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A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors

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Cytokines manifest their function through alteration of gene expression, However, target genes for signals from cytokine receptors are largely unknown. We therefore searched for immediate-early cytokine-responsive genes and isolated a novel gene, CIS (cytokine inducible SH2-containing protein) which is induced in hematopoietic cells by a subset of cytokines including interleukin 2 (IL2), IL3, granulocyte–macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO), but not by stem cell factor, granulocyte colony-stimulating factor and IL6. The CIS message encodes a polypeptide of 257 amino acids that contains an SH2 domain of 96 amino acids in the middle. To clarify the function of CIS in cytokine signal transduction, we expressed CIS in IL3-dependent hematopoietic cell lines under the control of a steroid-inducible promoter. The CIS product stably associated with the tyrosine-phosphorylated β chain of the IL3 receptor as well as the tyrosine-phosphorylated EPO receptor. Forced expression of CIS by steroid reduced the growth rate of these transformants, suggesting a negative role of CIS in signal transduction. CIS induction requires the membrane-proximal region of the cytoplasmic domain of the EPO receptor as well as that of the common β chain of the IL3, IL5 and GM-CSF receptor, whereas CIS binds to the receptor that is tyrosine phosphorylated by cytokine stimulation. Thus CIS appears to be a unique regulatory molecule for cytokine signal transduction.

Key words: cytokine/cytokine receptor/immediate-early gene/SH2 domain

Introduction

Growth, differentiation and functions of immune and hematopoietic cells are controlled by multiple cytokines, including interleukins (ILs) and colony-stimulating factors (CSFs)(Arai *et al.*, 1990). These cytokines exert their biological functions through specific receptors expressed on their target cells. The cytokine receptors are classified into several groups based on their structure, and the class I cytokine receptor family includes receptors for most of the ILs, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), leukemia-inhibitory factor (LIF) and erythropoietin (EPO) (Bazan, 1990; Miyajima *et al.*, 1992; Kishimoto *et al.*, 1994).

Although the cytoplasmic regions of these cytokine receptors have no intrinsic enzymatic activity, most of these cytokines induce the rapid tyrosine phosphorylation of several signaling molecules as well as of the receptor itself. Several shared signaling events activated by different cytokines have been identified. IL2, IL3, IL5, IL6, GM-CSF and EPO activate p21Ras as well as the Raf-MAP kinase cascade that leads to the induction of c-*fos* and c-*jun*. The expression of c-*myc* is also a common event induced by these cytokines, but it is mediated by a pathway that is distinct from the Raf-MAP kinase cascade (for review, see Sato and Miyajima, 1994). Deletion analysis of the IL2 receptor β chain, as well as the common β (β_c) chain of the GM-CSF, IL3 and IL5 receptors, has established that the membrane-proximal region of their cytoplasmic domains is essential for c-*myc* expression and that the distal region is required for the activation of Shc, Ras and the Raf-MAP kinase cascade (Shibuya *et al.*, 1992; Sato *et al.*, 1993). The membrane-proximal regions among various class I cytokine receptors have some sequence similarity and these regions are essential for proliferation. Recently Janus kinase-2 (JAK2) has been shown to be associated with this membrane-proximal region of the EPO receptor and β_c (Witthuhn *et al.*, 1993; Quelle *et al.*, 1994). Since JAK kinases directly activate transcription factors known as STATs (signal transducer and activator of transcription), this pathway may be important for cytokine-specific gene regulation (for reviews, see Darnell *et al.*, 1994; Ihle *et al.*, 1994). Although activation of the JAK/STAT pathway requires the same membrane-proximal region responsible for c-*myc* induction and proliferation, the relationship between JAK/STAT activation and proliferation is still unknown.

The membrane-distal region, on the other hand, contains tyrosine phosphorylation sites and is not required for proliferation under usual growth conditions containing serum. However, the distal region responsible for Ras activation seems to be required for the anti-apoptotic function of IL3 and GM-CSF under serum-free conditions, and long-term proliferation requires signals from both the membrane-proximal and the distal region (Okuda *et al.*, 1994; Kinoshita *et al.*, 1995). The Ras-MAP kinase cascade is also important for other functions such as IL6-dependent activation of the transcription factor NF-IL6 (Nakajima *et al.*, 1993). Tyrosine phosphorylation of the

cytokine receptors provides binding sites for proteins with *src* homology (SH)-2 domains such as Shc (Ravichandran and Burankoff, 1994), the 85 kDa regulatory subunit of phosphoinositide 3 (PI3) kinase (Miura *et al.,* 1994a) and hematopoietic cell phosphatase (HCP) (Yi *et al.,* 1993). Tyrosine phosphorylation of Shc probably recruits Grb2 and the Ras–guanine nucleotide exchange factor, SOS, into membranes, resulting in the activation of the Ras-MAP kinase pathway (Cutler *et al.,* 1993; Welham *et al.,* 1994a). Thus, signals from both the membrane-distal and -proximal regions are important for cytokine function.

Among the various signals produced by cytokine receptors, JAK/STAT activation is unique to cytokines. However, the target genes induced by this pathway have not been well defined. In this study, we isolated and characterized a novel immediate-early gene, CIS, which is a possible target gene for this pathway. CIS contains an SH2 domain and is induced via the membrane-proximal regions of the β_c chain and the EPO receptor. The CIS product stably associates with the tyrosine-phosphorylated β chain of the IL3 receptor as well as the tyrosine-phosphorylated EPO receptor. Thus, CIS is likely to be a regulatory protein of cytokine receptors that modifies signals mediated by the membrane-distal region or it may function as an adaptor for an unidentified signaling pathway.

Results

Isolation of genes induced by the membrane-proximal region of the cytokine receptors

To obtain genes whose induction is mediated by the membrane-proximal region of multiple cytokine receptors, we applied a novel cell line (Maruyama *et al.,* 1994; Ohashi *et al.,* 1994) and the cDNA library subtraction technique that we recently developed (Hara *et al.,* 1994). We showed that a chimeric receptor (EGFR/EPORH) containing the extracellular domain of the epidermal growth factor (EGF) receptor linked to the membrane-proximal region (~120 amino acids) of the cytoplasmic domain of the EPO receptor can support EGF-dependent growth and induce erythroid differentiation signals, which are indistinguishable from those of the wild-type EPO receptor (Maruyama *et al.,* 1994). The EGFR/EPORH activated JAK2 and induced c-*myc*, but marginally activated MAP kinases (data not shown). Since the EGFR/EPORH can generate signals for proliferation and differentiation more efficiently than the wild-type EPO receptor (Maruyama *et al.,* 1994), we used Ba/F3 cells expressing the EGFR/EPORH (BF-EGFR/EPORH) for subtraction.

We prepared a cDNA library of BF-EGFR/EPORH cells cultured in factor-free medium and subtracted this cDNA library from that of cells stimulated with EGF for 1 h in the presence of cycloheximide. To obtain an immediate-early gene which is commonly induced by cytokine receptors but not by receptor tyrosine kinases, clones isolated randomly from the subtracted library were further screened by Northern hybridization using RNAs from stimulated and unstimulated BF/$\alpha\beta_{626}$, BF-EGFR/EPORH and BF-EGFR cells. The BF/$\alpha\beta_{626}$ cell line is a Ba/F3 transfectant expressing the human GM-CSF receptor α chain and C-terminal truncated β_c chain (Sakamaki *et al.,*

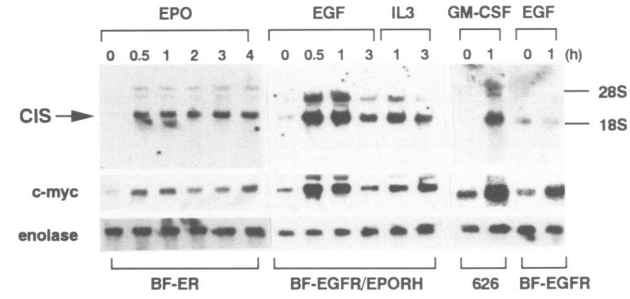

Fig. 1. Induction of the F17 (CIS) message in Ba/F3 transformants expressing various receptors. Ba/F3 cells expressing the EPO receptor (BF-ER), the EGFR/EPORH chimera (BF-EGFR/EPORH), the truncated human GM-CSF receptor (626) and the wild-type EGF receptor (BF-EGFR) were factor depleted for 4 h, then stimulated with various cytokines for indicated periods. Poly(A) RNA samples (0.5 μg/lane) from cells were separated on 1.0% agarose gels, transferred to a nylon membrane and hybridized with the probes for F17 (CIS) and c-*myc* as well as α-enolase, which is a housekeeping enzyme involved in glycolysis and used for verifying the amounts of RNA. The positions of 28S and 18S rRNA are indicated.

1992). Human GM-CSF stimulates c-*myc* expression and growth, but does not activate the Ras-MAP kinase pathway in these cells (Sato *et al.,* 1993). The BF-EGFR cell line is a Ba/F3 transfectant expressing the EGF receptor. EGF stimulates c-*fos* and c-*jun* expression in BF-EGFR cells but does not induce c-*myc* and does not support long-term cell growth (Shibuya *et al.,* 1992).

One of the genes, F17 (later named CIS), isolated by these procedures was induced in BF-$\alpha\beta_{626}$ and BF-EGFR/EPORH cells but not in BF-EGFR cells (Figure 1, 626 and EGFR in the right panel, and BF-EGFR/EPORH stimulated with EGF in the middle panel). This gene was induced through the wild-type EPO receptor as well as the endogenous IL3 receptor (Figure 1). Since the protein synthesis inhibitor cycloheximide did not affect the expression level of this gene (data not shown), *de novo* protein synthesis was not required for its induction. The major F17 transcript of 2 kb was induced within 30–60 min after stimulation and the expression was maintained thereafter. The induction kinetics of this gene were similar to that of c-*myc* (Figure 1). A minor transcript of ~4 kb was also detected at various levels (Figure 1).

Structure of protein encoded by the F17 cDNA

The initial F17 cDNA clone obtained from the subtracted library contained ~1.2 kb of the 3′ end of the mRNA. A panel of overlapping longer cDNA clones was obtained by rescreening, and the longest clone, 22F17, of 1.9 kb was sequenced completely (Figure 2). This clone contained an ATG codon followed by a single open reading frame, but had no in-frame stop codon upstream of the ATG. Thus, to obtain the 5′ end, rapid amplification of cDNA ends (RACE)-polymerase chain reaction (PCR) (Frohman, 1993) was performed. The PCR product was cloned into pUC18 vector and 10 clones were selected. The longest had a 5′ sequence that extended for ~140 bp, which is shown in Figure 2. The other nine clones contained a similarly sized cDNA, and three representative clones were sequenced. They started from nucleotide position 43 or 44 as shown in Figure 2. The sequence of nucleotides 1–50 was confirmed by genomic sequencing of the CIS gene (A.Yoshimura, unpublished results). Although there

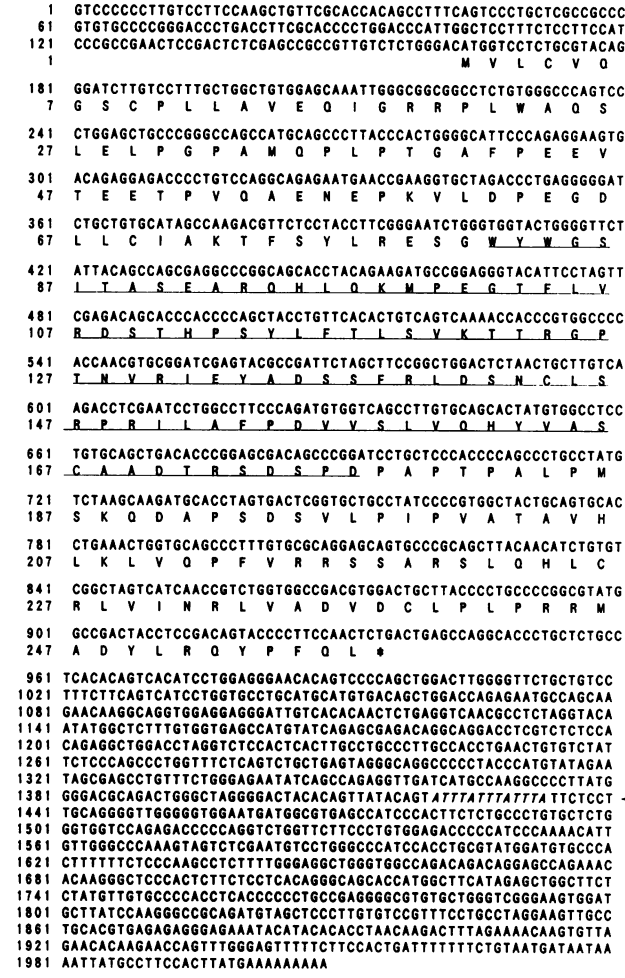

Fig. 2. The nucleotide and deduced amino acid sequences of CIS. Part of the 5′-untranslated flanking sequence (1–83) was obtained by RACE-PCR and the longest sequence is shown. Most of the RACE-PCR clones (9 out of 10) start at positions 43 or 44. Clone 22F17 starts from 84. The asterisk indicates the stop codon. The amino acid sequence of the SH domain is underlined. The italic sequence, ATTTATTTATTTA, in the line indicated by the arrowhead is an RNA degradation signal.

was still no in-frame stop codon in the upstream region, immunoblotting demonstrated that the size of the polypeptide encoded by the endogenous F17 gene was identical to that encoded by clone 22F17 (see Figure 7). Thus, the ATG codon at 163 bp is most likely the real start codon.

The F17 cDNA had a long 3′-untranslated region that contained three repeats of the pentameric mRNA destabilization signal, ATTTA (Figure 2, sequence in italics on the line with an arrowhead) (Shaw and Kamen, 1986). This sequence has been found in many immediate-early genes, including proto-oncogenes and lymphokine genes, providing further support for F17 as an immediate-early gene. The open reading frame encoded a 257 amino acid polypeptide. A comparison of this amino acid sequence with those in the NBRF protein database revealed that the middle of the sequence (codons 82–167) shows significant homology to SH2 domains (Figure 3). Three invariant residues, Trp (W) in the βA, Leu (L) in the αA and Arg (R) in the βB regions (Waksman *et al.,* 1993), are present in F17. Overall, the SH2 domain of F17 is most similar to the N-terminal SH2 domain of the PI3 kinase 85 kDa α subunit. However, the amino acid identity between them is only 31%. Furthermore, the αA2 and βD6 positions of the CIS SH2 domain are Ala (A) and Tyr (Y) respectively, whereas those of almost all other SH2 domains are Arg (R) or Lys (K) (Waksman *et al.,* 1993). Thus, the SH2 domain of CIS may belong to a unique class. As F17 is a novel cytokine-inducible SH2 protein, we refer to this gene as CIS.

There were no peptide sequences that were significantly homologous to the N- and C-terminal 80 amino acids of CIS in protein databases. No motif of an SH3 domain or any other known domain was found in CIS. Although there is no sequence similarity between the N- and C-terminal regions, these are rich in Pro (P) and Leu (L). The N-terminal region is also rich in Glu (E) and resembles the PEST sequences (Rogers *et al.,* 1986). This region may be involved in the rapid turnover of CIS protein (see Figure 7).

Chromosome mapping of the CIS gene

The chromosomal location of the CIS gene was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J×*Mus spretus*) F1×C57BL/6J mice. C57BL/6J and *M.spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a cDNA probe. The 8.0 and 6.1 kb *M.spretus Kpn*I RFLPs were used to follow the segregation of the *Cis* locus in backcross mice. The mapping results indicated that *Cis* is located in the distal region of mouse chromosome 9 linked to *Trf, Gnai2* and *Col7a1* (Figure 4). The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error] were *–Trf–*3.7 ± 1.6*–[Cis, Gnai2]–*2.7 ± 1.2*–Col7a1*. No recombinants were detected between *Cis* and *Gnai2* in 152 animals typed in common, suggesting that the two loci are within 2.0 cM of each other (upper 95% confidence limit).

The distal region of mouse chromosome 9 shares a region of homology with the short arm of human chromosome 3 (3p21). Since human *Trf, Gnai2* and *Col7a1* are mapped on this region, the human homolog of *Cis* will also reside on 3p21.

Expression of the CIS message in hematopoietic cells and tissues

CIS induction was examined by Northern blotting using other IL2-, IL3- and IL6-dependent cell lines (Figure 5). Since c-*myc* is induced via the membrane-proximal region of the GM-CSF receptor and IL2 receptor β chains (Shibuya *et al.,* 1992; Sato *et al.,* 1993), c-*myc* expression was examined as a control. IL3 induced CIS in several IL3-dependent hematopoietic cell lines including Ba/F3, FDC-P1, FDC-P2/kit and NFS60 (Figure 5). IL2 induced CIS as well as c-*myc* in an IL2-dependent CTLL2 cell line and the Ba/F3 transformant expressing the IL2 receptor β chain (Figure 5, CTLL and BF-IL2Rβ). The FDC-P2/*kit* cell line is a transfectant expressing c-*kit* and grows in response to either IL3 or stem cell factor (SCF) (Ohashi *et al.,* 1994). IL3 induced both c-*myc* and CIS in FDC-P2/*kit*, whereas SCF induced only c-*myc* but not CIS (Figure 5, FDC-P2/*kit*). NFS60 expresses the IL3 and the G-CSF receptors, and proliferates in response to either

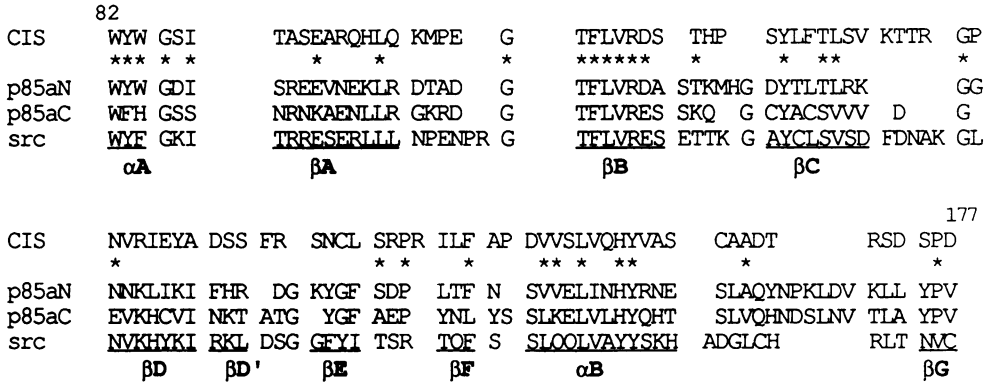

Fig. 3. Sequence alignment of the SH2 domain of CIS with other representative SH2 domains. The sequences of the SH2 domains from CIS, the p85 subunit of PI3 kinase and Src are aligned based upon the secondary structure (Waksman *et al.*, 1993). The asterisks show identical amino acids between CIS and the N-terminal SH2 domain of the p85 subunit of PI3 kinase. The sequences and positions of α helices (α) and β sheets (β) were aligned according to Waksman *et al.* (1993).

IL3 or G-CSF. Whereas G-CSF induced c-*myc* at a level comparable to that of IL3, the induction of CIS by G-CSF was only marginal compared with IL3 (Figure 5, NFS-60). These results indicated that the mechanism of CIS induction is different from that of c-*myc*. Neither CIS nor c-*myc* was induced by IL6 in the IL6-dependent hybridoma cell line, MH60.BSF2 (Figure 5, MH60). In response to IL6, NFS-60 cells expressed c-*myc* weakly, but did not express CIS (Figure 5). Phorbol myristate acetate (PMA), which activates protein kinase C (PKC), had little effect on CIS induction (Figure 5, PMA). Genistein, a protein tyrosine kinase inhibitor, inhibited the induction of CIS as well as of c-*myc* in Ba/F3 cells (Figure 5, IL3+GS).

CIS expression in various mouse tissues was examined. Although CIS expression requires cytokine stimulation in hematopoietic cell lines, a high level of CIS was expressed in the kidney, lung and liver (Figure 6). A CIS message was also detected in the stomach and heart but not in the brain or spleen. The wide distribution of CIS suggests that it plays a role in various tissues in addition to its role in hematopoiesis.

The expression of CIS protein in hematopoietic and COS cells

The expression of the CIS product was examined using an antibody raised against a recombinant GST–CIS fusion protein produced in bacteria. The expression of CIS protein in FDC-P1 cells was examined by immunoblotting (Figure 7). A 4 h depletion of IL3 almost completely eliminated the CIS protein (Figure 7a, lane 5) and, within 1 h after IL3 stimulation, the CIS protein reappeared as 37 and 32 kDa proteins (Figure 7a, lane 6). Thereafter, the amount of the 37 kDa species decreased and the 32 kDa protein gradually became predominant (lane 9). In COS cells, the major form of the CIS proteins was 37 kDa, suggesting that CIS was produced as the 37 kDa form, then processed to the 32 kDa form. A small amount of another 45–48 kDa species of CIS was found in IL3-stimulated FDC-P1 cells (Figure 7b, lane 6), as well as in COS cells transfected with the 22F17 cDNA clone, but not in untransfected cells (Figure 7a and b, lanes 1 and 2). Thus, this species should not be a product of alternative splicing or an unidentified upstream initiation codon. The nature of this species is currently unknown. In COS cells, several other smaller

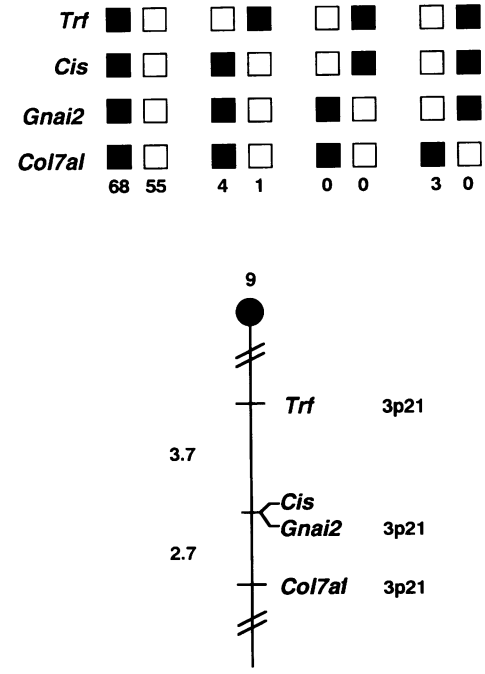

Fig. 4. Chromosome mapping of the CIS gene. The CIS gene was mapped by interspecific backcross as described in the text. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J×*M.spretus*) F1 parent. The shaded and white boxes represent the presence of C57BL/6J and *M.spretus* alleles, respectively. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 9 linkage map showing the location of CIS in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centiMorgans are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right.

fragments were seen, suggesting possible proteolytic degradation of CIS. The apparent molecular weight of CIS on SDS–PAGE was much higher than the value (29 kDa) calculated from the protein sequence, which could be due to an unusual structure of the CIS product or a modification such as ubiquitination. CIS was maximally induced at 1–10 ng/ml IL3 (Figure 7b), which is similar to the maximal response of cell proliferation to IL3 (data not shown).

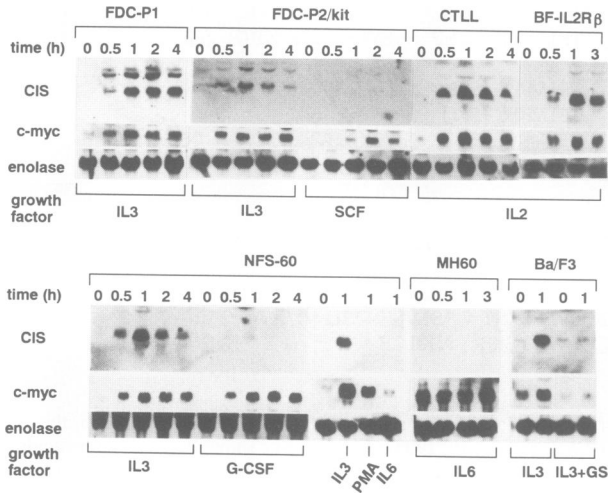

Fig. 5. Induction of CIS in factor-dependent cells. Ba/F3, FDC-P1, FDC-P2/*kit* (FDC-P2 cells expressing c-*kit*), CTLL2 and BF-IL2Rβ (Ba/F3 cells expressing IL2R β chain), NFS-60 and MH60.BSF2 (MH60) cells were factor depleted for 4 h, then stimulated with various cytokines for the indicated periods (h). Some NFS-60 cells were stimulated with 50 ng/ml PMA for 1 h. In Ba/F3 cells, 50 μg/ml of genistein was included throughout the factor depletion and IL3 stimulation (IL3+GS). The concentrations of cytokines are given in Materials and methods. Total RNA (10 μg/lane) was separated from the cells and blotted with CIS, c-*myc* and α-enolase probes.

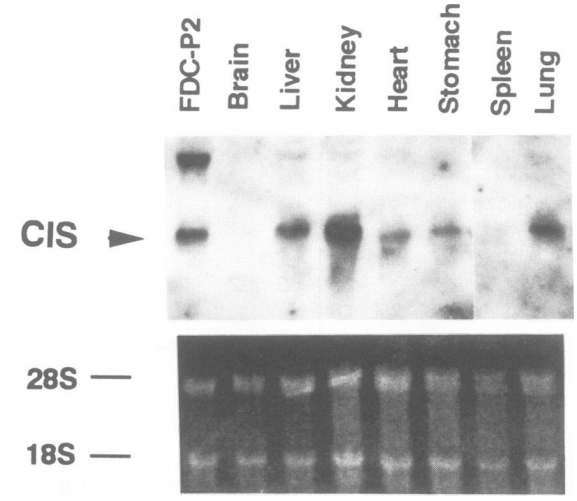

Fig. 6. The expression of CIS mRNA in tissues. Upper panel: total RNAs from various tissues (10 μg/lane) and IL3-stimulated FDC-P1 cells (5 μg/lane) were hybridized with the CIS probe. Lower panel: RNAs from various tissues were stained with ethidium bromide.

The association of CIS with the IL3 receptor β chain and the EPO receptor

As CIS contains an SH2 domain, we analyzed the tyrosine-phosphorylated proteins associated with CIS in IL3-dependent cells. Growth factor depletion or cell culture with a low concentration of cytokine is necessary to demonstrate the IL3-induced tyrosine phosphorylation of cellular proteins. However, as CIS expression depends on the presence of cytokine, this procedure also diminished CIS expression. Thus, to examine the interaction of CIS with IL3-induced tyrosine-phosphorylated proteins, we expressed the CIS cDNA using the mouse mammary tumor virus (MMTV) promoter in Ba/F3 and FDC-P1

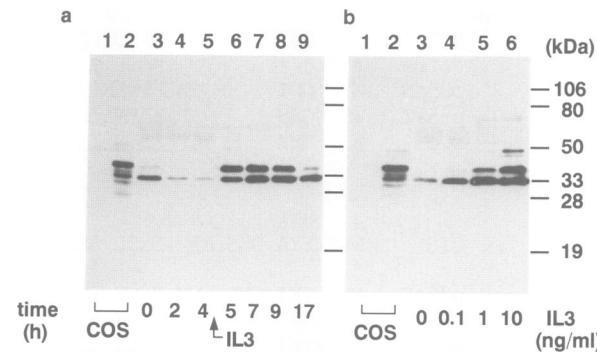

Fig. 7. IL3-dependent expression of the CIS product in FDC-P1 cells. (**a**) FDC-P1 cells were cultured in 10% WEHI conditioned medium. At the exponential growth stage, cells were harvested (time 0, lane 3), then transferred to factor-depleted medium for 2 and 4 h (lanes 4 and 5). After a 4 h factor depletion, 1 ng/ml IL3 was added to the medium, then cultured for a further 1, 2, 4 or 12 h (lanes 6–9). The period (h) from factor depletion is indicated. (**b**) exponentially growing FDC-P1 cells were transferred to the medium containing the indicated concentrations of IL3 (lanes 3–6) and cultured for 6 h. CIS product from 10^7 cells was detected by immunoblotting in combination with immunoprecipitation. In both A and B, extracts (10 μg protein/lane) from COS cells either transfected (lane 2) or untransfected (lane 1) with CIS cDNA in the pME vector (22F17) were also analyzed. Molecular weight standards (Bio Rad) are shown on the right.

cells. Dexamethasone (Dex) at a concentration of 10–1000 nM increased the CIS expression in transfectants (BF-CIS and FD-CIS), whereas it had little effect on CIS expression in parental FDC-P1 and Ba/F3 cells (see Figure 10, upper panel). FD-CIS and BF-CIS cells cultured in Dex maintained the CIS expression level even after IL3 withdrawal (data not shown).

After a 16 h incubation of the cells in a low concentration of IL3 (0.1 ng/ml) in the presence or absence of Dex, they were stimulated with IL3. More than 95% cells were viable under these conditions. Detergent extracts from equal numbers of cells were prepared and immunoprecipitated with polyclonal anti-CIS antibody (αCIS) or monoclonal anti-IL3 receptor β chain (AIC2A) antibody (9D3). The immunoprecipitates were further analyzed by immunoblotting with anti-phosphotyrosine antibody (αPY blot in Figure 8a), polyclonal anti-AIC2A (αAIC2A blot in Figure 8b) and αCIS (αCIS blot in Figure 8c). Figure 8 shows the results obtained with FD-CIS, and similar results were also obtained with BF-CIS (data not shown). Dex resulted in an ~5-fold increase in the expression of CIS (Figure 8c, lanes 9–12).

Consistent with other reports, IL3 induced the major tyrosine phosphorylation of 135, 95, 75, 60 and 55 kDa proteins in total cell extracts (Figure 8a, lanes 1–4). The broad 135 kDa phosphoprotein was the IL3 receptor β chain shown in lanes 5–8. A monoclonal antibody (9D3) against one of the IL3 receptor β chains, AIC2A, co-precipitated with CIS protein (Figure 8c, αCIS blot, lanes 5–8). Whereas equal amounts of the IL3 receptor β chain were precipitated with 9D3 antibody either with or without Dex (Figure 8b, αAIC2A blot, lanes 5–8), a significant amount of CIS was detected only when cells were incubated with Dex (lane 8) and only a trace amount of CIS was co-precipitated in the absence of Dex (lane 6), reflecting the expression levels of CIS (Figure 8c, lanes

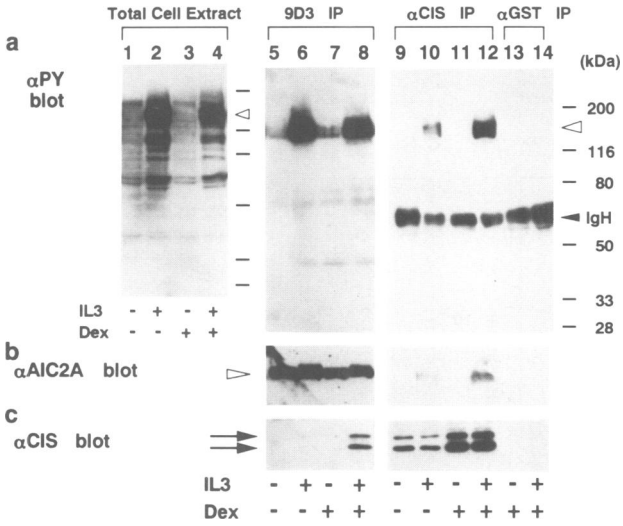

Fig. 8. Association of the IL3 receptor β chain and CIS. FD-CIS cells were cultured for 16 h in 0.1 ng/ml IL3 in the presence (lanes 3, 4, 7, 8 and 11–14) or absence (lanes 1, 2, 5, 6, 9 and 10) of 500 nM Dex. The cells were stimulated with (even numbered lanes) or without (odd numbered lanes) 50 ng/ml IL3 for 10 min and the cell lysates were prepared. The total cell extracts (50 μg protein/lane) (lanes 1–4) and the immunoprecipitates (IP) obtained with a monoclonal antibody (9D3) against the IL3 receptor β chain (lanes 5–6), anti-CIS (αCIS) (lanes 9–12) or control antiserum (αGST) (lanes 13 and 14) were resolved by SDS–PAGE and immunoblotted with 4G10 (αPY) (**a**), rabbit polyclonal anti-IL3 receptor β chain (αAIC2A) (**b**) and αCIS (**c**). Molecular weight standards are shown on the right. The open arrowheads indicate IL3 receptor β chain and the closed arrowheads indicate two major CIS bands. IgH indicates the IgG heavy chain.

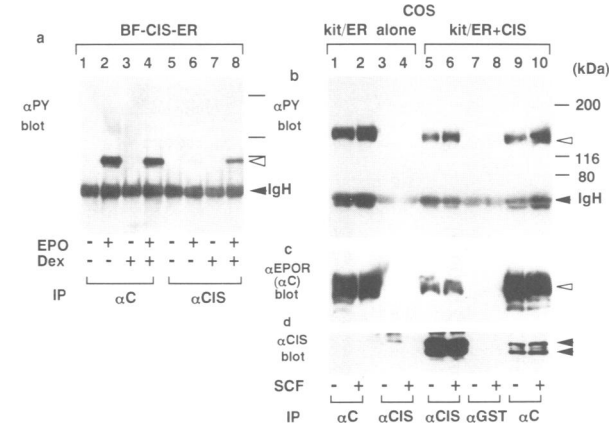

Fig. 9. Association of the EPO receptor and CIS. (**a**) BF-CIS-ER cells were cultured for 16 h in 0.1 ng/ml IL3 in the presence (lanes 3, 4, 7 and 8) or absence (lanes 1, 2, 5 and 6) of 500 nM Dex. The cells were stimulated with (even numbered lanes) or without (odd numbered lanes) 50 ng/ml EPO for 10 min and the cell lysates were prepared. The immunoprecipitates (IP) obtained with anti-EPO receptor (EPOR) (αC) (lanes 1–4) and anti-CIS (αCIS) (lanes 5–8) were resolved by SDS–PAGE and immunoblotted with 4G10 (αPY). (**b–d**) COS cells were transfected with kit/ER cDNA (lanes 1–4) or with kit/ER and CIS cDNAs (lanes 5–10), then stimulated with (even numbered lanes) or without (odd numbered lanes) 50 ng/ml SCF for 10 min. The immunoprecipitates (IP) obtained with αC (lanes 1, 2, 9 and 10), αCIS (lanes 3–6) or control antiserum (αGST) (lanes 7 and 8) were resolved by SDS–PAGE and immunoblotted with 4G10 (αPY) (b), αC (c) and αCIS (d). Molecular weight standards are shown on the right. The open arrowheads indicate the EPO receptor or kit/ER chimera and the closed arrowheads indicate two major CIS bands. IgH indicates the IgG heavy chain.

9–12). Both 37 and 32 kDa forms of CIS were associated with the β chain. This association was completely dependent upon IL3 stimulation, suggesting that CIS associated with only the tyrosine-phosphorylated β chain (compare lanes 7 and 8). Our antibodies used in this study only recognized AIC2A but not AIC2B, the common β chain for IL3, IL5 and GM-CSF. Antibodies against AIC2B were not available at this time. However, since GM-CSF, as well as IL3, stimulated CIS expression, it would be assumed that CIS interacts with both mouse β subunits.

Conversely, anti-CIS antibody precipitated a tyrosine-phosphorylated protein of ~135 kDa, which migrated with the same molecular mass as the IL3 receptor β chain (Figure 8a, lanes 6, 8, 10 and 12). Similar 135 kDa bands were recognized by a polyclonal antibody against the IL3 receptor β chain, AIC2A (Figure 8b, lanes 10 and 12). Thus, the 135 kDa tyrosine-phosphorylated protein was probably the IL3 receptor β chain. No other tyrosine-phosphorylated protein was detected in the αCIS immunoprecipitates, indicating that CIS exclusively bound to the tyrosine-phosphorylated β chain and that CIS itself was not tyrosine phosphorylated (Figure 8a, lanes 9–12, CIS protein located at the 37–32 kDa position). Anti-GST, a control antibody, precipitated neither CIS nor the β chain (Figure 8a, b, c, lanes 13 and 14). The amount of the β chain co-precipitated with CIS was increased by Dex treatment (Figure 8a and b, compare lanes 10 and 12), reflecting the expression levels of CIS (Figure 8c, lanes 10 and 12). This indicated that the co-precipitation of the β chain with CIS was due to a specific interaction between CIS and the β chain and not to cross-reactivity of anti-CIS antibody with other SH2-containing proteins associated with the IL3 receptor β chain. Again, co-immunoprecipitation of the β chain with CIS was completely IL3-dependent, suggesting that IL3-induced tyrosine phosphorylation of the β chain triggers the association between CIS and the β chain. These data suggested that CIS binds to the tyrosine-phosphorylated β chain via its SH2 domain.

The interaction between CIS and the EPO receptor was also examined (Figure 9). The EPO receptor cDNA was introduced into BF-CIS cells and an EPO-dependent clone (BF-CIS-ER) was selected. BF-CIS-ER cells stably expressed the EPO receptor and EPO induced tyrosine phosphorylation of the EPO receptor in these cells (Figure 9a, lanes 1–4). Anti-CIS antibody precipitated a similar tyrosine-phosphorylated 72 kDa protein (lanes 5–8). This band was diminished when the cell extracts were pre-cleared with the anti-EPO receptor antibody (data not shown). This suggested that CIS associates with the tyrosine-phosphorylated EPO receptor. The amount of the 72 kDa phosphoprotein co-precipitated with CIS was increased by Dex treatment (compare lanes 6 and 8), reflecting the expression levels of CIS (see Figure 8c). Immunoblotting was not sensitive enough to detect the EPO receptor protein in αCIS immunoprecipitates and the CIS protein in anti-EPO receptor (αC) immunoprecipitates, probably because of a very low level of cell surface expression of the EPO receptor and low affinity of our anti-EPO receptor antibody (Yoshimura *et al.*, 1990a). To obtain high level expression of the EPO receptor cytoplasmic domain, a chimeric receptor (kit/ER) containing the extracellular domain of c-*kit* and the cytoplasmic domain of the EPO receptor (Ohashi *et al.*,

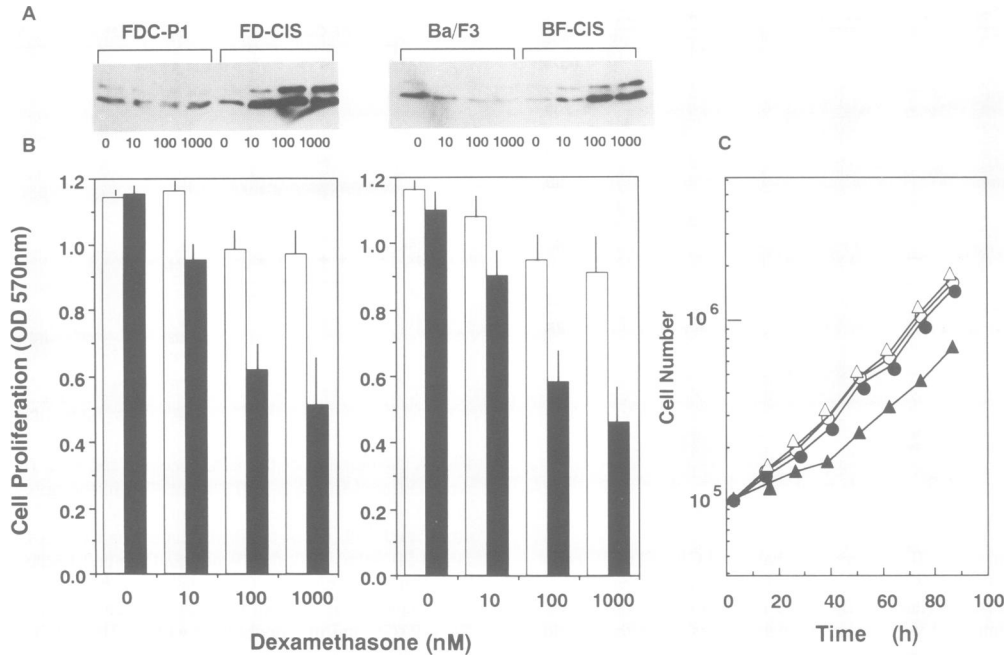

Fig. 10. Effect of dexamethasone (Dex) on the expression of CIS (**A**) and proliferation (**B** and **C**) in parental FDC-P1, Ba/F3 and their CIS cDNA transfected cells. In A, the CIS content was assessed by immunoblotting after cells were cultured in the presence of indicated concentrations of Dex under normal growth conditions (in 10% WEHI conditioned medium) for 2 days. In B, cells (2000/well) were cultured in the presence of indicated concentrations of Dex for 2 days and the cell numbers were compared using a colorimetric method as described (Yoshimura *et al.*, 1990a). Open bars indicate parental cells, FDC-P1 (left) and Ba/F3 (right); closed bars indicate CIS-transfected cells, FD-CIS (left) and BF-CIS (right). Error bars from duplicate experiments are also shown. In C, parental Ba/F3 (circles) and BF-CIS (triangles) cells were cultured at a density of 1×10^5/ml in the presence (closed symbols) or absence (open symbols) of 100 nM Dex, and the cell numbers were counted every 12 h.

1994) was transiently expressed in COS cells. SCF induced growth of Ba/F3 cells expressing kit/ER and concomitant tyrosine phosphorylation of kit/ER (Yoshimura, 1994). In COS cells, kit/ER was tyrosine phosphorylated even in the absence of SCF (Figure 9b, lanes 1 and 9), but SCF increased the level of tyrosine phosphorylation of kit/ER (lanes 2 and 10). Anti-CIS co-precipitated kit/ER protein when COS cells were co-transfected with kit/ER and CIS cDNAs (Figure 9c, lanes 5 and 6) but not when cells were transfected with kit/ER cDNA alone (lanes 3 and 4). The amount of the kit/ER protein co-precipitated with CIS was increased by SCF treatment (Figure 9c, compare lanes 5 and 6), suggesting that CIS preferentially associated with the tyrosine-phosphorylated kit/ER. Conversely, antibody against the cytoplasmic domain of the EPOR (αC) co-precipitated CIS in COS cells expressing both CIS and kit/ER, and SCF increased the amount of CIS associated with kit/ER (Figure 9d, lanes 9 and 10). Anti-GST precipitated neither CIS nor kit/ER (Figure 9b, c, d, lanes 7 and 8). These data strongly supported the proposal that CIS associates with the tyrosine-phosphorylated EPO receptor.

Forced expression of CIS in hematopoietic cells suppressed cell growth

First, we attempted to express CIS cDNA in Ba/F3 or FDC-P1 cells using strong constitutive promoters. We performed co-transfection of pME18S carrying CIS cDNA (clone 22F17) with a selection maker pSV2neo as well as transfection of pEFneo (Ohashi *et al.*, 1994) containing CIS cDNA. However, none of the G418-resistant clones obtained showed an increase in the expression of CIS. This could be due to a growth-inhibitory effect of CIS by constitutive overproduction. Therefore, we used the steroid-inducible MMTV promoter to express CIS, and compared growth of BF-CIS and FD-CIS cells in the presence or absence of Dex. Cells were cultured for 2 days in the presence of 10–1000 nM Dex under normal growth conditions (in 10% WEHI conditioned medium, equivalent to ~1 ng/ml IL3). As shown in Figure 10 upper panel, Dex at a concentration of 100–1000 nM significantly increased the CIS expression in transfectants, whereas it had little effect on CIS expression in parental FDC-P1 and Ba/F3 cells (Figure 10A). Incubating cells with 100–1000 nM of Dex suppressed the growth of FD-CIS and BF-CIS cells to ~50%, but that of FDC-P1 and Ba/F3 cells by only 20% (Figure 10B). Dex reduced the growth rate of transformants after 24 h (Figure 10C), but the cells appeared viable over a 4 day exposure to Dex, and growth suppression by Dex was reversible upon its withdrawal (data not shown). Dex also suppressed EPO-dependent growth of BF-CIS-ER cells (data not shown). Together with the failure of constitutive overproduction of CIS in FDC-P1 and Ba/F3 cells, these data suggest that CIS is a negative regulator of cell growth.

Discussion

We have isolated and characterized the novel immediate-early gene CIS, which is induced by a subset of cytokines. CIS induction is mediated by the membrane-proximal regions of the β chain of the GM-CSF receptor and of the EPO receptor. As the membrane-proximal region is responsible for JAK activation, CIS expression may be regulated by the JAK/STAT pathways. This pathway, originally found in interferon (IFN) signaling, is now

known to be a major signaling pathway regulating gene expression by various cytokines (Darnell *et al.*, 1994; Ihle *et al.*, 1994). Among several members of the JAK kinase family, JAK2 is activated by several cytokines including EPO, IL3, G-CSF and GM-CSF. Furthermore, JAK2 is associated with the membrane-proximal region of the EPO receptor and β_c of the IL3, IL5 and GM-CSF receptors (Witthuhn *et al.*, 1993; Quelle *et al.*, 1994). Although IL-2 does not activate JAK2, JAK1 and JAK3 have recently been found to be activated by IL-2 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). Thus, these JAK kinases or an unidentified JAK kinase may be involved in CIS induction. The inhibitory effect of genistein on CIS induction supports this notion.

Activated JAK kinases phosphorylate STATs, which then translocate to the nucleus where they bind to their specific target DNA elements. We recently found that STAT5 is activated by IL-2, IL-3, IL-5, GM-CSF and EPO (Mui *et al.*, 1995; Wakao *et al.*, 1995). We also found potential STAT5 binding sites in the promoter region of the CIS gene (A.Yoshimura, unpublished results). Thus, it is likely that STAT5 is involved in CIS induction. In contrast, the Ras-MAP kinase, PI3 kinase and PKC pathways are probably not involved in the induction of CIS, for the following reasons. The mutant GM-CSF receptor β chain and EPO receptor, which are truncated at their C-termini, do not activate the Ras-MAP kinase pathway (Sato *et al.*, 1993; Sato and Miyajima, 1994; data not shown), but do induce CIS (Figure 5). Activation of PI3 kinase also requires the C-terminal region of the GM-CSF and EPO receptors (Miura *et al.*, 1994a; Sato and Miyajima, 1994). Since PMA did not induce CIS, PKC may not be involved either. This is consistent with the inability of EGF and SCF, which activate these three pathways, to induce CIS.

CIS contained an SH2 domain, but no other motifs or enzymatic activities were found. It stably associated with the tyrosine-phosphorylated IL3 receptor β chain, probably via its SH2 domain. We also found that CIS associates with the tyrosine-phosphorylated EPO receptor. Tyrosine phosphorylation of the receptor triggers the activation of several signaling pathways through the interaction between phosphotyrosine and SH2 domains. The Grb2 adapter and the Shc/Grb2 complex associate with autophosphorylated receptor tyrosine kinases via their SH2 domains, and translocate SOS to the membrane (Lowenstein *et al.*, 1992; McCormick, 1993). This leads to the activation of the Ras-MAP kinase pathway, resulting in the induction of c-*fos* and c-*jun*. Although CIS does not contain an SH3 domain, its overall structure resembles that of Grb2, and CIS may function analogously to Grb2. However, whereas Grb2 tightly associates with receptor tyrosine kinases and we could not see strong association of Grb2 with the IL-3 receptor, CIS appears to associate selectively with cytokine receptors (A.Yoshimura., unpublished data). Thus, CIS may act as a specific adapter for cytokine receptors. While it is not known at present whether CIS shuttles any signaling molecules to the membrane, it is an attractive hypothesis. In this context, it is notable that the N- and C-terminal 80 amino acids of CIS are rich in Pro and Leu, which are often involved in protein–protein interaction. If these domains are involved in recruiting some molecules, the search for molecules associated with CIS via its N- and C-terminal regions may provide a new insight into the signaling pathway of cytokine receptors.

In our experimental system, forced expression of CIS suppressed IL3- and EPO-dependent cell proliferation (Figure 10 and data not shown). Since CIS is induced by IL3 and EPO, and associates with their receptors, it can be a negative feedback regulator, or it may be involved in ligand-dependent down-modulation of the receptors. This idea is consistent with the fact that deletion of the membrane-distal region of the EPO receptor enhances signal transduction of the receptor for growth and differentiation (Yoshimura and Lodish, 1992; De La Chapelle *et al.*, 1993; Maruyama *et al.*, 1994). A tyrosine phosphatase HCP, which associates with the tyrosine-phosphorylated IL3 receptor β chain and EPO receptor, has been postulated to be involved in such negative regulation (Yi *et al.*, 1993). CIS may also play a role in down-regulation of cytokine receptors. It may also be involved in cross-talk among cytokine receptors. For example, response to EPO is often reduced when hematopoietic cells are cultured in IL3 (Krosl *et al.*, 1995; A.Yoshimura, unpublished). Likewise, G-CSF-induced differentiation is blocked by IL3 (Fukunaga *et al.*, 1993). CIS induced by IL3 could associate with the other activated cytokine receptors and down-modulate their function.

The negative effect of CIS on cytokine receptor function could be explained in several ways. First of all, CIS may work as an adapter protein for an unidentified signaling pathway which causes growth inhibition. Alternatively, the major function of CIS may be to mask phosphotyrosine residues of activated receptors and thereby down-modulate the signaling involved in SH2 domains. In support of this idea, the overexpression of CIS reduced the level of tyrosine phosphorylation of several cellular proteins induced by IL3 (see Figure 8). In particular, we found that IL3-dependent tyrosine phosphorylation as well as activation of MAP kinase (ERK2) were reduced in Dex-treated FD-CIS cells to ~50% of the level in untreated cells. Tyrosine phosphorylation of Shc and Shc-associated p145 was also reduced in Dex-treated FD-CIS cells (data not shown). As Shc is known to be upstream of Ras, the observed down-modulation of MAP kinase activity may have resulted from inhibition of Ras activation. This is consistent with the study by Okuda *et al.* (1994) that showed growth suppression by dominant negative Ras in an IL3-dependent 32D cell line, and that the Ras pathway is required for long-term proliferation (Kinoshita *et al.*, 1995). However, other studies indicate that the Ras-MAP kinase pathway is not essential for cytokine-dependent cell proliferation (Miura *et al.*, 1994b; Welham *et al.*, 1994b). Further analysis is required to clarify the relationship between reduced activation of MAP kinase and growth suppression caused by CIS overexpression. Other signal transduction pathways involved in SH2 domains, such as phospholipase C and PI3 kinase, remain to be examined.

Another possibility is that CIS is a scavenger of tyrosine-phosphorylated proteins, because CIS is degraded very quickly. The target molecule containing phosphotyrosine residues may become a substrate of the proteolytic machinery by binding to CIS. Ligand-induced down-regulation or desensitization of cell surface receptors has been well documented, but the precise molecular

mechanism has not been elucidated. Binding of CIS to the activated receptor may be a trigger for its proteolytic breakdown. Previously, Mui *et al.* (1992) reported that tyrosine phosphorylation of the IL3 receptor β chain is the signal for its proteolytic cleavage to a 70 kDa fragment. CIS could be involved in such a proteolytic process. We also found that addition of high concentrations of IL3 reduced the number of the IL3 receptors. Dex treatment accelerated this process in FD-CIS and BF-CIS cells. This is consistent with the idea that CIS could induce degradation of the β chain.

CIS is expressed in a wide variety of tissues. It may associate with tyrosine-phosphorylated molecules other than cytokine receptors in those tissues, and function as a masking protein or a scavenger of signaling molecules, or as an adapter molecule. In our preliminary experiments, several tyrosine-phosphorylated proteins were associated with CIS in fibroblast cells stably expressing CIS. Overexpression of CIS in fibroblasts also resulted in reduction of their growth rate (A.Yoshimura, unpublished data). Thus, CIS seems to modulate intracellular signaling pathways and cell proliferation not only in hematopoietic cells but also in non-hematopoietic cells. The putative CIS chromosomal locus in humans, 3p21, is frequently lost or rearranged in renal cell carcinomas and lung cancer (Yamakawa *et al.*, 1992; Yokoyama *et al.*, 1992). If human CIS is mapped to this locus and is lost in cancer cells, CIS may function as a tumor suppressor gene, consistent with our data that CIS suppresses the growth of hematopoietic and non-hematopoietic cells.

In conclusion, CIS is a unique regulatory protein involved in cytokine receptor signaling, since it is induced through the membrane-proximal region of the receptor but binds to the membrane-distal region that contains phosphotyrosine residues. CIS appears to function as a negative regulator of cytokine signaling for growth, either by down-modulating the receptor function or by recruiting another negative modulator to the membrane. The precise function of CIS *in vivo* also remains to be investigated.

Materials and methods

Cells

Murine IL3-dependent lymphoid Ba/F3 cells, myeloid FDC-P1, FDC-P2 and NFS-60 cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from the WEHI-3B cell line as a source of IL3 (Yoshimura *et al.*, 1990a). The IL2-dependent T-cell line, CTLL2, was cultured in 1 nM IL2. FDC-P2 cells expressing c-*kit* (FDC-P2/*kit*) (Ohashi *et al.*, 1994), the IL6-dependent myeloma cell line, MH60.BSF2 (Matsuda *et al.*, 1988), Ba/F3 transformants expressing the EGF receptor (BF-EGFR), the EGFR/EPORH (BF-EGFR/EPORH) (Maruyama *et al.*, 1994), the human GM-CSF receptor α-chain and C-terminal truncated β chain (BF/$\alpha\beta_{626}$) (Sakamaki *et al.*, 1992), the EPO receptor (BF-ER) and the IL2 receptor β chain (BF-IL2Rβ) (Hatakeyama *et al.*, 1989) were as described elsewhere.

Library subtraction

BF-EGFR/EPORH cells were factor depleted for 5 h in RPMI medium containing 1% bovine serum albumin (BSA). Poly(A) RNAs from unstimulated cells and from those incubated with 100 ng/ml EGF in the presence of 10 μg/ml cycloheximide for 1 h were purified using a Quick-prep mRNA isolation kit (Pharmacia). cDNA library construction and library subtraction were performed as described (Hara *et al.*, 1994). Briefly, 100 μg purified plasmid DNA from the library of unstimulated BF-EGFR/EPORH cells was completely digested with *Eco*RI, *Not*I and *Sca*I, then biotinylated using Photoprobe biotin (Vector Labs). Five micrograms of purified plasmid DNA of the library from stimulated BF-EGFR/EPORH cells was digested with *Eco*RI and *Not*I, followed by hybridization with the digested and biotinylated library DNA of unstimulated BF-EGFR/EPORH cells in hybridization buffer (0.75 M NaCl, 5 mM EDTA, 25 mM HEPES buffer pH 7.5,0.1% SDS) containing 0.25 mg/ml of *Escherichia coli* tRNA at 68°C for 20 h. Biotinylated DNA was removed by incubation with streptavidin and by four extractions with phenol/chloroform. The subtracted DNA was hybridized for 2 h, using the same biotinylated DNA. After removing biotinylated double-stranded DNAs, residual DNA was ligated into the pME18S vector digested with *Eco*RI and *Not*I, and used to transform electro-competent *E.coli* to generate the subtracted library. To remove self-ligated vector and very small cDNA inserts, size-selected (0.5–2.0 kb) cDNA fragments of the subtracted cDNA library were religated into pCRII vector (Invitrogen). Randomly isolated clones were further screened by direct sequencing and by Northern hybridization using poly(A) RNA from stimulated and unstimulated BF/$\alpha\beta_{626}$, BF-EGFR/EPORH and BF-EGFR cells.

Isolation of the full length cDNA

The partial cDNA fragment of CIS obtained by library subtraction was labeled with digoxigenin (DIG) using a DIG–DNA labeling kit (Boehringer Mannheim) and used for colony hybridization screening of the cDNA library from stimulated BF-EGFR/EPORH cells according to the manufacturer's instructions. The longest clone (22F17) was fully sequenced using synthetic oligonucleotide primers. To isolate the 5′ end, RACE (Frohman, 1993) was performed using a 5′-AmpliFINDER-RACE kit (Clontech).

Transformants carrying the dexamethasone (Dex)-inducible CIS gene

A *Xho*I–*Sph*I-digested fragment of CIS cDNA containing the entire coding region, but not the non-coding region, was subcloned into pMAMneo (Clontech). G418-resistant transformants of FDC-P1 or Ba/F3 cells were isolated, then Dex-responsive clones (FD-CIS and BF-CIS) were screened by immunoblotting using anti-CIS. To obtain BF-CIS cells expressing the EPO receptor (BF-CIS-ER), the EPO receptor cDNA in pXM expression vector was transfected into BF-CIS cells, then cells were selected in EPO as described (Yoshimura *et al.*, 1990b).

Northern hybridization

The cells were factor-depleted for 4 h in RPMI medium containing 1% BSA, then stimulated with cytokines at the following concentrations for various periods: IL2, 10 ng/ml; IL3, 5 ng/ml; IL6, 5 ng/ml; GM-CSF, 10 ng/ml; G-CSF, 20 ng/ml; EPO, 10 ng/ml; EGF, 100 ng/ml; SCF, 25 ng/ml. For Northern blotting, poly(A) or total RNA was separated on 1.0% agarose gels containing 2.4% formaldehyde, then transferred electrophoretically to positively charged nylon membranes. After fixation under calibrated UV irradiation, the membranes were hybridized with DIG-labeled riboprobes prepared using a DIG–RNA labeling kit (Boehringer Mannheim). The blot was detected using alkaline phosphatase-labeled anti-DIG antibody and a chemiluminescent substrate according to the manufacturer's instructions.

Antibodies

An *Xma*I fragment from clone 22F17 containing the SH2 and C-terminal domains was ligated into an *Xma*I-digested pGEX3 vector (Pharmacia). The GST–CIS fusion protein was purified by preparative SDS–PAGE and used for raising rabbit polyclonal antiserum. Control antibody against GST (αGST) was produced in a similar manner. A rat monoclonal antibody (9D3) against the IL3 receptor β chain and rabbit polyclonal anti-AIC2A (αAIC2A, a gift from Dr A.Mui, DNAX Institute) were as described (Mui *et al.*, 1992; Ogorochi *et al.*, 1992). Both antibodies react with AIC2A but not AIC2B. 9D3 does not work for immunoblotting.

Immunoblot analysis

CIS protein in FDC-P1 or Ba/F3 cells was detected by a combination of immunoprecipitation and immunoblotting as described (Ohashi *et al.*, 1994). Briefly, 10^7 cells were solubilized with 1 ml of 1.0% NP-40 in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% aprotinin. After centrifugation, the cleared lysates were incubated with 100 μl of 100 mg/ml BSA and 4 μl of αCIS for 1 h, then with 50 μl of protein A–Sepharose (50% v/v, Pharmacia) for a further 30 min at 4°C. The immune complex was resolved by 12.5% SDS–PAGE and immunoblotted with a 1:2000 dilution of αCIS. The blots were visualized using peroxidase-conjugated protein A and Enhanced Chemiluminescence (ECL) detection reagents (Amersham).

Association of the IL3 receptor β chain and the EPO receptor with CIS

BF-CIS or FD-CIS cells were cultured in 0.1 ng/ml IL3 for 16 h in the presence or absence of 500 nM Dex. Cells (5×10^7/sample) were then incubated in serum-free RPMI medium containing 0.5% BSA and 0.2 mM vanadate for 15 min at 37°C, and stimulated with 50 ng/ml IL3 at 25°C for 10 min. Cells were then solubilized with 1 ml of 1.5% Triton X-100 in PBS containing 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM PMSF and 1% aprotinin. After centrifugation, cleared lysates were incubated with 4 μl of αCIS or αGST (control antiserum), or 3 μg of 9D3 monoclonal antibody for 1 h at 4°C. The immune complex adsorbed with protein A–Sepharose (for αCIS or αGST) or anti-rat IgG-conjugated agarose (for 9D3) was divided into three aliquots, and resolved on 9.5% or 12.5% SDS–PAGE in three separate gels. The two 9.5% gels were immunoblotted with 4G10 and αAIC2A and the 12.5% gel was immunoblotted with αCIS. Association of CIS and the EPO receptor was detected in a similar manner using BF-CIS-ER cells as well as COS cells transiently expressing CIS and kit/ER chimeric receptor (Ohashi *et al.*, 1994).

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J×*M.spretus*) F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the CIS locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins, 1982). Fragments of 8.8 and 6.1 kb were detected in *Kpn*I-digested *M.spretus* DNA. The presence or absence of the two co-segregated *M.spretus*-specific *Kpn*I fragments was followed in backcross mice. The probes and RFLPs for the loci linked to CIS including transferrin (*Trf*), guanine nucleotide binding protein, α inhibiting 2 (*Gnai2*) and procollagen type VII, α1 (*Col7a1*) have been described (Li *et al.*, 1993). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution pattern. Our interspecific map of chromosome 9 was compared with a composite mouse linkage map (The Jackson Laboratory, Bar Harbor, ME). References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H.Welch Medical Library of The Johns Hopkins University, Baltimore, MD).

Nucleotide accession number

The nucleotide sequence reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D31943.

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