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CIS Associates with the Interleukin-2 Receptor β Chain and Inhibits Interleukin-2-dependent Signaling*

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CIS is a cytokine-induced SH2-containing protein that was originally cloned as an interleukin (IL)-3-inducible gene. CIS is known to associate with the IL-3 receptor β chain and erythropoietin receptor and to inhibit signaling mediated by IL-3 and erythropoietin. We now demonstrate that CIS also interacts with the IL-2 receptor β chain (IL-2Rβ). This interaction requires the A region of IL-2Rβ (residues 313–382), which also mediates the association of IL-2Rβ with Lck and Jak3. Correspondingly, CIS inhibits functions associated with both of these kinases: Lck-mediated phosphorylation of IL-2Rβ and IL-2-mediated activation of Stat5. Thus, we demonstrate that CIS can negatively control at least two independent IL-2 signaling pathways. Although a functional SH2 binding domain of CIS was not required for its interaction with IL-2Rβ *in vitro*, its phosphotyrosine binding capability was essential for the inhibitory action of CIS. On this basis, we have generated a mutant form of CIS protein with an altered SH2 domain that acts as a dominant negative and should prove useful in further understanding CIS action.

Following antigen encounter, the magnitude and duration of the subsequent T-cell immune response is critically controlled by the interaction of IL-2[1] with specific high affinity receptors (1, 2). High affinity IL-2 receptors (IL-2Rs) are composed of three chains, denoted IL-2Rα, IL-2Rβ, and the common cytokine receptor γ chain, γ_c. IL-2 induces the heterodimerization of IL-2Rβ and γ_c, which together are necessary and sufficient for IL-2 signaling (3, 4). Although neither IL-2Rβ nor γ_c have intrinsic protein-tyrosine kinase catalytic activity, IL-2 rapidly induces tyrosine phosphorylation of these chains and of intracellular proteins (1, 2). This is accomplished through activation of receptor-associated tyrosine kinases, which in turn phosphorylate cellular substrates responsible for the transmission of IL-2-induced signals. Two principal groups of kinases have been reported to associate with the IL-2 receptor subunits: the Src family kinase Lck (1) and the Janus kinases Jak1 and Jak3 (5–7), which activate the transcription factors Stat5a, Stab5b, and Stat3 (2). Jak1 (5–7) and Lck (1) associate with IL-2Rβ, whereas Jak3 associates primarily with γ_c (5–7) but also can interact with IL-2Rβ following stimulation with IL-2 (6, 8). Syk has also been reported to associate with IL-2Rβ, although mice lacking Syk do not have defects related to IL-2 signaling (discussed in Ref. 2). In addition to these kinases, other signaling molecules can also associate with the IL-2 receptor. For example, IL-2Rβ associates with Shc (9, 10) and phosphatidylinositol 3-kinase (11).

Given the diverse array of molecules associating with cytokine receptors such as the IL-2 receptor, it is clear that the different signals they exert must be carefully controlled. In general, the functional outcome of biochemical events triggered by cytokines represents a balance between activating and inhibitory signals believed to function as part of negative feedback loop(s). The inhibitory signals play an important role in the control of the magnitude and duration of the cellular response to extracellular stimuli. In the past few years, several mechanisms for this negative regulation have been elucidated. These include the activation of phosphatases (12, 13), SIRPs (14), and a recently discovered family of small SH2-containing proteins including CIS (cytokine-inducible SH2-containing protein) (15), JAB (Jak-binding protein) (16, 17), SOCS (suppressor of cytokine signaling) (18, 19), and SSI (STAT-induced STAT inhibitors) (20, 21) proteins (reviewed in Ref. 22).

CIS (now also denoted as CIS-1) is the prototype member of the CIS/JAB/SOCS/SSI family of proteins (reviewed in Ref. 22). It is induced in hematopoietic cells within 30 min of stimulation by IL-2, IL-3, granulocyte-macrophage colony-stimulating factor, and erythropoietin, but not by stem cell factor, granulocyte colony-stimulating factor, or IL-6 (15). STAT response elements have been identified in the promoter region of CIS, allowing its induction by a variety of cytokines including IL-2 (23). Once expressed, CIS/JAB/SOCS/SSI proteins interfere with signaling events and suppress cytokine-specific cellular responses. JAB/SOCS-1/SSI-1 has been shown to associate with Jak kinases and to inhibit their catalytic activities.

Previously, it was demonstrated that CIS could associate with the IL-3 receptor β chain and erythropoietin receptor upon appropriate stimulation. Furthermore, CIS was shown to reduce the proliferative responsiveness of cells to IL-3 (15) and to partially inhibit erythropoietin-induced Stat5 phosphorylation and transactivation in HEK293 cells reconstituted with the erythropoietin receptor and Stat5 (23). We now demonstrate that CIS can associate with IL-2Rβ and that it can inhibit more than one IL-2-related signaling pathway.

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[1] The abbreviations used are: IL, interleukin; IL-2R, interleukin-2 receptor; PBL, peripheral blood lymphocytes; mAb, monoclonal antibody; PHA, phytohemagglutinin; SH2, Src homology 2; STAT, signal transducers and activators of transcription.

MATERIALS AND METHODS

Cells, Transfections, and Reporter Assays—Peripheral blood lymphocytes (PBL) were prepared from normal donors using standard methods. To generate "preactivated PBL," freshly isolated PBL were cultured for 72 h in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml each of penicillin and streptomycin ("complete medium"), and 2 μg/ml PHA-L (Roche Molecular Biochemicals), and then washed and rested for 24 h in complete medium. NK-like YT cells were cultured and maintained in complete medium. 32D cells were maintained in complete medium supplemented with 10^{-5} M 2-mercaptoethanol and 5% WEHI-3B conditioned medium as a source of IL-3. Transfectants expressing IL-2Rβ were generated by electroporating cells (5×10^6 cells/400 μl) with linearized pCDNA3zeo (InVitrogen) containing IL-2Rβ using a Gene Pulser (Bio-Rad; 300 V, 960 microfarads; average time constant = 30 ms). After 24 h, cells were aliquoted into a 24-well plate and selected in 0.8 mg/ml Zeocin™ (InVitrogen). Resistant clones were tested for IL-2Rβ expression by Western blotting using goat anti-human IL-2Rβ antiserum (R & D Systems, Minneapolis, MN). 293T$^+$ cells were cultured in Dulbecco's modified Eagle's medium (Biofluids) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 293T$^+$ cells at 50% confluency were transfected using calcium phosphate precipitation reagents (5 Prime → 3 Prime, Inc., Boulder, CO), as described previously (24).

Transient transfections of YT and 32D-IL-2Rβ cells for luciferase reporter assays were performed using the DEAE-dextran method. Briefly, 1–2 $\times$ 10^6 cells were incubated with up to 10 μg of plasmid DNA and 200 μg of DEAE-dextran in 1 ml of STBS buffer (25 mM Tris, pH 7.4, 137 mM NaCl, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 5 mM KCl) at 37 °C for 30 min, followed by washing once with medium. For YT cells, 18 h after transfection, cells were stimulated with 2 nM IL-2 for an additional 12–24 h and then harvested. 32D-IL-2Rβ cells were incubated with either 0.05% WEHI-3B conditioned medium (which is sufficient to maintain cell viability but not growth) or with 2 nM IL-2 or 5% WEHI-3B conditioned medium for 24–36 h. For luciferase assays, lysates were prepared using the Luciferase Assay System kit (Promega). Protein concentrations were measured with a protein assay kit (Bio-Rad), and 5–20 μg of protein were used for luciferase assays according to the manufacturer's instructions (Promega). Luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Ba/F3 cells were stably transfected with IL-2Rβ to create Ba/F3-IL-2Rβ cells. Ba/F3-IL-2Rβ-CIS cells additionally express CIS in pMAMneo (so that its expression can be induced by steroids; see Ref. 15).

RNA Preparation and Northern Blot Analyses—Poly(A)$^+$ RNA was extracted from PBL using the FastTrack 2.0 Kit (InVitrogen). Northern blotting was performed using 2 μg/lane of poly(A)$^+$ RNA on 0.8% formaldehyde-agarose gels as described previously (25), using CIS or control *pHe7* ^{32}P-labeled probes. *pHe7* (26) is a "housekeeping" gene whose expression is similar before and after IL-2 stimulation (27).

Constructs and Mutagenesis—The full-length murine CIS cDNA was subcloned into the mammalian expression vector pME18S. FLAG-tagged wild type CIS and truncation mutants of CIS were generated by polymerase chain reaction, subcloned into pME18S, and sequenced. A point mutation converting arginine 107 to lysine (R107K) in the SH2 domain was introduced into the CIS cDNA using standard polymerase chain reaction-based techniques, and the sequence was confirmed by DNA sequencing. The wild type human IL-2Rβ and IL-2Rβ mutant constructs used in this study have been described (8, 10). Myc-tagged wild type Lck (Lck^{wt}) and Lck^{Y505F} were provided by Dr. J. Ashwell (NCI, National Institutes of Health). The NF-κB-responsive chloramphenicol acetyltransferase reporter construct, pTKκB3, containing three copies of the human immunodeficiency virus κB site, has been described previously (28). The Stat5a, Stat5b, IL-2Rβ, γ_c, and Jak3 cDNAs were all human cDNAs. The β-casein reporter construct was generated by cloning three repeats of the GAS motif from the β-casein promoter and the cytomegalovirus minimal promoter into pGL-2basic (Promega).

Antibodies, Immunoprecipitations, and Western Blotting—Polyclonal rabbit anti-CIS antiserum was prepared as described previously (15). Anti-Myc mAb 9E10 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-FLAG M2 mAb was from Eastman Kodak Co.; anti-phosphotyrosine mAbs 4G10 and PY20 were from Upstate Biotechnology, Inc. (Lake Placid, NY) and Transduction Laboratories. Polyclonal rabbit anti-Lck antiserum was a gift of Dr. L. Samelson (NICHD, National Institutes of Health). Mikβ1 mAb to IL-2Rβ was provided by Drs. M. Tsudo (Tokyo Metropolitan Institute) and J. Hakimi (Hoffmann-La Roche), and Mikβ3 mAb to IL-2Rβ was provided by M. Tsudo. Cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 5 mM NaF, 10 μg/ml each leupeptin and aprotonin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) and centrifuged at 14,000 $\times$ *g* at 4 °C for 15 min. Lysates were boiled in reducing SDS sample buffer and immunoblotted or were immunoprecipitated for 1–2 h at 4 °C using specific antibodies and protein A-Sepharose beads.

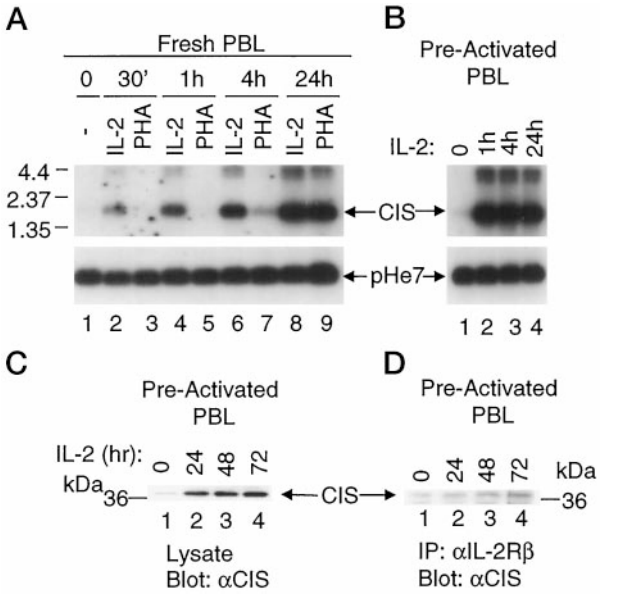

FIG. 1. **Induction of CIS mRNA and protein expression by IL-2 and association of CIS with IL-2Rβ.** *A*, fresh PBL were stimulated with either 2 nM IL-2 (*lanes 1*, *3*, *5*, and *7*) or 2 μg/ml PHA (*lanes 2*, *4*, *6*, and *8*) for the indicated times. Poly(A)$^+$ RNA was extracted and analyzed by Northern blotting using a ^{32}P-labeled murine CIS as a probe (*upper panel*). The blot was stripped and reprobed using *pHe7* (*lower panel*) to control for variations in loading. In addition to the major CIS transcript, a minor species of approximately 4.3 kilobases was detected. The basis for this form is unclear, but it may result from utilization of an alternative polyadenylation signal. *B*, preactivated PBL were unstimulated (*lane 1*) or stimulated with IL-2 for 1, 4, or 24 h (*lanes 2–4*). The blot was then hybridized with CIS and *pHe7* as in *A*. *C*, preactivated PBL were stimulated with 2 nM IL-2, and CIS protein expression was analyzed by Western blotting. *D*, lysates were immunoprecipitated with an anti-IL-2Rβ antibody, Mikβ3, and then Western blotted with anti-CIS antibody.

RESULTS

CIS mRNA and Protein Are Potently Induced by IL-2—To investigate the potential role of CIS in IL-2 signaling, we first examined the expression of CIS mRNA in response to IL-2 and phytohemagglutinin (PHA) in normal human PBL. In unstimulated freshly isolated PBL, CIS mRNA was not detected (Fig. 1*A*, *lane 1*); however, IL-2 induced CIS mRNA within 30 min (*lane 2*), and the levels of CIS mRNA increased with time, with high level expression being sustained for at least 24 h (*lanes 4*, *6*, and *8*). Stimulation of PBL with PHA also induced CIS, but with a slower time course so that it was 4 h before even very low levels of CIS mRNA were detected (compare *lanes 3*, *5*, and *7* with *lane 1*). However, by 24 h, the level of CIS expression was comparable with that seen with IL-2 (*lane 9*). These data suggest that PHA may not induce CIS expression directly but rather indirectly through induction of IL-2 production, given that at least 4 h is typically needed before appreciable levels of IL-2 protein can be detected after PHA activation. When PBL were preactivated with PHA for 3 days and then rested overnight in IL-2-free medium, a treatment that induces maximal expression of high affinity IL-2 receptors and primes cells for potent cellular responsiveness to IL-2, CIS mRNA expression was rapidly induced by IL-2 and sustained at a high level for at

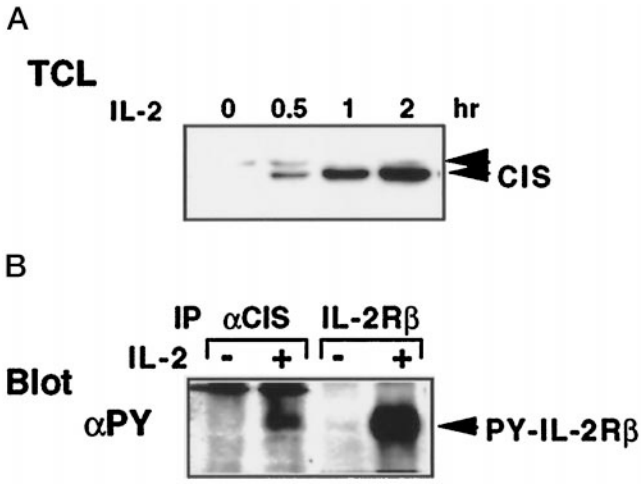

FIG. 2. **Association of CIS with IL-2Rβ in Ba/F3-IL-2Rβ cells.** *A*, induction of CIS protein by IL-2 in Ba/F3 cells. Cells were cultured for 0, 0.5, 1, or 2 h in IL-2, and then total cell lysates were Western blotted with anti-CIS antisera. *B*, cells were not stimulated or stimulated with IL-2 for 1 h. Lysates were then immunoprecipitated with anti-CIS or anti-IL-2Rβ antibodies and Western blotted with anti-phosphotyrosine. The position of IL-2Rβ was determined by alignment with an anti-IL-2Rβ immunoprecipitation.

least 24 h (Fig. 1*B*, *lanes 1–4*). We also investigated the expression pattern of CIS protein in these cells. CIS protein was potently expressed within 24 h, and high levels were maintained for at least 72 h (Fig. 1*C*, *lanes 1–4*).

CIS Physically Associates with the "A" Region (Amino Acids 313–382) of IL-2Rβ in a Fashion That Does Not Require a Classical SH2-Phosphotyrosine-mediated Interaction—As noted above, CIS is induced by both erythropoietin and IL-3 and can associate with both the erythropoietin receptor and the murine IL-3 receptor β chain (15). We therefore tested the ability of CIS to associate with the IL-2Rβ chain. Lysates from IL-2-stimulated, preactivated PBL were immunoprecipitated with Mikβ3 mAb to IL-2Rβ and then Western blotted with anti-CIS antibodies. As shown in Fig. 1*D*, CIS coprecipitated with IL-2Rβ, indicating a physical interaction between these two proteins. Although the degree of apparent interaction was inducible (Fig. 1*D*), it was not as marked as was the inducibility of CIS (Fig. 1*C*). To further investigate this situation, we also evaluated coprecipitation in Ba/F3 cells stably transfected with IL-2Rβ (Fig. 2). In these cells, IL-2 stimulates CIS expression (Fig. 2*A*). However, as in PBL, it was difficult to detect a CIS-IL-2Rβ interaction with anti-IL-2Rβ antibodies (data not shown), but this interaction was revealed with more sensitive anti-phosphotyrosine antibodies (Fig. 2*B*). The relatively weak coprecipitation of CIS and IL-2Rβ could reflect low stoichiometry; alternatively, a higher stoichiometry might exist but might not be readily detected given that neither the anti-CIS nor anti-IL-2Rβ antibodies are particularly robust. It is also possible that the weak coprecipitation could also reflect a comparatively low affinity or transient interactions.

To map the region of IL-2Rβ required for its interaction with CIS, we performed coimmunoprecipitation experiments in 293 T^+ cells transfected with murine CIS plus wild type IL-2Rβ or IL-2Rβ mutants containing various internal deletions or C-terminal truncations (see Fig. 3*A* for a schematic of IL-2Rβ, including the locations of the A (residues 313–382) and S (residues 267–323). For unclear reasons, we consistently observed lower CIS expression when CIS was co-expressed with mutants of IL-2Rβ lacking either the A or S regions. Therefore, we increased the amount of the CIS plasmid cotransfected with IL-2RβΔA or IL-2RβΔS in order to achieve more similar levels of CIS expression (Fig. 3*B*, *lanes 4* and *5 versus lane 1*; *middle panel*; note that murine CIS is routinely detected as a doublet). When CIS and wild type IL-2Rβ were cotransfected (*lane 1*) and then immunoprecipitated with Mikβ1 mAb to IL-2Rβ, CIS was efficiently coprecipitated (*lane 1*, *top panel*). Deletion of the S region partially decreased the degree of association of IL-2Rβ with CIS (*lanes 3* and *5*), whereas deletion of the A region of IL-2Rβ abrogated CIS interaction (*lanes 2* and *4*; note that no CIS was coprecipitated in lane 4 (*upper panel*) although expression of CIS in this lane was higher than in *lane 3* (*middle panel*), where CIS weakly coprecipitated with IL-2RβΔS).

We next analyzed several C-terminal IL-2Rβ truncation mutants for their abilities to bind CIS. These experiments revealed that the sequences beyond amino acid 350 were dispensable for this interaction, while truncation at amino acid 330 abrogated the association (Fig. 3*C*, *upper panel*). Therefore, these results identify amino acids 330–350 as critical for CIS binding, complementing the deletion analysis. The fact that a mutant lacking the S region (residues 267–323) showed reduced association with CIS (Fig. 3*B*) suggests the presence of an additional direct or indirect contact point within the S region or suggests that deletion of the S region has conformational effects resulting in reduced association.

To clarify the region of CIS that mediates its interaction with IL-2Rβ, we generated wild type CIS and C-terminal truncation mutants of CIS that were FLAG-tagged at their C termini. The truncation mutants contained either the first 82 amino acids (the residues N-terminal to the SH2 domain, denoted CIS^{NT}) or the first 177 amino acids (retaining the N-terminal region as well as the SH2 domain, denoted $CIS^{\Delta CT}$)(Fig. 4*A*). Following coexpression of these constructs with wild type IL-2Rβ in 293 T^+ cells, immunoprecipitation experiments were performed (Fig. 4*B*, *top panel*, *lanes 1–3*). Whereas $CIS^{\Delta CT}$ could associate with IL-2Rβ as well as wild type CIS (Fig. 4*B*, *middle panel*, *lane 1 versus lane 3*), CIS^{NT} (which lacks the SH2 domain) could not (*lane 2*), suggesting that the interaction of CIS with IL-2Rβ might involve a classical SH2/phosphotyrosine interaction. This hypothesis is consistent with the suggestion that tyrosine-phosphorylated forms of IL-3Rβ and the erythropoietin receptor could associate with CIS (15). Surprisingly, however, the association of CIS with IL-2Rβ did not appear to require the tyrosine phosphorylation of IL-2Rβ as demonstrated by the ability of CIS to associate with an IL-2Rβ mutant in which all six cytoplasmic tyrosines were mutated to phenylalanines ($IL\text{-}2R\beta^{FFFFFF}$) (Fig. 4*C*, *middle panel*, *lane 3 versus lane 1*). Even wild type IL-2Rβ does not appear to be phosphorylated in these 293 transfections (data not shown and shown below in Fig. 6*B*, *top panel*, *lane 1*). However, we used the $IL\text{-}2R\beta^{FFFFFF}$ construct to exclude the possibility that a low but undetectable level of tyrosine phosphorylation of IL-2Rβ played a role in the interaction seen in Fig. 4*C*. To more directly assess the role of the CIS SH2 domain in the CIS-IL-2Rβ interaction, we prepared a CIS mutant in which arginine 107 in the phosphotyrosine binding FLVR motif was changed to lysine (CIS^{R107K}). While this type of mutation is known to disrupt the ability of SH2 domains to bind phosphotyrosine (29–31), it had little effect on the ability of CIS to associate with IL-2Rβ (Fig. 4*C*, *middle panel*, *lane 2*). Together, the above results indicate that at least *in vitro* the CIS-IL-2Rβ interaction does not require a classical SH2-phosphotyrosine interaction between CIS and IL-2Rβ. Therefore, the ability of IL-2Rβ to interact with $CIS^{\Delta CT}$ but not CIS^{NT} suggests either that other residues distinct from the FLVR motif in the SH2 region are important for binding or that the CIS^{NT} construct has a severely altered structure resulting from deletion of the SH2 domain. The above results do not exclude a partial contribution of an SH2-medi-

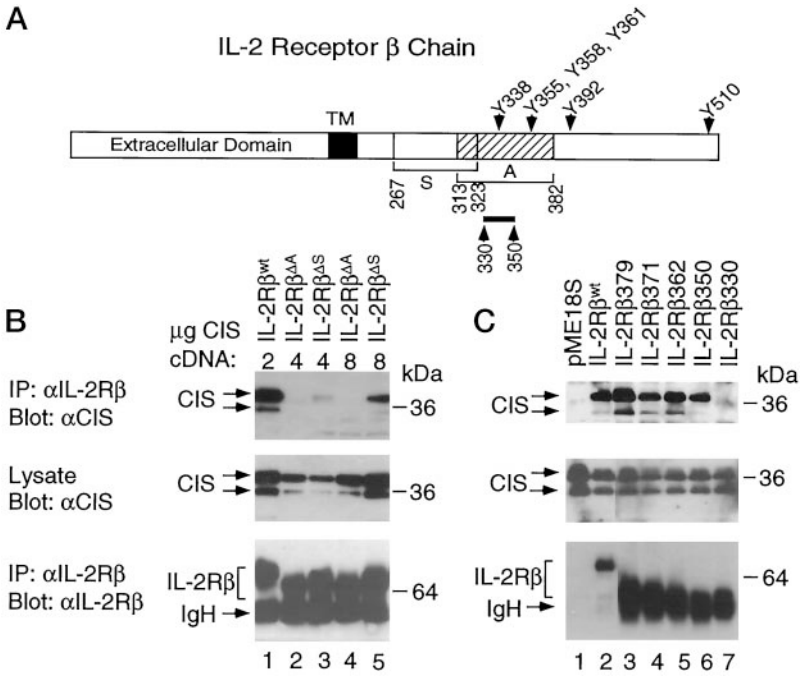

FIG. 3. **CIS association with IL-2Rβ requires the A region (amino acids 313–382) of IL-2Rβ.** *A*, schematic of IL-2Rβ, showing the S region, A region, and locations of tyrosines. 293T$^+$ cells were co-transfected with CIS and either wild type IL-2Rβ or the IL-2Rβ internal deletion mutants IL-2Rβ$^{\Delta A}$ and IL-2Rβ$^{\Delta S}$ (*B*) or the indicated C-terminal truncation mutants of IL-2Rβ (*C*). Lysates were immunoprecipitated with Mikβ1 anti-IL-2Rβ mAb and then Western blotted with anti-CIS (*upper panels*) or a polyclonal antiserum to IL-2Rβ (*lower panels*). Lysates were also Western blotted with anti-CIS to confirm the expression of CIS in different transfectants (*middle panels*).

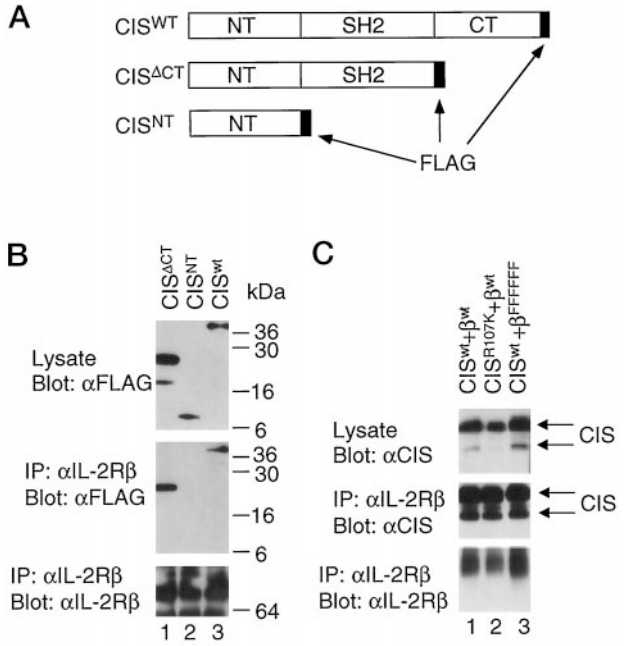

FIG. 4. **The CIS-IL-2Rβ association is not mediated by a classical SH2-phosphotyrosine-mediated interaction.** *A*, schematic of CIS, showing the location of the SH2 domain and the regions comprising the CIS$^{\Delta CT}$ and CIS^{NT} constructs. *B*, 293T$^+$ cells were transfected with wild type IL-2Rβ along with FLAG-tagged forms of wild type CIS (*lane 3*) or the indicated truncation mutants of CIS (*lanes 1* and *2*). Expression of each construct was confirmed by Western blotting with anti-FLAG antibodies (*M2*, *top panel*). Lysates were immunoprecipitated with Mikβ1 antibody and then Western blotted with anti-FLAG (*middle panel*) or polyclonal antiserum to IL-2Rβ (*lower panel*). *C*, 293T$^+$ cells were transfected with the indicated constructs, and the IL-2Rβ/CIS association was analyzed as described in *B*.

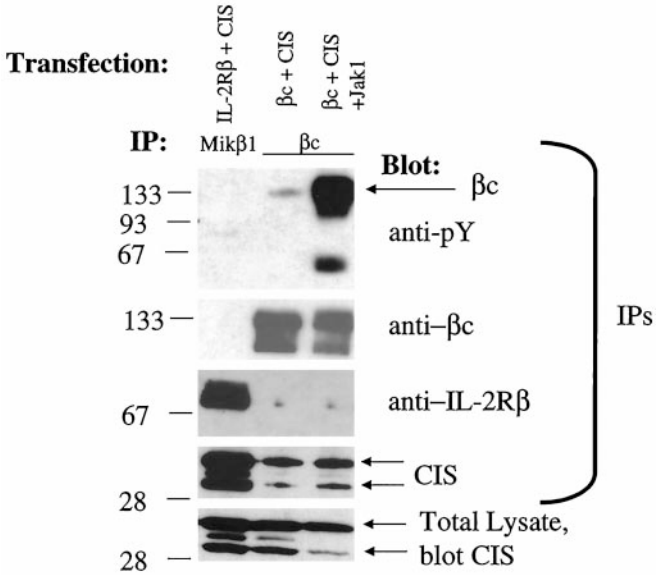

FIG. 5. **Association of β_c and CIS is not substantially affected by Jak1-mediated tyrosine phosphorylation of β_c in 293T$^+$ cells.** 293T$^+$ cells were transfected with CIS plus either IL-2Rβ, β_c, or β_c plus Jak1. Lysates were then immunoprecipitated with antibodies to IL-2Rβ or β_c followed by Western blotting with antibodies to phosphotyrosine, β_c, IL-2Rβ, or CIS.

ated interaction of CIS with IL-2Rβ; they instead demonstrate that non-SH2-mediated interactions also exist. Interestingly, in 293 cells, the common β chain (β_c) of the IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor receptors associated with CIS and augmenting its phosphorylation by cotransfecting Jak1 did not significantly increase its association with CIS (Fig. 5). Previously, CIS was shown to associate with IL-3Rβ and the EPO receptor after ligand stimulation (15). However, the role of ligand requirement for this induction may have been in part related to the stronger induction of CIS in the presence of ligand. Thus, like IL-2Rβ, β_c may at least in part associate with CIS independently of a phosphotyrosine-SH2 interaction.

CIS Inhibits Lck-mediated, but Not Jak1-mediated, Tyrosine Phosphorylation of IL-2Rβ—Because CIS serves as a negative regulator of IL-3-mediated signaling, we investigated the ability of CIS to inhibit IL-2 signaling. Since the region of IL-2Rβ (the A region) that binds CIS is also known to mediate the interaction of IL-2Rβ with Lck (1) and Jak3 (8), we focused on IL-2 signaling effects associated with these kinases. Although the functional effects of Lck for IL-2 signaling remain unclear,

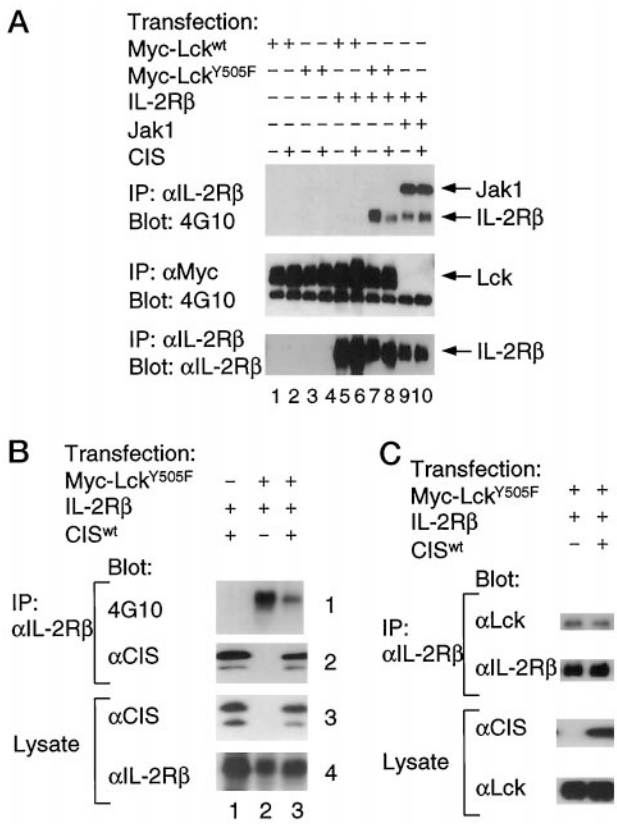

FIG. 6. **CIS inhibits Lck-mediated phosphorylation of IL-2Rβ.** *A*, 293T$^+$ cells were transfected with the indicated plasmids (wild type or constitutively activated forms of Lck, both tagged with Myc epitopes, IL-2Rβ, Jak1, and/or wild type CIS or CIS^{R107K}), and lysed. Lysates were immunoprecipitated using Mikβ1 or 9E10 (anti-Myc) mAbs. Samples were run on gels and Western blotted with the indicated antibodies (4G10 or anti-IL-2Rβ). Note that analogous to the lack of effect of CIS on Jak1 phosphorylation of IL-2Rβ, there is no effect of CIS on Jak1 autophosphorylation (*lanes 9* and *10*, *upper panel*). *B* and *C*, IL-2Rβ and LckY505F were cotransfected with CIS or Lck^{Y505F}. Lysates were either directly blotted as indicated (*lower panels*) or were first immunoprecipitated with anti-IL-2Rβ and then blotted as indicated (*upper panels*).

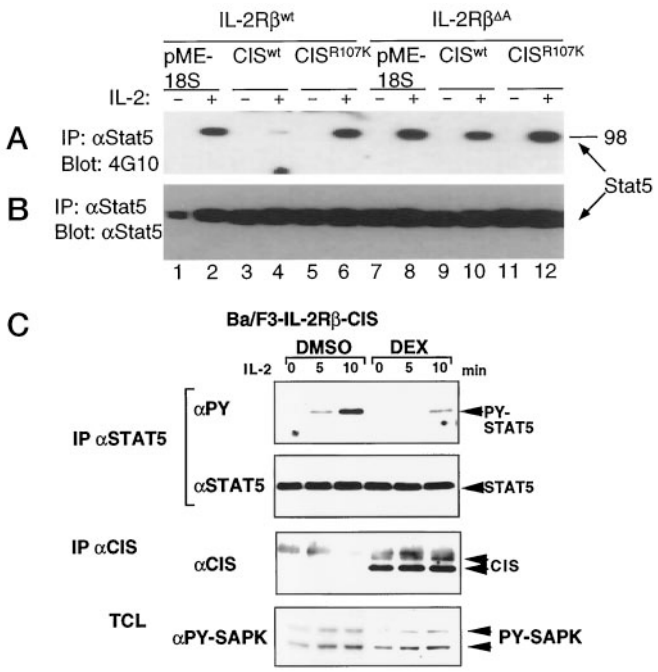

FIG. 7. **CIS inhibits phosphorylation of Stat5.** *A* and *B*, 293T$^+$ cells were transfected with γ_c, Stat5a, Stat5b, Jak3, and either IL-2Rβ^{wt} or IL-2Rβ$^{\Delta A}$ along with pME18S, CIS^{wt}, or CIS^{R107K}. After 16 h, cells from each transfection were plated in duplicate. 24 h later, one set of cells was stimulated with 2 nM IL-2 for 15 min. Cells were then harvested, and cytoplasmic and nuclear extracts were prepared. Stat5 was immunoprecipitated from lysates using a mixture of polyclonal antibodies to Stat5a and Stat5b and subjected to Western blotting with 4G10 (*A*) or anti-Stat5 (*B*). In *A*, note that IL-2-induced Stat5 tyrosine phosphorylation is seen even in the absence of the A region in this overexpression system, presumably because Jak3 is recruited via γ_c. *C*, Ba/F3-IL-2Rβ-CIS cells were treated with Me_2SO (*DMSO*) or dexamethasone (to induce CIS) and stimulated with IL-2 for 0, 5, or 10 min. Lysates were then immunoprecipitated and Western blotted with the indicated antibodies. As a control, it is shown that CIS expression did not affect phosphorylation of stress-activated protein kinase (*SAPK*).

it can associate with and phosphorylate IL-2Rβ (1). We therefore examined the effect of CIS on the tyrosine phosphorylation of IL-2Rβ mediated by Lck; as a control, we also examined the effect of CIS on the phosphorylation of IL-2Rβ by Jak1, a kinase known to phosphorylate IL-2Rβ but which associates primarily with the S region (reviewed in Refs. 1 and 2) (see Fig. 6*A*, *lanes 9* and *10 versus lanes 1* and *2*). Interestingly, coexpression experiments in 293T$^+$ cells revealed that CIS did not inhibit Jak1-mediated phosphorylation of IL-2Rβ (Fig. 6*A*, *lane 10 versus lane 9*), but it reproducibly could partially inhibit the ability of a constitutively active form of Lck (Lck^{Y505F}) to mediate phosphorylation of IL-2Rβ (Fig. 6*A*, *lane 8 versus lane 7*, *upper panel*; Fig. 6*B*, *lane 3 versus lane 2*, *upper panel*). This effect was specific, since CIS did not significantly affect Lck autophosphorylation (Fig. 6*A*, *lane 8 versus lane 7* and *lane 4 versus lane 3*, *middle panel*) or phosphorylation of an exogenous peptide substrate, NH_2-KVEKIGEGTYGVVKK-COOH, known to be efficiently phosphorylated by Src family kinases (Upstate Biotechnology Src family kinase assay kit; data not shown). When wild type Lck was used in place of Lck^{Y505F}, phosphorylation of IL-2Rβ was more difficult to detect (*lanes 5* and *6*); however, phosphorylation seen after longer exposures was also inhibited by CIS (data not shown). We considered the possibility that CIS might inhibit the binding of Lck to IL-2Rβ, since both proteins bind to the A region (1); however, when Lck and IL-2Rβ were expressed with CIS in 293T$^+$ cells, Lck binding to IL-2Rβ was either not affected or only slightly diminished (Fig. 6*C*, *panel 1*, *lane 2 versus lane 1*, and data not shown), suggesting that competition for binding cannot explain this inhibitory effect of CIS.

CIS Inhibits IL-2-mediated Activation of Stat5—Like Lck, Jak3 also associates with the A region of IL-2Rβ (8). Given the importance of Jak3 for Stat5 activation (32), we investigated whether CIS inhibited IL-2-mediated activation of Stat5 (which is important for the transcription of a number of IL-2-responsive genes, such as CIS (23), oncostatin M (33), and the IL-2 receptor α chain (34–37)). We first evaluated whether CIS could inhibit IL-2-induced tyrosine phosphorylation of Stat5 using an *in vitro* reconstitution system similar to that previously described (8, 32). 293T$^+$ cells were transfected with cDNAs encoding wild type or mutant forms of human IL-2Rβ, γ_c, Jak3, Stat5a, and Stat5b, with or without wild type CIS or CIS^{R107K}. When cells were transfected with the vector control (pME18S), as expected, we observed potent IL-2-induced phosphorylation of Stat5 (Fig. 7*A*, *lane 2 versus lane 1*). This activity was markedly reduced in the presence of wild type CIS (Fig. 7*A*, *lane 4 versus lane 2*); in contrast, the CIS^{R107K} mutant had no significant inhibitory effect (*lanes 5* and *6*). These data suggested a critical functional role for the phosphotyrosine binding activity of the SH2 domain of CIS in the inhibition of IL-2-induced Stat5 tyrosine phosphorylation, in contrast to its dispensability for receptor binding *in vitro*. When IL-2RβΔA, which did not associate with CIS (Fig. 3*B*), was used in place of

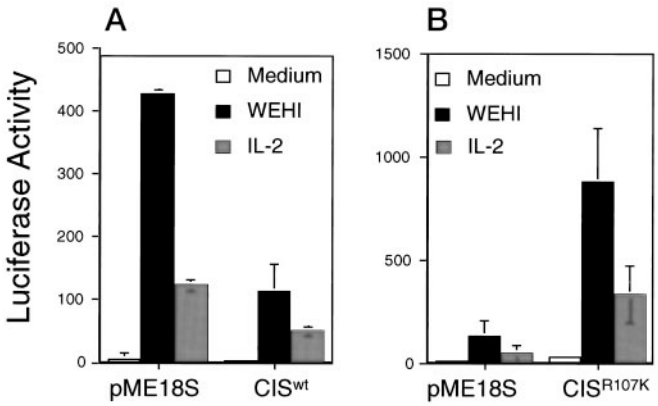

FIG. 8. **IL-2-induced Stat5-dependent transcription is repressed by CIS^{wt} and enhanced by CIS^{R107K}.** 32D-IL-2Rβ cells were transfected with the β-casein-luciferase reporter construct and either empty pME18S vector or wild type or mutant CIS. Transfectants were cultured with medium alone, with WEHI conditioned medium (for 32D-IL-2Rβ cells), or with IL-2, as indicated. Luciferase activity was measured 48 h after transfection.

wild type IL-2Rβ, CIS had much less of an inhibitory effect on Stat5 phosphorylation (*lanes 9* and *10 versus lanes 7* and *8*). Coupled with the receptor binding data, these results demonstrate that at least two functional regions of CIS are involved in the negative regulation of IL-2 signaling, one for receptor binding and one for binding phosphotyrosines. The ability of CIS to inhibit Stat5 tyrosine phosphorylation was confirmed in Ba/F3-IL-2Rβ-CIS cells, in which CIS expression is under control of a glucocorticoid-responsive promoter. As shown in Fig. 7*C*, treatment with dexamethasone induced CIS expression (*third panel*, *lanes 4–6*) and correspondingly diminished the tyrosine phosphorylation of Stat5 by IL-2 (Fig. 7*C*, *top panel*). However, such treatment did not affect tyrosine phosphorylation of stress-activated protein kinase (*SAPK*) (*both panels*).

Given the inhibition of IL-2-mediated tyrosine phosphorylation of Stat5, we next investigated the effect of CIS on Stat5-dependent transcription using a β-casein luciferase reporter construct. As shown in Fig. 8*A*, both IL-3 and IL-2 could induce the activity of this reporter construct in 32D-IL-2Rβ cells (32D cells stably transfected with IL-2Rβ). Although the effect of IL-3 was consistently greater than that of IL-2 in these cells, CIS similarly inhibited the effects of both of these cytokines. Interestingly, transfection of cells with CIS^{R107K} markedly enhanced Stat5 transcriptional activity (Fig. 8*B*), indicating that it could act as a dominant negative CIS mutant by competing with endogenous CIS protein present in 32D cells. Note that because CIS is a negative regulator, its "dominant negative" mutant actually enhances activity.

To exclude the formal possibility that CIS was nonspecifically inhibiting transcription, we examined the effect of CIS on NF-κB-dependent transcription in YT cells using a construct containing three repeats of the human immunodeficiency virus-NF-κB binding element upstream of the TK promoter in a chloramphenicol acetyltransferase reporter construct (pTKκB; Ref. 28). As shown in Fig. 9, PMA plus ionomycin induced NF-κB activity, and this induction was not inhibited by CIS. Similar results (data not shown) were obtained with a reporter construct containing IL-2 receptor α chain promoter PRRI element (38) that consists of an NF-κB and an SRE site (39). These data show that the reduced transcriptional activity of the Stat5-reporter construct was due to specific targeting of this IL-2-mediated signaling pathway by CIS rather than a nonspecific toxic effect.

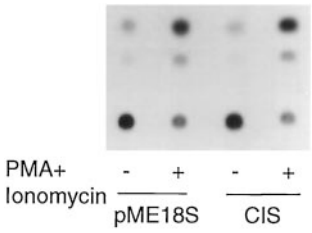

FIG. 9. **NF-κB activity is not inhibited by CIS.** YT cells were transfected with the pTKκB3HIVCAT reporter construct along with either empty pME18S vector or CIS. Transfectants were cultured with or without PMA plus ionomycin, and chloramphenicol acetyltransferase activity was analyzed by thin layer chromatography after 48 h.

DISCUSSION

Cytokines comprise a large number of diverse molecules that induce a broad range of signals. Signaling by interferons and by cytokines whose receptors are members of the cytokine receptor superfamily, also known as type I cytokine receptors, involve the activation of Jak kinases and STAT proteins (40, 41). To counteract these positive regulatory signals, a number of potential negative regulatory mechanisms exist, including protein degradation, phosphatase activation, and induction of the CIS/JAB/SOCS/SSI proteins (reviewed in Refs. 12 and 22).

Although the available data are still limited, most CIS/JAB/SOCS/SSI family proteins that have been studied exert negative regulatory activities (16, 18, 20, 22). However, comparatively little is known about the mechanisms by which these proteins can act. The presence of an SH2 domain in CIS/JAB/SOCS/SSI family proteins suggests that phosphotyrosine binding is likely to be important for the actions of these proteins, and in this regard, it was previously reported that tyrosine-phosphorylated forms of IL-3Rβ and the erythropoietin receptors associate with CIS (15). In the current study, we found that CIS could associate with IL-2Rβ; analysis of internal deletion and C-terminal truncation mutants suggested that the amino acid 330–350 region of IL-2Rβ is important for its interaction with CIS. Surprisingly, although this region contains a tyrosine, substantial CIS-IL-2Rβ association occurred *in vitro* even following mutation of the critical arginine (Arg^{107}) in the FLVR sequence of the SH2 domain of CIS or when all of the tyrosines in the IL-2Rβ cytoplasmic domain were mutated. These data therefore indicate that the interaction of CIS and IL-2Rβ *in vitro* does not require tyrosine phosphorylation. Although it is formally possible that at physiological levels of CIS, tyrosine phosphorylation of IL-2Rβ might enhance the interaction, the sequence surrounding Tyr^{338} (NQGYFFFH) is more typical of a motif for binding PTB phosphotyrosine binding domains than SH2 domains. Consistent with this notion, it has been demonstrated that the phosphorylated Tyr^{338} motif binds to Shc via the Shc PTB domain rather than through the Shc SH2 domain (42). Nevertheless, the fact that IL-2 induces both CIS expression and tyrosine phosphorylation of IL-2Rβ suggests that much of the physiologically induced interaction will be between CIS and phosphorylated IL-2Rβ.

In addition to defining the IL-2Rβ-CIS interaction and clarifying the time course of CIS induction in normal human PBL, we have demonstrated that CIS can inhibit two signaling pathways: 1) Lck-mediated (but not Jak1-mediated) phosphorylation of IL-2Rβ and 2) Stat5-dependent transcription. Given that CIS is itself regulated by Stat5 (23), the latter result indicates that CIS negatively regulates its own production as well as that of other Stat5-dependent proteins. Although the SH2 domain of CIS was not required for its interaction with IL-2Rβ, it was essential for the ability of CIS to act as a negative regulator. This was demonstrated by the ability of the CIS^{R107K} mutant (containing a mutation in the FLVR sequence

of the SH2 domain) to function as a dominant negative, enhancing Stat5-dependent transcription in 32D-IL-2Rβ cells, presumably by reversing the inhibitory effect of the endogenous CIS produced by these cells. The fact that CIS^{R107K} did not increase tyrosine phosphorylation of Stat5 in 293 T^+ cells can be explained by the lack of endogenous CIS in these cells (*i.e.* the "dominant" negative effect was, as expected, only seen in a setting where endogenous wild type CIS was present). To our knowledge, our data provide the first direct evidence for the functional importance of the CIS SH2 domain.

Our study raises a number of general questions. First, given the large number of CIS/JAB/SOCS/SSI family proteins, how many of these other proteins will potentially contribute to IL-2-dependent signaling? Second, within other settings, how many of these proteins will exert effects related to inhibition of Src family kinases and/or STAT proteins? Finally, what is the mechanism of action of CIS? Although the mechanism is not fully understood, we demonstrate that the negative regulatory effects of CIS are dependent on both receptor binding and on the integrity of the SH2 domain. Furthermore, we demonstrate that an SH2 mutant of CIS can act as a dominant negative. Because the phosphotyrosine binding ability of the SH2 domain is not required for CIS binding to IL-2Rβ, our data suggest that the CIS SH2 domain may bind other critical phosphoproteins with which CIS must interact in order to exert its inhibitory function on STAT protein activation. CIS may therefore be a novel type of adaptor protein that contains a single SH2 domain and lacks SH3 domains. The SH2 domain of CIS therefore may prove to be a valuable probe for identifying interacting proteins that help mediate CIS's negative regulatory effect.

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Note Added in Proof—Since submission, of this manuscript, Matsumoto *et al*. (Matsumoto, A., Seki, Y., Kubo, M., Ohtsuka, S., Suzuki, A., Hayashi, I., Tsuji, K., Nakahata, T., Okabe, M., Yamada, S., and Yoshimura, A. (1999) *Mol. Cell Biol*. **9,** 6396–6407) reported suppression of IL-2-induced Il-2Rα up-regulation as well as proliferation of T cells from CIS transgenic mice. These findings are consistent with our report on the negative regulation of IL-2 signaling by CIS.

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