CIS3 and JAB have different regulatory roles in interleukin-6 mediated differentiation and STAT3 activation in M1 leukemia cells

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We have reported JAK-signaling modulators, CIS1 (cytokine-inducible SH2 protein-1), CIS3 and JAB (JAK2 binding protein), which are structurally related. In M1 myeloid leukemia cells, CIS3 was induced by neither interleukin 6 (IL6) nor interferon γ (IFNγ), while JAB was induced strongly by IFNγ and slightly by IL6 and leukemia inhibitory factor (ILF). Forced expression of CIS3 and JAB in M1 cells prevented IL6- or LIF-induced growth arrest and differentiation, even when their expression levels were comparable to endogenous ones in several cell lines such as HEL, UT-7, IFNγ-treated M1, and CTLL2 cells. Pretreatment of parental M1 cells with IFNγ but not IFNβ resulted in suppression of LIF-induced STAT3 activation and differentiation, further supporting that physiological level of JAB is sufficient to inhibit LIF-signaling. However, unlike JAB, CIS3 did not inhibit IFNγ-induced growth arrest, suggesting a difference in cytokine specificity between CIS3 and JAB. CIS3 inhibited STAT3 activation with slower kinetics than JAB and allowed rapid c-fos induction and partial FcγRI expression in response to IL6. In 293 cells, CIS3 as well as JAB bound to JAK2 tyrosine kinase domain (JH1), and inhibited its kinase activity, however, the effect of CIS3 on tyrosine kinase activity was weaker than that of JAB, indicating that CIS3 possesses lower affinity to JAK kinases than JAB. These findings suggest that CIS3 is a weaker inhibitor than JAB against JAK signaling, and JAB and CIS3 possess different regulatory roles in cytokine signaling.

Keywords: interleukin 6; STAT; JAK; tyrosine kinase; regulation

Introduction

Growth, differentiation and functions of immune and hematopoietic cells are controlled by multiple cytokines, including interleukins (ILs) and colony stimulating factors (CSFs) (Arai et al., 1990). 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 (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 et al., 1995). Among these proteins are members of the STAT family of transcription factors (Ihle et al., 1996). The tyrosine-phosphorylated STATs form homo- or hetero-dimers and translocate into the nucleus where they bind to their specific target sequences, most of which are related to gamma interferon (IFNγ) activated sites (GAS), a key regulatory element in the promoter of IFNγ-inducible genes (Darnell et al., 1994).

Regulation of JAK-STAT signaling by the cytokine network has been shown to be important for inflammation, immunity, hematopoiesis and cancer. We cloned a cytokine-inducible SH2 protein, CIS (Yoshimura et al., 1995). CIS gene is a direct target of STAT5 and its product tightly binds to the tyrosine-phosphorylated IL3 receptor and EPO receptor. It partially suppresses STAT5 activation, probably through masking of STAT5 docking sites on the receptor (Matsumoto et al., 1997). Thus, CIS acts as a kind of negative feedback regulator of the JAK-STAT5 pathway. We recently cloned another CIS family member, JAB, which directly binds to the JAK2 tyrosine kinase domain and inhibits JAK tyrosine kinase activity (Endo et al., 1997). Overexpression of JAB resulted in the suppression of all cytokine signaling utilizing JAKs. We also found five additional CIS family members (CIS2-CIS6) and the original CIS has been referred to as CIS1 (Masuhara et al., 1997). Among CIS family members, CIS3 is closest to JAB, and CIS3 also binds to JAK2-JH1 tyrosine kinase domain. Two other groups have reported on related genes: one referred to JAB, CIS2, and CIS3 as SOCS-1, SOCS-2 and SOCS-3 (Starr et al., 1997), respectively and the other as SSI-1, SSI-2 and SSI-3 respectively (Naka et al., 1997; Minamoto et al., 1997). Since the CIS family genes (CISs) appear to be involved in regulation of cytokine signaling, we examined their role in IL6- or leukemia inhibitory factor (LIF)-dependent differentiation of M1 cells. We found that forced expression of CIS3 and JAB but not CIS1 and CIS2 in M1 leukemia cells resulted in the suppression of IL6- and LIF-dependent STAT3 activation, differentiation and growth arrest. However, inhibition of STAT3 activation by CIS3 was slower than that by JAB, reflecting the lower affinity of CIS3 to JH1 than that of JAB. Furthermore, IL6-dependent expression of a set of genes in M1 transformants expressing CIS3 was regulated differently from that in transformants expressing JAB. Expression of CIS3 did not inhibit whole gp130-mediated signals, but rather modulated their intensity in M1 cells. We also found that CIS3 did not prevent IFNγ-induced growth arrest of M1 cells, while JAB did. Although CIS3 and JAB can bind to

JAK kinases, these two genes seem to have different physiological functions.

Results

Effect of CIS3 and JAB on IL6 or LIF-induced differentiation and growth arrest of M1 cells

M1 leukemia cells have been used for the study of IL6 or LIF signaling, since IL6 and LIF has marked effects: it induces growth arrest, apoptosis and macrophage-differentiation. Forced expression of JAB (referred to as SOCS-1 and SSI-1) or CIS3 (SOCS-3 and SSI-3) has been shown to inhibit LIF and IL6-induced differentiation of M1 cells (Masuhara et al., 1997; Starr et al., 1997; Naka et al., 1997). First, we compared mRNA levels of exogenously expressed CIS3 and JAB with those endogenously expressed in M1 cells and several hematopoietic cells at the exponential growth stage (Figure 1). We used CMV promoter for stable expression, since it could not achieve very high levels of expression in M1 cells. Endogenous levels of CIS3 and JAB mRNAs were undetectable by Northern hybridization in M1 cells without cytokine stimulation. CIS3 mRNA was not induced by IL6, LIF, IFNγ or IFNβ in M1 cells (Figure 1, lanes 2–5 in the CIS3 panel). High expression of CIS3 mRNA was detected in human erythroleukemia (HEL) cells and granulocyte-macrophage colony stimulating factor (GM-CSF)-

dependent UT7 cells (lanes 10, 11 in CIS3 panel), and the level of exogenously expressed CIS3 in M1 cells was comparable to these levels.

Endogenous JAB mRNA was marginally induced by IL6 and LIF, and strongly induced by IFNγ (compare lanes 2 and 3 with lane 5 in the JAB panel). IFNβ did not induce JAB expression (lane 4). Expression of JAB was detected after 1–3 h stimulation with IFNγ and lasted as long as IFNγ was present for at least 24 h after stimulation (data not shown). Interestingly, the level of JAB exogenously expressed by constitutive promoter in M1 cells was comparable to or less than that induced by IFNγ in M1 cells (compare lanes 5,7 with lane 8). In JAB transformants, IFNγ did not induce JAB expression further (lane 9), suggesting that IFNγ signaling was inhibited by exogenously expressed JAB. We found that the level of expression of JAB was very high in CTLL2 without IFNγ stimulation (lane 12). These results indicate that expression levels of CIS3 and JAB in our stable M1 transformants were not extremely high; they were comparable to or less than those found in several hematopoietic cell lines or IFNγ-treated M1 cells.

Next we examined the effect of forced expression of CIS3 and JAB on IL6, LIF and IFNγ-induced growth arrest of M1 cells. Immunoblotting with anti-Myc revealed that the expression levels of CIS3 and JAB were similar (see Figure 5a, αMyc). Transformants expressing JAB and CIS3 but not those expressing CIS1 or CIS2 (see ref. 8) were resistant to IL6 or LIF-mediated growth inhibition (Figure 2). Interestingly, JAB transformants were also resistant to IFNγ-induced growth arrest, while CIS3 transformants were as sensitive as parental M1 cells (Figure 2). Morphologically, cells expressing JAB or CIS3 were similar to untreated M1 cells, while IL6 or LIF-treated parental M1 cells or transformants expressing CIS1 or CIS2 became vacuolated and exhibited chromatin condensa-
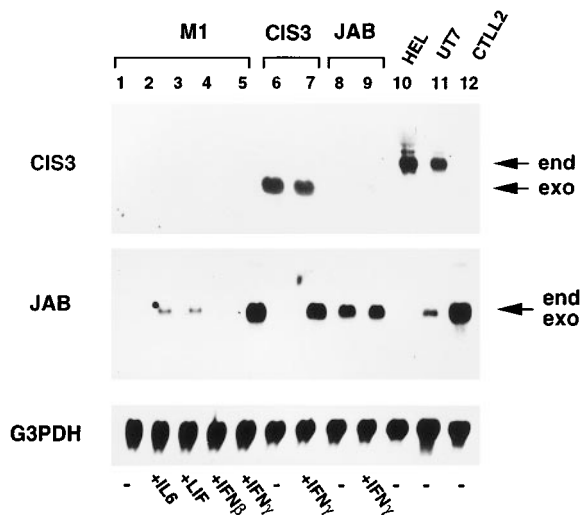
Figure 1 Expression of CIS3 and JAB in M1 transformants and several cell lines. Total RNA was extracted from parental M1 cells (M1; lanes 1–5) or transformants expressing human CIS3 (CIS3; lanes 6 and 7) or murine JAB (JAB; lanes 8 and 9) treated without (−; lanes 1, 6 and 8) or with 100 ng/ml IL6 (+IL6; lane 2), 10 ng/ml LIF (+LIF; lane 3), 1000 unit/ml IFNγ (+IFNγ; lanes 5, 8 and 9) or 1000 unit/ml IFNβ (+IFNβ; lane 4) for 6 h. RNA was also extracted from exponentially growing human erythroleukemia cell line, HEL (lane 10), myeloid cell lines, UT7 grown in the presence of 5 ng/ml GM-CSF (lane 11) and mouse T cell line CTLL2 (lane 12) grown in the presence of 10 ng/ml IL2. Northern hybridization with cDNA probes for coding regions of human CIS3 and murine JAB, and control G3PDH was performed. Similar result was obtained using human JAB cDNA probe. Positions for exogenous (exo) and endogenous (end) CIS3 and JAB are indicated by arrowheads. In size, exogenous JAB is indistinguishable from endogenous JAB
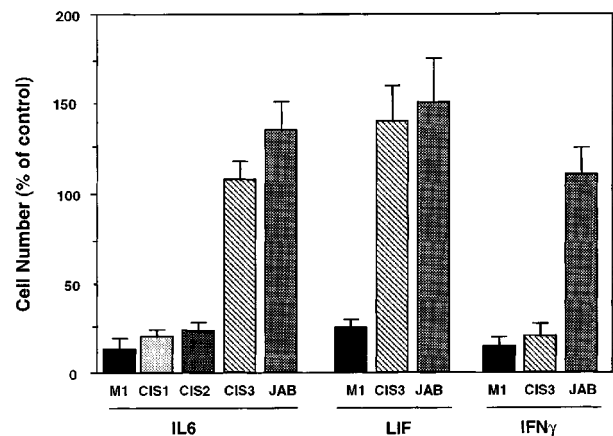

Figure 2 Inhibition of IL6, LIF and IFNγ-induced growth arrest of M1 cells by JAB and CIS3. 1×10^5 parental M1 cells (M1) and transformants expressing CIS1, CIS2, CIS3 and JAB (CIS1, CIS2, CIS3, JAB) were cultured in medium containing 10% horse serum supplemented with 100 ng/ml IL6, 1 ng/ml LIF or 1000 unit/ml IFNγ for 5 days, then viable cells determined by trypan blue exclusion were scored. Cell number values are expressed relative to the number cultured without cytokines for 5 days. Similar results were obtained for at least two independent clones for each transformation

tion (data not shown). Similar results were obtained for at least three independent clones for CIS3 and JAB.

Inhibition of STAT3 activation and differentiation in IFNγ-treated M1 cells

To address biological consequences of high level of JAB induction by IFNγ, we examined LIF-induced differentiation and STAT3 activation in parental M1 cells pretreated with IFNγ or IFNβ. Consistent with a report by another group (Hoffmann-Liebermann et al., 1991), IFNβ and IFNγ suppressed growth of M1 cells (data not shown), but did not induce morphological changes (Figure 3c,e). LIF-treatment resulted in vacuolation and enlargement of cytoplasm in M1 cells (Figure 3b), while such morphological changes were not apparent in cells preincubated with IFNγ (Figure 3d). IFNβ pretreatment did not prevent LIF-induced morphological changes (Figure 3f), which correlates with its inability of JAB-induction (see Figure 1). As shown previously, STAT3 plays a central role in LIF or IL6-induced macrophage differentiation of M1 cells (Nakajima et al., 1996; Minami et al., 1996). The effect of IFNγ on LIF-induced STAT3 activation was examined by immunoblotting with anti-phospho-STAT3 antibody (Figure 4). A high level of tyrosine phosphorylation of STAT3 was maintained in the presence of LIF in untreated M1 cells (lanes 2 – 5). LIF-induced STAT3 phosphorylation was apparent at 0.5 – 1 h in IFNγ-treated cells (lanes 7 and 8), however, it decreased rapidly. While STAT3 was normally phosphorylated in response to LIF in IFNβ-treated
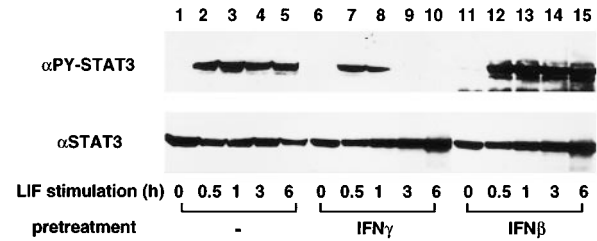
Figure 4 Suppression of LIF-STAT3 phosphorylation in IFNγ pretreated M1 cells. M1 cells pretreated without (lanes 1 – 5) or with 1000 unit/ml IFNγ (lanes 6 – 10) or IFNβ (lanes 11 – 15) for 12 h were stimulated with 2 ng/ml LIF for indicated periods. Cells extracts were prepared and immunoblotting with anti-phospho-STAT3 (αPY-STAT3) or anti-STAT3 (αSTAT3) antibodies was performed

cells (lanes 11 – 15). These data suggest that induction of JAB correlates with inhibition of LIF-induced STAT3 activation and differentiation of M1 cells.

## Different inhibition of STAT3 activation by CIS3 and JAB in M1 cells

To elucidate the mechanism of inhibition of IL6-mediated differentiation and growth arrest of M1 cells by CIS3, we examined IL6-dependent activation of STAT3 and gp130. Unfortunately, we could not detect JAK tyrosine phosphorylation in response to IL6 in M1 cells, probably because of very low levels of expression of JAKs or only a small fraction of JAKs were activated. M1 cells or transformants were stimulated with or without IL6, then DNA binding activity of STATs was assessed by electrophoretic mobility shift assay (EMSA) using the IRF-1 GAS probe (Fujii et al., 1995) (Figure 5a, the EMSA panel). IL6-induced DNA-binding activity of STAT3 was markedly reduced in M1 transformants expressing JAB (lanes 9 and 10). STAT3-DNA binding activity was also reduced in CIS3 transformants but to a lesser extent than in JAB transformants (lanes 7 and 8). STAT3-DNA binding activity was not affected by expression of CIS1 and CIS2 (lanes 3 – 6).

To detect the tyrosine phosphorylation of STAT3, cell extracts were blotted with antibody specific to tyrosine phosphorylated STAT3 (Figure 5a, αPY-STAT3) or to STAT3 (Figure 5a, αSTAT3). Expression of CISs and JAB was confirmed by immunoblotting with anti-Myc (Figure 5a, αMyc). STAT3 was tyrosine phosphorylated in response to IL6 in parental M1 cells and transformants expressing CIS1 and CIS2 (lanes 2, 4 and 6). IL6-induced tyrosine phosphorylation of STAT3 was markedly reduced in M1 transformants expressing JAB (lanes 9 and 10), while CIS3 weakly inhibited IL6-induced STAT3 phosphorylation (lanes 7 and 8). We also examined the effect of CIS3 and JAB on IL6-induced tyrosine phosphorylation of gp130 (Figure 5b). Similar to the case of STAT3 phosphorylation, JAB almost completely inhibited gp130 phosphorylation (lane 6), while CIS3 significantly, but not completely, reduced phosphorylation (lane 4).

To confirm different effects of CIS3 and JAB on STAT3 activity, we compared the time course of IL6-dependent phosphorylation of STAT3 (Figure 6). As
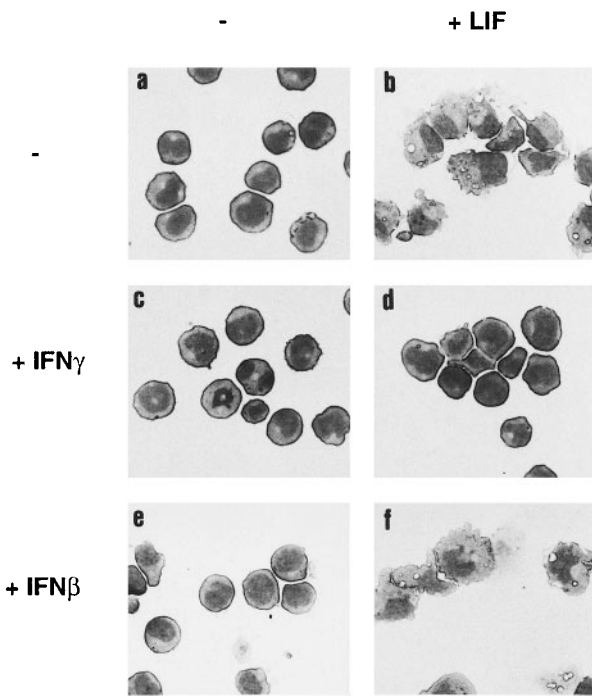
Figure 3 Suppression of LIF-mediated morphological changes in parental M1 cells treated with IFNγ. M1 cells were incubated without (−; **a,b**) or with 1000 unit/ml IFNγ (+IFNγ; **c,d**) or IFNβ (+IFNβ; **e,f**) for 12 h then 1 ng/ml LIF (**b,d,f**) or saline (**a,c,e**) was added. After 5 days culture, cells were spun down on slide glass using a cytospin and examined after May – Grunwals-Giemsa staining
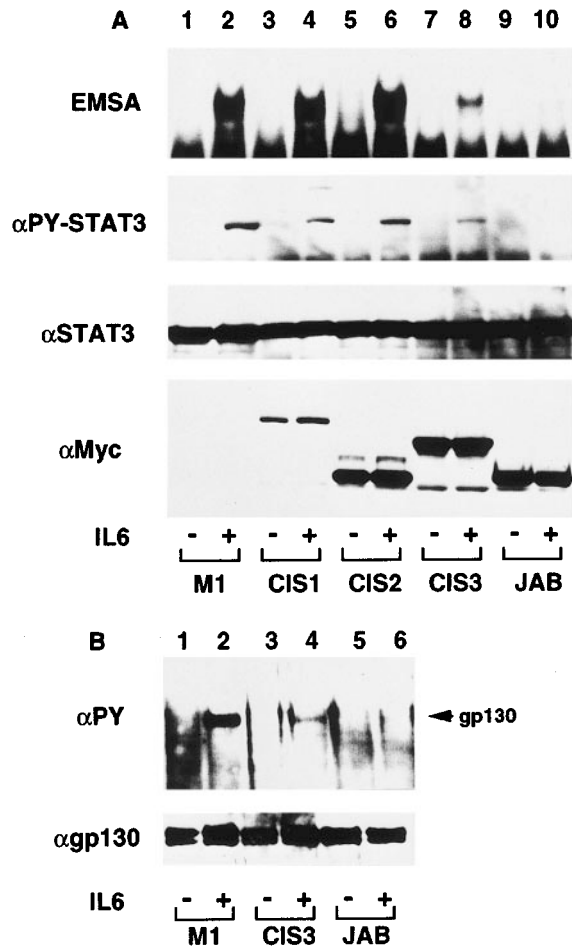
Figure 5 Effect of CISs on STAT3 activation and gp130 phosphorylation in M1 cells. Parental M1 cells (lanes 1 and 2) and transformants expressing CIS1 (lanes 3 and 4), CIS2 (lanes 5 and 6), CIS3 (lanes 7 and 8) and JAB (lanes 9 and 10) were incubated without (−) or with (+) 100 ng/ml IL6 for 60 min at 37°C. In (**a**) in the upper panel (EMSA), total cell extracts were incubated with ^{32}P-labeled IRF-1 GAS probe. Complexes were resolved by separation on 4% acrylamide gels and detected by autoradiography. Total cell extracts were also analysed by immunoblotting with anti-phospho-STAT3 (αPY-STAT3), anti-STAT3 (αSTAT3) or anti-Myc (αMyc) antibodies. In (**b**) immunoprecipitates with anti-gp130 from stimulated (lanes 2, 4 and 6) and unstimulated cells (lanes 1, 3 and 5) were blotted with anti-phosphotyrosine (αPY) or anti-gp130 (αgp130). The arrowhead indicates tyrosine phosphorylated gp130

shown previously (Nakajima *et al.*, 1996), STAT3 was activated for over 20 h in parental M1 cells, and such long term STAT3 activation seemed to be necessary for differentiation. In M1 transformants expressing CIS3 or JAB, IL6-induced STAT3 phosphorylation was reduced, however, the time course of inhibition of STAT3 phosphorylation was quite different between these two transformants. IL6-induced STAT3 phosphorylation appeared in CIS3 transformants after 15–30 min stimulation (lanes 8 and 9), then gradually decreased at 1–18 h stimulation. On the other hand, in JAB transformants, STAT3 was weakly phosphorylated at 15 min stimulation (lane 14) and phosphorylation was not detected thereafter. These findings could reflect a short term activation of JAKs, followed by rapid and slow inhibition of their kinase activity by JAB and CIS3 respectively. Similar results were obtained for LIF-induced activation of STAT3 (data not shown). This is consistent with the fact that tyrosine phosphorylation of JAKs is prerequisite for binding of JAB or CIS3 (Endo *et al.*, 1997; Masuhara *et al.*, 1997), since binding depends on SH2-phosphotyrosine interaction. The difference between JAB and CIS3 in the STAT3 inhibition kinetics may be due to the difference in their affinity to tyrosine phosphorylated JAKs.

Modulation of IL6-induced gene expression by CIS3

Next, we addressed how differently CIS3 and JAB affect IL6-induced gene expression in M1 cells (Figure 7). IL6-induced growth arrest of M1 cells has been shown to be accompanied by induction of Interferon Regulatory Factor (IRF)-1 and cyclin-dependent kinase (cdk) inhibitor p19^{INK4D} (Narimatsu *et al.*, 1997). These two genes are shown to be regulated by STAT3 (Narimatsu *et al.*, 1997). FcγRI has been used as a marker of early differentiation and is probably a target of STAT3 (Yamanaka *et al.*, 1996; Minami *et al.*, 1996). Proto-oncogene c-fos is known to be rapidly and transiently induced by most cytokines probably through pathways other than STATs. Reduction of c-myc and c-myb expression has been also shown to be accompanied by differentiation of M1 cells, although their downregulation may not be necessary for growth suppression (Hoffman-Liebermann *et al.*, 1991; Selvakumaran *et al.*, 1992). We compared the expression of
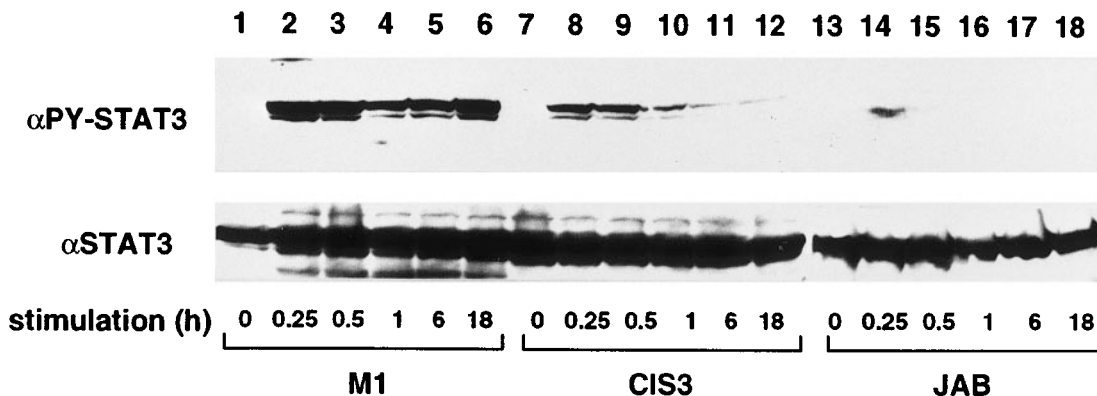
Figure 6 Time course of IL6-induced activation of STAT3 in M1 cells and transformants. Parental M1 cells (lanes 1–6) and transformants expressing CIS3 (lanes 7–12) or JAB (lanes 13–18) were stimulated with 100 ng/ml IL6 for indicated periods (h). Total cell extracts were analysed by immunoblotting with anti-phospho-STAT3 (αPY-STAT3) or anti-STAT3 (αSTAT3) antibodies

these genes in parental M1 cells and transformants expressing CIS3 or JAB (Figure 7).

First, IL6 rapidly enhanced expression of p19^{INK4D} in parental M1 cells. Cdk4 expression decreased in parental M1 cells after 48 h incubation with IL6. No such increase in p19^{INK4D} or reduction in cdk4 expression was seen in CIS3 or JAB transforms (Figure 7a), suggesting normal growth of these cells. IL6 enhanced the expression of IRF-1 in parental M1 cells by 3–5-fold within 1 h and a high level of expression was maintained thereafter. In contrast, IRF-1 expression was only marginally elevated in M1 transformants expressing CIS3 and JAB (Figure 7b). Thus, IRF-1 was also correlated to suppression of cell growth. FcγRI expression was gradually induced from 1 h after stimulation in parental M1 cells, whereas it was not induced within 6 h in CIS3 and JAB transformants. However, IL6 induced a low but significant level of expression of FcγRI after 24 h stimulation in CIS3 transformants but not in JAB transformants. This may be due to an incomplete block of STAT3 activation in CIS3 transformants (see Figures 5 and 6). In parental M1 cells, c-*myc* and c-

myb expression decreased after 24 h stimulation (lane 5). JAB suppressed IL6-dependent repression of c-*myc* and c-*myb* (lane 15), consistent with strong inhibition of IL6-signaling. However, repression of c-*myc* and c-*myb* at 24 h occurred normally in CIS3 transformants (lane 10). These findings indicate that CIS3 inhibits growth arrest and terminal differentiation as characterized by enhancement of IRF-1 and p19^{INK4D} expression and morphological changes, but not partial differentiation as characterized by expression of FcγRI and repression of c-*myc* and c-*myb*. On the other hand, JAB inhibited all of these changes, suggesting that CIS3 and JAB have different effects on IL6 signaling pathways.

Like other cytokines, IL6 also induced rapid and transient expression of c-*fos* in parental M1 cells (Figure 7, the c-*fos* panel). Since c-*fos* induction is rapid, it reflects early activation of JAKs by IL6. Induction of c-*fos* at 30 min in transformants expressing CIS3 was indistinguishable from that in parental M1 cells (Figure 7, lane 2), while that in transformants expressing JAB was reduced to about 50% the level of parental M1 cells (Figure 7, lane 12), suggesting that CIS3 and JAB do not inhibit early JAK activation, however, JAB suppresses the activation more rapidly and strongly than CIS3. This is consistent with the time course of JAK activation estimated by STAT3 phosphorylation (Figure 6). At 24 h stimulation, c-*fos* was evident in parental M1 cells and CIS3 transformants, but not detectable in JAB transformants. The mechanism of c-*fos* re-expression at 24 h stimulation is not clear at present.

Effect of CIS3 and JAB on JAK/STAT3 signaling in 293 cells

To confirm different effect of CIS3 and JAB on JAK kinase activity, we compared the dose effect of CIS3 and JAB on LIF-induced reporter activation. Various amounts of JAB and CIS3 plasmids were transiently introduced together with a constant amount of STAT3-reporter gene into 293 cells (Figure 8). CIS3 and JAB inhibited LIF-induced reporter gene activation in a dose-dependent manner, however, JAB inhibited it much more efficiently than CIS3 (Figure 8).

Next, we examined the effect of CIS3 and JAB on JAK2-JH1 tyrosine kinase activity in 293 cells (Figure 9). We used GST-JH1 instead of full length JAK2, because GST-JH1 caused higher levels of tyrosine phosphorylation of GST-JH1 itself and cellular proteins than full length JAK2 (data not shown), and GST-JH1 can be purified easily with immobilized GSH. CIS3 and JAB bound to GST-JH1 in 293 cells (Figure 9, αMyc). However, binding of CIS3 to GST-JH1 was much weaker than that of JAB (compare lanes 11 and 15, or lanes 12 and 16 in the αMyc panel). Tyrosine phosphorylation of cellular proteins rather than GST-JH1 was blocked by co-expression of JAB and CIS3 in a dose-dependent manner (Figure 9, αPY). However, inhibition of tyrosine phosphorylation required a higher level of CIS3 than JAB expression (compare lanes 4 and 7 in the αPY panel). These results suggest that JAB can more efficiently bind to and inhibit JAK kinases than CIS3, thereby inhibiting STAT3 more efficiently than CIS3.
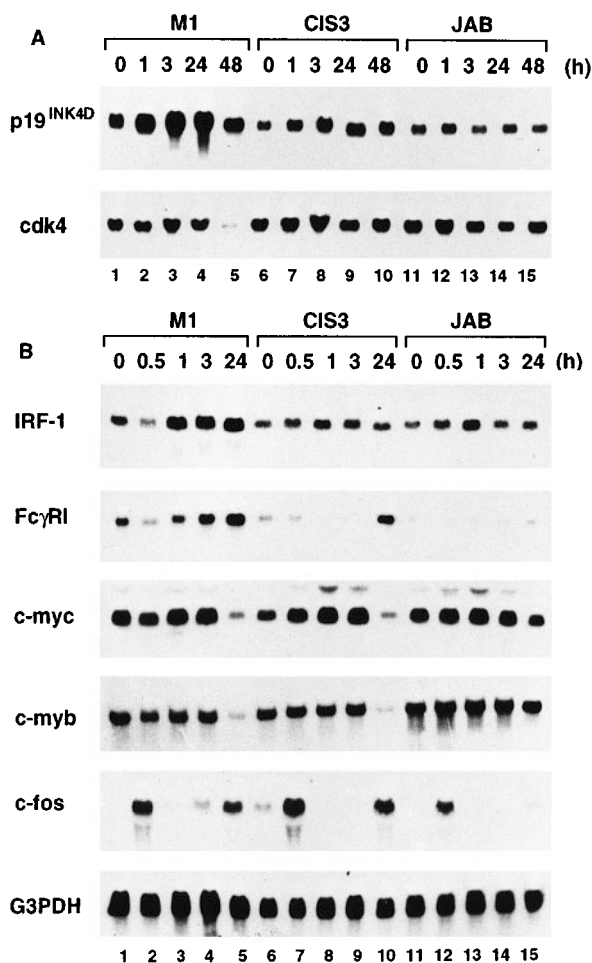
Figure 7 Regulation of IL6-induced gene expressions by CIS3 and JAB. Parental M1 cells (M1) and transformants expressing CIS3 (CIS3) or JAB (JAB) were stimulated with 100 ng/ml IL6 for indicated periods (h). Total RNA was extracted and the expression of p19^{INK4D}, cdk4, IRF-1, FcγRI, c-*myb*, c-*myc*, c-*fos* and G3PDH was analysed by Northern blotting
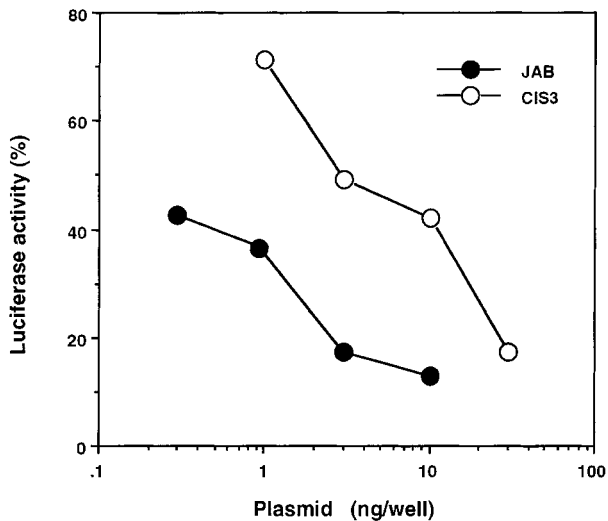
Figure 8 Dose-dependence of JAB and CIS3 for the inhibition of LIF-induced STAT3 reporter gene activation. STAT3-reporter plasmid, β-galactosidase gene and the indicated amount of pcDNA3-JAB (closed circle) or pcDNA3-CIS3 (open circle) plasmid were transfected into 293 cells which were then stimulated with 10 ng/ml of LIF. After 6 h stimulation, luciferase activity and β-galactosidase activity were measured. Relative luciferase activity (without JAB or CIS3 as 100%) is shown
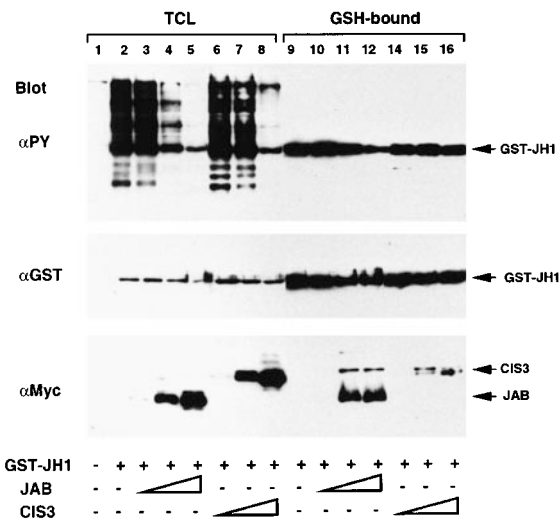
Figure 9 Inhibition of JAK2 kinase activity by CIS3 and JAB in 293 cells. Empty vector (lane 1), 5 μg of GST-JH1 in pcDNA3 alone (lanes 2 and 9) or pcDNA3-GST-JH1 plus different amounts of pcDNA3 carrying Myc-epitope tagged CIS3 (lanes 3–5, 10–12) or JAB (lanes 6–8, 14–16) were transiently expressed in 293 cells, and total cell extracts (lanes 1–8) or proteins bound to GSH-Sepharose (lanes 9–16) were resolved by SDS–PAGE, then immunoblotted with anti-phosphotyrosine (αPY), anti-Myc (αMyc) and anti-GST (αGST). The amounts of JAB and CIS3 plasmids were as follows; lanes 3, 10; 0.03 μg, lanes 4, 11; 0.1 μg, lanes 5, 12; 0.3 μg, lanes 6, 14; 0.1 μg, lanes 7, 15; 0.3 μg, lanes 8, 16; 1.0 μg per transfection for 10 cm dishes

Discussion

IL6 and IFNγ are known to be a central mediator of host defense responses, affecting growth and differentiation of a wide spectrum of cells. Here we showed

that the expression of CIS3 and JAB in M1 cells at close to endogenous levels can modulate IL6 or LIF signaling; it suppressed STAT3 activation, IRF-1 induction, terminal differentiation and growth arrest. However, while JAB rapidly and almost completely inhibited IL6 signaling, CIS3 allowed a relatively long period of activation of STAT3 (and probably JAKs), resulting in the partial induction of FcγRI, repression of c-*myc* and c-*myb* and normal c-*fos* induction. These differences could be explained by the difference in the affinity of JAB and CIS3 to JAKs. Indeed, inhibition of JAK2 tyrosine kinase and STAT3 activities in 293 cells required a higher concentration of CIS3 than JAB. Furthermore, IL6-induced tyrosine phosphorylation of STAT3 was strongly suppressed in M1 transformants expressing JAB, while it was partially and slowly inhibited in CIS3 transformants. From the analogy of cdk inhibitors, such as p21, p27, p16 and p19, inhibition of JAK activity by JAB and CIS3 probably depends on their affinity to JAKs and expression levels as well as the amount of activated JAKs. We also found that IFNγ-induced activation of JAK1 and JAK2 was inhibited in NIH3T3 transformants expressing JAB but not in cells expressing CIS3 (Sakamoto *et al.*, unpublished data), further supporting that the ability of CIS3 for inhibiting JAK activity is lower than that of JAB.

The presence of two JAK signaling inhibitors with different efficiency may be useful for modulating cytokine signaling at different magnitudes. As shown here, CIS3 inhibits IL6- and LIF-induced growth arrest but not IFNγ-induced JAB expression and growth arrest, while JAB can inhibit both in M1 cells. We found that forced expression of JAB but not CIS3 inhibited IFNγ-induced STAT1 phosphorylation as well as IRF-1 induction in M1 cells (Sakamoto *et al.*, unpublished data). Thus, CIS3 and JAB clearly possess different effects against different cytokines, which may be explained by their different affinities to JAKs. JAB and CIS3 can selectively inhibit cytokine actions, depending on receptor numbers and the amount of activated JAKs. The extent of suppression of JAKs/STAT1 activation by CIS3 may not be enough to inhibit IFNγ-mediated growth arrest, while only partial suppression of STAT3 is enough to inhibit LIF or IL6-induced growth arrest and differentiation in M1 cells.

Previously, we and two other groups reported that overexpression of JAB/SOCS1/SSI-1 and CIS3/SSI-3 in M1 cells inhibit LIF and IL6-induced differentiation and growth arrest. However, there remained a concern that such inhibition could be due to non-physiological 'overexpression'. In the present study, we found that the levels of JAB and CIS3 in M1 transformants were comparable to or less than those in hematopoietic cell lines. This is the first example showing that JAB and CIS3 can exhibit biological function at physiological expression levels. In particular, IFNγ induces JAB expression in M1 cells at a level probably high enough to inhibit IL6- or LIF-signaling. Indeed, pretreatment of M1 cell with IFNγ but not IFNβ (which does not induce JAB) suppressed LIF-induced STAT3 activation and morphological changes (Figures 2 and 3). Thus, our results raise the intriguing possibility that JAB is part, if not all, of a mechanism of antagonistic effect of IFNγ against LIF. When Figure 3 and Figure 6 are

compared, inhibition of STAT3 activation in IFNγ-treated cells was weaker and required longer than in JAB-transformants. The reason for this is not clear at present, but it could be a higher level of activation of JAKs because of stimulation by both IFNγ and LIF in IFNγ-treated cells.

JAB and CIS3 seem not to be simple negative 'feedback' regulators; but rather may be involved in cytokine unresponsiveness induced by other cytokines, including IFNγ. IFNγ is known as an inhibitory cytokine, suppressing many cytokine responses. For example, IL4-induced IgE synthesis and germline ε transcription was suppressed in the presence of IFNγ in B cells (Xu and Rothman, 1994). JAB could be involved in such antagonistic effect of IFNγ. Similarly, CIS3 may be able to modulate other cytokine signaling. We have shown that CIS3 and JAB were induced by GM-CSF in UT7 cells (Masuhara et al., 1997). Sengupta et al. reported that pre-incubation of cells with GM-CSF interrupts IL6-dependent STAT3 activation in blood mononuclear cells and monocytes (Sengupta et al., 1996). Interestingly, their results suggest that inhibition of STAT3 is caused by the suppression of JAK1 kinase activity. Moreover, this inhibition requires new RNA and protein synthesis. Therefore, JAB, CIS3 or other CIS family members are good candidates for a factor that contributes to such inhibition.

It is also quite interesting that the level of JAB is very high in IL2-dependent CTLL2, since it has been shown that exogenously expressed EPO receptor and G-CSF receptor are not functional in this cell line (Fukunaga et al., 1991; Yamamura et al., 1992). As expected, CTLL2 cells were also highly resistant to LIF and IFNγ-mediated growth arrest (Sakamoto et al., unpublished data). The molecular basis of such a selective inhibition of particular cytokine receptor signaling has not been elucidated. Although EPO does not induce tyrosine phosphorylation of JAK2 in CTLL2 cells expressing exogenous EPO receptor (Wakao et al., 1995), the reason why CTLL2 still can respond to IL2 is not clear at present. JAB may have a lower affinity to JAK3 than to JAK1 and JAK2, thereby allowing activation of IL2 signaling but not EPO or G-CSF signaling.

The molecular mechanism of differentiation and growth arrest of M1 cells has been extensively studied. Dominant negative forms of STAT3 block IL6-induced differentiation and growth arrest of M1 cells (Nakajima et al., 1996; Minami et al., 1996) and gp130 mutants which do not activate STAT3 cannot induce differentiation (Yamanaka et al., 1996). These findings suggest an essential role for STAT3 in terminal differentiation and growth arrest of M1 cells. In particular, long term activation of STAT3 well correlates with induction of terminal differentiation (Nakajima et al., 1996; Yamanaka et al., 1996). However, the molecular mechanism of growth arrest and apoptosis induced by STAT3 has not been elucidated. IRF-1 has been shown to play an important role in cell growth and apoptosis (Tanaka et al., 1996; Tamura et al., 1995). The growth inhibition by IL-6 or LIF is partially abrogated via the use of IRF-1 antisense oligomers in M1 cells (Abdollahi et al., 1991). Induction of cdk inhibitor p19^{INK4D} has also shown to play an important role in

IL6/STAT3-dependent growth arrest of M1 cells (Narimatsu et al., 1997). In M1 transformants expressing JAB and CIS3, IL6 only slightly induced IRF-1 and p19^{INK4D}, confirming the importance of these genes in growth arrest. On the other hand, repression of c-myc and c-myb has been implicated in differentiation but not growth arrest of M1 cells (Hoffman-Liebermann and Liebermann, 1991). In CIS3 transformants, repression of c-myc and c-myb occurred normally but growth arrest did not occur, which is consistent with this notion. Similar phenomena, block of IL6-induced growth arrest but repression of c-myc and c-myb, were observed in M1 transformants expressing a dominant negative STAT3 which lacks C-terminal transactivation domain (Minami et al., 1996). In these cases, endogenous STAT3 may not be completely blocked by the dominant negative STAT3 or CIS3, allowing repression of c-myc and c-myb. Partial inhibition of STAT3 seems to be sufficient for blocking terminal differentiation and growth arrest of M1 cells (Nakajima et al., 1996; Minami et al., 1996). Since CIS3 but not JAB is highly expressed in monocytes (Suzuki et al., unpublished data), modulatory action of CIS3 on IL6-signaling may be important for monocyte function and/or differentiation.

Materials and methods

Cells

293 cells were cultured in DMEM containing 10% calf serum. M1 cells were cultured in DMEM containing 10% horse serum. M1 transformants were maintained in the presence of 0.5 mg/ml G418. Other hematopoietic cells were maintained as described (Masuhara et al., 1997).

Luciferase assay

IL6 responsive APRE promoter-luciferase reporter gene has been described previously (Endo et al., 1997; Nakajima et al., 1996). Plasmids of reporter genes (0.1 μg/transfection) with either vector alone or indicated amount of pcDNA3-Myc-CIS3 or JAB were introduced into 293 cells in six well-dishes using the calcium-phosphate method. In addition, 0.1 μg of plasmid pCH110 encoding the β-galactosidase gene under the control of the SV40 promoter was included in each transfection as an internal control. The DNA was adjusted to 1.0 μg with empty pcDNA3. After 48 h, the cells were stimulated with 100 ng/ml human IL6 or 10 ng/ml human leukemia inhibitory factor (LIF) (PeproTech) for 6 h. Cell extracts were prepared and luciferase activity was measured as described (Matsumoto et al., 1997).

Effect of CIS3 and JAB on GST-JAK2 tyrosine kinase activity in 293 cells

Glutathione-S transferase (GST)-JAK2 JH1 (GST-JH1) was created by subcloning the fragment coding the JAK2 JH1 domain into pGEX-4T1, then the cDNA encoding the entire fusion protein was further subcloned into pcDNA3 (Masuhara et al., 1997). Plasmids for pcDNA3-GST-JH1 (5 μg/transfection), and Myc-tagged CIS3 or JAB in pcDNA3 at indicated amounts were transfected into 293 cells grown in 10-cm dishes using the calcium phosphate co-precipitation method. After 48 h, cell extracts were precipitated with GSH-Sepharose (Pharmacia) and analysed by immunoblotting with anti-PY (4G10), anti-Myc (9E10) and anti-GST as described (Masuhara et al., 1997).

M1 transformants and IL6 or LIF-signaling assays

M1 transformants were obtained by electroporation with pcDNA3 carrying Myc tagged full length CISs and selection with 0.8 mg/ml G418. Two to four independent clones were tested for LIF, IL6 or IFNγ-induced growth arrest. Briefly, 1×10^5 parental M1 cells and transformants expressing full length CISs were cultured in medium containing 10% horse serum supplemented without, or with the indicated amount of IL6, LIF or IFNγ for 5 days, then the viable cells were determined by trypan blue exclusion and counted in a hemocytometer. Immunoblotting with anti-phosphotyrosine, anti-STAT3 (C20; Santa Cruz) or anti-tyrosine-phosphorylated STAT3 (New England BioLabs) was performed as described (Yoshimura *et al.*, 1995; Endo *et al.*, 1997), after cells were stimulated with either saline or 100 ng/ml IL6 for 15 min – 18 h at 37°C. Electrophoretic mobility shift assay (EMSA) was carried out as described (Fujii *et al.*, 1995). Immunoprecipitation with anti-gp130 (Santa Cruz) from 2×10^7 cells and immunoblotting with anti-phosphotyrosine were performed as described (Yoshimura *et al.*, 1995; Endo *et al.*, 1997).

Northern hybridization

Parental M1 and transformants were stimulated with 100 ng/ml IL6, 10 ng/ml LIF, 1000 unit/ml murine IFNβ (TORAY Research Lab., Japan) or 1000 unit/ml murine IFNγ (Hayashibara Biochemical Lab., Japan) for indicated periods. For Northern blotting, total RNA (5 μg) was separated on 1.0% agarose gels containing 2.4% formaldehyde, then transferred to positively charged nylon membranes. Probe cDNAs and hybridization have been described previously (Matsumoto *et al.*, 1997; Yamanaka *et al.*, 1996). Glyceraldehyde-3 phosphate dehydrogenase (G3PDH) was used as internal control.

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