Cloning and Characterization of Novel CIS Family Genes

Masaaki Masuhara,* Hiroshi Sakamoto,* Akira Matsumoto,* Ritsu Suzuki,*† Hideo Yasukawa,* Kaoru Mitsui,* Toru Wakioka,*† Shyu Tanimura,*† Atsuo Sasaki,* Hiroyuki Misawa,* Masahiro Yokouchi,*† Motoaki Ohtsubo,* and Akihiko Yoshimura*1

*Institute of Life Science, Kurume University, Aikawa-machi 2432-3 Kurume 839, Japan; and †Department of Orthopaedic Surgery and ‡Department of Surgery, Faculty of Medicine, Kurume University, Asahi-machi, Kurume 830 Japan

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We have reported two JAK-signaling modulators, CIS (cytokine-inducible SH2 protein) and JAB (JAK2 binding protein), which are structurally related. Here we cloned three additional CIS family genes (CIS2, CIS3, and CIS4) on the basis of an expression sequence tag (EST) database search. We also found at least two additional candidates of this gene family in the database. These genes were induced by erythropoietin and granulocyte-macrophage colony stimulating factor in certain hematopoietic cell lines. The SH2 domain and a C-terminal 40 amino acid region, designated the CIS homology domain (CH domain), are highly conserved in this family, while the N-terminal regions of these proteins share little similarity. A yeast two-hybrid assay and in vitro and in vivo binding assays revealed that in addition to JAB, CIS3 bound to the JAK2 tyrosine kinase domain (J H1), although the interaction of CIS3 with the JAK2-J H1 domain was much weaker than that of JAB. Transient expression of JAB and CIS3, but not other CISs, strongly inhibited leukemia inhibitory factor (LIF)-induced STAT3-reporter gene activation in 293 cells. Furthermore, constitutive overexpression of JAB and CIS3 in M1 leukemia cells prevented LIF-induced differentiation and growth arrest. Although the physiological function remains to be investigated, CIS family genes could play a role in the negative regulation of cytokine signaling by interacting with specific targets.

Key Words: JAK; STAT; cytokine; CIS; J AB; SH2.

Growth, differentiation and functions of immune and hematopoietic cells are controlled by multiple cytokines, including interleukins (ILs) and colony stimulating factors (CSFs) [1]. 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 [2]. Among these proteins are members of the STAT family of transcription factors [3]. The tyrosine-phosphorylated STATs form homodimers 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 [4].

Several factors seem to be involved in regulation of the JAK-STAT pathway. For example, cAMP, calcium ionophore, and granulocyte-macrophage colony stimulating factor (GM-CSF) have been shown to inhibit JAK1-STAT1 or STAT3 pathways [5], although the molecular basis of such modulation has not been well characterized. Tyrosine phosphatases containing SH2 domain also regulate cytokine signaling, both positively and negatively [6, 7]. Degradation of STATs and receptors should be involved in regulation of signaling [8, 9]. Naturally occurring dominant negative variants of STAT5 have also been reported [10]. We cloned a cytokine-inducible SH2 protein, CIS [11]. The CIS gene is a direct target of STAT5, and its product tightly binds to the tyrosine-phosphorylated IL3 receptor and erythropoietin (EPO) receptor. It partially suppresses STAT5 activation, probably by masking STAT5 docking sites on the receptor [12]. Thus, CIS acts as a kind of negative feedback regulator of the JAK-STAT5 pathway. We and others recently cloned another CIS family member, JAB, which directly binds to the JAK2 tyrosine kinase domain and inhibits JAKs tyrosine kinase activity [13, 14, 15]. Overexpression of JAB resulted in suppression of all cytokine signaling utilizing JAKs. By
a database search we found 5 additional member of this family. Expression of these genes was induced by a subset of cytokines, of which one (CIS3) showed inhibitory action against JAK signaling similar to JAB. CIS3 also bound to JAK2 tyrosine kinase domain. The discovery of the CIS family suggests the presence of a complicated regulatory system in the JAK signaling pathways, and an important regulatory role for CISs in the action of cytokines.

MATERIALS AND METHODS

Cells. 293 cells were cultured in DMEM containing 10% calf serum (CS). Mo7e, UT-7 (from Dr. Komatsu, Jichi Medical School) and F36E [16] were cultured in RPMI containing 10% fetal calf serum (FCS) in the presence of 1 ng/ml GM-CSF (for Mo7e and UT-7) or 1 unit/ml EPO (for F36E). M1 cells were cultured in DMEM containing 10% horse serum.

Two-hybrid analysis. To detect interaction of CIS family members (CISs) with receptors and tyrosine kinases, L40 yeast strain was transformed with pBTM116 carrying CISs cDNA and pGAD424 carrying indicated cDNAs and selected in medium lacking tryptophan and leucine. Full length JAB, CIS1, CIS2, and CIS3 were cloned into pBTM116. Since the CIS4 N-terminal region itself expressed in L40 induced β-galactosidase expression, N-terminal truncated CIS4 (codon 331-355) was used for bait. To examine the interaction between c-kit and CISs, the tyrosine kinase domain of c-kit was cloned in pBTM116 [17] and CISs were cloned in pGAD424. The regions used for two-hybrid assay are as follows; the EPO receptor cytoplasmic domain (codon 313-483), the IL2 receptor beta chain (codon 322-551), tyrosine kinase domains for JAK2 (codon 839-1127), lck (codon 254-514), c-kit (E814V; [18]), the FGF receptor (codon 384-736) and PYK2 (codon 169-1009). Transfomers were restreaked on a paper filter and stained with β-galactosidase assay.

Database search and cloning of CIS related genes. The nucleotide and predicted amino acid sequences of mouse JAB and CIS cDNA were compared to EST databases using the BLASTN algorithm [19]. The cDNA clones of the human CIS2 (accession number; AA033687 and AA115239), CIS3 (AA018793), and CIS4 (N39810) were obtained from Genomesystems Inc. (CA, USA). The 5' regions of CISs were obtained by rapid amplification of cDNA ends (RACE)-PCR using poly A (+) RNA from Mo7e cells using the 5'-RACE kit from Gibco-BRL. Sequencing was performed using an ABI automated sequencer according to the manufacturer’s instructions. Partial human CIS5 (AA284558, AA233668, and AA233607) and CIS6 (AA220816, AA054102, AA127708, RB1093) sequences were predicted from ESTs after correcting possible errors.

Binding of CIS3 to JAK2 H1 in vivo and in vitro. To create the Myc-tagged CISs (Myc-CISs), the PCR fragment of the full-length coding region of each CIS was subcloned into EcoRI/XhoI digested pcDNA3 carrying Myc-AB [13]. The resulting fusion protein contained Myc-tag (MEKLISEEDLNE, repeated five times) at the N-terminus.GST-JAK2 H1 (GST-J1); GST-J1 was created by subcloning the fragment coding the JAK2 J1 H1 domain into pGEX-4T1, then the cDNA encoding the entire fusion protein was further subcloned into pcDNA3. For the in vivo binding assay, GST-J1 cDNA in pcDNA3, and Myc-tagged CISs in pcDNA3 were transiently expressed in 293 cells grown in 10-cm dishes using the calcium phosphate co-precipitation method. Cell extracts were precipitated with GSH-Sepharose (Pharmacia) and analyzed by immunoblotting with anti-PY (4G10), anti-Myc (9E10) and anti-GST.

Luciferase assay. Leukemia inhibitory factor (LIF) responsive promoter-luciferase reporter gene, EPO-responsive CIS-promoter-luciferase assay, and β-galactosidase gene under the control of the SV40 promoter were included in each transfection as an internal control. The EPO receptor and STAT5 cDNA was also included for EPO-responsive CIS promoter assay. The DNA was adjusted to 10 μg with empty pcDNA3. After 48 h, the cells were stimulated with 10 ng/ml LIF (PeproTech), 10 U/ml EPO or 50 ng/ml basic FGF for 12 h. Cell extracts were prepared and luciferase activity was measured as described [12].

RESULTS

Cloning of Novel CIS Family Members

We cloned two novel SH2 proteins, CIS and JAB which can potentially modulate cytokine signaling. These two proteins were structurally related, suggesting the presence of a novel family which is involved in cytokine signaling. From a nucleotide sequence database search, we found five additional members of the CIS family, the full length cDNA of three of which was cloned. The original CIS was designated as CIS1. Fig.1 shows alignment of the predicted amino acid sequences of human CIS family members (CISs). While the N-terminal regions share little sequence similarity, all seven contain SH2 domain and a conserved C-terminal region that we designate as the CH (CIS-homology) domain. The consensus motif in the CH domain is LXXPXXRX5SLQHLCRXX1X6glX55LXXLXXYY/F(X; any amino acid), although the entire length of the C-terminal region was variable. The SH2 domain exhibits 23-58% identity among CISs. Relatively high identity was seen between CIS1 and CIS2 (about 50%), and between CIS4 and CIS5 (about 58%), and JAB and CIS3 (about 40%). The N-terminal region is only 40-80 amino acids in length except for CIS4 which has quite a long N-terminal region (about 380 amino acids).

The function of CH domain is not clear at present. However, database search revealed that a similar motif is present in the C-terminal region of the M60.7 gene product of C. elegans which contains ankyrin-like re-
FIG. 1. Amino acid sequence alignments of the seven human CIS family genes. Black boxes indicate positions at which at least four amino acids are identical, and gray boxes indicate those at which three amino acids are identical. Partial amino acid sequences of CIS5 and CIS6 are predicted from EST and lack the N-terminal region. Part of the SH2 domain of CIS6 was predicted from mouse gene.

Expression of CIS Family Members and Induction by Cytokines

We examined expression of CIS family genes in various hematopoietic cell lines by Northern hybridization (Fig. 2A). At an exponentially growing stage, CIS1-CIS4 and JAB were expressed in GM-CSF-dependent myeloid leukemic cells (Mo7e, UT-7 and TF-1). Expression levels of CIS1, CIS2, CIS3 and CIS4 were high in factor-independent chronic myelogenous leukemia (CMK) and human erythroleukemia (HEL) cell lines, but low in B cell lines, Raji and Daudi, monocytic leukemia cell line, U937 and myeloid cell line, HL60. JAB was relatively strongly expressed in B cell lines (Raji and Daudi), as well as GM-CSF-dependent leukemia cell lines, Mo7e, UT-7, and TF-1, but weakly so in HEL, CMK, U937 and HL60 cells. The high level of expression of CISs in cytokine-dependent hematopoietic cells
FIG. 2. Expression of CISs mRNA in various cell lines (A) and induction by GM-CSF and EPO (B). In A, total RNA from indicated cell lines (5 μg/lane) at exponentially growing stage was hybridized with the human CISs probe or control G3PDH probe. Mo7e, UT7 and TF-1 cells were cultured in the presence of 1 ng/ml GM-CSF. In B, UT-7 and F36E cells were factor-depleted for 12 h and then stimulated with 5 ng/ml GM-CSF (for UT-7) or 10 unit/ml EPO (for F36E) for indicated periods.

suggests their role in cytokine signaling. CIS3 was expressed in non-hematopoietic cells, hepatoma cell line, HepG2, and osteosarcoma cell line, HOS, while CIS1, CIS2, CIS4 and JAB were not.

To elucidate whether CISs are really cytokine-inducible genes, their expressions were examined in GM-CSF dependent UT-7 cells and EPO-dependent F36E cells. Cells were factor depleted for 12 h, then stimulated with GM-CSF or EPO. As shown in Fig. 2B, ex-

FIG. 3. Two-hybrid analysis of CISs. Yeast strains carrying LexA binding domain-CIS fusion gene were transformed with plasmids carrying a fusion between the GAL4 activating domain and the cytoplasmic domain of the IL2 receptor β chain (IL2R), the EPO receptor (EPOR), JAK2-JH1 domain, full length Ick (Ick-full), tyrosine kinase domain of Ick (Ick-kinase), the FGF receptor (FGFR), and PYK2. For c-kit, tyrosine kinase domain of c-kit (E814V) was fused to the binding domain and CISs were fused to the activation domain. Transformants were restreaked on a filter paper and stained by in situ β-galactosidase assay.

FIG. 4. Interaction between JAK2-JH1 and CISs in vitro. In A, empty vector (lanes 1 and 7) or plasmids encoding Myc-epitope tagged CIS1 (lanes 2,8), CIS2 (lanes 3,9), CIS3 (lanes 4,10), CIS4 (lanes 5,11) or JAB (lanes 6,12) were transiently expressed in 293 cells, and the cell extracts were incubated with 1 μg of purified GST-JH1 immobilized on GSH-Sepharose. Total cell extract (50 μg/lane)-(lanes 1-6, input) or the protein bound to the beads (lanes 7-12, bound) was resolved on 10% SDS-PAGE, which was then blotted and analyzed with anti-Myc antibody.
FIG. 5. Interaction between GST-JH1 and CISs in vivo. Empty vector (lanes 1), GST-JH1 in pcDNA3 alone (lanes 2, 8) or pcDNA3-GST-JH1 plus pcDNA3 carrying Myc-epitope tagged CIS1 (lanes 3, 9), CIS2 (lanes 4, 10), CIS3 (lanes 5, 11), CIS4 (lanes 6, 12) or JAB (lanes 7, 13) were transiently expressed in 293 cells, and total cell extracts (lanes 1-7; input) or proteins bound to GSH-Sepharose (lanes 8-13; bound) were resolved by SDS-PAGE, then immunoblotted with anti-phosphotyrosine (PY), anti-Myc, and anti-GST. Five μg pcDNA3-GST-JH1 and different amounts of plasmid carrying each CIS were used to yield similar levels of expression and described in Materials and Methods.

Expression of all CISs was induced within 1-2 hour by GM-CSF in UT-7 cells and by EPO in F36E cells. Thus, like CIS1, other CISs including JAB seem to be cytokine-inducible genes. A published sequence of the 5’ flanking region of the JAB gene [21] contains several GAS motifs, suggesting that STATs are involved in induction of JAB message.

Interaction of CIS Family Members with JAK2 and Other Tyrosine Kinases in Yeast

Using two-hybrid system, we examined interaction of CISs with the EPO receptor cytoplasmic domain, IL2 receptor β chain cytoplasmic domain, full length lck, and tyrosine kinase domains of lck (lacking the C-terminal negative-regulatory tyrosine residue), constitutively active c-kit (E814V), the FGF receptor, and PYK2 (Fig.3). Although CIS1 binds to the tyrosine phosphorylated EPO receptor [11], it did not interact with the cytoplasmic domain of the EPO receptor in yeast. This suggests that tyrosine phosphorylation is essential for the high affinity interaction between the receptor and CIS1. Other CISs also did not interact with the cytoplasmic domains of the EPO receptor or IL2 receptor β chain. While CIS1, CIS2 and CIS4 did not interact with any tyrosine kinases, JAB as well as CIS3 bound to JAK2-JH1. Interaction of JAK2-JH1 with JAB was much stronger than that with CIS3 in this assay (Fig.3). CIS3 also bound to the tyrosine kinase domains of the FGF receptor and PYK2. Interestingly, JAB bound to active c-kit tyrosine kinase domain, while CIS3 did not. In contrast, CIS3 bound to lck, while JAB did not. These findings indicate that JAB and CIS3 can directly interact with tyrosine kinases, however, they have some preferences.

Binding of JAB and CIS3 with JAK2-JH1 in Vitro and in Vivo

To assess the binding of CISs to JAK2-JH1 domain in vitro, recombinant JAK2-JH1 domain fused to the glutathione-S transferase (GST) gene (GST-JH1) was produced in E.coli. Purified GST-JH1 was heavily tyrosine phosphorylated [5]. We also created GST-fusion protein of JH1 domains of JAK1, JAK3 and Tyk2, however, they were not phosphorylated in E.coli (data not shown). Myc epitope tagged CISs were produced in 293 cells and the cell extracts were incubated with GST-JH1 immobilized on GSH-Sepharose. To achieve similar levels of expression for each CIS gene, the amount of plasmid DNA transfected into 293 cells was carefully calculated (Fig.4; input). Since the expression level of JAB was always much higher than that of other CISs, about 10 times less plasmid carrying JAB cDNA was used. As shown in Fig.4, CIS3 and JAB but not CIS1, CIS2 or CIS4 bound to recombinant GST-JH1, although the binding of CIS3 was much less than that of JAB (compare lanes 10 and 12).
To examine the binding in intact cells, GST-JH1 cloned in a mammalian expression vector was transiently expressed into 293 cells with or without CISs. GST-JH1 caused higher levels of tyrosine phosphorylation of GST-JH1 itself and cellular proteins than full length JAK2 did (data not shown). Consistent with our previous report using full length JAK2 [11], phosphorylation of GST-JH1 and cellular proteins was blocked by co-expression of JAB but not by other CISs (Fig. 5, anti-PY, lanes 7,13), although the inhibition was dependent on the expression level of JAB. When GST-JH1 was precipitated with GSH-Sepharose, JAB and CIS3 were co-precipitated. Consistent with the two-hybrid (Fig.3) and in vitro binding (Fig.4) assays, CIS3 was much less well precipitated with JH1 than JAB was, indicating that the interaction of CIS3 with JH1 was much weaker than that of JAB.

Inhibition of JAK Signaling Pathway by JAB and CIS3

To investigate the biological effect of CISs on JAK signaling, we examined EPO-mediated STAT5 activation by using CIS promoter-reporter gene [12](Fig.6A) and LIF-mediated STAT3 activation by using a reporter gene construct containing IL6- and LIF- responsive APRE (acute phase responsive element) (Fig.6B). Reporter gene constructs were transfected into 293 cells with or without Myc-epitope tagged CISs [13]. As reported previously [12], CIS1 partially suppressed EPO-mediated STAT5 activation, while JAB and CIS3 almost completely blocked reporter gene activation. CIS2 and CIS4 rather enhanced the reporter gene activation.

LIF exerts its biological effect through activation of JAK1, JAK2 and TYK2, and consequent activation of STAT3. A marked increase in the luciferase activity was detected in response to LIF, however, co-expression of JAB and CIS3 almost completely suppressed LIF-induced reporter gene activation (Fig.6B). CIS1, CIS2 and CIS4 showed little effect on reporter gene activation. Since JAB and CIS3 bound to the FGF receptor in yeast (Fig.3), we examined the effect of CISs on FGF-dependent c-fos promoter activation (Fig.6C). JAB and CIS3 did not inhibit FGF-induced c-fos promoter activation, suggesting that tyrosine kinase signal-inhibition by JAB and CIS3 is specific to JAKs.

Finally, we have explored the effect of CIS expression in a system in which a long term biological response can be measured. LIF has shown to induce growth arrest and differentiation of M1 leukemia cells. Myc tagged CIS1, CIS2, CIS3 and JAB were stably expressed in M1 cells (Fig.7A). The expression level of JAB was a little higher than that of other CISs, which is consistent with the results of the transient expression experiments in 293 cells. Transformants expressing JAB and CIS3, but not CIS1 and CIS2 were resistant to LIF-mediated growth inhibition (Fig.7B). Morphologically, cells expressing JAB or CIS3 were similar to untreated M1 cells, while LIF-treated parental M1 cells or transformants expressing CIS1 or CIS2 became large and vacuolated (data not shown). These results indicate that not only JAB but also CIS3 inhibited the
FIG. 7. Inhibition of LIF-induced differentiation and growth arrest of M1 cells by JAB and CIS3. In A, total cell extracts from parental M1 cells (parent) and transformants expressing CIS1, CIS2, CIS3, and JAB were immunoblotted with anti-Myc. In B, 1×10⁵ parental M1 cells and transformants were cultured in medium containing 10% horse serum supplemented without, or with indicated concentrations of LIF for 5 days, then viable cells were scored. Similar results were obtained for at least two independent clones for each transformation.

DISCUSSION

In this study we describe a family of novel proteins containing SH2 domain. The first member reported was CIS1, which is cloned as an immediate early gene induced by various cytokines. The second was JAB, cloned as a binding protein to the catalytic domain of JAK2 by yeast two-hybrid system. A database search extended the size of this gene family. Taking into consideration that CIS1 was the first to be identified, expression of CIS1,2,3,4 and JAB was induced by GM-CSF and EPO in certain cell lines, and all of the proteins contain SH2 domain, we propose that this family is called the CIS gene family. At present, the family contains seven members, although the number will probably increase in the near future, because we have already found several CH domain-containing EST sequences.

Since the completion of this study, two other groups have reported on JAB[14, 15]. One referred to it as SOCS-1 (Suppressor of Cytokine Signaling-1) and also reported on related molecules, SOCS-2 and SOCS-3 [14]. SOCS-1 was cloned by its ability to inhibit the macrophage differentiation of M1 cells in response to IL6. SOCS-2 and SOCS-3 were cloned by database search. SOCS-2 is identical to CIS2, and SOCS-3 to CIS3, although the SOCS genes are of mouse origin while ours are human. The function of SOCS-2 and SOCS-3 was not analyzed, however, it was reported that these two genes were induced by IL6 in liver, and by IFN-γ, EPO, GM-CSF, and IL3 in bone marrow cells. These findings are consistent with our observation that CISs were induced by GM-CSF and EPO in UT-7 and F36E cells.

We have reported that CIS1 participates in negative feedback regulation of JAK-STAT5 pathway by binding to the STAT5-binding region of the EPO receptor [11, 12]. However, the effect of CIS1 on STAT5-inhibition is partial and CIS1 does not affect other cytokine signaling such as IFN-α and LIF, which do not utilize STAT5. Among other CISs we examined, overexpression of JAB and CIS3 exhibited remarkable biological activity. They directly interacted with JAK2 JH1 kinase domain and inhibited EPO-, and LIF-mediated signaling. Direct inhibition of JAK kinases by JAB can explain why JAB inhibits so many cytokine signals utilizing different receptors and STATs. CIS3 can also inhibit a wide range of cytokine signals, however, did not efficiently inhibit JAK5 kinase reaction in 293 cells, although it weakly bound to the kinase (Fig.5 and Masuhara et al., unpublished data). Despite of extensive studies, we could not obtain any evidence suggesting the interaction between CIS3 and STAT5 or receptors. Thus, the inhibition mechanism of cytokine signaling...
by CIS3 remains open to question. The expression level we used in 293 and M1 cells may be sufficient to inhibit endogenous JAK kinase activity. However, more interesting possibility is that CIS3 has its own target other than JAKs. Src-like kinases or unidentified tyrosine kinases associated with the receptor might be a target of CIS3, since CIS3 but not JAB strongly interacts with Ick. Many reports indicate that src-like kinases in addition to JAKs are activated by various cytokines, although their function has not been darified. These src-like kinases may be important for full activity of STATs. Search of molecules which interact with CIS3 in response to cytokine stimulation may uncover an unidentified pathway which is essential for JAK-STAT signaling.

Since CIS1, CIS3 and JAB are induced by cytokines and negatively regulate cytokine signals, these genes act in a classic negative loop for cytokine signal transduction. The genes could also be involved in cross-talk among cytokine signals. CISs induced by one cytokine can modulate the intensity of other cytokine signals. For example, GM-CSF inhibits IL6- or IFNγ-dependent STATs activation [5]. This inhibition requires new DNA and protein synthesis, thus, CIS family members are good candidates for the factor that contributes to the inhibition. Some CIS family members may positively participate in cytokine signaling, because CIS4 possessed an N-terminal region large enough to create an unidentified signal. From the elucidation of CIS1, CIS3 and JAB, we believe that each CIS binds to its own target through SH2 domain. Finding of the target molecule is important for uncovering the physiological function of each CIS gene.

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