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CIS1 Interacts with the Y532 of the Prolactin Receptor and Suppresses Prolactin-Dependent STAT5 Activation

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Prolactin (PRL) interacts with a single-chain prolactin-specific receptor of the cytokine receptor superfamily. PRL triggers the activation of JAK2 kinase, which phosphorylates the PRL receptor itself, and of STAT5, a member of the family of signal transducers and activators of transcription (STAT). We have shown that the STAT5-dependent immediate early gene, CIS1 (Cytokine-Inducible SH2 domain–containing protein–1), suppresses PRL-induced STAT5 activation *in vitro* as well as in transgenic mice. To facilitate the study of the interactions between CIS1 and the PRL receptor, we have developed the yeast tri-hybrid system, a modification of the yeast two-hybrid system. We expressed CIS1 fused to the DNA-binding domain and PRL receptor cytoplasmic domain fused to the transcription activation domain in the presence or absence of the tyrosine kinase domain of JAK2 in yeast. CIS1 bound to the PRL receptor cytoplasmic domain in a JAK2-dependent manner. Moreover, we determined that the phosphorylated Y532 of the murine PRL receptor is the binding site for CIS1. Interestingly, Y532 has been shown to be unnecessary for STAT5 activation, although CIS1 overexpression suppressed PRL-induced STAT5 activation. These data suggest that the suppression of STAT5 activation by CIS1 is not due to a simple competition with STAT5 but rather to a modification of the receptor by CIS1 binding.

Key words: cytokine, JAK kinase, phosphorylation, SH2 domain, STAT5.

The signal transducers and activators of transcription (STAT) proteins are cytosolic latent transcription factors that are rapidly activated by interferons (IFNs), interleukins (ILs), and growth factors (*1–3*). A total of seven different STAT proteins have been identified, many of which play highly specific roles in innate and acquired immunity. Knockout mice of STAT1, STAT4, and STAT6 genes are viable but lack functions that are mediated by IFNs, interleukin (IL)12, or IL4/IL13, respectively. In contrast, STAT3 knockout mice exhibit fetal lethality, a finding consistent with the activation of STAT3 by many cytokines that are important for development, such as the leukemia inhibitory factor (LIF), cardiotrophin-1, and IL6. Highly related STAT5a and STAT5b are activated by various cytokines, including the growth hormone (GH), prolactin (PRL), erythropoietin (EPO), IL2, IL3, granulocyte-macrophage colony–stimulating factor (GM-CSF), and thrombopoietin. The phenotypes of STAT5a and STAT5b single-gene knockout mice as well as double knockout mice revealed an important role of STAT5 in PRL and GH signaling as well as in IL2-dependent T-cell functions (*4–9*). STAT5a is critical in PRL signaling (*6*), GM-CSF signaling (*4*), and IL2 signaling (*7*), while STAT5b is necessary to maintain the sexual dimorphism of body growth rates and liver gene expression (*9*), IL2-mediated T-cell proliferation, and NK-cell development (*5*, *8*).

The regulation of the *Janus* kinases (JAKs) and STATs is a central component in the control of cytokine signaling. Because of the critical role of cytokines in mediating inflammation and immunity, it has been proposed that the constitutive activation of JAKs could contribute to hematopoietic disorders, autoimmunity, and inflammatory diseases. However, the mechanisms that terminate or down-modulate the JAK/STAT pathway are not fully understood. The CIS (cytokine-inducible SH2 protein) family of proteins, also referred to as the SOCS (suppressors of cytokine signaling) or SSI (STAT-induced STAT inhibitor) family, has been implicated in regulating signal transduction by a variety of cytokines (see Ref. *10* for a review). The first member of this family, CIS1, was cloned as an immediate early gene responding to a number of cytokines including EPO, IL2, IL3, and GM-CSF (*11*). The CIS1 promoter contains two pairs of tandem TTCNNNGAA motifs that are capable of binding to STAT5 (*12*). The essential role of STAT5 in CIS1 expression was confirmed by the observation that CIS1 was not expressed in the ovaries of STAT5a and b double-gene knockout mice (*8*).

In addition to STAT5-dependent expression, CIS1 could negatively modulate STAT5 activation by binding

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to the cytokine receptors that activate STAT5, including EPO, IL3, and IL2 receptors (*11*). Forced expression of CIS1 partially suppressed cytokine-dependent STAT5 activation in 293 and Ba/F3 cells. Moreover, we have found a striking similarity in the phenotypes of CIS1 transgenic mice and STAT5 knockout mice. The CIS1 transgenic mice exhibited growth retardation and defects in mammary gland development as well as T- and NK-cell development (*13*). These results strongly suggest that CIS1 negatively regulates STAT5 activation *in vivo*. We also showed a decrease in STAT5 activation in response to GH, PRL, and IL2 in the tissues of CIS1-transgenic mice.

However, the mechanism of inhibition of STAT5 activation by CIS1 has not been clarified. The CIS1 protein has been shown to associate with the tyrosine-phosphorylated cytokine receptors, including EPO, IL2, and IL3 receptors. We reported that CIS1 binds to the region of the EPO receptor containing the second tyrosine residue (Y401) (*14*). One possible mechanism is the simple masking of the STAT5 binding sites on the receptor. This model is supported by our observation that overexpression of STAT5 can overcome the negative effect of CIS1. The other possibility is that CIS1 accelerates the degradation of the receptor-CIS1 complex by the ubiquitin-proteasome pathway (*14*, *15*). However, the CIS1-dependent ubiquitination of cytokine receptors has not been demonstrated.

In this paper, we identified the tyrosine residue of the PRL receptor that interacts with CIS1 by using a modified yeast two-hybrid system. The interaction depends on the intact SH2 domain of CIS1 and the tyrosine phosphorylation of Y532 of the PRL receptor. This tyrosine residue has been shown to be unnecessary for STAT5 activation, although CIS1 overexpression suppressed PRL-induced STAT5 activation. These data suggest that suppression of STAT5 activation by CIS1 is not due to a simple competition with STAT5 but rather to a steric hindrance or modification of the receptor by CIS1 binding. This study also demonstrates the usefulness of the modified yeast two-hybrid system in searching for a target of the SH2 domain–containing protein.

MATERIALS AND METHODS

Construction of a Tri-Hybrid Vector—The JAK2 tyrosine kinase (JH1) domain fused to bacterial glutathione S-transferase (GST-JH1) was described previously (*16*). GST-JH1 cDNA was subcloned into pAUR123 (Takara, Kyoto) (pAUR-JH1). Then the *Bam*HI fragment containing ADH (alcohol dehydrogenase) promoter, GST-JH1 cDNA, and ADH terminator from pAUR-JH1 was blunt-ligated into the *Pvu*II site of pBTM116 vector (pBTM-JH1). The CIS1-SH2 domain (codon 26–161) was amplified by polymerase chain reaction (PCR) using 5′-GACCCCGGGCCAGGCAGAGAATGAA-3′ and 5′-TCGCTCGAGACTGCTCCTGCGCAC-3′ as primers, then ligated into pBTM (pBTM-CIS1) and pBTM-JH1 (pBTM-JH1-CIS1) using *Xma*I and *Xho*I sites. CIS1 cDNA and the C-terminal SH2 domain of the PI3-kinase p85 subunit (616–723) were subcloned into pACT2 as described (*17*). To introduce the point mutations in the SH2 domain, site-directed mutagenesis was performed by a standard technique using the oligonucleotide 5′-GGTACATTCCTAGTTGAAGACAGCACCCACCC-3′ for the R107K mutant and the oligonucleotide 5′-GGTACATTCCTAGTTAAAGACAGCACCCACCC-3′ for the R107E mutant (*16*).

Tri-Hybrid Screening—Tri-hybrid screening was performed basically as described previously (*18*). For CIS1-EPO receptor interaction, pBTM/CIS1 and pACT-EPOR plasmids were introduced into yeast L40 with or without pAUR-JH1. Transformants were selected in a medium lacking histidine, tryptophane, and leucine in the presence or absence of 100 ng/ml Aureobasidin A. For library screening, the human fetal liver cDNA library in the pACT vector (Clontech) was introduced into the L40 transformant bearing pBTM-JH1-CIS1 with a LiCl protocol. Ten million transformants were selected in a synthetic medium lacking histidine, tryptophane, and leucine. The isolated colonies were subsequently assayed for β-galactosidase activity on filters. The positive plasmid DNA was isolated by transformation into HB101 and sequenced as described (*18*).

PRL Receptor and EPO Receptor Construction—The cDNA fragment for the cytoplasmic domain of the PRL receptor was amplified by PCR with 5′-CGCGAATTCCTGGGCCAAAAATAAA-3′ and 5′-TTCCTCGAGTCAGTGAAAGGAG-3′ as primers, then ligated in-frame into the *Eco*RI and *Sal*I sites of pACT2. Site-directed mutagenesis for tyrosine (Y) to phenylalanine (F) substitution was conducted as described (*16*). To create the Y532F mutant, the oligonucleotide 5′-CCTGAGAACAATAAGGAGTTTGCCAAGGTGTCCGG-3′ was used. To create the prey of the entire cytoplasmic domain of the EPO receptor, the PCR fragment of the EPO receptor cDNA (codon 273–507) was subcloned into the *Eco*RI/*Xho*I sites of pACT2.

Luciferase Assay—The STAT5 responsive promoter-luciferase reporter gene, which carries four repeats of the GAS sequence with the jun promoter, was described previously (*16*). The mouse PRL receptor cDNA in pECE was a generous gift from Dr. Wolfgang Doppler (Innsbruck University, Austria). For the luciferase assay, a murine PRL receptor instead of a human PRL receptor was used because it had a higher level of expression and response in 293 cells. PRL-dependent luciferase activity in 293 cells grown in 6-well plates was measured after transfection with the reporter gene (50 ng plasmid), the β-galactosidase gene (50 ng), murine PRL receptor cDNA (200 ng), and Flag-tagged CIS1 cDNA (200 ng) as previously described (*13*). Recombinant ovine PRL was a kind gift from Dr. Arieh Gertler (The Hebrew University of Jerusalem).

RESULTS AND DISCUSSION

CIS1 Binds to the Cytoplasmic Domain of the EPO Receptor in a JAK2-JH1–Dependent Manner in Yeast—We have shown that CIS1 binds to the phosphorylated EPO receptor *in vivo*. To investigate CIS1-EPO receptor interaction in yeast, the yeast L40 was transformed with two plasmids, CIS1/pBTM as bait and the EPO receptor cytoplasmic domain/pACT2 "as prey." However, we could not detect any interaction between CIS1 and the EPOR cytoplasmic domain in this system (Fig. 1A). Similarly, no interaction was observed between the SH2-domain of PI3 kinase p85 subunit and the EPO receptor. These data suggest that the interaction between the SH2 domain proteins and the EPO receptor requires phosphorylation

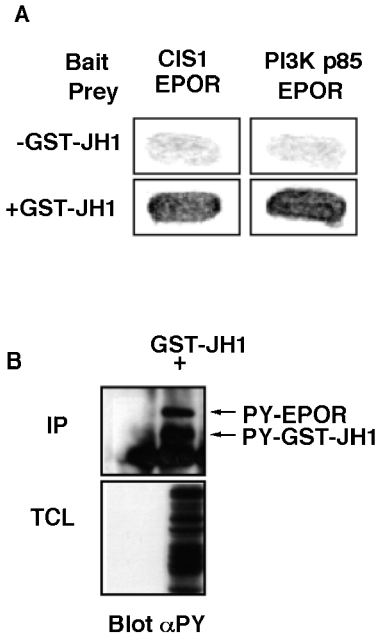

Fig. 1. **JAK2 kinase-dependent interaction between the EPO receptor (EPOR) cytoplasmic domain and SH2 proteins.** A: Yeast strains carrying pBTM-EPOR and pACT-CIS1 or the pACT-PI3kinase SH2 domain in the presence or absence of pAUR-JH1 were restreaked on a filter paper and assayed *in situ* for β-galactosidase. B: Tyrosine phosphorylation of the EPO receptor cytoplasmic domain in yeast carrying GST-JH1. Yeast strains carrying pBTM-EPOR with or without pAUR-JH1 were lyzed, and total cell extracts (TCL) and immunoprecipitates (IP) with an anti-EPOR antibody were immunoblotted with anti-phosphotyrosine antibody.

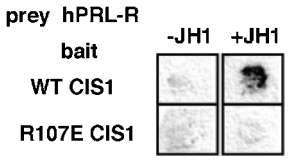

Fig. 2. **CIS1-PRL receptor (PRLR) interaction is dependent on the SH2 domain of CIS.** Yeast strains carrying pBTM-PRLR and pACT carrying wild-type (WT) CIS1 or R107E-CIS1 in the presence or absence of pAUR-JH1 were streaked on a filter paper and assayed *in situ* for β-galactosidase.

of the tyrosine residue of the receptor. Thus, we introduced a constitutively active tyrosine kinase to phosphorylate the cytoplasmic domain of the EPO receptor. For this purpose, we created a fusion protein between GST and the tyrosine-kinase (JH1) domain of JAK2, and this fusion gene (GST-JH1) was subcloned into a yeast expression vector, pAUR123, which contains a resistance marker against Aureobasigin A. Previously, we have shown that GST-JH1 is a constitutively active kinase, probably because of dimer formation through the GST molecule (*10*). The yeast carrying three plasmids, CIS1/pBTM, EPO receptor/pACT2, and GST-JH1/pAUR, was selected in a medium lacking leucine and tryptophane but containing Aureovacidine A. As shown in Fig. 1B, GST-JH1 was heavily phosphorylated in yeast, and the yeast proteins were also tyrosine-phosphorylated. The fusion protein of the cytoplasmic domain of the EPO receptor with the GAL4 activation domain was also tyrosine-phosphorylated (PY-EPOR). As illustrated in Fig. 1A, expression of GST-JH1 resulted in the interaction between CIS1 and the EPO receptor in yeast. Thus, CIS1 interacted with the cytoplasmic domain of the EPO receptor in a phosphorylation-dependent manner. Similar results were obtained for the interaction between the SH2-domain of PI3 kinase p85 subunit and the EPO receptor (Fig. 1A).

The PRL Receptor Interacts with CIS1 Tyrosine Phosphorylation-Dependently—First, we searched for CIS1-interacting proteins by use of a conventional two-hybrid system from the human fetal liver cDNA library, but no positive clone (His-resistant and lacZ-positive) was obtained. To find phosphorylation-dependent CIS1-interacting proteins, we carried out yeast tri-hybrid screening. For the screening, GST-JH1 was subcloned into the *Pvu*II site of the bait vector pBTM (Fig. 1A) so that the screening could be carried out without using expensive antibiotics and performing one additional transformation. In yeast carrying the pBTM-GST-JH1-CIS1 hybrid plasmid and yeast carrying pAUR-GST-JH1, similar GST-JH1 expression and tyrosine phosphorylation of cellular proteins were observed. We screened the same human fetal liver cDNA library and obtained two positive clones. These two encoded C-terminal regions of the human PRL receptor. As in the case of the EPO receptor, we confirmed the interaction of the PRL receptor cytoplasmic domain with CIS1, which is dependent on the presence of JH1 (Fig. 2). This is consistent with the previous biochemical demonstration of the binding between the PRL receptor and CIS1 (*19*).

The interaction between the CIS1 SH2 domain and the PRL receptor was examined more extensively using a tri-hybrid system. The interaction between CIS1 and the PRL receptor cytoplasmic domain clearly required the JH1 expression, suggesting that this interaction requires tyrosine phosphorylation (Fig. 2). Furthermore, the interaction between CIS1 and the PRL receptor required an intact SH2 domain, since the R105E mutation of CIS1, which disrupts the functional SH2 domain, abrogated the interaction between the CIS1 and the PRL receptor (Fig. 2). Therefore, the CIS1-PRL receptor interaction was dependent on the phosphotyrosine-SH2 domain interaction. We then constructed deletion mutants from the C-terminal end of the PRL receptor (Fig. 3A). As shown in Fig. 3B, CIS1 bound to the cytoplasmic domain of the PRL receptor with the C-terminal truncation lacking the eighth tyrosine residue (Y597 or Y8) but not with those lacking Y7 or further tyrosine residues. To confirm the importance of F7, we created substitution mutants. As shown in Fig. 3B, the Y7F8 mutant could still interact with CIS1; however, neither F7Y8 nor F7F8 interacted with CIS1. These data suggest that Y532 is necessary for the binding with CIS1.

Interaction of CIS1 with pY532 of the PRL Receptor Inhibits PRL-Induced STAT5 Activation—We have shown that CIS1 binds to Y431 of the EPO receptor, which is one of the major STAT5 binding sites. On the other hand, Y532 of the PRL receptor was not supposed to be a binding site for STAT5 (*20*, *21*). Therefore, we exam-

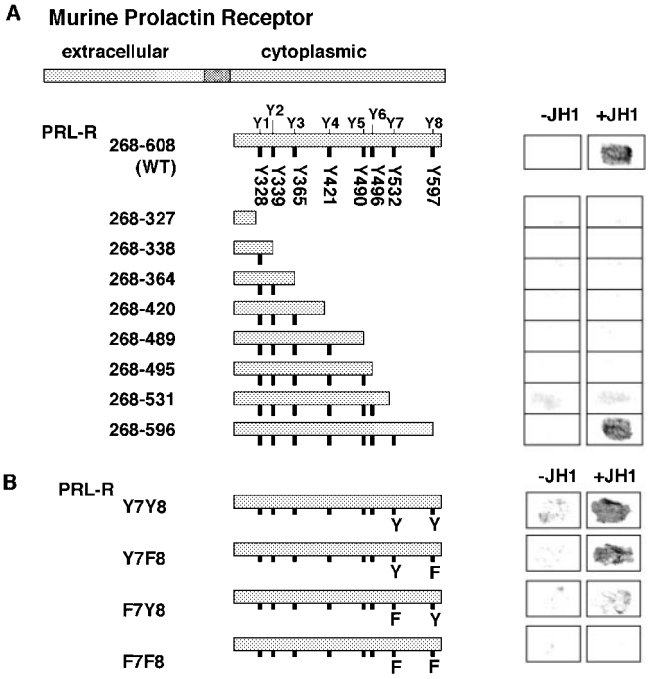

Fig. 3. **Y532 of the prolactin receptor is the binding site for CIS1.** Yeast strains carrying pBTM-CIS1 (–JH1) or pBTM-JH1-CIS1 (+JH1) and pACT containing indicated mutants of PRL receptor cytoplasmic domain cDNA were streaked on a filter paper and subjected to the *in situ* β-galactosidase assay. Representative results of at least three independent determinations are shown.

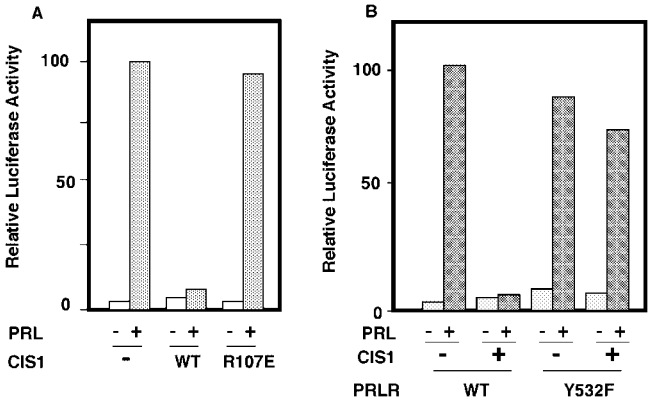

Fig. 4. **Y532 is necessary for the suppression of STAT5 activation by CIS.** 293 cells were co-transfected with plasmids encoding a wild-type PRL receptor (WT) or a mutated PRL receptor (Y532F) and luciferase reporter genes. The effect of CIS1 and its SH2 mutant (R105E) (A) and the effect of mutant prolactin receptor (B) were determined. Transactivation was determined after a 6-h stimulation with 50 ng/ml PRL. The activity observed in cells transfected with the WT PRL receptor in the absence of CIS1 was taken as 100%. Representative results of at least three independent determinations are shown.

ined the effect of Y532 mutation on the CIS1-dependent suppression of the PRL receptor–mediated STAT5 activation. To demonstrate a direct inhibition of PRL-induced STAT5 activity by CIS1, we used a transient reporter-gene assay in 293 cells (*12*, *13*). A STAT5 reporter-gene construct was transfected into 293 cells with the PRL receptor (Fig. 4) and CIS1 cDNAs. In agreement with an earlier report (*22*), wild-type CIS1 inhibited STAT5 activation in response to PRL (Fig. 4A). This suppression of STAT5 by CIS1 was dependent on the SH2-phosphotyrosine interaction, since the CIS1 R107E mutant, which lacks a functional SH2 domain, did not suppress PRL-induced STAT5 activation. As reported previously, the Y532F mutant PRL receptor still activated STAT5 (Fig. 4B). However, CIS1 did not inhibit STAT5 activation through the Y532F mutant PRL receptor. These data suggest that interaction between CIS1 and the prolactin receptor through Y532 inhibits STAT5 activation, although Y532 is not the binding site for STAT5.

Mechanism of the Suppression of STAT5 Activation by CIS1—Suppression of PRL-induced STAT5 activation by CIS1 was partially overcome by co-expression of STAT5 (*13*, and data not shown). This suggested competition for the binding to the PRL receptor between CIS1 and STAT5. Thus, we propose that CIS1 suppresses STAT5 activation by blocking the binding of STAT5 to the receptor binding sites either by steric hindrance or by inducing conformational changes of the receptor.

Recently, the possibility, that CIS1 accelerates the degradation of the receptor-CIS1 complex by the ubiquitin-proteasome pathway has been proposed (*14*). Ram and Waxman reported that CIS1 inhibits the growth hormone (GH) receptor-JAK2 signaling in a time-dependent manner that involves proteasome action in addition to competition between CIS1 and STAT5 for common GH receptor cytoplasmic tail phosphotyrosine-binding sites (*15*). They found that the proteasome inhibitor MG132 blocked CIS1 protein degradation as well as the inhibitory action of CIS1, but not that of SOCS-1 or SOCS-3, on GH-induced STAT5 signaling. Proteasome-dependent degradation of CIS1, most likely in the form of a (GH receptor-JAK2)-CIS1 complex, is therefore proposed to be an important step in the time-dependent CIS1 inhibition mechanism. Since proteasome inhibitors also stabilize the tyrosine-phosphorylated form of the EPO receptor as well as of STAT5, and CIS1 itself is rapidly ubiquitinated, this model is an attractive hypothesis (*14*). CIS1 has the SOCS-box, which was recently found to be involved in ubiquitination by recruiting Elongin B, C, Cul2, and the Rbx-1 complex (*23*, *24*). Furthermore, Waxman's group reported that the CIS R107E mutant function is a dominant negative form against CIS1 by blocking proteasome-dependent degradation of the GH receptor (*15*). However, we can not rule out the possibility that ubiquitination of the cytokine receptors prevents access of STAT5 to the receptor. Further study is necessary to elucidate the mechanism of STAT5 inhibition by CIS1.

This study has demonstrated the usefulness of a yeast tri-hybrid system for the detection of the SH2 domain-phosphopeptide interaction. This system can be applied for the screening of the target molecule of a given SH2 domain, such as the CIS family members, without modification of the conventional two-hybrid libraries. Furthermore, the peptide motifs that preferentially interact with an SH2 domain can be determined by using synthetic random peptide libraries containing tyrosine residues.

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