sufficient for the interaction of Jak2 with GM-CSFR β_c (Zhao *et al.*, 1995) and for Jak3 with γ_c (Chen *et al.*, 1997). However, the JH regions are loosely defined by an early clustering of Jak family sequences, and may not represent discrete folding or functional domains, such as SH2 or SH3 modules (Schlessinger, 1994; Pawson and Scott, 1997). Our finding that an N-terminal fragment of Jak3 encoding only amino acids 72–130 can bind γ_c suggests that the region sufficient for receptor interaction does not include the entire JH6 and JH7 domains. However, we also demonstrate that reconstitution of IL-2-induced signaling requires the complete JH6 and JH7 regions, suggesting that both of these domains are needed for an intact functional response.

Although the N-terminal JH6–JH7 domains of Jaks have been shown to interact with receptors in co-expression studies, it has been suggested that JH7–JH3 are required to mediate cytokine-induced signaling responses. For example, the JH3–JH7 domains of Tyk2 are required for stabilization of IFN- α R1 expression (Gauzzi *et al.*, 1997). In addition, studies with chimeric kinases have shown that IFN- γ -induced STAT1 activation was dependent on JH6–JH7 domains of Jak2, while the entire JH3–JH7 region of Jak1 was required to reconstitute STAT1 activation (Kohlhuber *et al.*, 1997). Likewise, it has been demonstrated that the JH4–JH7 domains of Jak3 are sufficient to induce signaling events in response to IL-2 (Chen *et al.*, 1997). Our results are in agreement with these data, and we have further defined a minimal functional receptor interaction domain of Jak3 (JH6–JH7; amino acids 1–256) that mediates γ_c binding and reconstitutes biochemical signaling in response to IL-2. In addition, we have shown that Jak3–Jak1 chimeras containing only Jak3 residues 1–130 or 1–193 can bind to γ_c but do not elicit downstream signaling events in response to cytokine. This suggests that while amino acid residues 1–130 (i.e. JH7 plus 5 amino acids of JH6) can couple to γ_c in co-expression studies, residues 1–256 (or the complete JH6/JH7 domains) are required for IL-2-induced Jak3 activation and induction of STAT5 tyrosine phosphorylation. Therefore, while the complete JH6–JH7 domains are not required for receptor interaction, they must play an important role in enhancing Jak activation and subsequent downstream signaling.

Mutational analysis of cytokine receptors has shown that the cytoplasmic membrane-proximal regions containing Box1 (proline-rich motif) and Box2 are essential for receptor function and Jak association. For example, deletion or mutation of Box1 proline residues abrogates the interaction of Jak2 with several receptor chains, including

gp130, GHR, EPOR and GM-CSFR β_c (Tanner *et al.*, 1995; Zhao *et al.*, 1995). Similarly, mutational analysis has shown that the Box1 region of γ_c is necessary for its interaction with Jak3, and deletion of Box1 abrogates Jak3-dependent proliferative signals (Goldsmith *et al.*, 1995; Nelson *et al.*, 1996). Moreover, a patient point mutation adjacent to Box1 in γ_c reduces Jak3 binding and results in XCID (Russell *et al.*, 1994; Schmalstieg *et al.*, 1995), suggesting that γ_c Box1 directly contacts Jak3. However, it has not been demonstrated that the conserved proline residues of γ_c Box1 participate in Jak3 binding. Our studies show that mutation of the Box1 proline residues 266 and 269 markedly reduces γ_c binding to Jak3 and further indicate that this Box1 region specifically interacts with the JH6–JH7 regions of Jak3. These data clearly suggest that the Janus kinases probably interact with receptor Box1 motifs through a sequence overlapping JH6–JH7 and that the specificity of the Jak kinase interaction is contained within this region. It is interesting to note that several unrelated protein structures have the ability to bind proline-rich motifs (e.g. SH3, WW and profilin-binding domains). The predicted $\alpha+\beta$ fold of the Box1-binding, N-terminal domain of Jaks may represent yet another departure from the observed folding motifs of these known proline-binding modules (Pawson and Scott, 1997).

Several autosomal SCID patients who express Jak3 protein have Jak3 mutations that result in constitutive tyrosine phosphorylation of the kinase, and in each case, cytokine-induced responses are impaired (Candotti *et al.*, 1998). Interestingly, the single amino acid substitution of Jak3 described here (Y100C) also results in a loss of proper regulation of basal tyrosine phosphorylation. These observations raise the possibility that the activity or basal tyrosine phosphorylation state of Jak kinases may be regulated by intramolecular interactions, similar to Tec and Src-family kinases (Andreotti, 1997; Moarefi, 1997). Autosomal SCID point mutations and deletions may disrupt a regulatory interaction that controls the basal phosphorylation state of the molecule, resulting in constitutive phosphorylation in cells derived from the patients, and possibly contributing to the SCID phenotype. However, we have demonstrated that the inability of Jak3 Y100C to interact with γ_c is not due to its constitutive phosphorylation, as a kinase-inactive derivative, Jak3 Y100C/K855A, that is not detectably tyrosine phosphorylated, also fails to interact with γ_c in 293T cells.

It is of note that a Jak3–Jak1 chimeric kinase can functionally substitute for Jak3 in the IL-2 receptor complex, as this suggests that the Jak1 catalytic domain can efficiently induce tyrosine phosphorylation of the IL-2R β chain and STAT5 in the absence of Jak3 catalytic activity. In agreement with previous reports (Chen *et al.*, 1997; Kohlhuber *et al.*, 1997), our data suggest that the specificity of cytokine responses is not dependent on the catalytic domain of the receptor-associated Jak kinase. Therefore, while the initiation of cytokine responses requires correct targeting of a Jak kinase catalytic domain to the receptor complex, the substrate specificity of the kinase does not appear to play a major role in the signaling response.

In summary, our data show that autosomal SCID can derive from a JH7 point mutation that disrupts the interaction of Jak3 with its cognate receptor. We also demonstrate that a segment overlapping two Janus homology domains, JH7 and JH6, defines a region sufficient for Jak3

interaction with the Box1 region of γ_c , and for functional targeting of Jak3 to the IL-2 receptor complex. Our studies, therefore, may provide a basis for the design of small molecule inhibitors of cytokine signaling, and allow an understanding of the molecular mechanism of some cases of autosomal SCID in which patients express Jak3 protein. It will be important to determine whether a receptor interaction domain can be mapped in all members of the Jak family of kinases.

Materials and methods

Cell lines and cytokines

293T cells (DuBridge *et al.*, 1987), IL-2R-expressing fibroblasts 3T3 $\alpha\beta\gamma$ (Chen *et al.*, 1997) and Phoenix A cells (Grignani *et al.*, 1998) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Bio-Whittaker, Walkersville, MD) and cultured at 37°C in 5% CO₂, and were transfected by the calcium phosphate method. All tissue culture media were supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Bio-Whittaker, Walkersville, MD). The EBV-transformed B-cell lines GM, JY and MC were maintained in RPMI 1640 (Bio-Whittaker, Walkersville, MD), supplemented as described above. IL-2 was purchased from R&D Systems (Minneapolis, MN).

Plasmids and constructs

Jak3 point mutations were generated by standard PCR techniques, using oligonucleotides encoding the mutant sequence. Jak3, Jak3 mutants and γ_c were FLAG- or myc-tagged at their C-termini by PCR. The stop codon of each cDNA was replaced by a *Clal* site (ATCGAT), and the cDNA was cloned into pME18S, in which expression is driven by the SR α promoter (Takebe *et al.*, 1988), and the pMX Neo retroviral vector (Onishi *et al.*, 1996), which contains either a single copy of the FLAG sequence or a pentamer of the myc-tag sequence. Jak3–Jak1 chimeras were generated by fusion PCR (Clackson and Winter, 1989), with the following junctions (amino acid position): J3 109/J1 105, J3 130/J1 140, J3 193/J1 209, J3 256/J1 278, J3 300/J1 326, J3 519/J1 570. The Jak3–Jak1 chimeras were FLAG-tagged at their C-termini by PCR, in which the 3' oligonucleotide encoded the FLAG sequence. Jak3 fragments were generated by PCR, introducing a *Clal* site after the coding sequence, and were cloned into pME18S encoding the C-terminal myc tag.

Cytokine stimulation, immunoblotting and immunoprecipitations

For the 293T co-immunoprecipitation experiments, cells were lysed in buffer containing 300 mM NaCl, 50 mM Tris–HCl, 2 mM EDTA, 0.875% Brij 97 (Sigma, St Louis, MO), 0.125% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF, 1 mM Na₃VO₄, and 1 mM NaF. Lysates were centrifuged at 12 000 g, and NaCl was added to 500 mM final concentration. Lysates were immunoprecipitated with the appropriate antibody. Rabbit polyclonal, mouse monoclonal (M2), and Sepharose-conjugated M2 anti-FLAG antibodies were purchased from Kodak Scientific Imaging Systems (Rochester, NY). Goat anti- γ_c antibody was purchased from R&D (Minneapolis, MN). Lysates and immunoprecipitates were boiled, subjected to 8% SDS–PAGE or 12% SDS–PAGE for experiments with Jak3 fragments, and electrotransferred onto nylon membranes (Immobilon-P, Millipore, Bradford, MA). Membranes were probed with one of the following antibodies: mouse monoclonal M2 anti-FLAG antibody; rabbit polyclonal anti- γ_c antibody; or mouse monoclonal anti-myc tag antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA). For the immunoprecipitations with EBV-transformed B-cell lines and the IL-2R-expressing fibroblasts, cells were lysed as described above, except with a final NaCl concentration of 150 mM. Lysates were immunoprecipitated with monoclonal M2 anti-FLAG antibody, rabbit polyclonal antibody directed against the C-terminus of Jak3 (α -Jak3; Kawamura *et al.*, 1994) or rabbit polyclonal antibody specific for STAT5b (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were then washed, boiled and resolved by SDS–PAGE. Membranes were immunoblotted with anti-phosphotyrosine mouse monoclonal antibody [α -PY (4G10); Upstate Biotechnology Inc., Lake Placid, NY], or reprobed with the original immunoprecipitation antibody as described (Kawamura *et al.*, 1994). Detection was then performed with chemiluminescent substrate (Pierce, Rockport, IL).

Retroviral infections and stable cell lines

IL-2R-expressing fibroblasts were infected with supernatant from the Phoenix A packaging cell line that had been transfected with wild-type Jak3, mutant Jak3, Jak3–Jak1 chimera or STAT5b cDNAs cloned onto the retroviral expression vector pMX-Neo (Onishi *et al.*, 1996). Stably expressing cell lines were selected in 0.5 mg/ml G418 (Gibco-BRL, Gaithersburg, MD).

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