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The Pseudokinase Domain Is Required for Suppression of Basal Activity of Jak2 and Jak3 Tyrosine Kinases and for Cytokine-inducible Activation of Signal Transduction*

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Janus (Jak) tyrosine kinases contain a tyrosine kinase (JH1) domain adjacent to a catalytically inactive pseudokinase domain (JH2). The JH2 domain has been implicated in regulation of Jak activity, but its function remains poorly understood. Here, we found that the JH2 domain negatively regulates the activity of Jak2 and Jak3. Deletion of JH2 resulted in increased tyrosine phosphorylation of the Jak2- and Jak3-JH2 deletion mutants as well as of coexpressed STAT5. In cytokine receptor signaling, the deletion of the Jak2- and Jak3-JH2 domains resulted in interferon-γ and interleukin-2-independent STAT activation, respectively. However, cytokine stimulations did not further induce the JH2 deletion mutant-mediated STAT activation. The deletion of the Jak2 JH2 domain also abolished interferon-γ-inducible kinase activation, although it did not affect the reciprocal Jak1-Jak2 interaction in 293T cells. Chimeric constructs, where the JH2 domains were swapped between Jak2 and Jak3, retained low basal activity and cytokine inducible signaling, indicating functional conservation between the two JH2 domains. However, the basal activity of Jak2 was significantly lower than that of Jak3, suggesting differences in the regulation of Jak2 and Jak3 activity. In conclusion, we found that the JH2 domain has a conserved function in Jak2 and Jak3. The JH2 domain is required for two distinct functions in cytokine signaling: (i) inhibition of the basal activity of Jak2 and Jak3, and (ii) cytokine-inducible activation of signaling. The Jak-JH2 deletion mutants are catalytically active, activate STAT5, and interact with another Jak kinase, but the JH2 domain is required to connect these signaling events to receptor activation. Thus, we propose that the JH2 domain contributes to both the uninduced and ligand-induced Jak-receptor complex, where it acts as a cytokine-inducible switch to regulate signal transduction.

Janus (Jak)[1] tyrosine kinases are essential mediators of cytokine-induced signal transduction (1). The Jak kinases bind to the cytoplasmic tails of receptors belonging to the hematopoietic receptor superfamily. Ligand-induced receptor aggregation results in tyrosine phosphorylation of Jak kinases and their substrate proteins, such as transcription factors called signal transducers and activators of transcription, STATs (2, 3). Each Jak kinase specifically binds to only a subset of cytokine receptors. Jak2 associates with single chain receptors for erythropoietin, growth hormone and prolactin, type two cytokine receptors (IFNγRII, IL-10R), and the β chain of receptors for IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (1). Lack of Jak2 causes embryonic lethality in mice because of absence of definitive erythropoiesis, and these cells also fail to respond to interferon-γ (IFN-γ), IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (4, 5). On the other hand, Jak3 has been found as the only signaling molecule so far to associate with the common γ chain of IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (6). Lack of functional Jak3 results in severe combined immunodeficiency (SCID) with varying defects on T and B cells both in human and mice (7–10).

The Jak kinases are characterized by the presence of seven regions of sequence similarity found between Jak kinases and designated as Jak homology (JH) domains (11). JH1 is a catalytically active tyrosine kinase domain located in the C terminus of Jak kinases and next to it is JH2, also called the pseudokinase or kinase-like domain. The JH2 domain has conserved sequence motifs found in protein kinases, but these motifs are subtly modified and consequently, the JH2 domain is catalytically inactive. Interestingly, the modifications are conserved between the JH2 domains of different Jak kinases, suggesting that the JH2 domain has an important function in Jak kinases. Recently, sequence-based predictions have indicated the existence of an SH2-like and a FERM domain in the JH3-JH4 and JH4-JH7 regions of Jak kinases, respectively (12–15). The FERM domain mediates specific binding of Jak kinases to cytokine receptors (16–18) and the FERM domains of Jak1 and Jak2 have been found to assist in cell surface expression of cytokine receptors (19, 20). The FERM domain of Jak3 was recently also found to positively regulate kinase activity, whereas no such function has been assigned to this domain in Jak2 or Tyk2 (21–24). The function of the SH2 domain in Jak kinases is currently unknown.

In cytokine receptors, Jak activation is achieved through ligand-induced aggregation of receptor chains bringing the associated Jak kinases in close contact. Ligand binding most

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[1] The abbreviations used are: Jak, Janus; IFN, interferon; IL, interleukin; JH, Jak homology region; PH, pleckstrin homology; HA, hemagglutinin; SCID, severe combined immunodeficiency; SH, Src homology; STAT, signal transducer and activator of transcription; KN, kinase negative.

likely induces a conformational change in the receptor cytoplasmic domains allowing formation of a multimeric receptor complex and further induction of signal transduction. The activity of Jak kinases is induced by phosphorylation of tyrosine residues in the kinase activation loop (A-loop) through auto- or transphosphorylation by another Jak kinase. The A-loop in Jak kinases contains a conserved YY motif and the functional role of these tyrosines varies between different Jaks. Phosphorylation of the first tyrosine in the YY motif greatly increases the activity of Jak1 and Jak2, but has a less pronounced effect in Jak3 (22, 25, 26). Mutation of the second tyrosine in the YY motif increases the activity of Jak3, whereas similar mutations in Jak1 or Jak2 have no effect (22, 25, 26). The double tyrosine motif is also a target for negative regulation of Jaks. Suppressor of cytokine signaling (SOCS)-1 and SOCS-3 directly bind to the activation loop of Jak2 to down-regulate kinase activity, and in the case of SOCS-1 interaction has been shown to induce ubiquitination of Jak2 (27–29). Also, the protein-tyrosine phosphatase PTP1B down-regulates IFN-γ and IFN-α signaling by recognizing and specifically dephosphorylating the double tyrosine motifs of Jak2 and Tyk2 (30). Jak3 and Jak1, on the other hand, are negatively regulated through the T cell protein-tyrosine phosphatase (TCPTP) (31).

Although catalytically inactive, the JH2 domain has been found to be a critical regulator of Jak kinases. We have previously found that JH2 is a negative regulatory domain in Jak2, and deletion of JH2 leads to activation of Jak2 (23). A mutation in the JH2 domain of *Drosophila* Jak kinase, Hop, has been found to up-regulate kinase activity and result in leukemia in the fly, also supporting a role for JH2 in negative regulation of Jaks (32). On the other hand, mutations in the Jak3-JH2 domain have been found to inactivate the kinase, and result in abrogation of IL-2 signaling leading to SCID (33, 34). Similarly, mutations in the JH2 domain of Tyk2 have been shown to interfere with Tyk2 activity, and deletion of JH2 resulted in inactivation of the kinase (35, 36). Thus, the JH2 domain has been found to regulate Jak activity either positively or negatively, leaving the role of the JH2 domain in Jaks still unclear. Furthermore, it is currently not known if the JH2 domain has a conserved function in regulating Jak activity or if the role of the JH2 domain is distinct in different Jaks.

In this study we have compared the JH2-mediated regulation of Jak2 and Jak3 to elucidate the function of the JH2 domain in cytokine signaling. Our results demonstrate that the JH2 domain is required to maintain low basal activity of both Jak2 and Jak3. Furthermore, the results show that JH2 specifically mediates cytokine receptor-induced Jak activation and cytokine-inducible activation of downstream signaling. Chimeric constructs, where the JH2 domains are swapped between Jak2 and Jak3, show low basal activity and cytokine-inducible signaling, indicating functional conservation of the two JH2 domains. In the absence of JH2, the Jak JH2 deletion mutants are catalytically active, activate STAT5, and interact with another Jak kinase, but are still unable to respond to cytokine stimulation. Based on these findings we propose that in addition to suppressing basal Jak activity in the uninduced Jak-receptor complex, the JH2 domain is an integral part of the ligand-activated receptor complex by mediating specific protein-protein interactions required for cytokine responsiveness of cells.

EXPERIMENTAL PROCEDURES

Reagents, Cell Culture, and Transfections—293T, COS, 293 (American Type Culture Collection, Manassas, VA) and γ2A (Jak2-deficient fibrosarcoma cell line (37)) cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. The cells were transfected using the FuGENE™ 6 transfection reagent (Roche Molecular Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. 0.5–5 μg of specific cDNAs, depending on the experiment, were used to transfect 60% confluent 10-cm plates of 293T or COS cells, and 100 ng of specific cDNA was used for transfection of a 6-well plate well of γ2A or 293 cells. The amount of each cDNA transfected was adjusted within a single experiment to obtain similar expression levels of the different cDNA constructs verified by immunoblotting. The cells were stimulated with IFN-γ (R&D Systems, Minneapolis, MN) or IL-2 (R&D Systems). The cells were harvested 72 h after transfection for immunoprecipitation and after 25 h for luciferase assay. The following antibodies were used: anti-phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY), anti-hemagglutinin (anti-HA, 16B12, Covance, Princeton, NJ), anti-STAT5 (ST5a-2H2, Zymed Laboratories Inc.), anti-Jak1 (Transduction Laboratories, Lexington, KY), and polyclonal anti-Jak2 and anti-Jak3 antibodies, a kind gift from Dr. James Ihle (38, 39).

DNA Constructs—The amino acids encoded by the Jak2 and Jak3 constructs are shown in Figs. 3*A*, 4*A*, and 5*A* and the numbering refers to mouse Jak2 (GenBank™ accession number L16956) and Jak3 (GenBank™ accession number L32955) sequences. Expression vectors for Jak2, JH2Δ-Jak2, and JH1-Jak2 have been described previously and they contain the HA tag in their C terminus (23). The mouse Jak3 plasmid was described previously, except for a HA tag, which was added to the C terminus of Jak3 (40). Jak2 and Jak3 constructs were prepared using PCR and cloned into the pCIneo expression vector (Promega, Madison, WI). New translation initiation codons were introduced by PCR into HA-tagged Jak3 to create JH1-2-Jak3 and JH1-Jak3. JH2Δ-Jak3 and Jak323 were constructed using recombinant PCR creating a deletion and a chimeric construct, respectively. Jak232 was constructed using recombinant PCR creating a chimeric construct with 1 amino acid change (P841S) in the linker between the kinase and pseudokinase domains. Prediction of the protein domains was done using the Smart program (41). All PCR products were confirmed by sequencing (Applied Biosystems). Expression vector for STAT5A and the luciferase reporter construct containing the STAT5 binding site from the promoter of the serine protease inhibitor gene (pSPI-luc2) were kind gifts from Dr. Tim Wood (42). Luciferase reporter construct containing the STAT1 binding site from the promoter of the IRF-1 gene was a kind gift from Dr. Richard Pine (43).

Immunoblotting, Immunoprecipitation, Kinase, and Luciferase Assay—Cells were lysed in kinase lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 20% glycerol, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitors, and the lysates were used for immunoprecipitation or directly for Western blotting. The immunoprecipitation protocol has been described (44). The immunoprecipitates were subjected to Western blotting or used for kinase assay. For kinase assay, the immunoprecipitates were washed four times with kinase lysis buffer and twice with kinase assay buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM NaF, 0.1 mM Na_3VO_4). The immunoprecipitates were suspended in kinase assay buffer containing dithiothreitol (1 mM). The following peptides (1 mg/ml) were used as substrates: STAT5 (AKAADGYVKPQIKQVV), Jak1YY (AIETDKEYYTVKDDRDS), Jak2YY (VLPQDKEYYKVKEPGES), or Jak3YY (LLPLDKDYYVVREPGQK). 10 μCi of [γ-^{33}P]ATP was added to the reactions followed by a 10-min incubation at room temperature and boiling in reducing Laemmli sample buffer. The reactions were separated in 20% SDS-PAGE followed by quantification of radioactivity using PhosphorImager (Fuji). Equal amounts of protein from cell lysates were always used for immunoprecipitations and Western blotting of cell lysates. Protein concentrations were determined using the Bio-Rad protein assay system. Immunodetection was performed using specific primary antibodies, biotinylated anti-mouse, or anti-rabbit secondary antibodies (Dako A/S, Glostrup, Denmark), and streptavidin-biotin horseradish peroxidase conjugate (Amersham Biosciences) followed by ECL. Luciferase activity was determined using dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. The STAT-dependent luciferase activity was normalized to the activity of the co-transfected plasmid constitutively expressing *Renilla* luciferase.

RESULTS

Jak2 Has Low Kinase Activity When Compared with Jak1 or Jak3—We have previously investigated the role of the different JH domains in regulation of Jak2 and found that the pseudokinase domain, JH2, negatively regulates the activity of Jak2 (23). Because the JH2 domain is conserved within the Jak family, we wanted to investigate the function of the JH2 domain also in other Jak kinases. In addition, we sought to

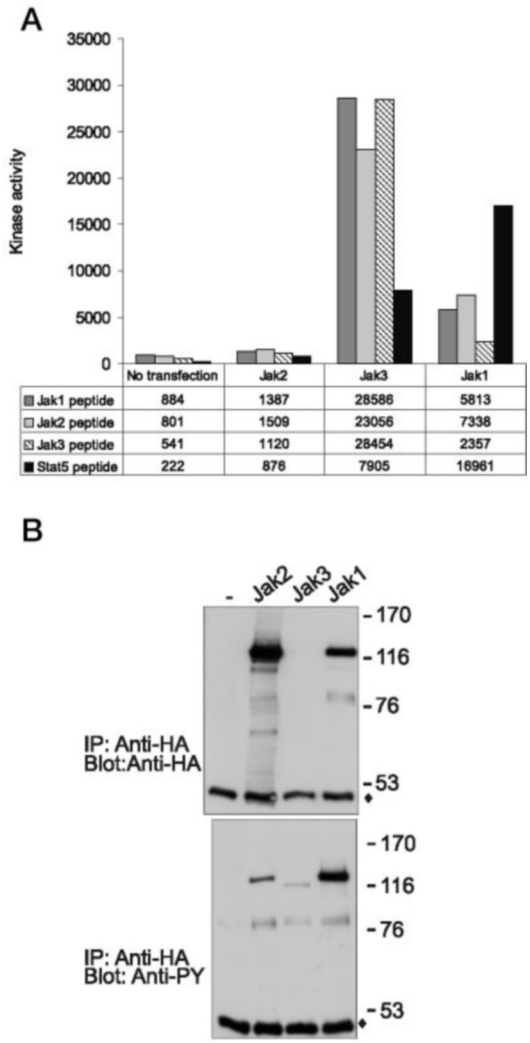

FIG. 1. **Comparison of kinase activity of Jak kinases.** *A*, expression plasmids for HA-tagged mouse Jak1, Jak2, and Jak3 kinases were transfected into 293T cells and the cell lysates were immunoprecipitated (*IP*) using anti-HA antibody. Aliquots of the immunoprecipitates were subjected to the *in vitro* kinase assay using [γ-^{33}P]ATP and as substrate four different peptides containing the double tyrosine motif phosphorylated in Jak1, Jak2, or Jak3 or tyrosine 694 phosphorylated in STAT5. The peptides were separated in 20% SDS-PAGE followed by quantification using PhosphorImager. *B*, aliquots of the immunoprecipitates from *A* were separated in 7.5% SDS-PAGE and analyzed in anti-HA (*top panel*) and anti-phosphotyrosine (*bottom panel*) immunoblots. ♦, immunoglobulin chains. The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*.

determine whether the JH2 domains regulate different Jak kinases similarly or if this domain mediates distinct functions in different Jak kinases.

We started the analysis by comparing the activities of Jak1, Jak2, and Jak3. HA-tagged Jak1, Jak2, and Jak3 kinases were expressed in 293T cells and the Jak proteins were immunoprecipitated using anti-HA antibody. The immunoprecipitates were subjected to *in vitro* kinase assay using four different peptide substrates corresponding to the double tyrosine motifs in the kinase activation loop of Jak1, Jak2, and Jak3 and the tyrosine phosphorylation site in STAT5 (Y694) (22, 25, 26, 45). The activity of Jak2 toward any of the substrate peptides was extremely low when compared with the activities of either Jak1 or Jak3 (Fig. 1*A*), although Jak2 was expressed at much higher levels than Jak1 or Jak3 (Fig. 1*B*). The expression of Jak3 was repeatedly very low, and difficult to detect using the monoclonal anti-HA antibody, but could be detected using polyclonal Jak3 antiserum (not shown). Despite the lower level of expression, tyrosine phosphorylation of Jak3 was very similar to that of Jak2, indicating that Jak3 was phosphorylated at a higher level than Jak2 (Fig. 1*B*). Also Jak1 had higher level of tyrosine phosphorylation than Jak2. Interestingly, Jak1 and Jak3 showed clear differences in phosphorylation of the substrate peptides. The STAT5-derived peptide was the best substrate for Jak1, whereas Jak3 showed preference for the A-loop-derived peptides of Jak1, Jak2, and Jak3 kinases (Fig. 1*A*).

The results from the kinase assay indicated that the activity of Jak2 was clearly lower than the activity of either Jak1 or Jak3. We considered two possible explanations for this finding. The difference in kinase activity might be because of different activities of the tyrosine kinase domains. Alternatively, but not exclusively, the kinase domains might be differentially regulated by other domains, such as the pseudokinase domain, in full-length Jak kinases. We first analyzed the activities of the isolated tyrosine kinase domains, and chose to compare the activities of Jak2 and Jak3, which showed the greatest difference in the kinase assay. A construct coding for the tyrosine kinase domain of Jak3, JH1-Jak3, and JH1-Jak2 (23), was expressed in 293T cells. The JH1 domains were immunoprecipitated using anti-HA antibody and subjected to *in vitro* kinase assay with STAT5 peptide as a substrate (Fig. 2*A*) or to immunoblotting with anti-HA or anti-phosphotyrosine antibodies (Fig. 2*B*). Despite lower level of expression, JH1-Jak3 had both higher kinase activity and higher level of tyrosine phosphorylation than JH1-Jak2, indicating that the tyrosine kinase domain of Jak3 was more active than that of Jak2.

The JH2 Domain Negatively Regulates Jak3—We previously found that deletion of JH2 resulted in activation of Jak2. To compare the pseudokinase domain-mediated regulation of Jak2 and Jak3, we created deletion constructs including JH2Δ-Jak3, a Jak3 protein lacking the pseudokinase domain, JH1-2-Jak3 coding for the double kinase domain, and JH1-Jak3 coding for the tyrosine kinase domain of Jak3 (Fig. 3*A*). Jak3, JH2Δ-Jak3, JH1-Jak3, and JH1-2-Jak3 constructs were expressed in COS cells, Jak3 proteins were immunoprecipitated, and the immunoprecipitates were analyzed by anti-phosphotyrosine immunoblotting (Fig. 3, *B* and *C*). The protein levels in the immunoprecipitates as well as in the cell lysates were found to be equal. JH2Δ-Jak3, lacking the pseudokinase domain, as well as JH1-Jak3 showed increased tyrosine phosphorylation when compared with Jak3 (Fig. 3, *B* and *C*). However, the presence of the pseudokinase domain in JH1-2-Jak3 resulted in phosphorylation comparable with Jak3 (Fig. 3*C*). This result indicated that the pseudokinase domain acted to inhibit tyrosine phosphorylation of Jak3.

To analyze if the JH2 domain also inhibited downstream signaling by Jak3, we co-expressed the different Jak3 deletion mutants with STAT5A. The ability of the Jak3 kinases to mediate tyrosine phosphorylation of STAT5 was analyzed after STAT5 immunoprecipitation by anti-phosphotyrosine immunoblotting (Fig. 3*D*). When compared with Jak3, JH2Δ-Jak3 as well as JH1-Jak3 induced increased tyrosine phosphorylation of STAT5 (Fig. 3*D*). The JH1-2-Jak3, on the other hand, resulted in STAT5 phosphorylation comparable with Jak3 (Fig. 3*D*). Thus, the JH2 domain also inhibited the ability of Jak3 to activate STAT5.

We next analyzed if the increase in tyrosine phosphorylation of the Jak3 mutants lacking JH2 correlated with their *in vitro* kinase activity. Jak3 and the Jak3 deletion constructs, JH2Δ-Jak3, JH1-Jak3, and JH1-2-Jak3, were expressed in COS cells, and the Jak3 immunoprecipitates were analyzed in *in vitro* kinase assay using as a substrate a peptide containing the A-loop double tyrosine motif of Jak3 (Fig. 3*E*). Immunoblotting

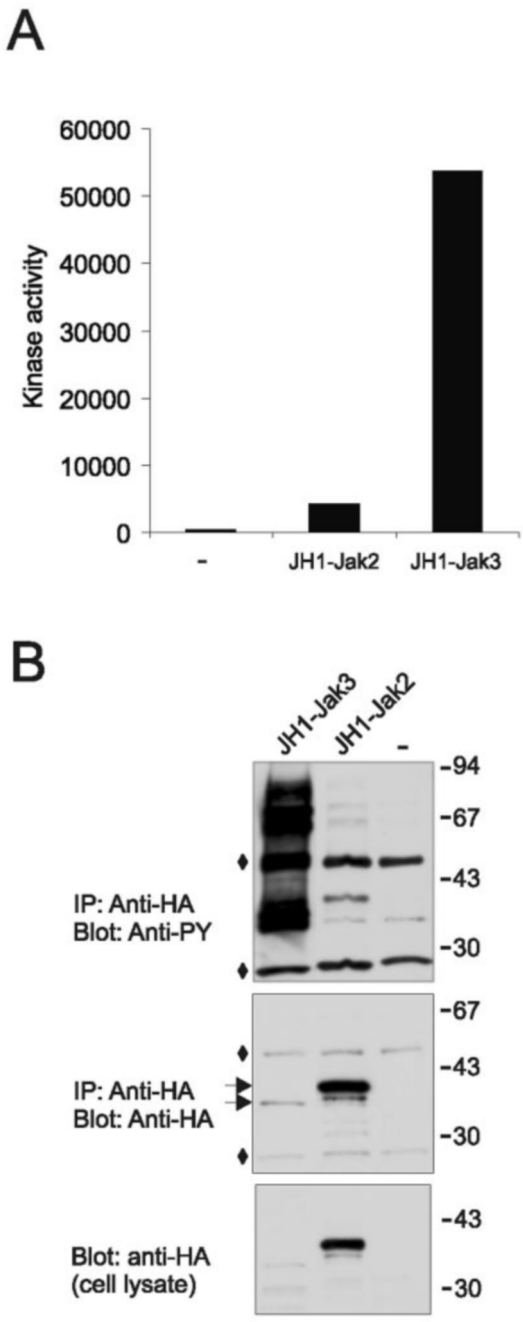

FIG. 2. **Comparison of kinase activity of the isolated kinase domains of Jak2 and Jak3.** *A*, expression plasmids for the HA-tagged kinase domains of Jak2 (*JH1-Jak2*) and Jak3 (*JH1-Jak3*) were transfected into 293T cells and cell lysates were immunoprecipitated (*IP*) using anti-HA antibody. Aliquots of the immunoprecipitates were subjected to *in vitro* kinase assay using [γ-^{33}P]ATP and STAT5-derived peptide as a substrate. The peptides were separated in 20% SDS-PAGE followed by quantification using PhosphorImager. *B*, aliquots of the immunoprecipitates from *A* were separated in 4–15% SDS-PAGE and analyzed in anti-phosphotyrosine (*top panel*) and anti-HA (*middle panel*) immunoblots. Cell lysates were separated in 4–15% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody (*bottom panel*). ♦, immunoglobulin chains. The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*. *Arrows* indicate migration of JH1-Jak2 and JH1-Jak3.

with anti-Jak3 antibody confirmed equal protein levels of the different Jak3 kinases in immunoprecipitates and total cell lysates (not shown). Compared with untransfected control, Jak3 showed clear phosphorylation of the peptide substrate (Fig. 3*E*). A slight increase in kinase activity was seen with the JH2Δ-Jak3 when compared with Jak3 (Fig. 3*E*). Also, the activity of JH1-Jak3 was increased over 2-fold when compared with Jak3. However, the activity of JH1-2-Jak3 was less than one-third of the activity of JH1-Jak3, indicating that the presence of JH2 reduced the activity of JH1-Jak3 (Fig. 3*E*). The lower activity of JH1-2-Jak3 compared with Jak3 is in line with the results showing that the N-terminal FERM domain positively regulates Jak3 (24). In summary, the results from Fig. 3 indicate that Jak3 is negatively regulated by its pseudokinase domain.

The JH2 Domains of Jak2 and Jak3 Are Interchangeable—The pseudokinase domain-mediated negative regulation of Jak3 showed analogy to our previous results with Jak2. However, deletion of JH2 increased the kinase activity of Jak3 only slightly, whereas we previously found a robust increase in kinase activity of Jak2 upon deletion of the JH2 domain. Thus, we considered the possibility that the JH2 domain of Jak3 was not as efficient in inhibiting Jak activity as Jak2-JH2 and decided to investigate how similar the individual pseudokinase domains of Jak2 and Jak3 are functionally. We cloned chimeric constructs; one where the JH2 domain of Jak2 was replaced by that of Jak3 (Fig. 4*A*) and one where the JH2 domain of Jak3 was replaced by that of Jak2 (Fig. 5*A*), generating the Jak232 and Jak323 constructs, respectively. These constructs left intact the entire tyrosine kinase domains and the adjacent linker regions between the kinase and pseudokinase domains.

To analyze if the pseudokinase domain of Jak3 was able to complement the pseudokinase domain of Jak2, the Jak232 chimeric construct was transiently expressed. As a control we expressed both Jak2 and JH2Δ-Jak2, a Jak2 kinase lacking the pseudokinase domain (Fig. 4*A*) (23). The Jak2 proteins were immunoprecipitated and analyzed by anti-phosphotyrosine immunoblotting. Whereas JH2Δ-Jak2 showed significantly increased tyrosine phosphorylation when compared with Jak2, tyrosine phosphorylation of Jak232 was equal to Jak2 (Fig. 4*B*). We also analyzed the signaling properties of the Jak232 chimera by co-expressing it with STAT5. Jak232 and Jak2 phosphorylated STAT5 at similar levels, whereas JH2Δ-Jak2 induced significantly increased tyrosine phosphorylation of STAT5 (Fig. 4*C*). Thus, the pseudokinase domain of Jak3 was able to restore wild type function in Jak2, suggesting that it was functionally similar to the pseudokinase domain of Jak2.

Similarly, the function of the Jak2-JH2 in the context of Jak3 was analyzed by transiently expressing the Jak323 chimeric construct (Fig. 5*A*). As a control we expressed either wild type Jak3 or JH2Δ-Jak3. Tyrosine phosphorylation of Jak323 was found to be equal to Jak3, whereas JH2Δ-Jak3 showed significantly increased tyrosine phosphorylation (Fig. 5*B*). The ability of Jak323 to induce downstream signaling was analyzed by co-expressing it with STAT5. The results show that Jak323 and Jak3 phosphorylated STAT5 at similar levels, whereas JH2Δ-Jak3 induced increased STAT5 phosphorylation (Fig. 5*C*). Thus, the pseudokinase domain of Jak2 was able to inhibit Jak3 function in the chimeric Jak323 kinase.

The JH2 Domain Is Required for Inhibition of the Basal Activity of Jak2 and Jak3 Kinases and for Cytokine-inducible Signal Transduction—We next wanted to analyze the function of the pseudokinase domain of Jak3 in cytokine receptor signaling, where activation of Jak3 is dependent on cytokine-induced receptor dimerization. We chose to use the IL-2 receptor, where Jak3 is an essential kinase for downstream signaling. We created an IL-2-dependent receptor system in 293 cells, which lack endogenous Jak3, by expressing β and γ chains of the IL-2 receptor together with STAT5 and the different Jak3 kinase constructs. Activation of STAT5 was monitored by STAT5-dependent luciferase reporter. As shown in

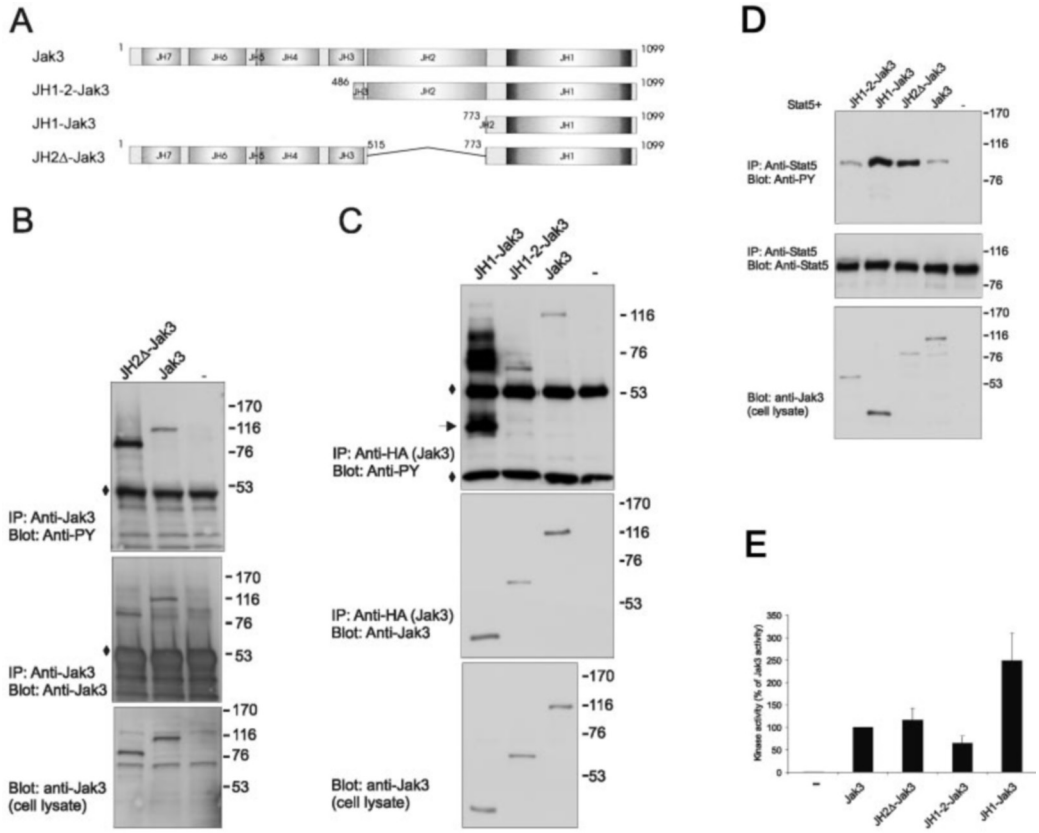

FIG. 3. **The effect of JH2 on the activity of Jak3 kinase.** *A*, Jak3 constructs coding for mouse Jak3 kinase (*Jak3*), tyrosine kinase domain (*JH1-Jak3*), pseudokinase + kinase domain (*JH1–2-Jak3*), and Jak3, where the pseudokinase domain was deleted (*JH2Δ-Jak3*) are shown schematically and amino acids are numbered according to the mouse Jak3 sequence. The cDNAs carry a HA tag in the C terminus. *B*, Jak3 and JH2Δ-Jak3 expression plasmids were transfected into COS cells and cell lysates were immunoprecipitated using anti-Jak3 antibody. The immunoprecipitates were separated in 4–15% SDS-PAGE and aliquots were analyzed in anti-phosphotyrosine (*top panel*) and anti-Jak3 immunoblot (*middle panel*). Cell lysates were separated in 4–15% SDS-PAGE and analyzed by immunoblotting with anti-Jak3 antibody (*bottom panel*). *C*, JH1-Jak3, JH1-2-Jak3, and Jak3 expression plasmids were transfected into COS cells and cell lysates were immunoprecipitated using anti-HA antibody. The immunoprecipitates were separated in 4–15% SDS-PAGE, and aliquots were analyzed in immunoblots using anti-phosphotyrosine (*top panel*) or anti-Jak3 antibody (*middle panel*). Cell lysates were separated in 4–15% SDS-PAGE and immunoblotted using anti-Jak3 antibody (*bottom panel*). The *arrow* indicates migration of the JH1-Jak3. *D*, STAT5A expression plasmid was transfected either alone or together with expression plasmids for Jak3, JH1-Jak3, JH1-2-Jak3, or JH2Δ-Jak3 into COS cells and cell lysates were immunoprecipitated using anti-STAT5 antibody. The immunoprecipitates were separated in 7.5% SDS-PAGE and aliquots were analyzed in anti-phosphotyrosine (*top panel*) and anti-STAT5 (*middle panel*) immunoblot. Cell lysates were separated in 4–15% SDS-PAGE and analyzed by immunoblotting with anti-Jak3 antibody (*bottom panel*). *E*, JH1-Jak3, JH1-2-Jak3, JH2Δ-Jak3, and Jak3 expression plasmids were transfected into COS cells and cell lysates were immunoprecipitated using anti-HA antibody. Aliquots of the immunoprecipitates were subjected to *in vitro* kinase assay using [γ-^{33}P]ATP and a peptide containing the double tyrosine motif of Jak3 as a substrate. The peptides were separated in 20% SDS-PAGE followed by quantification using PhosphorImager. The relative catalytic activities are shown as percentages of the activity of Jak3. Shown is the mean of at least three experiments and S.E., except that JH2Δ-Jak3 shows the mean of two experiments. The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*.

Fig. 6*A*, IL-2 did not induce signaling in the absence of Jak3, confirming that also in the reconstituted system signaling through the IL-2 receptor was dependent on Jak3. Stimulation of Jak3-transfected cells with IL-2 resulted in a significant increase in STAT5-dependent luciferase activity (Fig. 6*A*). In contrast, transfection of JH2Δ-Jak3 resulted in slightly increased STAT5 activation in the absence of IL-2, and notably, this activity did not increase following IL-2 treatment. Jak323 was able to induce STAT5 activation upon IL-2 stimulation, although not to the level of wild type Jak3. Thus, the results from the IL-2 receptor system also supported a role for JH2 in inhibition of the basal activity of Jak3. Furthermore, the JH2 domain was essential for IL-2 inducible activation of STAT5. This function was restored in the chimeric Jak3 construct containing the JH2 domain of Jak2, although not to the level of Jak3.

To test the function of the Jak3 pseudokinase domain in Jak2 in IFN-γ signaling, we transiently expressed Jak2, JH2Δ-Jak2, or Jak232 together with a STAT1-dependent luciferase reporter construct in a Jak2 negative cell line, γ2A. We previously found that expression of JH2Δ-Jak2 in γ2A cells results in cytokine-independent activation of STAT1 (23). Transfection of Jak2 had no effect on STAT1 activation, but stimulation of the Jak2-transfected cells with IFN-γ resulted in significant STAT1 activation (Fig. 6*B*). JH2Δ-Jak2 induced significantly increased, constitutive activation of STAT1 in the absence of IFN-γ, and this activity was not further increased by IFN-γ stimulation. The Jak232 protein behaved as wild type Jak2 having low basal activity and inducing strong STAT1 activation in response to IFN-γ stimulation. Thus, the JH2 domain of Jak3 was able to restore wild type regulation of Jak2 in IFN-γ signaling, demonstrating functional similarity between the JH2 domains of Jak2 and Jak3. In summary, our results indicate that a functional pseudokinase domain is required for suppression of the basal activity of Jak2 and Jak3 kinases. Moreover, the pseudokinase domain is required to render Jaks competent to respond to cytokine stimulation with induction of STAT activation.

We next directly analyzed the role of JH2 on cytokine-induced Jak activation. Jak2 and JH2Δ-Jak2 were expressed in

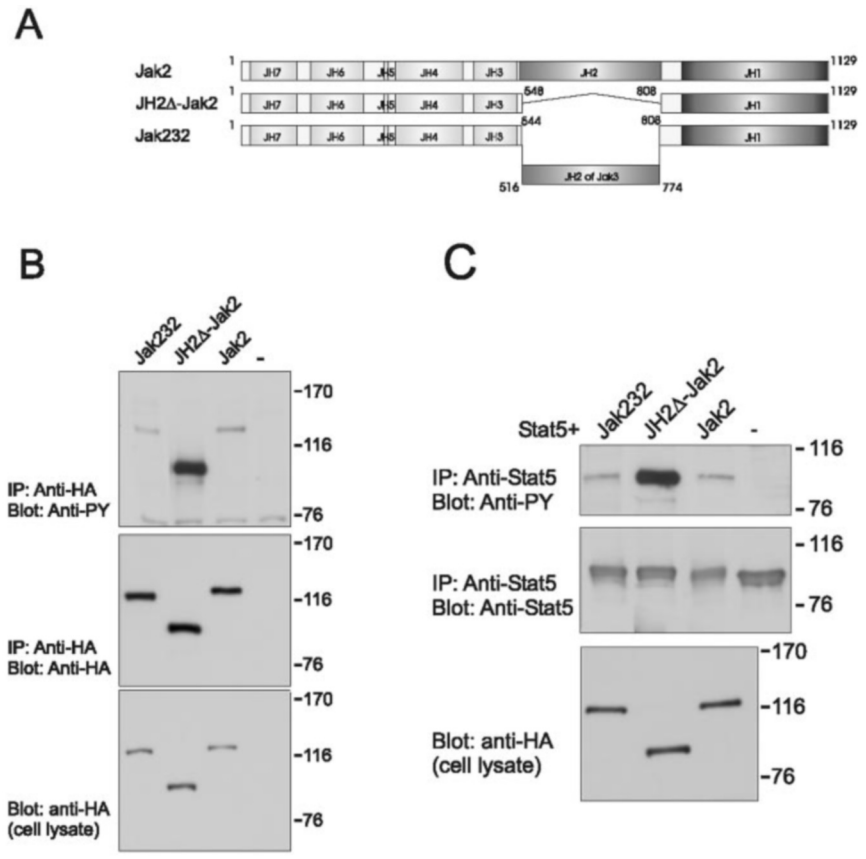

FIG. 4. **Analysis of the chimeric Jak2 construct containing the JH2 domain of Jak3.** *A*, the Jak2 constructs coding for the mouse Jak2 kinase (*Jak2*), Jak2 where the JH2 domain has been deleted (*JH2Δ-Jak2*), and Jak232 chimera containing the JH2 domain of Jak3 (*Jak232*) are shown schematically and amino acids are numbered according to the mouse Jak2 and Jak3 sequences. The cDNAs carry a HA tag in the C terminus. *B*, expression plasmids for Jak2, JH2Δ-Jak2, and Jak232 were transfected into 293T cells and cell lysates were immunoprecipitated using anti-HA antibody. The immunoprecipitates were separated in 7.5% SDS-PAGE and analyzed in anti-phosphotyrosine (*top panel*) and anti-HA immunoblot (*middle panel*). Cell lysates were separated in 7.5% SDS-PAGE and analyzed by immunoblotting with anti-HA antibody (*bottom panel*). *C*, 293T cells were transfected with STAT5 expression plasmid alone or together with expression plasmids for Jak2, JH2Δ-Jak2, and Jak232. STAT5 was immunoprecipitated using anti-STAT5 antibody and aliquots of the immunoprecipitates were analyzed in 7.5% SDS-PAGE followed by anti-phosphotyrosine (*top panel*) and anti-STAT5 immunoblotting (*middle panel*). Aliquots of cell lysates were analyzed in 7.5% SDS-PAGE followed by anti-HA immunoblotting (*bottom panel*). The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*.

γ2A cells, and activation of the Jak2 kinases was analyzed after IFN-γ stimulation by anti-phosphotyrosine immunoblotting (Fig. 7). Tyrosine phosphorylation of Jak2 was induced upon IFN-γ treatment, whereas JH2Δ-Jak2 showed constitutive phosphorylation, which was unresponsive to IFN-γ (Fig. 7, *left panel*). When the tyrosine phosphorylation levels of Jak2 and JH2Δ-Jak2 were normalized to the protein levels shown in anti-HA immunoblot (Fig. 7, *right panel*), tyrosine phosphorylation of JH2Δ-Jak2 was found to be much higher than that of Jak2. Thus, the JH2 domain was required for inhibition of basal Jak2 activity and for IFN-γ-induced activation of Jak2.

To elucidate the mechanism of JH2-mediated induction of Jak2 activity, we analyzed if the JH2 domain was required for the reciprocal interaction and subsequent activation of Jak1 and Jak2 kinases. We coexpressed kinase inactive forms of Jak2 (KN-Jak2) and JH2Δ-Jak2 (KN-JH2Δ-Jak2) with wild type Jak1. The kinase inactive Jak2 proteins were immunoprecipitated, and their tyrosine phosphorylation was analyzed. Jak1 was found to equally phosphorylate the KN-Jak2 and KN-JH2Δ-Jak2 proteins (Fig. 7*B*). The tyrosine phosphorylation of the kinase-inactive Jak2 proteins was decreased with increasing Jak2 protein levels, most likely because of inhibition of Jak1 activity by kinase-inactive Jak2 proteins. Thus, Jak1 phosphorylates both kinase negative Jak2 proteins, and the Jak1-Jak2 interaction does not require the JH2 domain. However, in Jak receptor complexes (Fig. 7*A*) inducible Jak activation is prevented in the absence of JH2, suggesting that the JH2 domain contributes to signaling interactions in the ligand-bound receptor-Jak complexes.

DISCUSSION

The Jak kinases form a distinct family of cytoplasmic tyrosine kinases characterized by the double kinase domain motif. Since the cloning of the four known Jak kinases the function of the second, inactive kinase domain has been a question of interest. Recent reports have shown that the JH2 domain is differentially involved in regulation of the various Jak kinases, raising the question if the JH2 domains regulate Jak kinases similarly or if the JH2 domains mediate distinct functions in different Jak kinases (23, 34, 36). Likewise, the exact mechanisms of the JH2-mediated regulation have remained elusive. In this study, we found that the function of the JH2 domain is, to a high degree, conserved between Jak2 and Jak3. In addition, we show that the JH2 domain has a critical role in regulating cytokine responsiveness of cells and propose a novel function for the JH2 domain in Jak-receptor complexes.

Deletion of JH2 was found to increase tyrosine phosphorylation of Jak3 itself and result in constitutive activation of STAT5, thus identifying a negative regulatory role for the JH2 domain in Jak3. These findings are in line with our earlier results regarding regulation of Jak2 (23), and indicate similar regulation of Jak2 and Jak3 by their JH2 domains. Also, a chimeric Jak2 kinase containing the JH2 domain of Jak3 behaved as wild type Jak2, indicating that the Jak3-JH2 domain was very similar in function to the JH2 domain of Jak2. Notably, despite increased basal activity, the Jak2- and Jak3-JH2 deletion mutants were unresponsive to cytokine stimulation and failed to further increase STAT activation following IFN-γ or IL-2 stimulation, respectively. Our results thus indicate that the JH2 domain is in a critical position to regulate Jak activity in response to cytokine stimulation: both by inhibiting basal activity of Jaks in the absence of cytokine and by inducing increased activity following cytokine stimulation. An analogous model has been recently proposed for the regulation of the Btk tyrosine kinase. Deletion of the pleckstrin homology (PH) domain resulted in increased activation of Btk, indicating that the PH domain normally suppressed kinase activity (46). Binding of phosphatidylinositol 3,4,5-trisphosphate to the PH do-

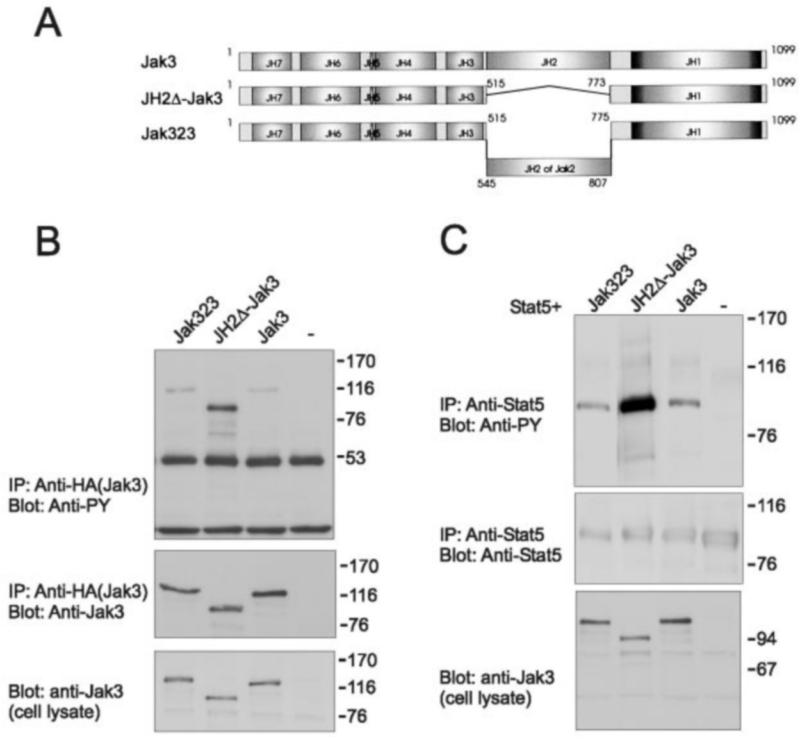

FIG. 5. **Analysis of the chimeric Jak3 construct containing the JH2 domain of Jak2.** *A*, the Jak3 constructs coding for the mouse Jak3 kinase (*Jak3*), Jak3 where the JH2 domain has been deleted (*JH2Δ-Jak3*), and Jak323 chimera containing the JH2 domain of Jak2 (*Jak323*) are shown schematically and amino acids are numbered according to the mouse Jak3 and Jak2 sequences. The cDNAs carry a HA tag in the C terminus. *B*, Jak3, JH2Δ-Jak3, and Jak323 expression plasmids were transfected into COS cells and cell lysates were immunoprecipitated using anti-HA antibody. The immunoprecipitates were separated in 7.5% SDS-PAGE and aliquots were analyzed in anti-phosphotyrosine (*top panel*) and anti-Jak3 immunoblot (*middle panel*). Cell lysates were separated in 7.5% SDS-PAGE and analyzed by immunoblotting with anti-Jak3 antibody (*bottom panel*). *C*, COS cells were transfected with STAT5 expression plasmid alone or together with Jak3, JH2Δ-Jak3, and Jak323 expression plasmids. STAT5 was immunoprecipitated using anti-STAT5 antibody and aliquots of the immunoprecipitates were analyzed in 7.5% SDS-PAGE followed by anti-phosphotyrosine (*top panel*) and anti-STAT5 immunoblotting (*middle panel*). Aliquots of cell lysates were analyzed in 7.5% SDS-PAGE followed by anti-Jak3 immunoblotting (*bottom panel*). The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*.

main increased Btk activity, suggesting that phosphatidylinositol 3,4,5-trisphosphate disrupted PH domain-mediated inhibition. However, without the PH domain Btk was unable to signal through B cell receptor (46).

Our results show that although JH2 was essential for cytokine-inducible activation of signaling, JH2 was not required for kinase activity of Jak3 or Jak2 as such, because Jak kinases lacking JH2 retained catalytic activity. Previously, either deletion or point mutations in the JH2 domains of Jak3 and Tyk2 have been found to prevent cytokine signaling, possibly because of lack of kinase activity of these mutant kinases (34–36). These seemingly contradictory results may be explained in regard to the central function of the JH2 domain in Jak kinases. It is evident that the JH2 domain is tightly connected to the function of the following tyrosine kinase domain. Thus, various mutations and small differences in the deleted JH2 regions appear to produce Jak mutants with different activities, and in certain Jaks, the JH2 domain may not be deleted without disturbing the JH1 function. Here, using murine Jak2 and Jak3 we have been able to delete JH2, while retaining catalytic activity of these kinases. Thus, our results indicate a novel function for the JH2 domain, which cannot be explained by the lack of kinase activity of the deletion mutants. Interestingly, despite similar inhibitory JH2 domains, the basal activity of Jak2 was much lower than that of Jak3, indicating that the two kinases are differentially regulated. Our results indicate that the JH1 domains may have inherently different activities, which is in line with the different role of A-loop tyrosines in different Jaks. These results also support the conclusion that the deletion of JH2 may have distinct effects in different Jaks because of differential activation requirements of the JH1 domains.

Previously, the JH2 domain has been suggested to inhibit the kinase domain through intramolecular interaction by preventing access of substrates and ATP to the catalytic cleft in the absence of cytokine stimulation (23, 34). Cytokine stimulation leading to juxtaposition of Jaks would relieve this inhibition, and our results indicate that in cytokine receptor signaling the subsequent activation of Jaks as well as STATs requires the JH2 domain. Our results showing that (i) the Jak JH2 deletion mutants were catalytically active, (ii) were able to activate STAT5 and interact with other Jaks, and (iii) the results from other groups that JH2 is not required for coupling Jaks to cytokine receptors, collectively indicate that Jaks are able to interact with all components of the signaling pathway in the absence of JH2, but the JH2 domain is required to connect cytokine receptor activation to Jak activation and induction of signaling. Thus, the functions of JH2 are important in the context of the Jak-receptor complex. Induction of negative feedback regulation by the constitutively active JH2 deletion mutants, for example, through expression of SOCS proteins, is one plausible explanation for the lack of cytokine responsiveness. However, the relatively low activity of JH2Δ-Jak3 in the IL-2 receptor model is unlikely to result in prevention of further signaling by IL-2. Alternatively, the JH2 domain may be required for the JH1 domain to acquire its fully active conformation. However, the Jak2 JH1 domain is extremely active compared with full-length Jak2, making this explanation less likely.

A more likely explanation for the JH2 requirement for cyto-

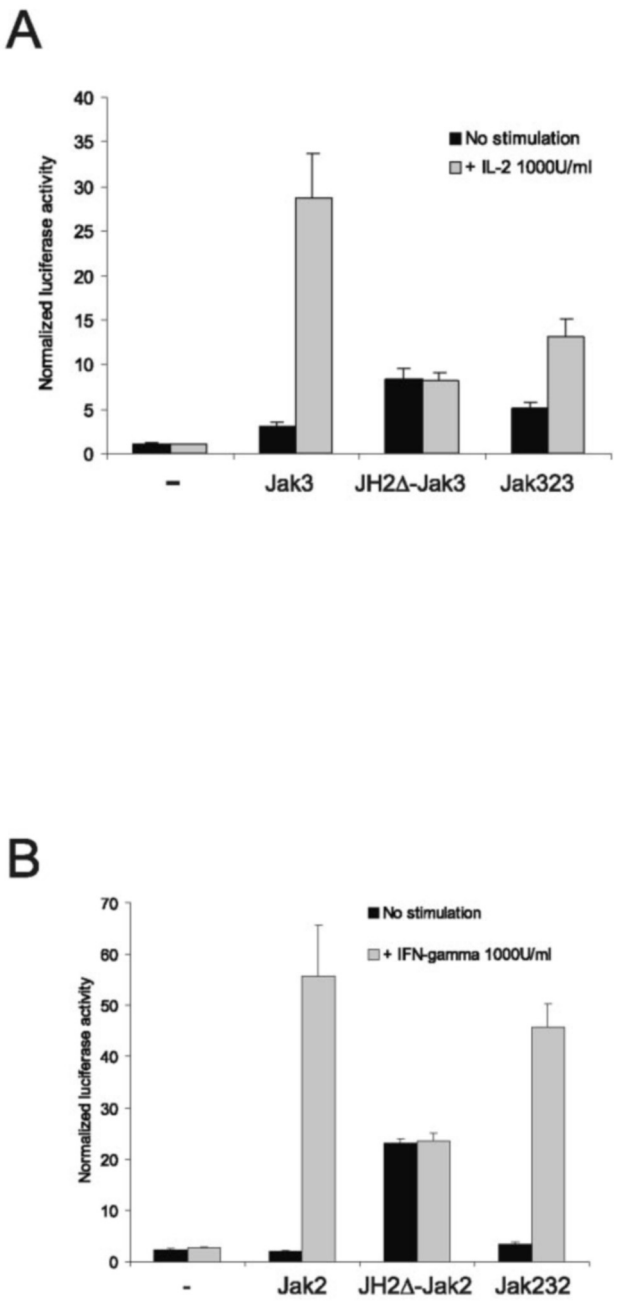

FIG. 6. **The effect of the JH2 domain on cytokine signal transduction.** *A*, 293 cells were transfected with STAT5-dependent luciferase reporter vector, pRLTK control vector, and expression vectors for STAT5A, IL2Rβ, and IL2Rγ. In addition the cells were transfected with Jak3, ΔJH2-HA, or Jak323 expression plasmids or with empty vector as a control. 5 h after transfection the cells were changed into serum-free medium and either stimulated with IL-2 (1000 units/ml) for 20 h or left unstimulated. Luciferase activity was measured as described under "Experimental Procedures." Shown is the mean from three independent experiments and S.E. *B*, γ2A cells were transfected with STAT1-dependent luciferase reporter vector, pRLTK control vector, and Jak2, ΔJH2, or Jak232 constructs or with empty vector as a control. 5 h after transfection the cells were changed into serum-free medium and starved for 15 h. The cells were stimulated with IFN-γ (1000 units/ml) for 5 h or left unstimulated. Luciferase activity was measured as described under "Experimental Procedures." Shown is the mean from three independent experiments and S.E.

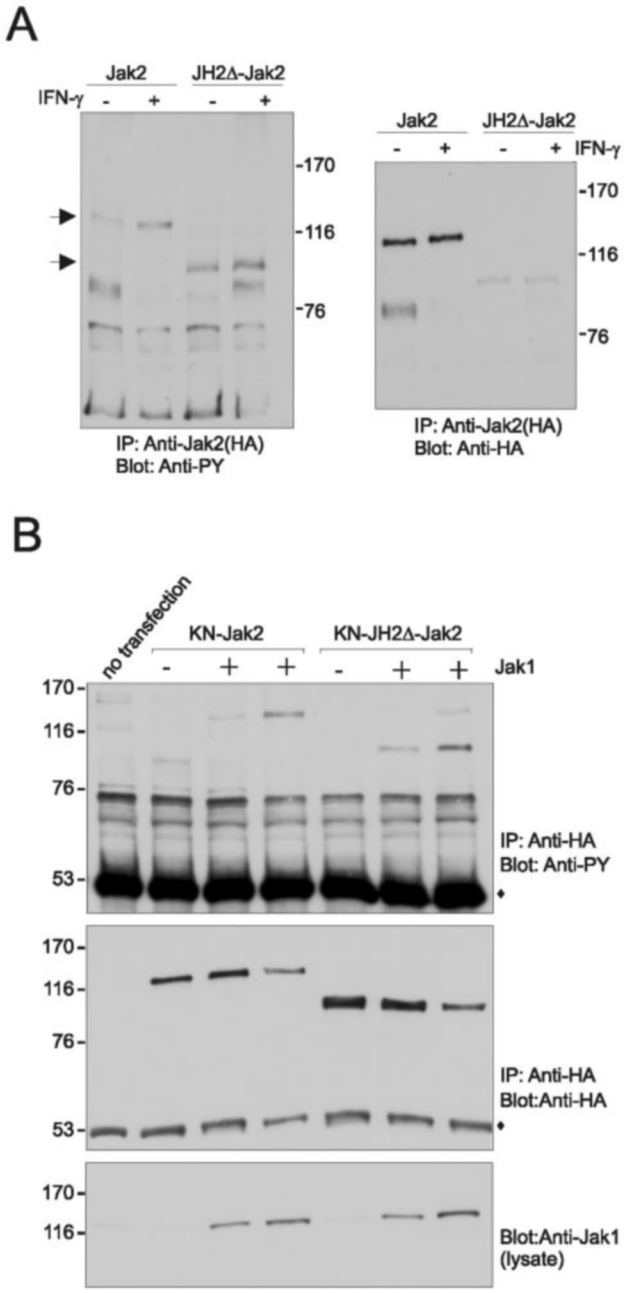

FIG. 7. **The effect of the JH2 domain on IFN-γ-induced activation of Jak2.** *A*, γ2A cells were transfected with expression plasmids for HA-tagged Jak2 or ΔJH2-Jak2. Before lysis, the cells were changed into serum-free medium and starved for 15 h. The cells were stimulated with IFN-γ (1000 units/ml), or left unstimulated. Jak2 proteins were immunoprecipitated and the immunoprecipitates were analyzed in 7.5% SDS-PAGE followed by anti-phosphotyrosine (*left panel*) and anti-HA immunoblotting (*right panel*). The mobilities of the molecular mass markers (in kilodaltons) are shown on the *right*. *Arrows* indicate migration of Jak2 and JH2Δ-Jak2. *B*, 293T cells were transfected with expression plasmids for HA-tagged KN-Jak2 or KN-ΔJH2-Jak2 alone or together with Jak1. Jak2 proteins were immunoprecipitated and the immunoprecipitates were analyzed in 7.5% SDS-PAGE followed by anti-phosphotyrosine (*top panel*) and anti-HA immunoblotting (*middle panel*). Aliquots of cell lysates were analyzed in 7.5% SDS-PAGE followed by anti-Jak1 immunoblotting (*bottom panel*). The mobilities of the molecular mass markers (in kilodaltons) are shown on the *left*.

kine-inducible signaling would be that the JH2 domain has an active structural role in Jak-receptor signaling complexes by contributing to the formation of the active configuration of the receptor complex following cytokine binding. In the resting state JH2 mediates inhibitory interaction with JH1, but ligand-induced conformational changes in the receptor may trigger interactions between JH2 and other protein domains in the receptor complex. Interestingly, Jaks have been found to be essential structural components of cytokine receptors. Specifi-

cally, Jaks have been found to interact with the receptor in multiple ways, one interaction being required for cell surface expression of certain cytokine receptors and for initial binding of a Jak kinase to its receptor, and the other one required for Jak activation (19, 20). Recently, receptor epitopes required for efficient activation of Jaks were identified that are not involved in the initial binding of Jaks to the receptor (19, 47, 48). These studies indicated that the cytoplasmic domain of the receptor must adopt a highly ordered structure to allow Jak activation (47, 49). Interestingly, activation of Jak2 by erythropoietin requires a membrane proximal hydrophobic motif in the erythropoietin receptor, which is suggested to interact with the C-terminal JH1-JH2 region of Jak2 (19, 47). This result, and our data on the role of JH2 in induction of Jak activity following cytokine treatment, suggest that cytokine-induced change in the configuration of the receptor complex enables JH2-mediated interactions, which are required for progression of signal transduction.

Although the JH2 domains mediated similar regulatory functions in Jak2 and Jak3, the JH2 domains functioned slightly differentially in signaling through the IFN-γ and IL-2 receptors. Deletion of JH2 resulted in constitutive STAT activation, but this activation was lower in IL-2 signaling than in IFN-γ signaling. Similarly, the Jak2 and Jak3 chimeras restored inducible STAT activation by IFN-γ and IL-2, respectively, but whereas the Jak232 chimera behaved as wild type Jak2 in IFN-γ signaling, the Jak323 chimera was not as potent in inducing STAT activation by IL-2 as wild type Jak3. On the other hand, in a receptor-independent system, the Jak3 chimera behaved as wild type Jak3. These results suggested that the JH2 domain was more critical for signaling through the IL-2 receptor than through the IFN-γ receptor, and that the JH2 domain of Jak3 may have a very specific role in the IL-2 receptor, which cannot be totally complemented by the JH2 domain of Jak2.

In line with our results, mutations in JH2 causing SCID resulted in constitutive tyrosine phosphorylation of Jak3, but failure of Jak3 to mediate significant downstream signaling from the IL-2 receptor (33, 34). The SCID JH2 domains were found to be more potent inhibitors of the Jak3-JH1 domain than the wild type JH2 domain, thus explaining the abrogation of signaling by the SCID mutations. Because our constructs do not contain JH2, the lack of inducible signaling in our experiments is not because of increased inhibition by JH2. Thus, our results on the critical role of the JH2 domain in regulating basal as well as IL-2-inducible Jak3 activation may give an additional explanation for the SCID phenotype of JH2 mutations in Jak3, which may contribute to disease pathogenesis in the SCID patients by enforcing the effects of the SCID JH2 mutations. The Jak3 mutants causing SCID were capable of inducing IL-4 signaling, although at a reduced level when compared with wild type Jak3 (33). Thus the role of Jak3 JH2 is particularly critical in the IL-2 receptor and less in IL-4 signaling, where STAT activation can be induced also in the absence of Jak3 (50). Our results suggest that JH2 interacts with other protein domains in receptor complexes to regulate cytokine responsiveness, and it is possible that these interactions are receptor-specific, thereby explaining the distinct functions of JH2 domains in different cytokine receptors.

The result that Jak3 showed preference for Jak autophosphorylation peptides, whereas the STAT5-derived peptide was a better substrate for Jak1, support a model for IL-2 signaling, where Jak3 activates Jak1, which then phosphorylates STAT5. However, Jak3 lacking functional JH2 is expected to be defective in activation of Jak1, which might perturb downstream signaling, thus explaining the critical role of JH2 in IL-2 signaling. In line with this notion, STAT5 has been previously found to be a better substrate for Jak1 than for Jak3 (22). Furthermore, deletion of JH2 abolished IFN-γ-inducible Jak2 activation, indicating that the reciprocal activation of Jaks was prevented upon JH2 deletion. In IFN-γ signaling, Jak2 may be the STAT1 activating kinase, whereas Jak1 would be needed for activation of Jak2, and this activation step could be circumvented by activation of Jak2 through JH2 deletion. This concept is supported by our results indicating that STAT1 can be activated by JH2Δ-Jak2 in the absence of Jak1, whereas wild type Jak2 requires Jak1 in IFN-γ signaling.[2]

In conclusion, our results show that the JH2 domain has a conserved function in Jak2 and Jak3 by regulating both basal as well as inducible Jak activity in Jak-receptor complexes. The JH2 domain may be viewed as an inducible switch in Jak kinases enabling initiation of signal transduction in response to cytokine stimulation. This regulation most likely requires interaction of the JH2 domain with other proteins in Jak-receptor complexes. These interactions remain to be identified, but the conserved, α-helical membrane proximal domain in ligand-bound cytokine receptors is one potential interaction site (47). Prevention of the JH2-mediated interaction essential for cytokine signaling may give an alternative for development of a more specific inhibitor for cytokine-induced Jak signaling.

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