CIS3/SOCS-3 suppresses erythropoietin signaling by binding the EPO receptor and JAK2

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Running title: Molecular mechanism of EPO signal inhibition by CIS3/SOCS3.
Abstract

The cytokine-inducible SH2 protein-3 (CIS3/SOCS-3/SSI-3) has been shown to inhibit the JAK/STAT pathway and act as a negative regulator of fetal liver erythropoiesis. Here, we studied the molecular mechanisms by which CIS3 regulates the erythropoietin (EPO) receptor (EPOR) signaling in erythroid progenitors and Ba/F3 cells expressing the EPOR (BF-ER). CIS3 binds directly to the EPOR as well as JAK2 and inhibits EPO-dependent proliferation and STAT5 activation. We have identified the region containing Y401 in the cytoplasmic domain of the EPOR as a direct binding site for CIS3. Deletion of the Y401 region of the EPOR reduced the inhibitory effect of CIS3, suggesting that binding of CIS3 to the EPOR augmented the negative effect of CIS3. Both N- and C-terminal regions adjacent to the SH2 domain of CIS3 were necessary for binding to EPOR and JAK2. In the N-terminal region of CIS3, the amino acid G45 was critical for binding to the EPOR but not to JAK2, while L22 was critical for binding to JAK2. The mutation of G45A partially reduced ability of CIS3 to inhibit EPO-dependent proliferation and STAT5 activation, while L22D mutant CIS3 was completely unable to suppress EPOR signaling. Moreover, overexpression of STAT5, which also binds to Y401, reduced the binding of CIS3 to the EPOR, and inhibitory effect of CIS3 against EPO signaling, while it did not affect JAB/SOCS-1/SSI-1. These data demonstrate that binding of CIS3 to the EPOR augments the inhibitory effect of CIS3. CIS3 binding to both EPOR and JAK2 may explain a specific regulatory role of CIS3 in erythropoiesis.

Keywords: CIS3/EPO/EPOR/JAK2/SOCS-3
Introduction

Erythropoietin (EPO) is a glycoprotein hormone required for the survival, proliferation and differentiation of committed erythroid progenitor cells. The EPO receptor (EPOR) belongs to the cytokine receptor superfamily which includes receptors for other hematopoietic growth factors such as interleukins (ILs), colony-stimulating factors (CSFs), and growth hormone (GH). A family of protein tyrosine kinases, known as Janus kinases (JAKs), has been shown to associate with cytokine receptors and play an important role in cytokine-dependent cell proliferation and regulation of gene expression. EPO induces receptor dimerization, which leads to the activation of JAK2 and rapid tyrosine phosphorylation of the cytoplasmic domain of the EPOR. Subsequently, various signaling proteins including STAT5 (signal transducer and activator of transcription-5) are recruited to the receptor complex, which ultimately regulates gene expression in the nucleus (1-4).

At least three distinct stages of erythropoiesis have been defined in embryonic development. The first stage, primitive erythropoiesis, occurs in the blood islands in the yolk sac at approximately day 7 and does not require EPO (5). The second stage, definitive erythropoiesis, starts at around day 10 of gestation, and a drastic expansion of erythroid lineage cells occurs in the fetal liver, which is highly dependent upon EPO, the EPO receptor, and JAK2 (6, 7). Finally, hematopoietic stem cells move to the bone marrow, where adult-type erythropoiesis begins. Adult erythropoiesis was suggested to be negatively regulated by tyrosine phosphatase SHP-1 (8).

In addition to the EPO-EPOR-JAK2 system, a number of genes have been involved in regulating erythropoiesis, which include a variety of transcription factors (9, 10), c-kit tyrosine kinase (11) and various apoptosis regulators such as FADD (13), and Caspase-8 (14). We have recently demonstrated that CIS3 acts as a negative regulator of fetal liver erythropoiesis (15).

CIS3 belongs to the CIS (for cytokine-inducible SH2-protein) or SOCS (for suppressor of cytokine signaling) or SSI (for STAT-induced STAT inhibitor) family, which has been shown to play a regulatory role in the signal transduction of a variety
of cytokines (16-21). Initially, the first member of this family, CIS1, was cloned as an immediate early gene induced by a number of cytokines including EPO, IL-2, IL-3, and granulocyte macrophage-colony-stimulating factor (GM-CSF) (22). CIS1 tightly binds to tyrosine-phosphorylated IL-2, IL-3, and EPO receptors and negatively regulates their signals when overexpressed (22-25). Subsequently, the second family member was independently cloned by three groups and is identified as JAB, SOCS-1, or SSI-1 (26-28). CIS1 and JAB were structurally related in both the SH2 domain and the C-terminal region (CH domain/SOCS box), and a database search identified additional CIS family members (29-31). Among these members, CIS3 is most similar to JAB. Both CIS3 and JAB bind and inhibit the JAK/STAT pathway in cells treated with EPO, GH, leptin, leukemia inhibitory factor (LIF), IL-2, IL-6, interferons (IFNs) or prolactin (PRL) (32-37). Mutational analysis and biochemical characterization of CIS proteins revealed a novel type of inhibition of tyrosine kinase activity. Two independent binding sites of JAB and CIS3 were identified: the N-terminal kinase inhibitory region (KIR) bound to the catalytic groove of the kinase (JH1) domain and the SH2 domain bound to the critical tyrosine residue in the activation loop of the kinase (38-40). Although CIS3 and JAB had similar inhibitory effects on cytokine signaling in vivo, the affinity of CIS3 for binding to JAK2 was much weaker than that of JAB, and CIS3 hardly inhibited JAK2 kinase activity in vitro (38, 39). These data suggest that the mechanisms of action between CIS3 and JAB are different.

Here we found that CIS3 bound to the EPOR as well as JAK2 in erythroid progenitors and Ba/F3 cells expressing the EPOR. The SH2 domain of CIS3 was essential for binding to the EPOR as well as JAK2. Binding of CIS3 to JAK2 was prerequisite for EPO signal inhibition, while binding to the EPOR augmented the negative effect of CIS3 on EPO signaling such as EPO-dependent proliferation of Ba/F3 cells and JAK2-STAT5 activation. CIS3 binding to both EPOR and JAK2 may explain the similar inhibitory effects between CIS3 and JAB on the JAK2 signaling in living cells.
Materials and methods

**Luciferase assay**

The STAT3 and STAT5 responsive promoter-luciferase reporter gene, which carries four repeats of the GAS sequence with the jun promoter, has been described previously (29). EPO-dependent luciferase activity in 293 cells transfected with Myc- or Flag-tagged wild-type or mutant CIS3 cDNA was measured as described (29, 36, 40). Ras-MAP kinase activity was measured using Elk-1 reporter assay (PathDetect in vivo signal transduction pathway trans-reporting system, STRATAGENE). Briefly, 293 cells were transfected with 0.05ug of pFA-Elk1, 0.1ug of pFR-Luc, and 0.2ug of pCH110 encoding the β-galactosidase, and Flag-tagged CIS3 or JAB cDNA. After 24h, cells were treated with 10ng/ml EGF for 4h, then luciferase activity was measured. In all reporter assays, 1x10^5 of 293 cells were plated on 6 well dishes and transfected by calcium phosphate method.

**Plasmids and constructs**

CIS3 mutants were generated by the standard PCR method, as described previously (39). Wild-type CIS3 and mutants were subcloned into pcDNA3 for Myc epitope tagging, pCMV2 for Flag epitope tagging (39), or pMX-IRES-EGFP for retroviral gene transfer (41). The wild-type and mutant EPOR cDNAs were subcloned in pXM, as described previously (42).

**Expression of CIS3 in CFU-E-enriched splenocytes and Ba/F3 cells**

CFU-E-enriched splenocytes were obtained according to the procedure of Spangler and Sytkowski (43). Briefly, C57BL/6 mice were injected subcutaneously on day 1 with a sterile solution of phenylhydrazine/ml in PBS to achieve a dose of 60 mg/kg. The injection was repeated on day 2, and the mice were killed on day 5 by cervical dislocation. The enlarged spleens were excised, and isolated splenocytes were added cold ammonium chloride solution, then washed with RPMI-2%FCS twice.
After being incubated in RPMI-10%FCS for 6 h, cells were stimulated with 20 unit/ml EPO for indicated periods. Approximately 5X10^7 cells/sample were used for immunoprecipitation with 10 μl of anti-SOCS3 obtained from Santa Cruz Biotechnology (M-20, goat), then immunoblotted with anti-CIS3 from Immuno-Biological Laboratories CO., LTD., Fujioka, Japan (anti-C, rabbit). Ba/F3 transformants stably expressing the EPO receptor (BF-ER) (44) were maintained in 1 unit/ml EPO. BF-ER cells (1x10^7/sample) were cultured without EPO for 8 h and then stimulated with 20 unit/ml EPO for 2 h in the presence of 50 μM MG132 (PEPTIDE Institute Inc., Osaka, Japan), a proteasome inhibitor, and 0.1 mM sodium vanadate. Cells were lysed in buffer A (20 mM Hepes, pH 7.3, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM DTT, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin) containing 25 μM MG132 and then centrifuged. Total cell extracts and immunoprecipitates with 10 μl of anti-SOCS3 were analyzed by immunoblotting with anti-PY, anti-JAK2 (HR-758; Santa Cruz Biotechnology), and rabbit anti-CIS3 antibodies as described previously (39).

In the re-immunoprecipitation experiment, the immunoprecipitates with goat anti-SOCS3 (Santa Cruz Biotechnology; M-20) or 20 μl of goat anti-LexA (D-19) were boiled in a buffer containing 125 mM Tris (pH 6.8), 20% glycerol, 20 mM DTT and 4% SDS and then diluted 40-fold with buffer B (50 mM Tris, pH6.8, 1% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate). The supernatant was re-immunoprecipitated with anti-EPOR or anti-JAK2 antibodies. The immunoprecipitates were analyzed by immunoblotting with anti-PY antibody.

**Northern hybridization**

Cells were stimulated with or without EPO 10U/ml for indicated periods. For Northern blotting, total RNA (20 μg) was separated on 1% agarose gels containing 2.4% formaldehyde and then transferred to positively charged nylon membranes. After fixation under calibrated ultraviolet irradiation, the membranes were hybridized with DIG-labeled riboprobes and visualized using alkaline-phosphatase-labeled
anti-DIG antibody according to the manufacturer’s instructions (Boehringer, Mannheim, Germany). Probe cDNAs for mouse CIS3 correspond to the full-length coding region. G3PDH have been described previously (36).

**Binding of CIS3 mutants to GST-EPOR or phosphopeptide in vitro**

The GST-EPOR cytoplasmic domain (codon 301-406), which contains two tyrosine residues (YY), or tyrosine-to-phenylalanine substitution mutants (FY, YF, and FF) at Y343 or Y401 was produced in TKB-1 bacteria (Stratagene, CA) and purified on GSH-Sepharose (Pharmacia-Amersham Biotech Japan, Tokyo) as described previously (45). The purified protein was dialyzed against 10 mM Tris-buffered saline (TBS) and concentrated to 2 mg/ml. Y401 and pY401 peptides of the EPOR were synthesized and coupled to AFFI-GEL 15 (BIO RAD) according to the manufacture’s instructions. pY1007 phosphopeptide conjugated to agarose has been described previously (40). Flag-tagged CIS3 constructs were transiently expressed in 293 cells grown in 10-cm dishes. Before the precipitation experiments, bovine serum albumin (BSA) was added to a final concentration of 5 mg/ml. Cell extracts were incubated with 20 µl (50% v/v) of phosphopeptide-conjugated agarose (40) or GST-EPOR-conjugated Sepharose, then washed extensively with buffer A, and finally analyzed by immunoblotting with anti-Flag (M2) antibody.

**EPO-induced JAK2 phosphorylation in 293 cells**

Plasmids for Flag-tagged JAK2, YF, or YY EPOR mutants and Flag-CIS3 were transfected into 293 cells. After 24-h transfection, cells were treated with or without 20 units/ml EPO for 10 min and lysed in 0.5 ml of lysis buffer A, followed by immunoprecipitation with 5 µl anti-Flag antibody. The cell extracts were analyzed by immunoblotting with anti-PY, anti-JAK2, and anti-Flag antibody.

**Retrovirus infection**

The CIS3 cDNA subcloned in pMX-IRES-EGFP was transfected into a
packaging cell line using the FUGENE6 (Boehringer Mannheim) to obtain the viruses. The substitution mutations of CIS3 were introduced into the dC40 backbone, since we could not achieve sufficient expression of full-length CIS3 in Ba/F3 cells for unknown reasons. Ba/F3 cells expressing the EGFR-EPOR chimeras were infected with viruses on a RetroNectin (TaKaRa) coated plate for 24 h in the presence of 1 ng/ml IL-3. Then, after being washed 3 times with PBS, cells were resuspended in RPMI-10% FCS containing either 10 ng /ml EGF or 1 ng /ml IL-3 and incubated for indicated periods. Infected cells expressing EGFP were analyzed by a fluorescence-activated cell sorter (FACS).
Results

**CIS3 binds to JAK2 and inhibits EPO-JAK2 signaling through KIR and SH2 domain**

We have shown that CIS3 binds to phosphorylated Y1007 of the JAK2 activation loop and inhibits JAK2 activation through the N-terminal KIR domain (39). The critical amino acids necessary for the tight binding to JAK2 and the inhibition of kinase activity were located in the KIR domain and were conserved between CIS3 and JAB (Fig. 1A). Here we examined the effect of expression of CIS3 and its mutants on EPOR phosphorylation and EPO-induced JAK/STAT pathway activation. Wild-type (WT) CIS3 inhibited tyrosine phosphorylation of the EPOR as well as STAT5 activation in response to EPO (Fig. 1B and 1C). On the contrary, CIS3 carrying a point mutation in either the KIR (F25A) or the SH2 domain (R71E) failed to inhibit EPO-induced receptor phosphorylation and STAT5 activation (Fig. 1C).

Suppression of EPO signaling by CIS3 is probably due to inhibition of JAK2 kinase activity, because phosphorylation of the EPOR was suppressed (Fig. 1B). To show direct inhibition of JAK2 activation, we co-expressed JAK2 and CIS3 in 293 cells. As shown in Fig. 1D, WT-CIS3 bound to JAK2 and inhibited autophosphorylation of JAK2, while F25A and R71E mutants did not. Mutation of other conserved amino acids in the KIR region (L22, E30, and Y31) also failed to inhibit EPO-induced receptor phosphorylation and STAT5 activation (data not shown). These data confirmed that both the KIR and SH2 domains are required for binding and inhibition of JAK2 signaling in response to EPO.

**CIS3 binds to the EPOR and JAK2**

Since CIS3 was indicated as a negative regulator in fetal erythropoiesis (15), we further investigated the precise mechanism of EPO signal inhibition by CIS3 using erythroid progenitor (CFU-E)-enriched splenocytes from phenylhydrazine-treated anemic mice. CFU-E-enriched splenocytes highly expressed CIS3 mRNA immediately after harvest, and the expression decreased by EPO starvation (Fig. 2A). EPO treatment rapidly induced the CIS3 mRNA to the level observed at the harvest.
We also detected endogenous CIS3 protein that was accumulated after EPO stimulation in the splenocytes (Fig. 2B-b, lanes 3,4). Two major tyrosine-phosphorylated proteins (130kDa and 72kDa) were co-precipitated with CIS3 (Fig. 2B-a: arrowheads). Similar 130kDa and 72kDa proteins were co-precipitated with endogenous CIS3 in Ba/F3 cells expressing the EPOR (BF-ER) after stimulation with EPO (Fig. 2C-a: arrowheads). The 130kDa and 72kDa proteins migrated at the same positions as JAK2 and the EPOR, and anti-JAK2 blot revealed that the 130kDa protein was phosphorylated JAK2 (Fig. 2D-b).

A re-immunoprecipitation experiment demonstrated that the 130kDa phosphoprotein was JAK2 (Fig. 2E, lane 4) and the 72kDa phosphoprotein was the EPOR (Fig. 2E, lane 3). We could not detect the EPOR in the CIS3 immunoprecipitates by immunoblotting with anti-EPOR antibody (Fig. 2D-c). This suggested that the sensitivity of our anti-EPOR antibody for immunoblotting was too low to detect such a low level of the EPOR. Co-precipitation of the EPOR with CIS3 does not seem to be due to binding of the EPOR to JAK2 because the phosphorylated EPOR was not co-precipitated with JAK2 (Fig. 2D-a, lane 6). These data indicate that CIS3 bound to the phosphorylated EPOR as well as JAK2 in both CFU-E and BF-ER cells.

**CIS3 binds to a region containing Y401 of the EPOR**

We next determined the CIS3 binding site in the EPOR cytoplasmic domain by using transient expression in 293 cells. CIS3 was found in a complex with the EPOR in unstimulated cells and EPO stimulation further enhanced co-precipitation of CIS3 with the EPOR (Fig. 3A, lane 4). Binding of CIS3 to the unphosphorylated EPOR (lane 3) suggests a weak affinity of CIS3 to the EPOR without phosphorylation. Such phosphorylation-independent binding was also observed in CIS1-EPOR interaction (Matsumoto et al. unpublished data). To determine the binding site of the EPOR for CIS3, we expressed the EPOR and CIS3 with JAK3 to introduce phosphorylation in the EPOR, since CIS3 had little effect on JAK3 kinase activity (37). Using different
truncation mutants of the EPOR, we could show that the deletion of the C-terminal 135 amino-acid (truncation Y) abrogated CIS3 binding to EPOR (Fig. 3B-lane3).

To characterize CIS3-EPOR interaction in more detail, we performed an in vitro binding assay using GST fusion proteins carrying the phosphorylated EPOR cytoplasmic domains (Fig. 3C). GST fusion protein was expressed in TKB-1 bacteria carrying an active tyrosine kinase, and fusion proteins were thereby tyrosine-phosphorylated (Fig. 3D-d). CIS3 did not bind to the GST or GST-Y truncation mutant but strongly bound to GST-FY (Y343F mutant) and GST-YY (Fig. 3D-a, lanes 2, 3). Interestingly, CIS3 also bound to GST-FF and GST-YF (Y401F mutant), although this binding was weaker than that to GST-FY and GST-YY. The interaction between GST-EPOR and CIS3 was dependent on the intact SH2 domain of CIS3, because disruption of the functional SH2 domain by point mutation (R71E) abrogated the interaction (panel b). We measured binding of phospholipase-Cγ (PLCγ) as a control. As shown in Fig. 3D-c, PLCγ bound to Y343 but not to Y401. In this case, binding was strictly dependent on the phosphorylation of Y343. In contrast, as shown in Fig. 3E, CIS3 bound to the tyrosine-phosphorylated 12 mer peptide containing Y401, but not to non-phosphorylated peptide. Thus, interaction of CIS3 with non-phosphorylated EPOR may require a wider region around Y401 or some specific conformation of the EPOR cytoplasmic domain.

Taken together, we have detected a weak interaction between the CIS3 SH2 domain and the non-phosphorylated Y401 region of EPOR. However, CIS3 SH2 domain binding to the EPOR was significantly increased following Y401 phosphorylation.

**Decreased inhibitory effects of CIS3 against EPO signaling by Y401F mutation of the EPOR**

To evaluate the functional role of CIS3-EPOR interaction in EPO-induced signaling, we compared the ability of CIS3 to suppress EPO-dependent JAK2 and STAT5 activation in cells expressing the YF (Y401F) and FY (Y343F) mutant EPORs.
293 cells were transfected with mutant EPORs, Flag-tagged CIS3, and STAT5 reporter gene. STAT5 activation by the FY mutant EPOR was suppressed more efficiently than by the YF mutant EPOR, suggesting that enhanced binding of CIS3 to phosphorylated Y401 contributed to the efficient suppression of EPO-induced STAT5 activation (Fig. 4a). We next compared the effect of CIS3 on the tyrosine phosphorylation of JAK2 through YF and YY mutant EPORs. As shown in Fig. 4B, EPO-dependent JAK2 phosphorylation was suppressed by CIS3, but the suppression was more profound in the YY EPOR than in the YF mutant EPOR. These data suggested that interaction between CIS3 and the EPOR enhanced the inhibitory effect of CIS3 against JAK2 kinase.

To evaluate the biological effects of CIS3-EPOR interaction further, we used retrovirus vectors carrying a bicistronic expression system of enhanced green fluorescent protein (EGFP) (41). Since the individual infected cells expressed both EGFP and CIS3, the population of infected cells could be monitored by FACS analysis. Retroviruses encoding dN10 or dN25 CIS3-IRES-EGFP were prepared by a packaging cell line and added to the Ba/F3 cells stably expressing a chimeric receptor bearing the extracellular domain of the EGF receptor linked to the whole cytoplasmic domain of the EPOR (EGFR/wtEPOR) (Fig. 5A). Ba/F3 cells expressing a chimera between the EGF receptor and the EPOR cytoplasmic domain lacking C-terminal 135 amino acids (EGFR/-135Y) were also created by stable transfection (42). The expression level of these hybrid receptors on the cell surface was similar, as judged by FACS analysis (data not shown). dN10, which contains KIR and the SH2 domain, suppressed EPO-dependent STAT5 activation like wild-type CIS3, while dN25, which lacks functional KIR, did not inhibit EPO-dependent STAT5 activation (39). After infection, cells were cultured in either EGF or IL-3, and the EGFP positive cells were counted by FACS. Typical results are illustrated in Fig. 5B. In the presence of EGF, the number of Ba/F3 cells expressing EGFR/wtEPOR decreased by dN10 CIS3 expression with a rate similar to that in the presence of IL3. However, the population of infected EGFR/-135Y-expressing cells decreased more slowly in EGF.
than in IL-3. Infection of dN25 CIS3 did not affect the growth of Ba/F3 cells in EGF or IL3. The expression levels of CIS3 were similar after 1-day infection (Figs. 5B-e, -f). These data confirmed that the EPOR cytoplasmic domain lacking the CIS3 interacting region was less sensitive to the negative effect of CIS3 on the EPO-mediated proliferation than the wild-type EPOR.

**Requirements of the SH2 domain and the N-terminal region of CIS3 for binding to the EPOR**

To investigate the structural requirement of CIS3 for the interaction with the EPOR, we created series of deletion mutants from N- and C-termini of CIS3 (Figs. 6A and B). These deletion mutants were transfected into 293 cells and cell extracts were subjected to an *in vitro* binding assay using phosphorylated GST-YY or pY1007 JAK2 phosphopeptide conjugated to agarose. In addition to the SH2 domain, N-terminal 12 amino acids as well as C-terminal 42 amino acids next to the SH2 domain were necessary for binding to the pY1007 phosphopeptide which corresponds to the JAK2 activation loop (Fig. 6A). We therefore called these regions N- and C-terminal extended SH2 subdomains (N-ESS and C-ESS) (39). Similar requirement for N-ESS and C-ESS domains were observed for the interaction between CIS3 and GST-YY (Fig. 6A and B). In addition, amino acids 25-35 next to the N-ESS were necessary for the interaction between CIS3 and GST-YY (Fig. 6B).

Next, we created a number of substitution mutants of CIS3 and identified four different types of mutants: L22D mutation retained the affinity for GST-YY but lost the binding to GST-JH1; F25A mutation in KIR lost the binding activity to both GST-YY and GST-JH1; G45A mutation in ESS abolished the binding to the GST-YY but retained the binding activity to GST-JH1; and R71E mutation in the SH2 domain abolished the binding activity to GST-YY, GST-JH1, and pY1007 (Fig. 6C). L22D, F25A, and G45A still bound to pY1007 (Fig. 6C).

Glycine at the position 45 in CIS3 is located next to the SH2 domain and is conserved among CIS family members. We have shown that CIS1 also binds to the
Y401 region of the EPOR (24). Therefore, we examined whether point mutation in conserved glycine residue of CIS1 (G81) affects CIS1-EPOR interaction as well as CIS3 (Fig. 6D). The CIS1 G81A mutant failed to bind phosphorylated Y401 of the EPOR, indicating that the conserved G45 of CIS3 and G81 of CIS1 are critical for the binding to the EPOR Y401 region.

**G45A mutation decreased the inhibitory effect of CIS3 on EPO signaling but not on LIF signaling**

CIS3 mutants which differently bound to JAK2 and EPOR were further evaluated the importance of CIS3-EPOR interaction on EPO-mediated signaling. L22D mutants, that bound to the EPOR but not bind to JAK2, failed to inhibit EPO-dependent STAT5 activation (39) and the EPOR-mediated proliferation of BF-ER cells as well as R71E mutant (Fig. 7B). This indicates that the binding of CIS3 to JAK2 is prerequisite for signal inhibition. On the other hand, the G45A mutant, that binds to the JAK2 but does not interact with EPOR, suppressed EPO-dependent STAT5 activation and EPOR-mediated proliferation (Figs. 7A and B). However, the inhibitory effect of CIS3 G45A mutant required a higher level of expression (Fig. 7A) or longer time (Fig. 7B) to achieve a suppressive effect comparable to wild-type CIS3. This indicates that the CIS3 mutant which binds to JAK2, but not to the EPOR, possessed less potent inhibitory activity to the EPOR signaling than the wild-type CIS3.

To examine the role of G45A mutation in other cytokine mediated signal, we compared the effect of wild-type CIS3 and G45A mutant on LIF-gp130-dependent STAT3 activation. As shown in Fig. 7C, LIF-induced STAT3 activation was suppressed by the G45A mutant with similar efficiency to that by wild-type CIS3. These data suggest that G45 of CIS3 is not important for gp130 signal inhibition but important for EPO signal inhibition. Previous studies also have shown that either E30R or Y31A mutation of CIS3 loses the inhibitory effect on EPO signal but not on LIF signal (38, 39). These suggest that there are some differences in the structure of
CIS3 for inhibiting EPO and LIF signaling.

**CIS3 suppresses EPO signaling differently from JAB**

We and others have shown that JAB binds to JAK2 more tightly than CIS3 but that their inhibition of cytokine signaling was very similar (38, 39). In addition, CIS3 bound very weakly to JAK2 *in vitro* (39) but tightly to JAK2 *in vivo* after stimulation with EPO (see Fig. 2). Therefore, we hypothesize that the receptor binding of CIS3 enhances the accessibility of CIS3 to JAK2, thereby increasing the inhibitory effect against JAK2.

To test this hypothesis, we first compared the inhibitory mechanism between CIS3 and JAB. CIS1 and CIS3, but not JAB nor CIS2, bound to the EPOR (Fig. 8A). Since CIS3 bound to Y401, one of the two major STAT5 binding sites of the EPOR (Y343 and Y401), we overexpressed STAT5 to inhibit the binding of CIS3 to the receptor by competition. As shown in Fig. 8B, co-expression of STAT5 suppressed the binding of CIS3 to the receptor. Next, we examined the inhibition of EPO-induced STAT5 activation by CIS3 or JAB in the presence of excess STAT5. There was little difference in the inhibitory effect between CIS3 and JAB without STAT5 transfection (Fig. 8C). However, CIS3 expressed at low levels (30-100 pg cDNA transfection) lost the inhibitory effect on EPO-induced STAT5 activity in the presence of excess STAT5, while JAB exhibited a similar inhibitory effect regardless on STAT5 expression (compare Fig. 8C, b and c). CIS3 and JAB at the level of our transfection conditions did not affect EGF-induced Elk-1 activation which is mediated by the Ras-MAP kinase pathway (Fig. 8C-d) as well as EGF-induced STAT3 activation (data not shown). These data indicate that the binding of CIS3 to Y401 of the EPOR enhances the inhibitory effect of CIS3, especially when CIS3 level was not very high.

To address the question whether the level of CIS3 in our experimental conditions in 293 cells is relevant for inhibiting EPOR action, we compared the level of CIS3 between CFU-E cells and transfected 293 cells using CIS3 specific antibody. As shown in Fig. 8C-e and -f, the level of CIS3 in CFU-E enriched splenocytes after
stimulation with EPO was comparable to that in 293 cells transfected with 100 to 300 pg Flag-CIS3 cDNA (transfection efficiency was about 50%). In those range of plasmids (below 300 pg), Flag-CIS3 partially suppressed EPO-induced STAT5 activation in 293 cells, and binding to the EPOR enhances the inhibitory effect (Fig.8 C-b,c). Thus, CIS3 level in CFU-E cells might be high enough to modulate EPOR signaling activity.
Discussion

The process of fetal erythropoiesis is regulated by the EPO receptor and JAK/STAT signaling pathways during embryogenesis (5-7). Recent studies in CIS3 deficient mice indicated an important role for CIS3 in negative regulation of erythropoiesis (15). These findings prompted us to study the role of CIS3 in inhibition of EPOR/JAK/STAT signaling. We have previously shown that CIS3 interacts with JAK2 and inhibits its kinase activity by binding to phosphorylated Y1007 in the activation loop (39). In this report, we describe a novel mechanism by which CIS3 inhibits EPO-mediated proliferation and STAT5 activation. We show that CIS3 binds directly to a region containing phosphorylated Y401 of the EPOR and that this interaction is necessary for efficient inhibition of the wild-type EPOR. Furthermore, a CIS3 mutant (G45A) which is unable to bind EPOR, but still interacts and inhibits JAK2, exhibited less inhibitory effect on EPO-induced proliferation and STAT5 activation than the wild-type CIS3. Therefore, we speculate that CIS3-EPOR interaction augments the inhibitory effect of CIS3, probably by efficiently recruiting CIS3 to the EPOR/JAK2 complex. The required inhibition of both the EPOR and JAK2 by CIS3 may also explain a relatively specific negative effect of CIS3 in fetal liver hematopoiesis process that is controlled in part by EPOR signaling (15).

The interaction of CIS3 and the EPOR does not absolutely require tyrosine phosphorylation of EPOR, although phosphorylation additionally increased the binding of CIS3 to phosphorylated Y401. It is not clear whether this phosphorylation-independent interaction is due to overexpression of the two proteins. However, we could not see such phosphorylation-independent interaction using 12-mer peptide of the Y401 (Fig. 3E), which suggests that CIS3 recognized a wide region including Y401 of the EPOR. This is also supported by the fact that CIS3-Y401 interaction requires an additional region (KIR, N-ESS, and C-ESS) at the N- and C-termini of the SH2 domain. Most other SH2 proteins do not require such N- and C-terminal regions outside of the SH2 domain for the interaction with phosphotyrosine-containing peptides. Recently, a small SH2 protein SAP (also called SH2D1A) was
identified as the product of the gene mutated in the X-linked lymphoproliferative syndrome (XLP)(46). SAP has been shown to bind to the Y281 of SLAM, a self-ligand glycoprotein found on activated B and T cells, in a phosphorylation-independent manner. In this case, specific interaction of the SAP SH2 domain with residues at the N-terminal as well as the C-terminal of the Y281 of SLAM is required for their stable binding (47, 48). Structural analysis of the interaction between CIS3 and the EPOR will provide a new insight into a novel interaction mechanism between the SH2 domain and a polypeptide containing the tyrosine residue.

In this report, we show that CIS3 binds to the EPOR in addition to JAK2 in erythroid progenitor cells. Experiments using various mutants of CIS3 and the EPOR demonstrate that the CIS3 binding to JAK2 was prerequisite for EPO signal inhibition and the binding to the EPOR augmented the negative effect of CIS3. We have also shown that CIS3 was able to bind to the IL2 receptor β-chain (37). As in the case of the EPOR, CIS3-mediated inhibition of JAK1 phosphorylation was markedly enhanced in the presence of the IL-2 receptor β-chain, which showed by in vitro kinase assay. Another groups also showed that CIS3 was induced by GH and CIS3 could bind to the GST-fused GH receptor. They also suggested an augmentation of the negative effect of CIS3 by co-expression of the GH receptor with reporter gene assay (49, 50). Although all these experiments were performed in vitro or in 293 cells, CIS3 may also be a negative feedback regulator of IL2 and GH by binding to their respective receptors as well as JAK2. Our present study provides a molecular basis for these augmentations of negative effect of CIS3 on cytokine signaling.

Recently, two reports demonstrated that CIS3 bound to phosphorylated Y757 of gp130, and this CIS3-gp130 interaction augmented the inhibitory activity of CIS3 (51, 52). Interestingly, Y757 of gp130 is the binding site of SHP-2, a phosphotyrosine-phosphatase containing the SH2 domain. Y401 of the EPOR has been shown to be the binding site for SHP-2 (53). Therefore, CIS3 and SHP-2 may compete each other or may cooperatively down-modulate cytokine signaling.

Additional CIS family member, CIS1, was also shown to bind to Y401 of the
EPOR (24). However, CIS1 does not bind to JAKs but binds to other cytokine receptors that activate STAT5, including the IL2 receptor β-chain, and the IL3 receptor (22, 25, 29). CIS1 suppresses STAT5 activation, probably by masking the phosphotyrosine residues of the EPOR that are involved in STAT5 activation (23, 54). Mechanistically, CIS3 is an intermediate between CIS1 and JAB. This is also supported by their structural similarities. Both CIS3 and JAB, but not CIS1, have KIR in the N-terminal region, while CIS1 and CIS3, but not JAB, have a C-terminal extended SH2 region (C-ESS). The chimeric mutant containing CIS3-KIR and CIS1-SH2 domain, but not wild-type CIS1, was able to bind to JAK2-JH1 (39). These structural details and observation from chimera study suggest that strong preference of JAB for binding to JAK kinases and CIS1 for cytokine receptors, while CIS3 shows combined binding to both cytokine receptors and JAK kinases. CIS3 binding to EPOR and JAK2 may explain the similar inhibitory effects between CIS3 and JAB on the JAK2 signaling observed in living cells, which is in contrast to the in vitro evidence showing that JAB binds much stronger to JAK2 than CIS3 (38, 39).

Although CIS1 and CIS3 are induced by similar cytokines, their time course of induction is very different. For example, CIS3 induction by EPO or IL2 was very rapid and transient, while CIS1 induction was slower and remained at high levels as long as cytokines were present (ref.37 and unpublished data). It has been reported that the gene disruption of CIS1 in mice exhibited no drastic phenotype (15). Forced expression of CIS1 in transgenic mice demonstrated the ability of CIS1 for specific suppression of STAT5 activation in vivo (54). Our present study suggests that longer-term expression of CIS3 can compensate for the loss of CIS1 in CIS1-knockout mice. These results imply that CIS1 and CIS3 may cooperate in inhibiting cytokine signals. Different mechanisms of inhibitory actions of CIS family members combined with their cytokine-induced expression profiles may provide the specificity in negative regulation of cytokine signaling.
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Figure Legends

Fig. 1 The effect of KIR and SH2 mutants on EPO-JAK2 signaling. (A) Schematic model of the functional domains of CIS3. Essential amino acids in KIR which are conserved between CIS3 and JAB are shown as a hatched box. KIR: Kinase Inhibitory Region. ESS: Extended SH2 Subdomain. (B) Effect of KIR and SH2 mutations on the EPOR phosphorylation in response to EPO. Flag-tagged CIS3 mutants and the EPOR were transiently expressed in 293 cells and then stimulated with or without EPO (10U/ml) for 10 min. Cell extracts were analyzed with anti-PY (a), anti-EPOR (b), and anti-Flag antibody (c). (C) Effect of KIR and SH2 mutations on EPO-induced STAT5 activation. An EPO-dependent STAT5 reporter gene assay was carried out in the presence of the indicated CIS3 mutants in 293 cells. Data are normalized with the β-galactosidase activity from duplicate experiments. (D) Effect of KIR and SH2 mutations on the binding to JAK2 and the autophosphorylation of JAK2. JAK2 and each Flag-tagged CIS3 mutants were transiently expressed in 293 cells. Cell lysates were then immunoprecipitated with anti-JAK2 antibody and analyzed with anti-Flag (a and d), anti-PY (b), and anti-JAK2 (c) antibody.

Fig. 2 Expression and binding of endogenous CIS3 to the EPOR and JAK2 in vivo. (A) Induction of CIS3 mRNA by EPO treatment in CFU-E cells from anemic mice spleens. Splenocytes (1x10^8) were isolated from phenylhydrazine-treated mice (lane 1). Cells were cultured without EPO for 4 h (lane 2) and then stimulated with EPO for 1 or 3 h (lanes 3, 4). RNA samples (20 μg) were blotted with CIS3 (a) or G3PDH (b) riboprobes. (B) CFU-E cells were cultured without EPO for 6 h and then treated with or without 10 U/ml EPO for 1 h. After immunoprecipitation with anti-CIS3 antibody, total cell lysates (TCL) and immunoprecipitates were analyzed by immunoblotting with anti-PY (a) and anti-CIS3 antibodies (b). (C) BF-ER cells (1x10^7 cells/samples) were cultured without EPO for 8 h and then stimulated with 20U/ml EPO for indicated periods in the presence of 50 μM MG132 and 0.1 mM sodium vanadate. After lysis, the CIS3 complex was immunoprecipitated with anti-CIS3 antibody.
Immunoprecipitates were blotted with anti-PY (a) and anti-CIS3 (b) antibodies. (D) BF-ER cells were treated with or without EPO for 1 h and then cell extracts were immunoprecipitated with anti-EPOR (lanes 1 and 2), anti-CIS3 (lanes 3 and 4), and anti-JAK2 (lanes 5 and 6) antibodies. The immunoprecipitates were analyzed by immunoblotting with anti-PY (a), anti-JAK2 (b), anti-EPOR (c), and anti-CIS3 (d) antibodies. (E) EPO-stimulated BF-ER cell extracts were immunoprecipitated with control goat anti-LexA (lane 1) or goat anti-CIS3 (lane 2) antibodies. The anti-CIS3 immunoprecipitates were denatured by boiling in an SDS-sample buffer and then re-immunoprecipitated with anti-EPOR (lane 3) or anti-JAK2 (lane 4) antibodies. These immunoprecipitates were analyzed by immunoblotting with anti-PY antibody. The positions for JAK2 and EPOR are indicated by arrowheads.

**Fig. 3** CIS3 binds to the region containing Y401 of the EPOR. (A) 293 cells were transfected with (+) or without (-) plasmids carrying the EPOR, JAK2, and Flag-tagged CIS3 cDNAs. After stimulation with 20 units/ml of EPO for 5 min, cell extracts were immunoprecipitated with anti-EPOR. The immunoprecipitates (panels a, b, c) or total cell lysates (TCL) (panel d) were blotted with anti-Flag (a and d), anti-PY (b), or anti-EPOR (c) antibodies. Positions for CIS3, phosphorylated EPOR (PY-EPOR), and the EPOR are indicated. (B) Effect of EPOR mutations on the interaction between CIS3 and the EPOR. 293 cells were transfected with the wild-type EPOR (WT) or truncated EPOR containing Y343 and Y401 (YY) (deletion of C-terminal 76 amino acids), or Y343 (Y) (deletion of C-terminal 135 amino acids), Flag-tagged CIS3, and JAK3. Cell lysates were immunoprecipitated with anti-EPOR antibody. The immunoprecipitates (panels a, b, c) or total cell lysates (panel d) were analyzed with anti-Flag (a and d), anti-EPOR which recognize extracellular domain of the receptor (b) and anti-PY (c) antibodies. (C) Schematic structure of GST-EPOR constructs. The two tyrosine residues (Y343 and Y401) in the cytoplasmic portion of the EPOR (codon 301-406) were mutated to phenylalanine (F) residues. These cDNAs (YY, YF, FY, and FF) or the EPOR codon 301-348 containing Y343 was fused to the GST.
The region for box 2 is indicated. (D) GST-EPOR constructs were expressed in TKB-1, and recombinant proteins were purified on GSH-Sepharose and then stained with Coomassie Brilliant Blue (CBB) (panel e) or immunoblotted with anti-PY antibody (d). Extracts of the 293 cells expressing either Flag-tagged wild-type CIS3 (WT) or R71E mutant CIS3 (R71E) were incubated with 5 µg of immobilized GST-fusion proteins at 4 °C for 1 h. The complex with GST-fusion proteins was resolved by 13% SDS-PAGE and then immunoblotted with anti-Flag (a, b) or anti-PLC-γ1 (c) antibodies. (E) Sequences of phospho- (pY401) or non-phosphorylated (Y401) peptides of the EPOR are shown. Cell extract of the 293 cells expressing Flag-CIS3 was incubated with peptide conjugated beads. CIS3 and STAT5 were detected by immunoblotting with anti-Flag (a), and anti-STAT5 antibodies (b).

**Fig. 4** The effect of Y401 on JAK2 signal inhibition by CIS3. (A) EPO-dependent STAT5 reporter assay was carried out in 293 cells expressing indicated mutant EPORs. The following amounts of CIS3 plasmids were transfected: +; 0.4 ng, ++; 2 ng, +++; 10 ng. (B) 293 cells were transfected with the indicated plasmids of Flag-JAK2, EPOR mutants (YY or YF), and Flag-CIS3 (+; 0.1 µg, ++; 1.0 µg). After stimulation with 20 units/ml of EPO for 10 min, anti-Flag immunoprecipitates (a and b) were blotted with anti-PY (a) and anti-JAK2 (b) antibodies. Total cell lysates (TCL) were blotted with anti-Flag antibody (c). PY-JAK2, JAK2, and CIS3 are indicated.

**Fig. 5** The effect of CIS3-EPOR interaction on the EPO-dependent proliferation. (A) Structures of the chimeric receptors and retrovirus vector containing IRES-EGFP. CIS3 cDNA is introduced in front of the IRES-EGFP, allowing CIS3 expression together with EGFP. LTR: long terminal repeat. (B) The effect of deletion of the Y401 region on CIS3 function. Ba/F3 cells expressing chimeric receptors were infected with a retrovirus carrying dN10 or dN25 CIS3 mutants in the presence of IL-3. After 24 h, cells (1x10^5) were divided into half and cultured either in the presence of 10 ng/ml EGF (a and c) or 1 ng/ml of IL3 (b and d). Populations of EGFP-positive cells were
counted by FACS analysis on indicated days after infection. Cells (1x10^5) after 1-day infection were also immunoblotted with anti-Myc (e) and anti-STAT5 (f) antibodies. dN10 and dN25 indicate 10- and 25-amino acid deletion mutants of CIS3 from the N-terminus, respectively.

**Fig. 6** The effect of mutations in the CIS3 on the binding to the EPOR. Representative mutations are shown in (E). Substituted amino acids are indicated with asterisks under the amino acid sequence in the kinase inhibitory region (KIR) and extended SH2 subdomain (ESS). Flag-tagged wild-type (WT) CIS3 or N-terminal deletion mutants (A), C-terminal deletion mutants (B), or substitution mutants (C) were transiently expressed in 293 cells and then subjected to an *in vitro* binding assay with immobilized phosphorylated GST-YY (a) or pY1007 phosphopeptide (A-b, B-b, and C-c) or GST-JH1 (C-b). Total cell lysates were also immunoblotted with anti-Flag antibody (Input). **(D)** Critical role of G81 of CIS-1 for the binding to the Y401 of the EPOR. Conserved glycine at the N-terminal of the predicted SH2 domain is shown as a hatched box in the upper panel. Flag-tagged wild-type CIS1 or G81A substitution mutants were expressed in 293 cells and then subjected to an *in vitro* binding assay with phosphorylated GST-FY (a). Total cell lysates were also analyzed with anti-Flag antibody (b).

**Fig. 7** Effects of G45 mutation on the activity of CIS3. **(A)** An EPO-dependent STAT5 reporter assay was carried out for 293 cells expressing the EPOR and Flag-tagged wild-type CIS3 or G45A mutant (b). To achieve similar expression levels, 40 ng wild-type CIS3 or 200 ng G45A mutant plasmids were transfected at the highest amount, and CIS3 cDNA samples serially diluted 5-time were also transfected. The expression level of CIS3 proteins at the indicated amount of plasmids was analyzed with anti-Flag antibody (a). **(B)** Effects of CIS3 mutants on EPOR-mediated proliferation. Ba/F3 cells expressing EGFR/wt EPOR chimera were infected with a retrovirus encoding CIS3 mutants in the presence of IL-3. After 24 h, half of the cells
(1x10^5) were collected for Western blot analysis with anti-Myc (a) and anti-STAT5 (b) antibodies. The other half of the cells were cultured in the presence of 10 ng/ml EGF, and the populations of EGFP positive cells were counted by FACS after indicated days of culture (c). All substitutions were introduced into dC40 backbone to obtain higher level of CIS3 expression in Ba/F3 cells. (C) An LIF-dependent STAT3 reporter assay was carried out in 293 cells expressing myc-tagged wild-type CIS3 or G45A mutant (b). To achieve similar expression levels, 4 ng wild-type CIS3 or 20 ng G45A mutant plasmids were transfected at the highest amount, and serially diluted 5-times CIS3 cDNA samples were also transfected. The expression level of CIS3 proteins at the indicated amount of plasmids was analyzed with anti-Myc antibody (a).

**Fig. 8** Comparison of the negative effect on EPO induced STAT activation between CIS3 and JAB. (A) Binding of the CIS family proteins with the EPOR. 293 cells were transiently expressed with the EPOR and indicated Flag-tagged CIS family proteins, and cell extracts were precipitated with anti-EPOR antibody. The immunoprecipitates (a and b) or total cell lysates (c) were blotted with anti-Flag (a and c) or anti-EPOR antibodies (b). CIS2 is also referred as SOCS2 or SSI-2. (B) Suppression of CIS3 binding to the EPOR by overexpression of STAT5. 293 cells were transfected with Flag-CIS3 (1.0 µg) and STAT5 (-; 0 µg, +; 1.5 µg, ++; 9.0 µg). Cell extracts were incubated with immobilized phosphorylated GST-FY in vitro, and precipitates were analyzed with anti-Flag (a and c), anti-STAT5 (b) antibodies. (C) The effect of STAT5 overexpression on the activity of CIS3 and JAB. An EPO-dependent STAT5 reporter assay was carried out in 293 cells transfected with indicated amount of Flag-tagged CIS3 or JAB plasmids in the absence (b) or presence (c) of 400 ng STAT5 cDNA. An EGF-dependent Elk-1 reporter assay was also carried out in 293 cells transfected with the same amounts of Flag-CIS3 or Flag-JAB (d). To compare the expression level of Flag-CIS3 and Flag-JAB, the transfected samples were immunoblotted with anti-Flag antibody (a). Splenocytes were prepared as Fig.2B, and Flag-CIS3 was transfected as (Fig.8C-a). Each of cell extract (100ug) was
subjected to 13% SDS-PAGE and analyzed by rabbit anti-CIS3 antibody (e) or anti-STAT5 antibody (f). In Flag-CIS3 transfected 293 cells, two bands were detected by anti-CIS3 antibody; the upper one corresponds to full-length Flag-CIS3 and the other is translated from internal ATG of CIS3 cDNA.
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CIS3/SOCS3 suppresses erythropoietin signaling by binding the EPO receptor and JAK2

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