Northern analysis and reverse transcription. Purification of poly(A)$^+$ mRNA, and northern blot hybridization were performed essentially as described[22]. To assess the induction of SOCS genes by IL-6, mice (C57BL/6) were injected intravenously with 5 μg IL-6 followed by collection of the liver at the indicated timepoints after injection. M1 cells were cultured in the presence of 20 ng ml^{-1} IL-6 and collected at the indicated times. For RT-PCR analysis, bone marrow cells were collected as described[23] and stimulated for 1 h at 37 °C with 100 ng ml^{-1} of a range of cytokines. RT-PCR was performed on total RNA as described[23]. PCR products were resolved on an agarose gel and Southern blots were hybridized as described[23] with probes specific for each SOCS family member. Expression of β-actin was assessed to ensure uniformity of amplification.

Western blotting and electrophoretic mobility shift assays. M1 cells (10^7) or their derivatives were stimulated for 4 min at 37 °C with either saline or 100 ng ml^{-1} IL-6. Cells were lysed and 1 to 2 mg protein was immunoprecipitated with 4 μg anti-gp130 antibody (M20; Santa Cruz Biotechnology, Santa Cruz, CA) essentially as described[24]. Western blots were performed using anti-tyrosine phosphorylated STAT3 or anti-STAT3 (New England Biolabs, Beverly, MA), or anti-gp130 (Santa Cruz Biotechnology) as described[25]. Electrophoretic mobility shift assays were performed using the m67 oligonucleotide probe, as described[26].

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A new protein containing an SH2 domain that inhibits JAK kinases

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The proliferation and differentiation of cells of many lineages are regulated by secreted proteins known as cytokines. Cytokines exert their biological effect through binding to cell-surface receptors that are associated with one or more members of the JAK family of cytoplasmic tyrosine kinases. Cytokine-induced receptor dimerization leads to the activation of JAKs, rapid tyrosine-phosphorylation of the cytoplasmic domains, and subsequent recruitment of various signalling proteins, including members of the STAT family of transcription factors, to the receptor complex[1–5]. Using the yeast two-hybrid system, we have now isolated a new SH2-domain-containing protein, JAB, which is a JAK-binding protein that interacts with the Jak2 tyrosine-kinase JH1 domain[6]. JAB is structurally related to CIS, a cytokine-inducible SH2 protein[7,8]. Interaction of JAB with Jak1, Jak2 or Jak3 markedly reduces their tyrosine-kinase activity and suppresses the tyrosine-phosphorylation and activation of STATs. JAB and CIS appear to function as negative regulators in the JAK signalling pathway.

We screened several yeast two-hybrid complementary DNA libraries and found a single positive clone, JAB, in a human B-cell library that could interact with the Jak2 JH1 domain (Fig. 1a–c). A database search revealed that the mouse and rat JAB genes are located downstream of the protamine gene cluster[9]. The coding region of the gene for mouse JAB appears to contain no introns, and encodes a 212-amino-acid protein. The JAB protein contains a central SH2 domain (amino acids 79–170) which is most closely related (35% identical) to that of CIS, a cytokine-inducible SH2-containing protein (Fig. 1d) that we have cloned previously[7].

We examined the specificity of the interaction of JAB with the Jak2 JH1 domain using the yeast two-hybrid assay. We found no evidence for interaction of the Jak2 JH1 domain with other SH2-containing proteins such as Crk, the p85 subunit of phosphatidylinositol-3-OH kinase (PI(3)K), phospholipase C-γ (PLCγ) or a new

SH2 protein, APS, although these proteins did interact with another tyrosine kinase, a D814V mutant[10] oncogenic form of the c-Kit cytoplasmic domain (Fig. 1a). CIS, the closest relative of JAB, did not interact with the Jak2 JH1 domain (Fig. 1b). Furthermore, tyrosine-kinase activity of Jak2 was required for interaction with JAB in yeast, because JAB did not interact with a kinase-defective mutant (K882D) of the Jak2 JH1 domain (Fig. 1c).

We confirmed that JAB bound to Jak2 *in vitro* using a recombinant fusion protein of glutathione-*S*-transferase with the JAB SH2 domain (GST–SH2). As shown in Fig. 2a (upper panel, αGST blot), GST–SH2, but not GST, bound to Jak2 immobilized on beads. The fusion protein of GST with the Jak2 JH1 domain (GST–JH1) bound to the JAB SH2 domain fused to maltose-binding protein (MBP–SH2) immobilized on amylose resin but not to MBP alone (Fig. 2b, upper panel). ATP had little effect on the binding of Jak2 and GST–JH1 to the JAB SH2 domain, probably because they were already heavily tyrosine-phosphorylated (Fig. 2a,b: αPY blot). These results indicate that the JAB SH2 domain binds directly to the tyrosine-phosphorylated JH1 domain *in vitro*.

To confirm this interaction between JAK and JAB in intact cells, we co-expressed a Myc-epitope-tagged version of JAB (Myc-JAB) with Jak2 in 293 cells (Fig. 3). Jak2 was tyrosine-phosphorylated and active in the absence of cytokine stimulation in 293 cells because of its high level of production. The ability of antibodies against either the Myc epitope or Jak2 to precipitate a complex of JAK and JAB suggests that these two proteins interact in the cytoplasm (Fig. 3a,b). We noticed that tyrosine-phosphorylation of Jak2 was greatly reduced when it was co-expressed with JAB (Fig. 3c), suggesting that JAB inhibits tyrosine kinase activity upon binding to Jak2. Jak2 can be phosphorylated on several tyrosine residues, but phosphorylation of a single residue at Y1007 appears to be an early and critical requirement for catalytic activation[11]. We propose that JAB binds to Jak2 soon after Jak2 autophosphorylates Y1007, and blocks Jak2 kinase activity, thereby inhibiting further intra- and intermolecular phosphorylation.

To explore the downstream effects of the inhibition of Jak2 activity by JAB, we also expressed a physiological Jak2 substrate, Stat3, in 293 cells in the presence and absence of Jak2 and JAB (Fig. 3d). A rise in Stat3 phosphorylation was observed upon co-expression with Jak2. In the presence of JAB, however, there was a marked reduction in the level of tyrosine phosphorylation of Stat3, as well as Jak2 (Fig. 3d). Results were similar when Stat5 was used in place of Stat3 (data not shown). The specificity of the inhibition of Jak2 phosphorylation by JAB was suggested by the inability of the related molecule, CIS, to inhibit this Jak2 activation (Fig. 3e). The SH2 domain of JAB alone did not markedly inhibit the phosphorylation of Jak2, suggesting that the C-terminal or N-terminal region is needed for the kinase-inhibitory activity of JAB (Fig. 3e). In 293 cells, JAB inhibited autophosphorylation of not only Jak2 but also of Jak1 and Jak3 (Fig. 3f), indicating that JAB may be a general inhibitor of JAK kinases. Although JAB bound to the active c-Kit kinase domain in yeast (Fig. 1a), JAB did not inhibit phosphorylation of either c-Kit (Fig. 3f) or the epidermal growth factor (EGF) receptor (data not shown), suggesting that the kinase-inhibitory action of JAB may be specific to JAK.

We investigated the effect of JAB on JAK-dependent gene activation in three different reporter-gene assay systems: Stat5 activation by erythropoietin (EPO)[8] (Fig. 4a), Stat3 activation by interleukin (IL)-6 (ref. 12) (Fig. 4b), and c-*fos* promoter activation by IL-2 and IL-3 (ref. 13; Fig. 4c). Transient expression of JAB with the reporter gene inhibited reporter activation in response to both EPO and IL-6 (Fig. 4a,b). IL-2- and IL-3-induced c-*fos* promoter activation was

Figure 1 Two-hybrid analysis of Jak2 JH1 and JAB stained by *in situ* β-galactosidase assay (**a**–**c**) and sequence comparison between murine JAB and CIS (**d**). **a**, Yeast strains containing a LexA-binding domain (BD)–Jak2 JH1 fusion gene (Jak2), LexA BD alone (neg.), and BD-active c-Kit kinase domain (Kit) with the Gal4 activating domain (AD) fused to the SH2 domains from PI(3)Kp85, Crk, PLC-γ, APS, or JAB. Dark blue staining indicates a positive interaction. **b**, Yeast strains (three independent colonies) containing either BD-JAB and AD-Jak2 JH1 or BD-CIS (full-length) and AD-Jak2 JH1. **c**, Yeast strains containing either AD-JAB and BD-Jak2 JH1 (wild type), or BD-kinase-negative Jak2 JH1 (K882D). In **d**, asterisks indicate conserved amino acids. SH2 domains are boxed.

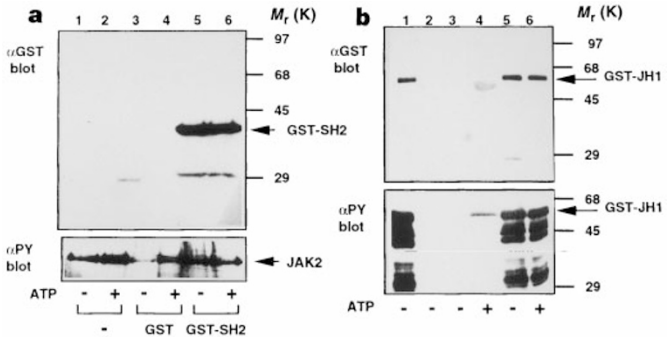

Figure 2 Interaction between Jak2 and JAB *in vitro*. **a**, Jak2–agarose was incubated with buffer alone (lanes 1, 2), GST (lanes 3, 4), or GST–JAB SH2-domain fusion protein (GST–SH2) (lanes 5, 6) in the presence (+) or absence (–) of ATP. **b**, Immobilized MBP (lanes 2, 4) or MBP–SH2 (lanes 3, 5, 6) were incubated with (lanes 4–6) or without (lanes 2, 3) GST–JH1 in the presence (lanes 4 and 6) or absence (lane 5) of ATP. Lane 1, purified GST–JH1 fusion protein. Precipitates were analysed by immunoblotting with anti-GST (αGST blot) and anti-PY (αPY blot).

almost completely inhibited by co-expression of full-length JAB (Fig. 4c). Although the c-*fos* gene may not be activated by STATs, the activation of JAKs is necessary for c-*fos* activation (see the effect of dominant-negative Jak3, J3M1, in Fig. 4c, and ref. 13). Thus, the decrease in c-*fos* activation in this assay therefore probably reflects inhibition of JAKs, rather than STATs, by JAB. Truncated JAB lacking both the C- and N-terminal domains had little effect on IL-2- and IL-3-induced c-*fos* activation (Fig. 4c, Myc-SH2), indicating that these regions are necessary for full JAK kinase inhibition by JAB. This is consistent with the results shown in Fig. 3e, which indicate that the JAB SH2 domain alone is unable to inhibit JAK activation. Co-expression of JAB did not inhibit activation of the

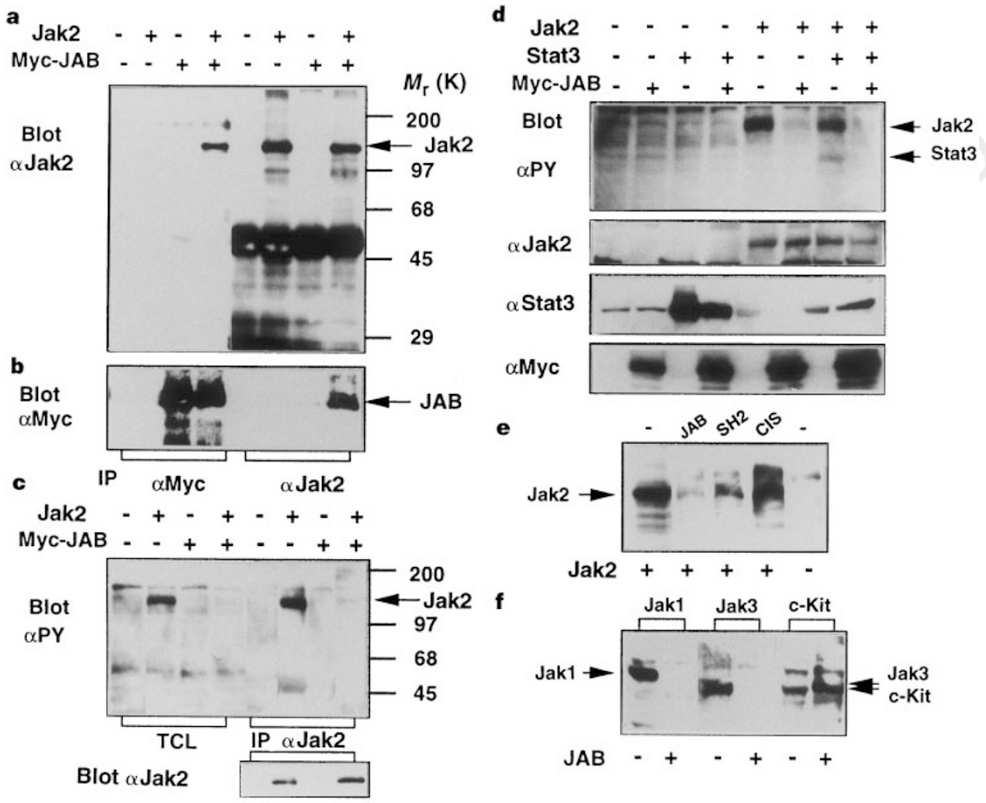

Figure 3 Association of Jak2 with JAB and inhibition of JAK kinase reaction *in vivo*. **a**–**c**, 293 cells were transfected with Jak2 and/or Myc–JAB constructs, and cell extracts were immunoprecipitated (IP) with antibodies specific for either Myc (IP αMyc) or Jak2 (IP αJak2), then immunoblotted with anti-Jak2 (blot αJak2) (**a**) or anti-Myc (blot αMyc) (**b**) antibodies. **c**, Total cell extracts (TCL) or anti-Jak2 immunoprecipitates (IP αJak2) were blotted with anti-PY (blot αPY) or anti-Jak2 (blot αJak2). **d**, Jak2, Stat3 and JAB constructs were transiently transfected into 293 cells as indicated, and total cell extracts were blotted with anti-PY (αPY), anti-Jak2 (αJak2), anti-Stat3 (αStat3), and anti-Myc (αMyc) antibodies. **e**, 293 cells were transfected with the Jak2 (constructs) (+) or vector (–), either alone or with pcDNA3 carrying full-length JAB (JAB), JAB SH2 domain (SH2), or full-length CIS (CIS). Cell extracts were immunoblotted with anti-PY. **f**, 293 transfected with Jak1, Jak3 or c-Kit(D814V) constructs, either alone (–) or with (+) pcDNA3-Myc JAB. Cell extracts were immunoblotted with anti-PY.

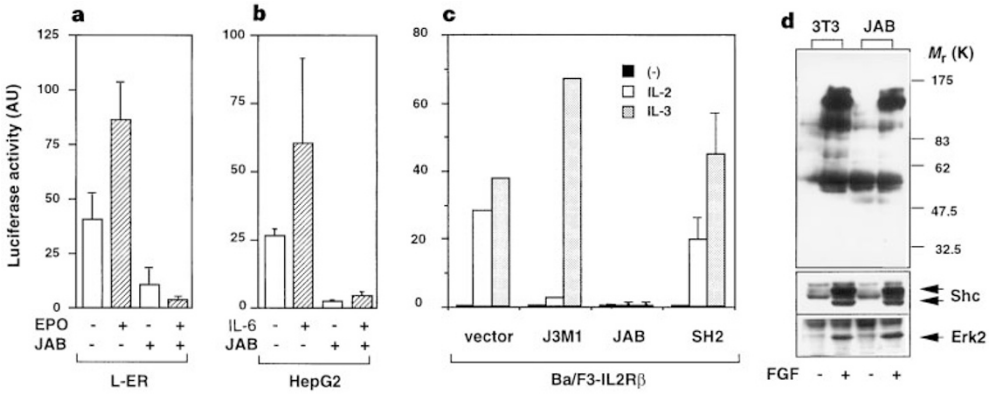

Figure 4 Inhibition of JAK activity by JAB in cells assessed by reporter assay. The indicated cells were transfected with reporter genes for Stat5 (**a**), Stat3 (**b**) or c-*fos* promoter (**c**) with pcDNA3 carrying JAB (+ or JAB), the SH2 domain of JAB (SH2), JH1-truncated Jak3 (J3M1), or vector alone (–). Luciferase activity (AU, arbitrary units) was measured after stimulation without (–) or with (+) 10 unit m^{-1} EPO (**a**), 20 ng ml^{-1} IL-6 (**b**), or either 0.5 nM IL-2 or IL-3 (**c**), for 10–12 h. In **d**, parental NIH3T3 cells and 3T3 cells expressing Myc–JAB (JAB) were stimulated for 10 min either with (+) or without (–) 500 ng m^{-1} FGF. Total cell lysates (upper panel) or anti-Shc or anti-MAP kinase (Erk2) immunoprecipitates (lower panels) were blotted with anti-PY antibody.

c-*fos* promoter stimulated by cyclic AMP (data not shown), suggesting that JAB does not inhibit any pathways other than those involving JAKs. Furthermore, constitutive overexpression of JAB in NIH3T3 cells did not inhibit basic fibroblast growth factor (FGF)-induced tyrosine-phosphorylation of cellular proteins, including Shc and Erk2 (Fig. 4d), whereas interferon-α-induced antiviral and antiproliferative effects were almost completely blocked in the JAB transformants (data not shown).

We have described the cloning of a cDNA encoding a protein that binds to the catalytic domain of the cytoplasmic tyrosine kinase Jak2. Our results indicate that interaction with JAB inhibits the catalytic activity of JAKs and thus the activation of signalling intermediates such as STATs. CIS also inhibits cytokine signal transduction by competing with Stat5 or other signalling molecules for docking sites on the receptor[7,8]. The potent inhibition by JAB and CIS of cytokine activity suggests that members of this family may be key regulators of haematopoiesis, of the response to infection and injury, and of development—all biological processes in which JAKs and STATs are important.

Note added in proof: Elsewhere in this issue, JAK inhibitors similar to JAB are reported under the names SOCS[18] and SSI-1 (ref. 19). □

Methods

Two-hybrid screening and cloning of JAB. The two-hybrid screen was done according to ref. 14. The hybrid bait consisted of the Jak2 JH1 domain, and the DNA-binding/dimerization domain of LexA allowed interchain tyrosine phosphorylation of the kinase domain in yeast. The murine Jak2 JH1 domain (amino acids 839–1,127; kindly provided by R. Fukunaga) or the oncogenic c-Kit kinase domain (amino acids 544–975, containing the D814V mutation[10]) in a pBTM116 vector was transfected into yeast strain L40. Kinase-negative JH1 was created by substitution of lysine (K) at position 882 to aspartic acid (D) as described[15]. A human B-lymphocyte cDNA library in a pACT vector[16] was screened.

Binding of JAB to Jak2 *in vitro*. About 1 μg purified Jak2 (Upstate Biotechnology) was incubated with 1 μg purified GST or GST-SH2 fusion protein (amino acids 78–180 of human JAB) in kinase buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ and 0.1 mM Na_3VO_4) in the presence or absence of 0.2 mM ATP for 2 h at room temperature. After washing twice with phosphate-buffered saline, protein bound to beads was analysed by immunoblotting with anti-phosphotyrosine (PY) (4G10) or goat anti-GST (Pharmacia).

Analysis of JAB / Jak2 interaction in intact cells. Jak2 cDNA in the expression vector pEF-BOS and Myc-epitope-tagged-JAB (Myc-JAB; ref. 17) in pcDNA3 (2.5 μg per transfection) were transiently expressed in 293 cells grown in 35-mm dishes using calcium phosphate co-precipitation. Cell extracts were immunoprecipitated with either monoclonal anti-Myc antibody (9E10) or polyclonal anti-Jak2 antibody as described[7]. Total cell lysate (50 μg protein) or the immune complex were further analysed by immunoblotting with anti-PY, anti-Myc, and anti-Jak2. For phosphorylation of Jak1 and Jak3, 293 cells were transfected with 2.5 μg Jak1 in pCDM8 (from A. F. Wilks), or with Jak3 in pSRα (from T. Shirasawa), or with murine oncogenic c-Kit carrying the D814V mutation in pEF-BOS[10] in the presence or absence of 2.5 μg JAB cDNA.

Reporter gene assay. Luciferase gene constructs containing an EPO/Stat5-responsive promoter from the CIS gene[8], an IL-6/Stat3-responsive promoter (based on the acute phase response element[12], or the c-*fos* promoter[13], were transfected into L929 cells expressing the EPO receptor (L-ER), the hepatocyte cell line HepG2, or into Ba/F3 cells expressing the IL-2 receptor β-chain (Ba/F3-IL2Rβ), respectively. Luciferase activity was measured as described[8].

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Structure and function of a new STAT-induced STAT inhibitor

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The signalling pathway that comprises JAK kinases and STAT proteins (for signal transducer and activator of transcription) is important for relaying signals from various cytokines outside the cell to the inside[1–3]. The feedback mechanism responsible for switching off the cytokine signal has not been elucidated. We now report the cloning and characterization of an inhibitor of STAT activation which we name SSI-1 (for STAT-induced STAT inhibitor-1). We found that SSI-1 messenger RNA was induced by the cytokines interleukins 4 and 6 (IL-4, IL-6), leukaemia-inhibitory factor (LIF), and granulocyte colony-stimulating factor (G-CSF). Stimulation by IL-6 or LIF of murine myeloid leukaemia cells (M1 cells) induced SSI-1 mRNA expression which was blocked by transfection of a dominant-negative mutant of Stat3,