The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop

Hideo Yasukawa1,2, Hiroyuki Misawa1,3, Hiroshi Sakamoto1, Masaaki Masuhara1, Atsuo Sasaki1, Toru Wakioka1,4, Satoshi Ohtsuka1, Tsutomu Imaizumi2, Akihiko Yoshimura1,7, Atsuo Sasaki1, Toru Wakioka1,4, Masaaki Masuhara1, Tadashi Matsuda5,6, James N.Ihle5 and Akihiko Yoshimura1,7

1Institute of Life Science, Kurume University, Aikawa-machi 2432-3 Kurume 839-0861, Departments of Internal Medicine (III) and Orthopedic Surgery, Faculty of Medicine, Kurume University, Asahi-machi, Kurume 830-0011, Japan and 2Department of Biochemistry, St Jude Children’s Research Hospital, Memphis, TN, USA
3Present address: Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, Shizuoka 422-8526, Japan
4Orthopedic Surgery, Faculty of Medicine, Kurume University, Kurume 839-0861, USA
5Present address: Department of Immunology, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan
7Corresponding author
 std: yosimura@lsi.kurume-u.ac.jp

The Janus family of protein tyrosine kinases (JAKs) regulate cellular processes involved in cell growth, differentiation and transformation through their association with cytokine receptors. However, compared with other kinases, little is known about cellular regulators of the JAKs. We have recently identified a JAK-binding protein (JAB) that inhibits JAK signaling in cells. In the studies presented here we demonstrate that JAB specifically binds to the tyrosine residue (Y1007) in the activation loop of JAK2, whose phosphorylation is required for activation of kinase activity. Binding to the phosphorylated activation loop requires the JAB SH2 domain and an additional N-terminal 12 amino acids (extended SH2 subdomain) containing two residues (Ile88 and Leu75) that are conserved in JAB-related proteins. An additional N-terminal 12-amino-acid region (kinase inhibitory region) of JAB also contributes to high-affinity binding to the JAK2 tyrosine kinase domain and is required for inhibition of JAK2 signaling and kinase activity. Our studies define a novel type of regulation of tyrosine kinases and might provide a basis for the design of specific tyrosine kinase inhibitors.

Keywords: activation loop/CIS/JAB/JAK/SH2 domain

Introduction

The growth, differentiation and functions of immune and hematopoietic cells are controlled by multiple cytokines, including interleukins (ILs) and colony stimulating factors (CSFs). Cytokines exert their biological effects through binding to cell-surface receptors that are associated with one or more members of the JAK family of cytoplasmic tyrosine kinases (JAKs). Cytokine-induced receptor dimerization leads to the activation of JAKs, rapid tyrosine phosphorylation of the cytoplasmic domains and subsequent recruitment of various signaling proteins to the receptor complex (Ihle, 1995). Among these proteins are members of the signal transduction and activators of transcription (STAT) family (Ihle, 1996; Darnell, 1997; O’Shea, 1997). The tyrosine-phosphorylated STATs form homo- or heterodimers and translocate into the nucleus, they then activate target genes.

The regulation of the JAKs is a central component in the regulation of cytokine signaling. Because of the critical role of cytokines in mediating inflammation and immunity, it could be proposed that constitutive activation of JAKs could contribute to hematopoietic disorders, autoimmunity and inflammatory diseases. Activation of JAK tyrosine kinase activity is positively regulated by transphosphorylation of a critical tyrosine within the activation loop of the kinase domain (Gauzzi et al., 1996; Feng et al., 1997; Liu et al., 1997; Zhou et al., 1997; Weiss and Schlessinger, 1998). However, little is known regarding the mechanisms that terminate or down-modulate JAK kinase activity in cytokine responses. Considerable evidence suggests that one mechanism involves the recruitment of a tyrosine-phosphatase-containing SH2 domain (SHP-1) to receptor complexes resulting in the dephosphorylation of JAKs (Klingmuller et al., 1995; Jiao et al., 1996). The potential importance of this mechanism is strongly suggested by the phenotype of motheaten (me/me) mice lacking SHP-1 which die from a disease with components of autoimmunity and inflammation (Shultz et al., 1993). However, SHP-1 has been shown to negatively regulate a number of receptor and non-receptor tyrosine kinases including c-kit and ZAP-70 (Plas et al., 1996; Kozlowski et al., 1998). Thus, the kinases specifically responsible for the phenotype remain to be determined. In addition, however, it has been suggested that a family of small SH2-domain-containing proteins may also be involved in the relatively specific regulation of cytokine signaling.

The CIS family of proteins, also referred to as the SOCS or SSI family has been implicated in regulating signal transduction by a variety of cytokines (Aman and Leonard, 1997; Adamus et al., 1998; Bjorbaek et al., 1998; Sakamoto et al., 1998). The first member of this family (CIS1) was cloned as an immediate-early gene in response to a number of cytokines including erythropoietin (EPO), IL-2, IL-3 and granulocyte-macrophage-colony stimulating factor (GM-CSF) (Yoshimura et al., 1995), and is regulated by STAT5 (Matsumoto et al., 1997). CIS1 tightly binds the tyrosine phosphorylated IL-3 and EPO receptors, and negatively regulates their signals when it is overexpressed (Yoshimura et al., 1995; Matsumoto et al., 1997). The second family member was independ-
H.Yasukawa et al.

Fig. 1. The role of each domain of JAB in the inhibition of JAK signaling. (A) Schematic structures of wild-type (WT) and mutated JAB proteins. dN and dC indicate deletion from the N- and C-terminus, respectively, and the number represents the length of each deletion. For example, dN39 indicates a 39-amino-acid deletion from the N-terminus. JN contains only the N-terminal region (codon 1–79). sssss indicates eight repeats of the serine residue. All constructs contain an N-terminal Myc epitope tag. (B) 293 cells were transfected with a plasmid mixture containing the reporter gene, the β-galactosidase gene, the EPOR, STAT5 and each JAB mutant, as described in Materials and methods. After transfection, cells were incubated in the presence (+) or absence (−) of 1 unit/ml EPO for 6 h, and luciferase activity was then measured. Data normalized with the β-galactosidase activity from duplicate experiments are shown. (C) Excess GST–JH1 was transiently expressed in 293 cells with wild-type or mutant JABs, then purified on GSH–Sepharose. The amounts of JAB and GST–JH1 in the precipitates were estimated by immunoblotting with anti-Myc (αMyc) and anti-GST (αGST), respectively. The expression level of each JAB mutant in the total cell lysate (TCL) was measured by immunoblotting with anti-Myc (TCL, αMyc).

ently cloned by three groups and is termed JAB, SOCS-1 or SSI-1 (Endo et al., 1997; Starr et al., 1997; Naka et al., 1997). We identified JAB by screening for proteins that bound to the JAK2 tyrosine kinase (JH1) domain (Endo et al., 1997). Starr et al. (1997) cloned SOCS-1 as an inhibitor of IL-6-induced differentiation and growth arrest of the murine monocytic leukemia cell line M1; and Naka et al. (1997) identified SSI-1 using an antibody that recognizes a common sequence of the SH2 domain of STATs. The potentially specific role for JAB in cytokine signaling is suggested by the observation that it does not inhibit activation of the fibroblast growth factor (FGF) receptor, the insulin receptor, Flt-3 or c-kit (Endo et al., 1997; Masuhara et al., 1997; Naka et al., 1997; Starr et al., 1997). Although JAB apparently inhibits JAK signaling when expressed ectopically, the molecular mechanism by which JAB specifically inhibits JAK tyrosine kinases has not as yet been determined. To address this question, we identified the domains of JAB that are required for inhibition and determined the tyrosine residue of JAK2 JH1 with which JAB associates. Results from mutational analysis and biochemical characterization of JAB demonstrate a novel type of inhibition of tyrosine kinase activity through the independent binding of JAB to both the critical tyrosine in the activation loop of the kinase domain and the catalytic groove. Elucidation of the molecular mechanism of kinase inhibition by JAB may provide a novel strategy for the design of specific tyrosine kinase inhibitors.

Results

**SH2 domain and sequences immediately N-terminal of JAB are necessary and sufficient for inhibiting JAK signaling**

We have reported previously that EPO-dependent STAT5-reporter gene activation in the 293-cell transient assay was inhibited completely by co-expression of JAB (Masuhara et al., 1997). In this assay, N-terminally or C-terminally Flag- or Myc-tagged proteins were as effective as wild-type JAB and consequently in the studies presented here N-terminal Myc-tagged versions of JAB were used throughout. As illustrated in Figure 1, mutants with deletions of sequences carboxyl to the SH2 domain (dC20,
Fig. 2. Suppression of JAK2 activation in vivo by N-terminal truncated JAB mutants. 293 cells were transfected with (+) or without (−) expression plasmids carrying the EPO receptor (EPOR), Flag-tagged JAK2 (F-JAK2) and truncated dN51JAB or dN60 JAB. After stimulation with 20 units/ml of EPO for 20 min, total cell extracts (TCL; a and b) or anti-Flag immunoprecipitates (αFlag IP; c–e) were blotted with anti-PY (αPY; a and c), anti-Myc (αMyc; b and d) or anti-Flag (αFlag; e). Positions for Flag-JAK2 (F-JAK2), the EPOR and Myc-JAB (M-JAB) are indicated by arrows.

dC40 and dC44) retained the ability to inhibit STAT5 activation of transcription. Importantly, this region contains sequences that are found in all CIS family members and is referred to as the CIS homology (CH) domain (Masuhara et al., 1997), SOCS box (Starr et al., 1997; Hilton et al., 1998) or SC motif (Minamoto et al., 1997).

Moreover, a mutant (dN51/dC40) that contains deletions of both the carboxyl sequences and 51 amino acids from the N-terminal region retained the ability to inhibit signaling. However, further deletion of the N-terminal sequences caused loss of activity (dN68; see Figure 6B for other N-terminal deletions). The importance of the SH2 domain is illustrated by the loss of activity by mutations of the phosphotyrosine-binding residue Arg105 to Lys (R105K) or Glu (R105E) or by deletion of the SH2 domain (JN).

The ability of the mutants to associate with the JAK2 kinase domain was also assessed using N-terminal Myc-tagged mutants and JAK2 JH1 domain fused to glutathione S-transferase (GST). GST–JH1 and JAB derivatives were transiently co-expressed in 293 cells, and GST–JH1 was then purified on GSH–Sepharose. Co-precipitation of JAB was measured by immunoblotting with anti-Myc (Figure 1C). Consistent with the biological assays shown in Figure 1B, EPO-dependent tyrosine phosphorylation of JAK2 and the EPOR was suppressed in the presence of dN51JAB (Figure 2a and c, lanes 2 and 4). Association of dN51JAB with F-JAK2 was dependent upon stimulation with EPO (Figure 2d, lanes 3 and 4), suggesting that phosphorylation of JAK2 is necessary for JAB binding. However, no such phosphorylation inhibition and binding increase was seen for the inactive dN60JAB (Figure 2a, c and d; lanes 5 and 6). These data indicate that sequences immediately N-terminal to the SH2 domain are important for JAK2 kinase inhibition in vivo.

JAB has been shown to prevent IL-6-induced or leukemia inhibitory factor (LIF)-induced growth arrest and differentiation of M1 cells (Masuhara et al., 1997; Naka et al., 1997; Starr et al., 1997). We therefore examined selected mutants for their ability to function in this assay to determine whether comparable determinants were required. As shown in Figure 3B, the C-terminal truncated mutant (dC40), as well as wild-type JAB, inhibited LIF-induced growth arrest in stably transfected cells. However, deletion of 64 amino acids of the N-terminal resulted in a loss of activity comparable with the transient EPO/STAT5 reporter assay, as well as JH1-binding assay (Figure 6B and C). The differences in activity were not due to (dN51 and dN60) were transiently expressed in 293 cells. Consistent with the reporter gene assay shown in Figure 1B, EPO-dependent tyrosine phosphorylation of JAK2 and the EPOR was suppressed in the presence of dN51JAB (Figure 2a and c, lanes 2 and 4). Association of dN51JAB with F-JAK2 was dependent upon stimulation with EPO (Figure 2d, lanes 3 and 4), suggesting that phosphorylation of JAK2 is necessary for JAB binding. However, no such phosphorylation inhibition and binding increase was seen for the inactive dN60JAB (Figure 2a, c and d; lanes 5 and 6). These data indicate that sequences immediately N-terminal to the SH2 domain are important for JAK2 kinase inhibition in vivo.

JAB has been shown to prevent IL-6-induced or leukemia inhibitory factor (LIF)-induced growth arrest and differentiation of M1 cells (Masuhara et al., 1997; Naka et al., 1997; Starr et al., 1997). We therefore examined selected mutants for their ability to function in this assay to determine whether comparable determinants were required. As shown in Figure 3B, the C-terminal truncated mutant (dC40), as well as wild-type JAB, inhibited LIF-induced growth arrest in stably transfected cells. However, deletion of 64 amino acids of the N-terminal resulted in a loss of activity comparable with the transient EPO/STAT5 reporter assay, as well as JH1-binding assay (Figure 6B and C). The differences in activity were not due to
The ability of various mutants to bind the phosphopeptide-containing pY1007 is illustrated in Figure 5B. Binding did not require the CH domain or S1 amino acids from the N-terminus (lanes 1–5). However, deletion of 68 amino acids from the N-terminal region disrupted binding, although this deletion did not include sequences normally associated with the SH2 domain (dN68; lane 6). The importance of the SH2 domain is indicated by the loss of binding activity on deletion of entire SH2 domain (JN; lane 7) or by mutations of the phosphotyrosine-binding Arg105 residue (R105K and R105E; lanes 8 and 9). Thus, both the SH2 domain and a region N-terminal to the SH2 domain are essential for the binding to pY1007.

### Twelve amino acids immediately N-terminal to the SH2 domain are essential for binding to pY1007

To elucidate the function of the N-terminal region of JAB, we created a series of deletion mutants of JAB from Gly52 to Gly79 next to the classical SH2 domain. First, we found that N-terminal 24 amino acids (F56–G79) in addition to the SH2 domain were essential for EPO-dependent STAT5 activation (Figure 6B). Two-hybrid analysis and an in vitro pY1007 peptide-binding assay indicate that 12 amino acids (I68–G79) immediately N-terminal to the SH2 domain were required for binding (Figure 6C and D). This 12-amino-acid region, which we termed the extended SH2 subdomain, is critical for the interaction of JAB with pY1007.

Among CIS family members, little similarity was found in the N-terminal region. However, Ile at position 68, Leu at 75 and Gly at 79 are highly conserved (boxed in Figure 6A). At codon 68, some CIS members possess Leu or Val instead of Ile. However, these amino acid residues are quite similar in nature. Interestingly, these three amino acid residues are also found at the same positions close to the SH2 domain of STATs (STAT1 and STAT3 sequence in Figure 6A). Similarity among the SH2 domains of CIS family members and those of STATs may not be surprising, because SSI-1/JAB was cloned using an antibody against common sequences in the SH2 domain of STATs (Naka et al., 1997). Substitution of conserved Ile68 with Glu (I68E) and Leu75 with Glu (L75E) abolished or markedly reduced EPO-dependent STAT5 signaling (Figure 6B) as well as the interaction with JH1 and pY1007 (Figure 6C and D), suggesting the essential role of Ile68 and Leu75 on binding to the phosphotyrosine residue. Gly79 was able to be replaced with Glu (G79E), although this amino acid is also conserved. Defined X-ray crystal structures of STAT1 and STAT3 were reported recently (Becker et al., 1998; Chen et al., 1998). The region of STAT1 (a10) including these conserved amino acids interacts with the phosphate-binding loop between BC and BD of the SH2 domain, and is indeed involved in phosphotyrosine binding (Chen et al., 1998).
Amino acids other than I68 and L75 in this region may not be so important because chimeric JAB mutants, C1J and C1JdN67, bound to pY1007 in vitro and JH1 in yeast (Figure 6C and D, in vitro binding of C1JdN67 is not shown). These chimeric mutants contain the entire N-terminal region or 12 amino acids of the extended SH2 subdomain of CIS1 in place of the JAB N-terminal region respectively. Again, substitution of I68E in C1JdN67 (C1JdN67,I68E) completely abolished binding (Figure 6C and data not shown for in vitro binding assay), further supporting the importance of the Ile residue at 68. These data raise the strong possibility that the extended SH2 subdomain is part of the SH2 domain in this family, and is essential for recognition of a specific phosphopeptide sequence.

**Additional 12 amino acids N-terminal to the extended SH2 subdomain are required for signal inhibition as well as high-affinity binding to JH1**

Although deletion mutants containing the extended SH2 subdomain (dN56–67) and the C1J chimeric molecule (C1J) effectively bind the pY1007 peptide, these mutants bound to GST–JH1 much more weakly than did shorter deletion mutants (dN51, dN54, dN55) (Figure 6C and D; compare pY1007 Bound with JH1 Bound). These 12 amino acids (codon 56–67) are also essential for inhibition of EPO/STAT5 signaling (Figure 6B). These observations suggest that the region between F56 and R67 is another site of interaction with JH1 and is important for signal inhibition. Within these 12 amino acids, eight are identical or closely related between JAB and CIS3, which also binds to JH1 and inhibits JAK signaling (Figure 6A), further suggesting the involvement of this region in JAK signal inhibition.

To address the function of this region, we mutated amino acid residues conserved between JAB and CIS3 in the context of the dN51 backbone (Figure 7A). Substitution of F56 with S or D, F59 with A or E, D64 with R, and Y65 with A abolished the ability of JAB to inhibit EPO/STAT5 signaling (Figure 7B). The Y65A mutation had a lesser effect. F56L and F59L substitutions had no
effect on JAB activity, suggesting that the hydrophobic nature of these positions is very important. The Y65F mutant was as effective as wild-type JAB. Relatively extensive changes at other positions (H55D, R57E, T58A, S61E, H62E) had no significant effects.

As shown in Figure 7C, all substitution mutants had no effect on binding to the pY1007 peptide (pY1007 Bound). Altering F56 to E or S (but not to L), F59 to A or E (but not to L), D64 to R and Y65A reduced the ability of JAB to bind to JH1 (Figure 7C, JH1 Bound). Therefore, all substitutions that resulted in reducing the affinity of JAB for JH1, decreased or diminished signal inhibition activity. F56, F59, D64 and Y65 were especially critical, and these were conserved between CIS3 and JAB. As shown in Figure 7C (JH1 Bound), F59 mutations exhibited a more profound effect on JH1 binding than F56, D64 and Y65 mutations, suggesting that F59 is the most critical residue in this region.

To quantify the affinity of JAB to the JH1 domain, we performed in vitro binding assays at various concentrations of JAB deletion mutants that did or did not inhibit JAK signaling (Figure 8). The concentration of Myc-JAB in cell lysates was semi-quantitatively estimated by immunoblotting using myc-tagged GST protein or recombinant JAB as a standard. As shown in Figure 8B, classical SH2 domain plus extended SH2 subdomain (dN56), as well as the shorter deletion mutant (dN39) that has full JAK signal inhibitory activity, bound to pY1007 with equal affinity ($K_d \approx ~50–100$ nM). However, dN39 binds to JH1 with very high affinity ($K_d \approx ~1–5$ nM), whereas dN56 binds to JH1 weakly with a $K_d$ of $\approx 50–100$ nM (Figure 8A). The mutant dN75, which lacked the extended SH2 subdomain, did not bind to JH1 and pY1007 at any concentration. The affinity of dN56 to JH1 was close to that of the pY1007 phosphopeptide, suggesting that the region containing classical plus extended SH2 subdomain binds to JH1 through only the phosphorylated activation loop. However, the fully active JAB that contains essential N-terminal 12 amino acids probably interacts with JH1 at two different sites; the phosphorylated activation loop by the SH2 domain and another JH1 region by the 12 amino acid region, resulting in a high-affinity binding.

**The SH2 domain and the kinase inhibitory region are necessary for inhibition of JH1 kinase activity**

JAB was isolated by its ability to bind to the kinase domain of JAK2 and is hypothesized to inhibit cytokine signaling by blocking kinase activity. To test this, purified GST–JH1 was incubated with 293 cell lysates containing several CIS family member proteins and wild-type or several mutant JAB proteins, an in vitro autophosphorylation assay was then carried out (Figure 9A and B). To exclude the possibility that JAB recruits an unidentified inhibitory molecule in 293 cells to JH1, an in vitro kinase assay using JAB proteins expressed in bacteria and GST–EPO receptor cytoplasmic domain fusion protein (GST–EPO) as a substrate was also performed (Figure 9C). Consistent with our hypothesis, JAB, but not other members of the family including CIS1, CIS2 or CIS3, inhibited JAK2-JH1 in vitro kinase activity (Figure 9A, lanes 2–4).

We have shown that CIS1 and CIS2 do not bind to GST–JH1 in vitro (Masuhara et al., 1997), which is consistent with their inability to inhibit kinase activity. CIS3 has been shown to interact with JH1 weakly and to inhibit cytokine signaling, however, we could not detect the effect of CIS3 on JAK2 tyrosine kinase activity using this assay. This is probably because the affinity of CIS3 to JH1 is too weak to bind to JH1 at a sufficient level for kinase inhibition in vitro (Masuhara et al., 1997).

Deletion of the C-terminal sequences (dC40) did not affect the ability to bind to JH1 in vitro and to block kinase activity, neither did the deletion of 51 amino acids from the N-terminus (dN51) (Figure 9B, a and b, lanes 2 and 4). Blotting with anti-GST or anti-PY indicated that the inhibition of kinase activity was not due to proteolytic degradation or dephosphorylation of GST–JH1 (Figure 9B, d and e). Similar results were obtained by using recombinant proteins from bacteria and GST–EPO as a polypeptide substrate (Figure 9C, a). Wild-type JAB, dN51, as well as dN51/dC40, efficiently inhibited JH1 tyrosine kinase activity (Figure 9C lanes 2, 4 and 6).

However, R105E (Figure 9C, lane 3), dN60 (Figure 9B, lane 3) and F59E (Figure 9B and C, lane 5) mutants did not affect kinase activity. These are consistent with the results of a biological assay using reporter gene (Figures 1B, 6B and 7B) and indicate the essential role of the SH2 domain and N-terminal region close to the extended SH2 subdomain. Thus, we propose to term this 12-amino-acid region (F56–R67) the ‘kinase inhibitory region’, because it is critical for inhibition of JAK signaling as well as kinase activity.

**Discussion**

In the present study, we have focused on the molecular mechanism of JAK inhibition by JAB. Previous reports indicate that a certain level of JAB expression can potentially block many cytokine functions, including LIF,
Fig. 7. The effect of amino acid substitution in the kinase inhibitory region of JAB. (A) Unchanged amino acids are shown as dots, substituted amino acids by a one letter code. All substitutions except Y65F were introduced into the dN51 backbone. The Y65F mutation was introduced into full-length JAB. All mutants contain an N-terminal Myc-epitope tag. (B) EPO-dependent reporter gene assay was carried out in the presence of the indicated substitution mutants in 293 cells. Data normalized with the β-galactosidase activity from duplicate experiments are shown. (C) Binding of mutated JAB to JH1. Upper panel: each mutated JAB was transiently expressed in 293 cells and subjected to pY1007 phosphopeptide-binding assay (pY1007 Bound). Middle and lower panels: GST–JH1 and each JAB mutant were co-expressed in 293 cells and precipitated with GSH–Sepharose. Myc-JAB was detected by immunoblotting with anti-Myc in the total cell lysate (TCL) or GSH-bound materials (JH1 Bound).

interferon-γ, GM-CSF, leptin, IL-2 and EPO (Masuhara et al., 1997; Naka et al., 1997; Starr et al., 1997; Adams et al., 1998; Bjorbaek et al., 1998; Sakamoto et al., 1998). JAB specifically inhibits members of the JAK family of tyrosine kinases relative to a number of other receptor kinases and cytoplasmic kinases, including the FGF-receptor, c-kit, Flt-3, the insulin receptor and syk (Endo et al., 1997; Masuhara et al., 1997; Naka et al., 1997 and unpublished data). Although the physiological functions of JAB in vivo are not clear at present, the molecular mechanism of specific inhibition of JAK2 tyrosine kinase activity by JAB is interesting from a biochemical point of view and may provide useful insights for developing agents that specifically inhibit tyrosine kinases.

Our studies demonstrate the existence of three functional domains: the kinase inhibitory region, the extended SH2 subdomain and the SH2 domain. The function of the C-terminal region has not been clarified in this study, but it is apparently not necessary for JAK signal inhibition. The SH2 domain and extended SH2 subdomain are both
sufficient and necessary for binding to the pY1007 phosphopeptide, indicating that the extended SH2 subdomain is a part of the SH2 domain, which is important for phosphotyrosine-containing peptide binding. Two branched-chain amino acids, I68 and L75 conserved among the CIS family and STATs were essential for binding to pY1007. The kinase inhibitory region is responsible for high-affinity binding to JH1, which is necessary for signal and kinase inhibition. Furthermore, we also identified four important amino acids that are necessary for JAK2 signal inhibition, F56, F59, D64 and Y65. These are summarized in Figure 10A.

Role of the kinase inhibitory region in JAK inhibition

Without the kinase inhibitory region, JAB binds to JH1 with similar affinity to pY1007 phosphopeptide (Figure 8), suggesting that this region is a second binding site for JH1. Some kinase inhibitory region mutants, such as F56D and dN58, can still bind to JH1, however, they never inhibit EPO/STAT5 signaling even at very high levels of expression (H.Yasukawa, data not shown), suggesting that a simple binding of the JAB SH2 domain to the activation loop is not enough to inhibit kinase activity. We noticed that the kinase inhibitory region resembles somewhat the activation loop of JAKs (Figure 10B). Therefore, we hypothesized that the kinase inhibitory region may mimic the activation loop by functioning as a pseudosubstrate. Consistent with this idea, a synthetic 15mer peptide corresponding to this region was phosphorylated well by GST–JH1 in vitro, and also suppressed JH1 kinase activity in a competitive manner against substrate (H.Yasukawa, data not shown). These data indicate that the kinase inhibitory region can potentially interact with the catalytic groove of the JH1 kinase domain. Since we did not observe tyrosine phosphorylation of the intact JAB molecule by JAK2, the kinase inhibitory region may function as a non-phosphorylatable ‘pseudosubstrate’ or mimic activation loop, thereby preventing the access of substrates and/or ATP to the catalytic pocket (Figure 10C). This hypothesis must be verified by kinetic analysis or more directly by co-crystal structure analysis using purified JAB and JH1.

Comparison with Ser/Thr kinase inhibitors

The molecular mechanism underlying the inhibition of serine/threonine kinases by intrinsic kinase inhibitors is well understood. For example, cyclin-dependent kinase inhibitors (CDKIs) are well characterized both biochemically and physiologically. The crystal structure of the p27Kip1 bound to the cyclin A–Cdk2 complex has been resolved (Russo et al., 1996). The C-terminal region of p27 inserts into the catalytic cleft, and F87 and R90 in this region mimic ATP, thereby inhibiting kinase activity. Recently, crystal structures of the complex of cyclin D-dependent kinase Cdk6 bound to the cell-cycle inhibitor p16INK4a have also been determined (Brotherton et al., 1998; Russo et al., 1998). The structures reveal that the INK4 inhibitors bind next to the ATP-binding site of the catalytic cleft, thereby preventing both productive binding of ATP and the cyclin-induced rearrangement of the kinase from an inactive to an active conformation. It is interesting that both Kip/Cip and INK4 inhibitors inhibit kinase activity by preventing ATP binding to the catalytic cleft, although they inhibit ATP binding by totally different mechanisms. The kinase inhibitory region of JAB possesses essential F56 and F59 for kinase


**Fig. 9.** The effect of JAB on GST–JH1 kinase activity *in vitro.* (A) Purified GST–JH1 bound to GSH–Sepharose was incubated without (−) or with extracts of cells transiently transfected with plasmids carrying CIS1 (lane 2), CIS2 (lane 3), CIS3 (lane 4) and JAB (lane 5). After washing, the beads were incubated with [γ-32P]ATP, then analyzed with SDS–PAGE and autoradiography (a). The arrowhead indicates 32P-labeled GST–JH1. Aliquots of the total cell extracts were blotted with anti-Myc (b). (B) GST–JH1 was incubated *in vitro* with extracts of cells transfected without (−; lane 1) or with the indicated JAB mutants (lanes 2–5). After washing, aliquots of samples were blotted with anti-Myc to measure binding to JH1 (b). The input JAB proteins were estimated by immunoblotting of total cell extracts with anti-Myc (c). To confirm the stability of GST–JH1 after incubation with the cell extracts, the same membrane was reprobed extracts with anti-Myc (d). (C) Lysate (1 ml) of *Escherichia coli* transformed with pQE vector carrying Myc-tagged full length JAB (lane 2), R105E (lane 3), dN51 (lane 4), dC40 (lane 5) or vector alone (lane 1) were incubated with GST–JH1 conjugated to GSH–Sepharose for 1 h at 4 °C. After washing, an *in vitro* kinase assay using GST–EPOR as substrate was performed (a). Aliquots of cell lysates were blotted with anti-Myc (αMyc) to show a similar level of JAB proteins was inoculated into the reaction (b).

inhibition. There is a possibility of the interaction of these Phe residues with the ATP-binding sites in the catalytic cleft such as p27.

Other well-characterized Ser/Thr kinase inhibitors are cAMP-dependent protein kinase (PKA) inhibitors, PKIs. PKIα and PKIβ work as pseudosubstrates and share a common sequence FXXXXXRXRXXI/L, which has been shown to be the core element of PKI activity and to be essential for high-affinity docking to the PKA catalytic site. The crystal structure of the catalytic subunit of the PKA and PKIα(5–22) complex has revealed that the specific residues of the catalytic subunit interact with each of these key residues in PKI (Knighton et al., 1991). Protein kinase C (PKC) also has a pseudosubstrate region (19–36) within its regulatory domain (House and Kemp, 1987). Both PKI and PKC pseudosubstrate regions are non-phosphorylatable substrates; substitution of Ala with Ser in the core region transforms the pseudosubstrates into potent substrates (Cheng et al., 1985, 1986; Scott et al., 1985; House and Kemp, 1987). Similarly, DY64,65 of JAB may be involved in recognition as a pseudosubstrate.

**Comparison with Grb10 and Grb14**

Recently, other SH2 proteins Grb10 and Grb14 have been shown to contain a second novel domain that interacts with the insulin receptor and insulin-like growth factor receptor in an activation loop-dependent manner (He et al., 1998; Kasus-Jacobi et al., 1998). These studies suggest that Grb10 and Grb14 may interact within or near the activation loop of the insulin-receptor-kinase domain, thereby physically blocking tyrosine kinase activity. Although there is no sequence similarity between the kinase inhibitory region of JAB and any domains of Grb10 or Grb14, it is interesting to note whether other tyrosine kinases possess their own specific inhibitory SH2 protein that interacts with the activation loop. It may also be possible to design a specific JAB-like tyrosine kinase inhibitor acting against a given tyrosine kinase, by modifying the SH2 domain to bind to the kinase activation loop, as well as the kinase-inhibitory region to the catalytic pocket of the kinase.

**Materials and methods**

**Cells and transfectants**

M1 myelogenous leukemia cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum. M1 transformants were obtained by electroporation with pcDNA3 carrying Myc-tagged full-length JAB, dN64 and dC40 and maintained in the presence of 0.5 mg/ml G418 as described previously (Masuhara et al., 1997). Growth of M1 cells in the presence of LIF was measured as described. 293 Cells were maintained in DMEM–10% calf serum (CS).

**Luciferase assay**

The STAT5 responsive promoter-luciferase reporter gene, which carries four repeats of the GAS sequence with the jun promoter, was described previously (Masuhara et al., 1997). The STAT5 responsive promoter-luciferase reporter gene, which carries four repeats of the GAS sequence with the jun promoter, was described previously (Masuhara et al., 1997). This reporter gene has been used for STAT3 activity measurement in an earlier study, but can also be used for STAT5 activity. EPO-dependent luciferase activity in 293 cells co-transfected with STAT5 and the EPO receptor cDNA was measured as previously (Masuhara et al., 1997).

**Mutant cDNA construction**

All cDNAs used herein were derived from murine JAB. Deletion, substitution and chimeric mutants were generated using standard poly-
merase chain reaction (PCR) methods. cDNA fragments were subcloned into EcoRI/XhoI sites of pcDNA3-Myc as described previously (Endo et al., 1997). Sequences of all mutant cDNAs were confirmed. This was essential because errors were frequently found in the PCR product probably because of the high GC content of JAB cDNA. For N-terminal and C-terminal Flag-tagging, JAB cDNA was cloned in a pFLAG-CMV2 or a pFLAG-CMV5 vector (Kodak, CT, USA). To produce His6 and single Myc-tagged JAB in bacteria, the PCR product of JAB cDNA carrying N-terminal Myc-tag was subcloned into pQE32 (Qiagen, CA, USA).

**Binding of JAB to GST–JH1 or phosphopeptides in vitro**

Peptides and phosphopeptides were synthesized and coupled to CNBr-activated Sepharose 4B according to the manufacturer’s instructions (Pharmacia, Uppsala, Sweden). Approximately 15 μmol of the peptide were coupled to 1 g dried Sepharose resin. GST–JH1 expressed in 293 cells (5 μg plasmid/transfection) grown in 10-cm dishes was purified in 100 μl (50% v/v) of GSH–Sepharose just before the experiment. Myc-tagged JAB and mutants in pcDNA3 (5 μg/transfection) were transiently expressed in 293 cells grown in 10-cm dishes. Cells were lysed in 1 ml of lysis buffer A (20 mM HEPES buffer pH 7.3, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and 1% aprotinin), then centrifuged. Before the precipitation experiments, bovine serum albumin (BSA) was added at a final concentration of 1 mg/ml. Cell extracts were incubated with 20 μl (50% v/v) of peptide-conjugated Sepharose or GST–JH1 Sepharose, then washed twice with phosphate-buffered saline (PBS) and analyzed by immunoblotting with anti-phosphotyrosine (PY) (4G10), anti-Myc (9E10), anti-Flag (M2; Kodak) and anti-PLCγ-1 (Santa Cruz, CA, USA) antibodies. For semi-quantitative analysis, cell extracts containing Myc-JAB were diluted with buffer A containing 1 mg/ml BSA, then used for in vitro binding assay. The concentration of JAB in the cell lysates was estimated roughly by immunoblotting with anti-Myc antibody using purified Myc-tagged GST produced in bacteria or with anti-SOCS1 antibody (SantaCruz) using purified recombinant JAB (kindly provided by Dr A.Gertler, The Hebrew University of Jerusalem, Israel) as a standard protein.

**JAK2–JH1 in vitro kinase assay**

A polypeptide substrate, GST–EPOR cytoplasmic domain (codon 325–430) containing two tyrosine residues was produced in NM522 bacteria and purified on GSH–Sepharose. Purified protein was dialyzed against 10 mM Tris-buffered saline (TBS) and concentrated to 2 mg/ml. GST–JH1 expressed in 293 cells (5 μg plasmid/transfection) grown in 10-cm dishes was purified in 100 μl (50% v/v) of GSH–Sepharose just before the experiment. The resin was diluted 10–100× with Sepharose S-200, because the kinase activity was too high. For the in vitro binding assay, 20 μl of resin was incubated with 1 ml of cell extract from 293 cells transiently transfected with each JAB mutant (1.0 μg plasmid/10 cm dish) or 1 ml of bacterial lysate (from 10 ml overnight culture) carrying His-tagged JAB or mutants at 4°C for 1 h, then washed twice with kinase reaction buffer (50 mM HEPES buffer pH 7.5, 50 mM NaCl, 5 mM MgCl2, 5 mM MnCl2 and 0.1 mM Na3VO4). The recombinant protein was produced in bacteria without isopropyl-β-D-thiogalactopyranoside (IPTG) treatment, because the addition of IPTG resulted in degradation and aggregation of JAB protein.
For the *in vitro* autophosphorylation assay, the beads resuspended in 20 μl of kinase reaction buffer were further incubated with [γ-32P]-ATP (10 μCi/sample; final concentration 10 μM) for 15 min at room temperature. After washing twice with PBS, the beads were subjected to 10% SDS-PAGE and autoradiography. For substrate phosphorylation, 2 μg GST–EPOR and 100 μM cold ATP were included in the reaction and substrate phosphorylation was measured by immunoblotting with anti-PY.

**Two-hybrid assay**

To detect the interaction of JAB mutants with JH1, L40 was transformed with pBTM116 carrying JH1 or the c-ikit tyrosine kinase domain (Endo et al., 1997) and pACT2 (Clontech, CA, USA) carrying the indicated mutant JAB cDNAs, then selected in medium lacking tryptophane and leucine as described previously (Masuhara et al., 1997). Transformants were restreaked on a paper filter and stained using an 1:320 dilution of 5-bromo-4-chloro-3-indolyl-phosphate and 1:5000 dilution of nitroblue tetrazolium.

**Acknowledgements**

We thank Ms H. Ohgusu and Ms M. Sasaki for excellent technical assistance, Ms M. Chikushi for preparing the manuscript, Drs W. Leonard, A. Mui and D. Waugh for critical reading of the manuscript. Part of this work was supported by grants from the Ministry of Science, Education and Culture of Japan, TORAY Research Foundation, Naito Memorial Foundation, Sumitomo Foundation and Kanae Research Foundation for A. Y., and grants from the National Heart, Lung and Blood Institute (PO HL53749) and American Lebanese Syrian Associated Charities (ALSAC) for J. N. I.

**References**


Received September 29, 1998; revised December 23, 1998; accepted December 31, 1998