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The Cytokine-inducible Scr Homology Domain-containing Protein Negatively Regulates Signaling by Promoting Apoptosis in Erythroid Progenitor Cells*

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The small cytokine-inducible SH2 domain-containing protein (CIS) has been implicated in the negative regulation of signaling through cytokine receptors. CIS reduces growth of erythropoietin receptor (EpoR)-dependent cell lines, but its role in proliferation, differentiation, and survival of erythroid progenitor cells has not been resolved. To dissect the function of CIS in cell lines and erythroid progenitor cells, we generated green fluorescent protein (GFP)-tagged versions of wild type CIS, a mutant harboring an inactivated SH2 domain (CIS R107K), and a mutant with a deletion of the SOCS Box (CISΔBox). Retroviral expression of the GFP fusion proteins in BaF3-EpoR cells revealed that both Tyr-401 in the EpoR and an intact SH2 domain within CIS are prerequisites for receptor recruitment. As a consequence, both are essential for the growth inhibitory effect of CIS, whereas the CIS SOCS box is dispensable. Accordingly, the retroviral expression of GFP-CIS but not GFP-CIS R107K impaired proliferation of erythroid progenitor cells in colony assays. Erythroid differentiation was unaffected by either protein. Interestingly, apoptosis of erythroid progenitor cells was increased upon GFP-CIS expression and this required the presence both of an intact SH2 domain and the SOCS box. Thus, CIS negatively regulates signaling at two levels, apoptosis and proliferation, and thereby sets a threshold for signal transduction.

Survival, proliferation, and differentiation of hematopoietic cells are regulated by multiple cytokines (1), and erythropoietin (Epo)[1] is essential for erythropoiesis. By binding to the erythropoietin receptor (EpoR), a member of the hematopoietic cytokine receptor superfamily, Epo prevents apoptosis of erythroid progenitor cells and promotes their proliferation and erythroid maturation (2). The formation of erythrocytes is tightly controlled by the coordinated activation of several signal-promoting and signal-terminating cascades activated by the EpoR (3). The receptor-associated tyrosine kinase JAK2 is essential for surface appearance of the EpoR (4) and becomes activated after Epo binding. JAK2 in turn phosphorylates several tyrosine residues on the EpoR-cytosolic domain and probably on JAK2 itself that serve as docking sites for SH2 or protein tyrosine binding domains of downstream signal transduction proteins such as STAT5, phosphatidylinositol 3-kinase, Shc, and tyrosine phosphatases SHP1 and SHP2 (5–10).

EpoR (phospho)tyrosine residues 343 and 401 serve as docking sites for the latent transcription factor STAT5 (5–7). STAT5 becomes tyrosine-phosphorylated upon receptor recruitment, homodimerizes, and migrates to the nucleus where it promotes the activation of target genes. STAT5 activates transcription of the *Bcl-x$_L$* gene (11, 12). Bcl-x$_L$ has an essential role in preventing apoptosis of primitive and definitive erythrocytes at the end of maturation (13), and the STAT5 Bcl-x$_L$ signaling pathway has been suggested to protect cells from apoptosis and to promote cell proliferation (11, 12). A *STAT5a/b* null mutation in fetal and neonatal mice leads to defects in erythroid maturation accompanied by increased apoptosis of erythroid progenitors (14), supporting the notion that the *STAT5* Bcl-x$_L$ pathway mediates anti-apoptotic effects in erythroid cells.

For the controlled production of erythroid cells, it is critical that the activation of signal-promoting cascades is counterbalanced by terminating events. In erythroid cells, these include activation of the tyrosine phosphatase SHP1 that dephosphorylates JAK2 (9) and induction of the negative regulatory protein CIS (cytokine-inducible SH2 domain-containing protein) (15). CIS lacks enzymatic activity and belongs to the family of suppressor of cytokine signaling (SOCS) proteins. These proteins possess a SH2 domain in the middle that mediates binding to phosphotyrosine residues and a SOCS Box at the C terminus that has been implicated in proteasomal degradation. By the use of a limited set of mutated EpoRs, tyrosine 401 in the cytoplasmic domain has been identified as the binding site for CIS (16). However, Tyr-401 is a multi-task residue that binds (besides CIS) other SOCS family members such as SOCS2 (17) and SOCS3 (18, 19) as well as the phosphatidylinositol phosphatase SHIP, the tyrosine phosphatase SHP2 (10), and STAT5 (5–7).

Forced expression of CIS inhibits proliferation of cell lines in response to Epo or interleukin-3 (IL-3) and results in reduced activation of STAT5 (20, 21). The molecular mechanism of this effect is poorly understood because CIS lacks the kinase inhib-

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[1] The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; SH2, Src homology; STAT, signal transducers and activators of transcription; CIS, cytokine-inducible SH2 domain-containing protein; SOCS, suppressor of cytokine signaling; IL, interleukin; GFP, green fluorescent protein; HA, hemagglutinin; PE, phycoerythrin; CFU, colony-forming unit; JAK2, Janus kinase 2; 7-AAD, 7-amino-actinomycin D; TUNEL, TdT-mediated dUTP-X nick-end labeling.

itory region that mediates JAK2 inactivation by SOCS1 and SOCS3 (18). It has been suggested that the CIS SH2 domain competes with STAT5 for binding to the EpoR (18), and the CIS SOCS Box has been proposed to mediate proteasome-dependent degradation of the EpoR (16). The physiological role of CIS for the regulation of erythropoiesis remains to be determined since analyses of the two transgenic animal models harboring CIS under the control of the β-casein promoter (22) or the CD4 promoter (23) were focused on development of the mammary gland and on T-cell signaling, respectively.

During embryonic development, CIS is first expressed in the fetal liver at embryonic day 12.5, the same stage as the EpoR (24). The role of CIS in fetal erythropoiesis has not been addressed yet since most studies on growth and survival have been undertaken in cell lines. Here we show that CIS overexpression mimics STAT5 loss-of-function in erythroid progenitor cells, and we determine which CIS domains are essential for this effect. To this end, we expressed GFP-tagged versions of the CIS protein in erythroid progenitor cells under the control of a retroviral promoter, which uncoupled CIS synthesis from activation of the JAK/STAT pathway. We show that elevated levels of CIS inhibit proliferation of erythroid progenitor cells. Although erythroid differentiation is unaffected, an intact CIS SH2 domain is essential for inhibition of proliferation. Similar to bone marrow-derived erythroid progenitor cells in STAT5 knock-out mice (14), erythroid progenitor cells from fetal liver overexpressing CIS show an increased tendency to undergo apoptosis, which requires both the CIS SH2 domain and the SOCS box. Thus, we propose that CIS negatively regulates signaling through the EpoR by two mechanisms. 1) Recruitment of CIS to the EpoR via its SH2 domain is sufficient to repress proliferative responses. 2) In addition, the SOCS Box mediates apoptotic effects of CIS expression in fetal liver erythroid progenitor cells.

EXPERIMENTAL PROCEDURES

Materials—Single or double Tyr to Phe mutant EpoRs were generated by PCR mutagenesis and inserted into the eukaryotic expression vector pXM as described previously (5, 9). EpoR Y401F was subcloned in frame as a *Apa*I- and *Eco*RI-digested fragment into the appropriate restriction sites of the retroviral expression vector pMX (puro)-EpoR. GFP-CIS fusion proteins were established by introducing an in-frame *Bgl*II restriction site at the 5′-end and a *Eco*RI restriction site at the 3′-end of the CIS cDNA and subcloning the DNA fragment in frame into the *Bam*HI and *Eco*RI restriction sites of the retroviral expression vector pMX-enhanced GFP or pOSdSV (25). The SOCS Box from CIS was deleted at amino acid 182, and the fragment was cloned via *Bgl*II and *Eco*RI into the *Bam*HI and *Eco*RI restriction sites of pMOWS-GFP (25). GFP-SOCS3 was generated by introducing *Bam*HI and *Not*I restriction sites at the 5′ and 3′ ends and subcloned in-frame into pMOWS-GFP. Arginine 107 localized within the SH2 domain of CIS was replaced by lysine using overlap extension PCR and subcloned into the *Bam*HI and *Eco*RI restriction sites of pMX-enhanced GFP.

Cell Lines and Cultures—BaF3 cell lines expressing single or double Tyr to Phe mutant EpoRs in pXM were generated as described previously (5, 9). By retroviral transduction, pMX-EpoR or pMX-EpoR Y401F was introduced into the IL-3-dependent pro-B cell line BaF3. BaF3 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 10% WEHI-conditioned medium. Pools of stable transfectants were selected in 1.5 μg/ml puromycin (Sigma). The retroviral packaging cell line Phoenix-eco was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum.

Fetal Liver Cell Preparation and Transduction—Fetal livers from 13.5-day-old BALB/c mouse embryos were prepared as described elsewhere (25). For Ter119 expression kinetics, the fetal liver cell preparation was incubated with anti-Ter119 (provided by Dr. Albrecht Müller) and subjected to AutoMACS depletion (Miltenyi Biotech, Bergisch-Gladbach, Germany). For TUNEL assay, the cells were depleted for hematopoietic lineages with an antibody mixture as described previously (25). Retroviral expression vectors were transiently transfected into Phoenix-eco cells using the Calcium-phosphate method (25). Twenty-four hours after transfection of Phoenix cells, the medium was changed to Iscove's modified Eagle's medium containing 50 μM β-mercaptoethanol and 30% fetal calf serum. Twenty-four hours later, 1 ml of virus-containing supernatant was harvested and filtered through a 0.45-μm filter. For spin infection, the supernatant was mixed with 1×10^4 (for colony assays) or 5×10^4 (for liquid culture) freshly prepared fetal liver cells and spun for 2 h in an Eppendorf centrifuge at 1800 rpm at room temperature. The cells were supplemented with 0.4 unit/ml Epo (Cilag-Jansen, Bad Hamburg, Germany) and plated in 0.8% methylcellulose (StemCell Technologies, Vancouver, Canada) or seeded in Iscove's modified Eagles's medium, 30% fetal calf serum, 50 μM β-mercaptoethanol supplemented with 0.4 unit/ml Epo. GFP-positive and hemoglobinized erythroid colonies were identified by benzidine staining.

Growth Assay—BaF3 cells expressing the indicated EpoRs in the context of pMX were washed three times in medium and plated at a density of 5×10^4 cells/well in 24-well plates. After 3 days in culture in the indicated concentrations of Epo or in 10% WEHI-conditioned medium, cell numbers were determined using a Coulter counter.

Immunoprecipitation and Immunoblotting—For BaF3 cells expressing the wild-type EpoR or EpoR, Y401F 1×10^7 cells were used per immunoprecipitation. Lysis and immunoprecipitation experiments were carried out as described previously (9) using the following antibodies: crude rabbit antiserum raised against the extracellular domain of the EpoR (9), rabbit antiserum raised against CIS (kindly provided by A. Yoshimura), and rabbit antiserum raised against a GST-GFP fusion protein. Proteins were detected by immunoblotting with anti-phosphotyrosine antiserum 4G10 (Upstate Biotechnology, Palo Alto, CA) followed by enhanced chemiluminescence (Amersham Biosciences). Membranes were denatured with β-mercaptoethanol/SDS before reprobing for control of equal protein loading.

FACScan Analysis—To determine surface expression of HA-tagged EpoR (26), BaF3 cells were transduced with retroviral supernatants and sorted for GFP-expression using MoFlo (Cytomation, Colorado). The sorted cells were incubated with rat anti-HA (Roche Molecular Biochemicals) as primary antibody and anti-rat IgG coupled to Cy5 (Dianova) as secondary antibody and analyzed for green and red fluorescence by flow cytometry. For apoptosis assay, the cells were stained with 5 μl of VIAprobe 7-AAD (BD Biosciences) and 5 μl of annexin V coupled to phycoerythrin (PE) or Cy5 (BD Biosciences) according to the manufacturer's instructions. For TUNEL assay, lineage negative fetal liver cells were cultivated for 24 h in serum-free medium PANserin401 (PANbiotech) supplemented with 0.05 unit/ml Epo. The cells were washed with phosphate-buffered saline, fixed with 2% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline. The TUNEL assay was performed using the TMR-Red *in situ* cell death kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Ter119 staining of fetal liver cells as a marker for erythroid differentiation (27) was performed with a rat anti-Ter119 antiserum (kindly provided by Dr. Albrecht Müller, Julius-Maximilian University, Würzburg, Germany) and a secondary anti-rat IgG coupled to Cy5. GFP, PE, Cy5, and TMR-Red fluorescence were detected with a Becton Dickinson FACScan (BD Biosciences) using the CellQuest software.

RESULTS

Importance of EpoR Tyrosine 401 for Proliferative Signaling and Survival—To confirm that Tyr-401 in the EpoR cytoplasmic domain represents the major CIS binding site, we expressed the wild type EpoR or a panel of EpoRs containing specific single or double tyrosine to phenylalanine mutations in the IL-3-dependent pro-B cell line BaF3 and tested in co-immunoprecipitation experiments for their ability to bind CIS. Despite lacking one or two tyrosine residues, the mutant EpoRs were tyrosine-phosphorylated upon Epo stimulation to the same extent as the wild type receptor as judged by immunoprecipitation with antiserum recognizing the EpoR followed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 1*A*). In agreement with the observation of others, tyrosine-phosphorylated mutant EpoR Y401F was greatly impaired in its ability to associate with CIS (Fig. 1*A*, *lower panel*, *lane 3*). Because a faint band representing the tyrosine-phosphorylated EpoR Y401F was consistently observed in these co-immunoprecipitation experiments, we conclude that Tyr-401 represents the major binding site for CIS on the EpoR but that other minor binding sites exist.

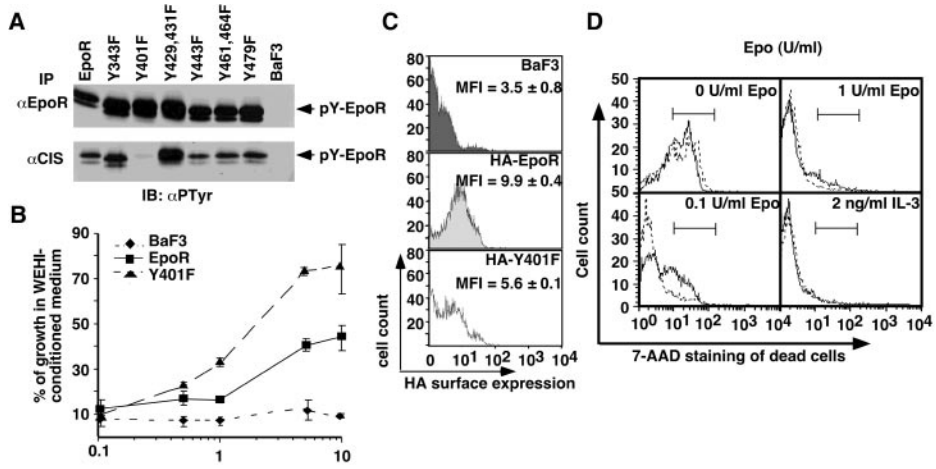

FIG. 1. **Proliferation and survival of BaF3 cells stably expressing the wild-type EpoR or the mutant EpoR Y401F.** *A*, BaF3 cells or BaF3 cells stably expressing the wild type EpoR or the various tyrosine to phenylalanine point mutant EpoRs were stimulated with 100 units/ml Epo for 5 min, lysed, and subjected to immunoprecipitation (*IP*) using antiserum directed against the EpoR (*αEpoR*) or CIS (*αCIS*). The immunoprecipitates were analyzed by SDS-PAGE and visualized by anti-phosphotyrosine immunoblotting (*IB*: *αPTyr*) followed by ECL. *B*, pools of BaF3-EpoR or BaF3-EpoR Y401F cells were seeded at a density of 5×10^4 cells/ml in the presence of either 0.1–10 units/ml Epo or in the presence of 10% WEHI-conditioned medium as a source of IL-3. After 3 days in culture, the cell number was determined using a Coulter counter. The cell numbers obtained were plotted as the percentage of growth obtained in the presence of WEHI-conditioned medium. For BaF3 cells stably expressing the mutant EpoR Y401F, three independent cell pools were analyzed and the results were reproduced at least three times. *C*, cell surface expression of HA-tagged EpoR and EpoR. Parental BaF3 cells (*dark gray*) or BaF3 cells stably expressing HA-EpoR (*medium gray*), HA-EpoR Y401F (*white*) were incubated with rat anti-HA antiserum followed by Cy5-labled anti-rat IgG and analyzed by flow cytometry. The mean fluorescence intensity (*MFI*) detected in three independent experiments ± S.D. is indicated. *D*, BaF3 cells stably expressing EpoR (*solid line*) or EpoR Y401F (*dashed line*) were stained with 7-AAD to determine by flow cytometry the number of dead cells after 24 h in culture in the indicated concentrations of Epo or IL-3.

Since Tyr-401 has been identified as the binding site for multiple signal-promoting and signal-terminating molecules including CIS, we asked whether the absence of Tyr-401 increased or decreased biological responsiveness of the EpoR. To examine proliferative signaling, BaF3 cells stably expressing the wild type EpoR or the mutant EpoR Y401F were cultivated in the presence of increasing concentrations of Epo ranging from 0.1 to 10 units/ml. After 3 days, the cell number was determined and normalized to the cell growth obtained in the presence of WEHI-conditioned medium. All cell pools analyzed showed comparable growth rates in WEHI-conditioned medium. When grown in Epo, half-maximal proliferation of BaF3 cells expressing the wild type EpoR was achieved at 1 unit/ml Epo and equaled 17% of the growth obtained in the presence of WEHI-conditioned medium (Fig. 1*B*). At 10 units/ml Epo, these cells grew to 45% of the number achieved in WEHI-conditioned medium. In contrast, although proliferation of BaF3 cells expressing EpoR Y401F remained Epo-dependent, these cells grew better at all Epo concentrations. At the highest Epo concentration employed, 76% of the growth observed in WEHI-conditioned medium was achieved compared with 45% obtained upon expression of the wild type EpoR. The expression of mutant EpoR Y401F allows cells to proliferate in Epo 2–4-fold less than normal and enables cells to grow to higher density. To confirm that the growth-promoting effects of EpoR Y401F are not caused by increased surface prevalence, we expressed HA-tagged forms of the wild type EpoR and EpoR Y401F in BaF3 cells and analyzed the extent of surface expression by flow cytometry (Fig. 1*C*). This analysis revealed that in comparison to the HA-tagged wild type receptor, the amount of HA-EpoR Y401F detectable on the cell surface is not enhanced but rather slightly reduced.

To determine whether the increased yield of BaF3 cells expressing EpoR Y401F correlates with increased cell survival, we performed viability staining of BaF3-EpoR and BaF3 EpoR Y401F cells. The cells were cultivated in the absence of growth factor or in the presence of 0.1 or 1 unit/ml Epo or, as a positive control, 2 ng/ml IL-3. The cells were stained with 7-AAD, a dye that stains dead cells. (Fig. 1*D*). As expected, in the absence of cytokine, the majority of cells (90.7% of BaF3-EpoR and 85.9% of BaF3-EpoR Y401F cells) underwent death within 24 h, whereas in the presence of IL-3, death was reduced to ~13% of the cells. Similarly, in 1 unit/ml Epo, only 20.5% BaF3-EpoR cells and 10.8% BaF3-EpoR Tyr-401 cells were 7-AAD-positive. At a lower Epo concentration (0.1 unit/ml), the number of 7-AAD-positive BaF3-EpoR cells increased to 50.0% but only 16.5% BaF3-EpoR Y401F cells were dead. Thus, the absence of Tyr-401 from the EpoR decreases sensitivity to undergo cell death and increases the proliferative yield, suggesting that EpoR Tyr-401 is important for activating a down-modulating signal.

Role of the CIS SH2 Domain for Binding to the EpoR—To determine the domains in CIS required for binding to the EpoR and for exerting biological functions, we generated GFP-tagged versions of wild type CIS and a mutant CIS harboring an inactivated SH2 domain (CIS R107K). To examine binding to (phospho)tyrosine 401 of the EpoR, GFP, GFP-CIS, and GFP-CIS R107K were stably expressed in parental BaF3, BaF3-EpoR, and BaF3-EpoR Y401F cells. The cells were stimulated with Epo, lysed, and subjected to immunoprecipitation using antisera against the EpoR or GFP. Tyrosine-phosphorylated EpoR complexed with GFP fusion proteins were subsequently identified by immunoblotting with anti-phosphotyrosine antiserum (Fig. 2). As expected, GFP alone was unable to bind either one of tyrosine-phosphorylated receptors (Fig. 2, *lanes 5* and *6*). However, compared with the total amount of phosphorylated EpoR (Fig. 2, *lanes 2* and *3*), GFP-CIS preferentially bound the tyrosine-phosphorylated wild type EpoR and to a lesser extent tyrosine-phosphorylated EpoR Y401F (Fig. 2, *lanes 8* and *9*). By testing a panel of tyrosine-to-phenylalanine mutant EpoRs (Fig. 1*A*), we could demonstrate that, despite the residual association of GFP-CIS with EpoR Y401F, Tyr-401 represents the major binding site for CIS in the EpoR (Fig. 2, *lane 8*). Conversely, mutating the critical arginine 107 in the CIS SH2 domain completely abrogated the ability of CIS to bind the tyrosine-phosphorylated EpoR (Fig. 2, *lanes 11* and *12*). This finding confirms the

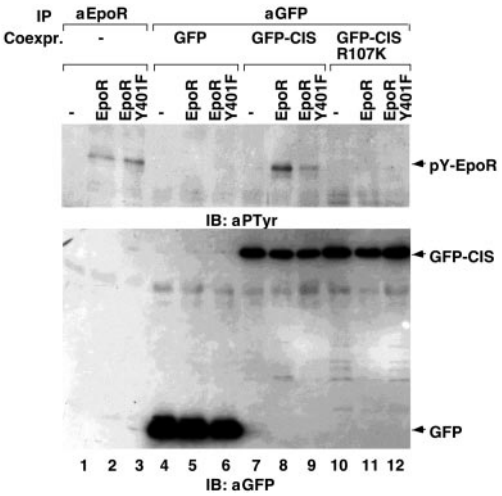

FIG. 2. **Association of a green fluorescent protein-tagged CIS with tyrosine phosphorylated EpoR.** Parental BaF3 cells or BaF3 cells stably expressing the wild type EpoR or the mutant EpoR Y401F were transduced with retroviral expression vectors pMX-GFP, pMX-GFP-CIS, or pMX-GFP-CIS R107K. In this retroviral vector, the transcription of the GFP-CIS gene is promoted by the long terminal repeats and transcription of the puromycin resistance gene is from an internal SV40 promoter. Cell pools expressing GFP or the GFP fusion proteins were selected in the presence of puromycin and grown in the presence of WEHI. After immunoprecipitation with anti-EpoR or anti-GFP antiserum, tyrosine-phosphorylated EpoR was detected by immunoblotting with anti-phosphotyrosine and anti-GFP antiserum. The positions of the tyrosine-phosphorylated EpoR (*pY-EpoR*), GFP-CIS, and GFP are indicated with *arrows*.

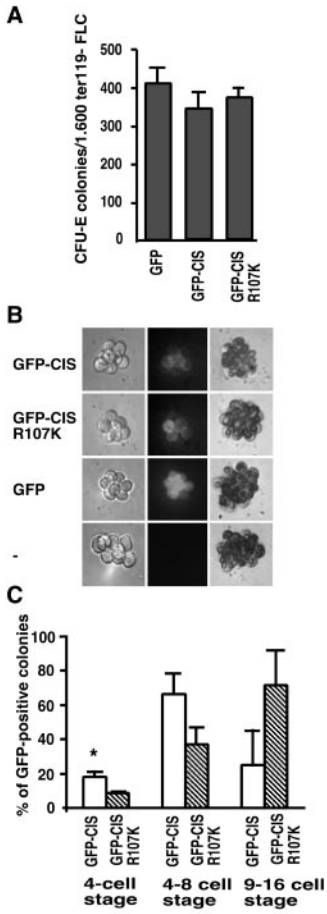

FIG. 3. **Effect of GFP-CIS fusion proteins on CFU-E colony formation and erythroid differentiation.** *A*, GFP, GFP-CIS, and GFP-CIS R107K were transduced into Ter119-depleted day 13.5 fetal liver cells and grown in methylcellulose for 3 days in 0.5 units/ml Epo. The colonies were stained with benzidine and benzidine-positive colonies with >8 cells/colony considered as CFU-E. *B*, after 23 h, GFP-positive erythroid colonies were identified by fluorescence microscopy (*middle panel*) and bright field microscopy (*left panel*) using a Zeiss Axiovert 100 microscope. After 72 h, hemoglobinization of the colonies was visualized by benzidine staining (*right panel*). Pictures of representative colonies are shown. *C*, after 23 h, GFP-positive erythroid colonies at the 4-, 8-, and 16-cell stage were identified by fluorescence microscopy and the number of cells within each GFP-positive colony was counted. The *white bars* represent values obtained for erythroid progenitors expressing GFP-CIS, whereas the *gray bars* indicate the values for GFP-CIS R107K. The numbers presented are the mean of three independent experiments ± S.D. The overall infection rate of erythroid colonies was 41 and 31% for GFP-CIS and GFP-CIS R107K, respectively. Single cells and two-cell colonies were not considered. Significances were calculated according to a two-sided paired Student's *t* test.

importance of the SH2 domain for receptor recruitment of CIS.

CIS Reduces Growth of Fetal Liver Erythroid Progenitor Cells—To elucidate whether binding of CIS to the EpoR negatively regulates erythropoiesis, GFP-CIS and GFP-CIS R107K were transduced by retroviral infection into fetal liver cells and tested for their effect on CFU-E colony formation. The transduction efficiency ranged from 30 to 50% and was the same for transduction of GFP-CIS and GFP-CIS R107K. As shown in Fig. 3*A*, the total number of CFU-E colonies expressing GFP-CIS was not significantly reduced compared with colonies expressing GFP-CIS R107K or GFP.

Colonies grown in methylcellulose supplemented with 0.4 unit/ml Epo, thus favoring growth of CFU-E colonies, were inspected by fluorescence microscopy to monitor GFP-positive CFU-E colonies. As shown in Fig. 3*B*, the expression of GFP-CIS and GFP-CIS R107K in erythroid progenitors is comparable with each other and is predominantly localized to the cytoplasm.

To determine the effect of unregulated CIS expression on proliferation of erythroid progenitor cells, we investigated the cell proliferation rate within each GFP-positive colony. After 20–25 h of culture in Epo, colonies containing 4–16 cells predominated. To detect alterations in colony size, the formed erythroid colonies were classified into three categories: 4 cell, 4–8 cell, and 9–16 cell. After 23 h in culture, the percentage of the largest (9–16 cell) colonies expressing GFP-CIS (25%) was reduced compared with the percentage of the 9–16 cell colonies expressing GFP-CIS R107K (71%) (Fig. 3*C*). The latter result was identical to the distribution of colony sizes after transduction of the control GFP protein (data not shown). Thus, the expression of GFP-CIS but not GFP-CIS R107K reduces proliferation of erythroid progenitor cells, indicating the importance of the CIS SH2 domain in this process.

To determine whether these erythroid colonies were defective in differentiation, we directly examined all GFP-positive colonies after 24 h of cultivation for the extent of benzidine staining and detected no difference among the fusion proteins (data not shown). We conclude that the observed colonies are of erythroid origin and that maturation of erythroid progenitors is normal even though proliferation is impaired as a consequence of CIS expression. Accordingly, benzidine staining of CFU-E colonies after 3 days in culture with Epo did not show a difference between cells expressing GFP-CIS, GFP-CIS R107K, or GFP (Fig. 3*B*, *right panel*). Similarly, as judged by fluorescence-activated cell sorter analysis, Ter119 expression occurred at normal rates in GFP-CIS, GFP-CIS R107K, and GFP-expressing cells, reaching a plateau of >80% positive cells after 12–18 h of culture (data not shown). Ter119 is closely associated with glycophorin, an erythroid-specific integral membrane protein (27). Thus, the recruitment of CIS via its SH2 domain to the EpoR negatively regulates proliferative

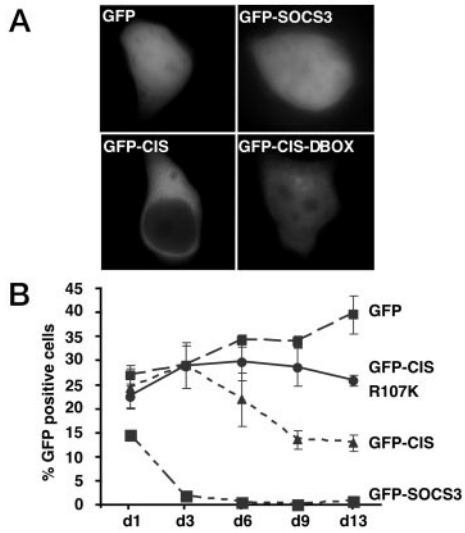

FIG. 4. **Effect of GFP-CIS on growth in BaF3 cells.** *A*, GFP, GFP-CIS, GFP-CISΔBox, or GFP-SOCS3 was transfected into the retroviral packaging cell line Phoenix-eco. Representative cells were photographed 48 h after transfection using a Zeiss Axiovert 100 fluorescence microscope. *B*, GFP-CIS, GFP-CIS R107K, GFP-SOCS3, and GFP as a control were transduced into BaF3 cells stably expressing the wild type EpoR and were cultivated in the presence of 1 unit/ml Epo. At the indicated time points, the expression of GFP or the GFP fusion proteins was evaluated by flow cytometry and used to calculate the number of transduced GFP-positive cells.

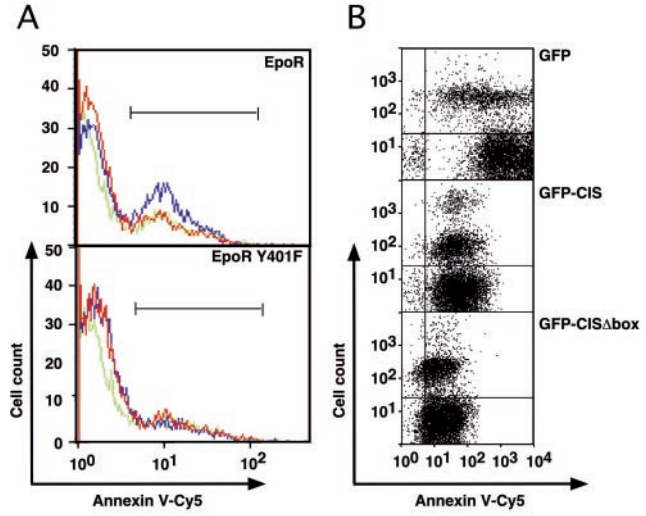

FIG. 5. **Effect of GFP-CIS on apoptosis in BaF3 cells.** *A*, BaF3 cells stably expressing EpoR or EpoR Y401F were transduced with GFP-CIS (*blue line*), GFP-CIS R107K (*red line*), and GFP (*green line*) and grown in 1 unit/ml Epo. After 24 h, the cells were stained with annexin V coupled to PE to determine the number of apoptotic cells and analyzed by flow cytometry. The panels are gated on GFP-positive cells. *B*, BaF3 cells stably expressing the wild type EpoR were transduced with GFP, GFP-CIS, or GFP-CISΔBox, were sorted for GFP expression, and cultivated in 0.01 unit/ml Epo. After 28 h, the cells were stained with annexin V coupled to Cy5 and analyzed by flow cytometry.

responses in erythroid progenitor cells, whereas erythroid differentiation is not affected.

SH2 Domain-mediated Receptor Recruitment of CIS Accelerates Apoptosis of BaF3 Cells—To further dissect the role of the domains present in CIS, we generated, in addition to the GFP-tagged CIS protein harboring an inactivated SH2 domain (GFP-CIS R107K), a GFP-CIS fusion protein lacking the C-terminal region including the SOCS Box that has been implicated in proteasomal degradation (GFP-CISΔBox). To gauge the effectiveness of CIS, we constructed a GFP-tagged SOCS3 protein since SOCS3 has been previously demonstrated to significantly reduce growth of Epo-dependent BaF3 cells (18). As in erythroid progenitor cells, the subcellular localization of GFP-CIS and GFP-CIS R107K (data not shown) in the adherent retroviral packaging cell line Phoenix-eco was comparable and exclusively cytosolic (Fig. 4*A*), whereas GFP and remarkably GFP-SOCS3 and GFP-CISΔBox were detected throughout the cell.

To determine the effect of the various GFP-tagged fusion proteins on proliferative responses, we used retroviral vectors to generate stable populations of BaF3-EpoR cells expressing GFP-CIS, GFP-CIS R107K, GFP-CISΔBox, and GFP and monitored the percentage of GFP-positive cells during 1–13 days in the presence of 1 unit/ml Epo. By applying comparable viral titers for each fusion protein, we routinely achieved transduction rates of 23–27% GFP-positive BaF3-EpoR cells. During the observation period, the fraction of GFP expressing BaF3-EpoR steadily increased from ~27 to 40%, showing that GFP expression had no general toxic effect on cell proliferation. The percentage of GFP-SOCS3-expressing cells was reduced to 15% just after 1 day of culture and reached 2.1% after 3 days (Fig. 4*B*). Thus, the expression of this SOCS3 fusion protein blocked cell proliferation. The expression of GFP-CIS was less inhibitory. After 3 days, the fraction of GFP-CIS-positive cells decreased to 29.2%, and this percentage decreased with time in culture to 13% after 9 days. Similar inhibition of proliferation was achieved by expression of GFP-CISΔBox in BaF3-EpoR cells (data not shown), confirming the notion that the SOCS Box is not essential for inhibition of cell proliferation. In contrast, the GFP-CIS R107K mutant was constantly expressed in 26–29% of the cells between days 3 and 13, indicating that it does not lead to inhibition of cell proliferation. Thus, the antiproliferative effect of CIS in BaF3 cells requires receptor binding of CIS via the SH2 domain, whereas proteasomal degradation facilitated by the CIS SOCS Box is of lesser importance.

To elucidate whether CIS binding to tyrosine 401 is involved in apoptosis, we transduced GFP, GFP-CIS, and GFP-CIS R107K into BaF3-EpoR and BaF3-EpoR Y401F cells and cultured the cells for 24 h in 0.1 unit/ml Epo (Fig. 5*A*). By flow cytometry, we quantified the amount of apoptotic cells in these cultures as annexin V-positive viable cells. The shown panels were gated on GFP-positive cells, and in both cell lines, ~53–65% of the cells expressed GFP-CIS and GFP-CIS R107K. Strikingly, the overexpression of GFP-CIS led to an increased number of annexin V-positive BaF3-EpoR cells (33.0%) compared with GFP-CIS R107K (11.9%) or GFP alone (10.4%). In contrast, in BaF3-EpoR Y401F cells, GFP-CIS had no effect on the number of apoptotic cells. Furthermore, BaF3-EpoR cells expressing GFP-CISΔBox showed an overall reduced GFP fluorescence compared with BaF3-EpoR cells expressing GFP-CIS but a similar annexin V-staining pattern that differed from BaF3-EpoR cells harboring GFP alone (Fig. 5*B*). Thus, we conclude that through the binding of its SH2 domain to tyrosine 401 of the EpoR, GFP-CIS promotes apoptotic cell death in BaF3-EpoR cells at low Epo concentrations.

Receptor Recruitment of CIS Decreases STAT5 Activation by the EpoR in BaF3 Cells—To elucidate mechanisms mediating pro-apoptotic effects of GFP-CIS in BaF3 cells, we asked whether the effect of GFP, GFP-CIS, and GFP-CIS R107K expression on apoptosis in BaF3 cells paralleled inhibition of Epo-induced STAT5 activation. To test this possibility, GFP, GFP-CIS, or GFP-CIS R107K were expressed in BaF3 cells stably expressing the wild type EpoR or the mutant EpoR Y401F. The ability of the obtained cell lines to activate STAT5 in response to increasing concentrations of Epo ranging from 0.5 to 10 units/ml Epo was tested by immunoprecipitation of STAT5 from the cellular lysates and subsequent analysis by

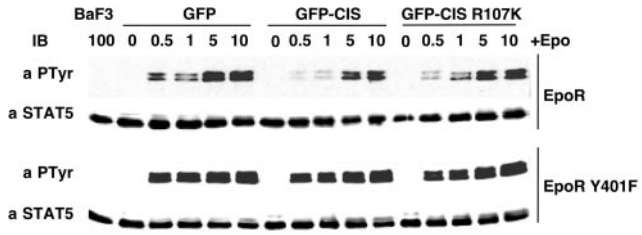

FIG. 6. **CIS reduces STAT5 activation in BaF3 cells.** BaF3 cells stably expressing the wild type EpoR (BaF3 EpoR) or the mutant EpoR Y401F (BaF3 EpoR Y401F) in conjunction with GFP or GFP-CIS or GFP-CIS R107K were left unstimulated or were stimulated for 5 min with 0.5, 1, 5, or 10 units/ml Epo, lysed, and subjected to immunoprecipitation with an anti-STAT5 antiserum. The analysis by immunoblotting was performed using an anti-phosphotyrosine monoclonal antibody (*αPTyr*). To verify equal loading, the blots were stripped and reprobed with anti-STAT5 antiserum. As control, parental BaF3 cells were stimulated with 10 units/ml Epo.

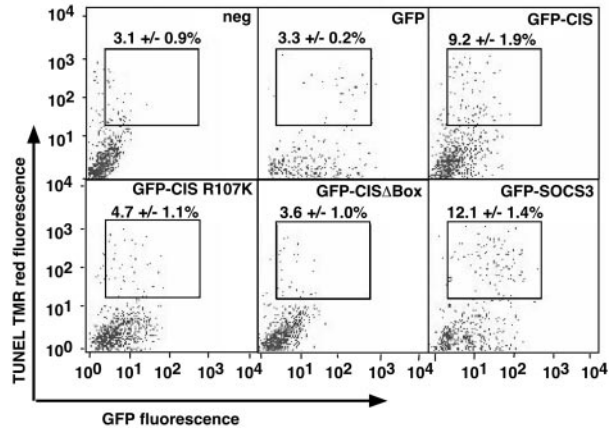

FIG. 7. **CIS enhances apoptosis in erythroid progenitor cells.** Fetal liver cells were depleted by MACS beads with antibodies raised against hematopoietic lineages, transduced with empty vector, GFP-CIS, GFP-CIS R107K, GFP-CISΔBox, and GFP-SOCS3, and cultured for 24 h in serum-free medium PANserin401 supplemented with 0.05 unit/ml Epo. Fixed and permeabilized cells were stained with the TMR-Red TUNEL assay kit, and apoptotic cells were analyzed concomitantly in GFP-positive cells by flow cytometry. The *boxes* indicate GFP and TMR-Red positive cells gated on viable cells according to forward scatter/side scatter discrimination given as percentage of all viable cells.

immunoblotting with anti-phosphotyrosine (Fig. 6). At low Epo concentrations (0.5 unit/ml Epo), STAT5 tyrosine phosphorylation was reduced by the expression of GFP-CIS to ~50% of the level achieved in BaF3 EpoR cells co-expressing GFP or GFP-CIS R107K. At higher Epo concentrations, the extent of STAT5 tyrosine phosphorylation was identical in cells expressing GFP, GFP-CIS, or GFP-CIS R107K. In contrast, in BaF3-EpoR Y401F cells, tyrosine phosphorylation of STAT5 was elevated ~2-fold at lower Epo concentrations and was not influenced in a concentration-dependent manner by the co-expression of GFP-CIS. Therefore, in comparison with the apoptosis-promoting effects, CIS-mediated inhibition of STAT5 phosphorylation depends upon the presence of an intact SH2 domain within CIS and the presence of (phospho)tyrosine 401 within the EpoR. This finding suggests that in BaF3 cells the recruitment of CIS to the EpoR can impair STAT5 activation and thereby accelerate the induction of apoptosis.

CIS SH2 Domain and SOCS Box Are Required to Promote Apoptosis in Erythroid Progenitor Cells—To investigate whether CIS promotes apoptosis in erythroid progenitor cells, we performed TUNEL staining of retroviral transduced fetal liver erythroid progenitor cells grown in serum-free medium supplemented with 0.05 unit/ml Epo. The flow cytometry analysis shown in Fig. 7 revealed that under these conditions ~3% untransduced or GFP-expressing erythroid progenitor cells were undergoing apoptosis. In contrast, of the green fluorescent cells expressing GFP-SOCS3, 12% were undergoing apoptosis. Similarly, the expression of GFP-CIS increased the proportion of apoptotic cells to 9%. This increase in apoptotic cells upon the expression of GFP-CIS was significant ($p < 0.05$) and reproducibly observed also with bicistronic expression using the pOS-IRES-GFP vector (data not shown). Importantly, CIS mutants defective in the SH2 domain (4.7%) or missing the SOCS Box (3.6%) did not promote apoptosis, thus indicating that both domains are essential to mediate the pro-apoptotic effect of CIS in erythroid progenitor cells.

DISCUSSION

At the molecular level, developmental processes such as maturation of erythrocytes are controlled by precise orchestration of activating and inhibiting intracellular signal transduction cascades. Our principal result is that the overexpression of the negative regulatory protein CIS reduces signaling through the EpoR both in cell lines and in primary erythroid progenitors. This results in reduced proliferation and increased apoptosis of erythroid progenitor cells, suggesting an important role for CIS in setting a threshold for erythrocyte production triggered by Epo.

SH2 Domain-mediated Receptor Recruitment of CIS and Inhibition of Cell Proliferation—Our results showed that the recruitment to the EpoR is essential for the negative regulatory function of CIS. In agreement with the studies of Verdier *et al.* (16), we identified (phospho)tyrosine residue 401 in the EpoR cytoplasmic domain as the principal binding site for the CIS SH2 domain (16). By analyzing an extended panel of Tyr-to-Phe mutant EpoRs, we confirmed that, despite a residual association of GFP-CIS to a mutant EpoR lacking Tyr-401 (EpoR Y401F), this residue represents the major CIS binding site. The negative regulatory effect of CIS binding to (phospho)tyrosine 401 is supported by our observation that the expression of EpoR Y401F in BaF3 cells confers hyper-responsiveness to Epo and decreases the sensitivity of cells to undergo apoptosis at low Epo concentrations.

It is possible that in other cell lines, this negative regulatory role of CIS is less important for signaling through the EpoR since Tauchi *et al.* (10) observed that expression in DA-3 cells of an EpoR lacking Tyr-401 resulted in reduced rather than enhanced proliferative responses (10). This observation supports the notion that the efficiency of signaling pathways strongly depends on the cellular background used for analysis. However, we showed that CIS proteins mutant in the SH2 domain and thus unable to bind to the EpoR also could not inhibit proliferation of primary erythroid progenitor cells. This finding supports the role of the CIS SH2 domain in negatively regulating signaling through the EpoR in primary cells.

Role of CIS SOCS Box for Apoptosis-promoting Effects—It was previously shown that CIS is ubiquitinated and that treatment of cells with the proteasome inhibitor LLnL protects the CIS·EpoR complex from degradation (16). This result suggested that CIS negatively regulates EpoR signaling by targeting the receptor for degradation. However, we showed that deletion of the CIS SOCS Box, the segment mediating the association of CIS with the ubiquitin degradation cascade, does not affect the ability of CIS to inhibit proliferation of BaF3 cells. Thus, our results show that whereas SH2 domain-mediated receptor recruitment of CIS is critical for its anti-proliferative effects in BaF3 cells, the SOCS Box is not required. However, in primary erythroid progenitors, the SOCS Box as well as the SH2 domain is essential for the ability of the CIS protein to promote apoptosis. This finding suggests that CIS utilizes different mechanisms to repress growth and to increase sensitivity for entering apoptosis.

Interaction of CIS with the STAT5 Bcl-x_L Pathway—In contrast to other members of the SOCS family of proteins, CIS does not directly interact with or inhibit the activity of Janus kinases (28). CIS binds via its single SH2 domain, specifically, to (phospho)tyrosine 401 in the activated EpoR and thereby could compete binding of other signal-transducing proteins including STAT5. Accordingly, it was observed that forced overexpression of CIS reduces the activation of STAT5 by the EpoR (20, 21). We show here that reduced activation of STAT5 depends on SH2 domain-mediated receptor recruitment of CIS. However, because the EpoR contains a second STAT5 binding site (phospho-Tyr-343) that is equally efficient in activating STAT5, it was proposed that the negative regulatory effect of CIS was amplified by targeting the activated EpoR for degradation (16). We show that cell surface expression of the mutant EpoR lacking the CIS binding site (EpoR Y401F) is slightly reduced compared with the wild type EpoR, suggesting that CIS binding to (phospho)tyrosine 401 does not down-regulate cell surface expression of the EpoR. In addition, our results show that a mutant CIS missing the SOCS Box is as efficient as wild type CIS in repressing the proliferative response of BaF3 cells, supporting the hypothesis that by binding to (phospho) tyrosine 401 CIS either sterically shields (phospho)tyrosine 343 or induces conformational changes in the receptor that impair STAT5 binding to (phospho)tyrosine 343.

CIS itself is transcriptionally induced by the STAT5-signaling cascade but not mitogen-activated protein kinase or phosphatidylinositol 3-kinase (21), and therefore CIS has been suggested to be part of a negative feedback loop controlling the activity of the pathway (15). Numerous reports show a role of STAT5 in proliferation and differentiation mediated by the EpoR (29). Furthermore, STAT5 has been implicated in protection from apoptosis by directly inducing the expression of the anti-apoptotic gene *Bcl-x_L* (12). Adult *STAT5a*$^{-/-}$/*STAT5b*$^{-/-}$ mice have persistent anemia despite a marked compensatory expansion in their erythropoietic tissues. Importantly, decreased expression of *Bcl-x_L* and increased apoptosis in adult and neonatal *STAT5a*$^{-/-}$/*STAT5b*$^{-/-}$ early erythroblasts correlated with the degree of anemia (14). Similarly, in fetal erythropoiesis, the absence of Bcl-x_L could be sufficient to render *STAT5a*$^{-/-}$/*STAT5b*$^{-/-}$ cells more sensitive to apoptosis. Mice deficient in Bcl-x_L have demonstrated an essential role for Bcl-x_L in preventing apoptosis at the end of maturation of both primitive and definitive erythrocytes (13).

In addition to the activation of Bcl-x_L, a role for NFκB has been proposed in EpoR signaling to prevent apoptosis (30). Epo-mediated activation of NFκB was induced by the same membrane-proximal tyrosines that serve as binding sites for STAT5 (30). An EpoR lacking all of the tyrosine residues in the cytoplasmic domain and an EpoR with a mutation of the JAK2 binding site have been demonstrated to induce apoptosis in BaF3 cells (30), supporting the idea that signals involved in protection from apoptosis emanate from the EpoR. We propose that forced expression of CIS impairs activation of the STAT5-signaling cascade and potentially the NFκB pathway and thereby increases the sensitivity of cells to undergo apoptosis. It has been shown that GATA1 cooperates with erythropoietin to regulate Bcl-x_L expression (31) and GATA1 can enhance Bcl-x_L expression by the EpoR/STAT5 pathway (32). The interplay between GATA1, NFκB, STAT5, and CIS clearly demands further investigations.

Two Functions of CIS in Primary Erythroid Cells—Previous studies investigating the role of CIS in erythropoiesis have been limited to analysis of proliferation in cell lines. Here, we have used retroviral expression to analyze the role of CIS in proliferation, differentiation, and apoptosis of primary erythroid progenitor cells. Upon expression of CIS in fetal liver cells, erythroid progenitors form smaller colonies and show a reduced growth rate and an increased frequency to undergo apoptosis. We propose that CIS exerts its negative regulatory function by two interconnected mechanisms. CIS represses recruitment of other signaling molecules to the EpoR, which is sufficient to suppress proliferative responses. However, the ability of CIS to enhance apoptosis in erythroid progenitor cells depends on the SOCS Box, and hence, the enhancement of apoptosis may require degradation of activated receptors by the proteasome. Thus, the amount of CIS induced in cells sets a threshold to EpoR signaling and therefore tightly controls the elicited response.

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The Cytokine-inducible Scr Homology Domain-containing Protein Negatively Regulates Signaling by Promoting Apoptosis in Erythroid Progenitor Cells

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