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Richard Monni, Susana Constantino Rosa Santos, Martine Mauchauffe, Roland Berger, Jacques Ghysdael, et al.. The TEL-Jak2 oncoprotein induces Socs1 expression and altered cytokine response in Ba/F3 cells. Oncogene, 2001, 20 (7), pp.849-858. 10.1038/sj.onc.1204201 . hal-02427368

HAL Id: hal-02427368

https://univ-tours.hal.science/hal-02427368

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Oncogene (2001) 20, 849–858
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www.nature.com/onc

The TEL-Jak2 oncoprotein induces Socs1 expression and altered cytokine response in Ba/F3 cells

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The leukemia-associated TEL-Jak2 fusion protein possesses a constitutive tyrosine kinase activity and transforming properties in hematopoietic cell lines and animal models. In the murine pro-B Ba/F3 cell line, this fusion constitutively activates the Signal Transducer and Activator of Transcription 5 (Stat5) factors and, as a consequence, induces the sustained expression of various Stat5-target genes including the Cytokine Inducible SH2-containing protein (Cis) gene, which codes for a member of the Suppressor of Cytokine Signaling (Socs) protein family. In TEL-Jak2-transformed Ba/F3 cells, we also observed the upregulation of the Socs1 gene, whose product has been reported to negatively regulate the Jak kinase activity. In transient transfection experiments, Socs1 physically interacts with TEL-Jak2 and interferes with the TEL-Jak2-induced phosphorylation and activation of Stat5 factors, probably through the Socs1-induced proteasome-mediated degradation of the fusion protein. Interestingly, TEL-Jak2-expressing Ba/F3 cells were found to be resistant to the anti-proliferative activities of gamma interferon (IFN-γ) seemingly as a consequence of Socs1 constitutive expression. These results indicate that the Socs1-dependent cytokine feedback loop, although active, is bypassed by the TEL-Jak2 fusion, but may play a role in the leukemogenic process by altering the cytokine responses of the leukemic cells. Our results also suggest that Socs1 plays a role in shutting down the signaling from the normally activated Jak2 kinase by inducing its proteasome-dependent degradation. *Oncogene* (2001) **20,** 849–858.

Keywords: leukemogenesis; TEL-Jak2; Socs1; Ba/F3; interferon-γ

Introduction

The Janus kinases (Jaks) are receptors-associated cytoplasmic tyrosine kinases activated upon stimulation with cytokines, growth factors and hormones. Jak activation by auto- or trans-phosphorylation initiates signaling cascades allowing adaptative cellular responses in regard to both ligands and cell types (reviewed in Schindler, 1999). Among the different substrates of activated Jaks, is the family of Signal Transducers and Activators of Transcription (Stat), that is composed of seven members (Stat1-4, 5A, 5B, 6). These cytoplasmic transcription factors dimerize upon phosphorylation by the Jaks, translocate into the nucleus and mediate the expression of a large array of responsive genes driving cellular processes, such as cell growth and survival. In hematopoietic cells, the Jak-Stat pathway has been implicated in the signaling from various cytokine receptors, as the receptors of interleukins (ILs) and interferons (IFNs), focusing on the major role of Jak and Stat activities in the homeostasis control.

Multiple levels of negative regulation of cytokine signal transduction have been identified, that include down-regulation of activated cytokine-receptors complexes and direct inhibition of components of the Jak-Stat pathway. Among them, the Cytokine Inducible SH2 protein/Suppressors of Cytokine Signaling (Cis/Socs) proteins are important modulators of Jak and Stat activities (for review see Gisselbrecht, 1999). Cis encodes a protein involved in switching off the cytokine signal by competing for Stat5 docking sites on the intracellular domains of phosphorylated cytokine receptors (Yoshimura *et al*., 1995). Cis appears to behave as a classical negative feedback of Stat5 signaling since functional Stat5 binding sites have been identified in the promoter sequences of the murine and human Cis genes that are responsible for its transcriptional induction in response to cytokine (Matsumoto *et al*., 1997; Verdier *et al*., 1998). Socs1 (also called JAB, SSI-1 or Tip3) acts as a negative regulator of the Jak-Stat signaling by interfering with Jak tyrosine kinase activity and the subsequent activation of Stat factors (Naka *et al*., 1997; Endo *et al*., 1997; Starr *et al*., 1997;

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Received 3 October 2000; revised 5 December 2000; accepted 19 December 2000

Ohya *et al*., 1997). The Socs1 gene is transcriptionally induced by a large panel of cytokines leading to the activation of Stat5, Stat3 and Stat1 and analysis of the 5′ region of the Socs1 gene identified interferon-stimulated responsive elements (ISRE) and interferon-γ-activated sites (GAS) elements known to bind Stat6 (Schluter *et al*., 2000). Accordingly, studies have focused on the pivotal role of Socs1 on IFN receptors signaling. Dysregulation of the IFN-γ receptor signaling is believed to be the major cause of organ failures and hematological abnormalities observed in mice lacking Socs1 (Starr *et al*., 1998; Naka *et al*., 1998; Metcalf *et al*., 1999; Alexander *et al*., 1999; Marine *et al*., 1999). Moreover, Socs1 has been shown to inhibit the cellular response to cytokines different from those responsible for its expression and to participate to the IFN-γ-mediated repression of IL-4-induced transcription (Venkataraman *et al*., 1999). Based on sequence homologies, additional Socs proteins have been identified that may also participate to negative feedback controls of cytokine signalings (Hilton *et al*., 1998; Nicholson and Hilton, 1998). Socs proteins share a common structure including a central Src homology 2 (SH2) domain interacting with the catalytic domain of Jaks, and for Socs1 with various tyrosine kinases and molecular adaptors (Ohya *et al*., 1997; De Sepulveda *et al*., 1999), and a conserved COOH-terminal region referred as the SOCS box and able to bind the ElonginBC complex (Kamura *et al*., 1998; Zhang *et al*., 1999). This latter interaction has been proposed to drive Socs and Socs-associated proteins to the ubiquitin-proteasome degradation pathway, as recently illustrated by the Socs1-mediated stimulation of polyubiquitination of vav proteins (De Sepulveda *et al*., 2000).

Inappropriately regulated forms of Jaks and Stats proteins have been recurrently associated with oncogenic processes (reviewed in Coffer *et al*., 2000; Bowman *et al*., 2000). The direct implication of the Jak-Stat signaling pathway in human hematological malignancies has been demonstrated by the identification of chromosomal translocations involving Jak and Stat encoding genes. For example, the Stat5B gene is fused to the retinoic alpha receptor (RARα) gene in an acute promyelocytic-like leukemia (Arnould *et al*., 1999) and several examples of chromosomal rearrangements involving Jak2 and TEL (Translocated Ets Leukemia/ETV6) genes have been described in leukemias (Lacronique *et al*., 1997; Peeters *et al*., 1997). The TEL-Jak2 chimeric proteins expressed as a result of these translocations are essentially formed by the fusion of the amino-terminal part of TEL to Jak2 sequences, always including its catalytic domain (JH1). TEL-mediated oligomerization of the TEL-Jak2 protein, results in constitutive tyrosine kinase activity and transforming properties in cytokine-dependent hematopoietic cell lines (Lacronique *et al*., 1997) and animal models (Schwaller *et al*., 1998; Carron *et al*., 2000). Fusion proteins containing the oligomerization domain of TEL and the tyrosine kinase domains of other mammalian Jaks (Jak1, Jak3 and Tyk2) were also able to confer growth factor independence to the murine pro-B IL-3-dependent Ba/F3 cell line, pointing to their potential transforming capacities in an hematopoietic context (Lacronique *et al*., 2000).

In this study, we analysed the inhibitory effect of Socs1 on the activity of the TEL-Jak2 fusion protein. Our results show that Socs1, whose gene is specifically upregulated in the Ba/F3 cells transformed by TEL-Jak2, is able to functionally interfere with the TEL-Jak2 catalytic activity and subsequent activation of Stat5. Additionally, in transient transfection experiments the overexpression of Socs1 appears responsible for the proteasome-dependent degradation of the fusion protein, while the overexpression of Socs3 do not. Moreover, TEL-Jak2-transformed Ba/F3 cells are resistant to the antiproliferative activities of IFN-γ seemingly as a consequence of Socs1 constitutive expression and suggesting a paradoxical role for Socs1 in the leukemogenic process.

Results

Socs1 expression is specifically upregulated in TEL-Jak2-transformed Ba/F3 cells

We previously established that expression of TEL-Jak2 and of fusion proteins containing the oligomerization domain of TEL and the tyrosine kinase domains of other mammalian Jaks (TEL-Jak1, TEL-Jak3 and TEL-Tyk2) efficiently substitutes for the survival and mitogenic signals induced by IL-3 in the murine IL-3-dependent Ba/F3 cells (Lacronique *et al*., 2000). Stat5 activation was shown to be a crucial step in this transforming process. In keeping, transcriptional activation of known Stat5-target genes (oncostatin M, pim-1 and Cis) was observed. The same set of genes was induced in Ba/F3 cells expressing the TEL-ABL fusion protein, presumably through Stat5 activation (Lacronique *et al*., 2000). The upregulation of Cis in both TEL-Jak2- and TEL-ABL-transformed cells prompted us to examine the expression of other members of the Cis/Socs family.

As shown Figure 1a, Northern blotting analysis revealed a strong induction of Cis in the IL-3-stimulated parental Ba/F3 cells (lanes 3–6). Cis was constitutively transcribed in the IL-3-independent TEL-Jak2-transformed Ba/F3 cell line (lane 7) and, at lower levels, in the TEL-ABL-transformed Ba/F3 cells (lane 8), as already reported (Lacronique *et al*., 2000). The same membrane, re-probed with a Socs1 specific probe, showed induction of Socs1 expression following IL-3 stimulation in parental Ba/F3 cells. Accumulation of Socs1 transcripts was readily observed in the TEL-Jak2-expressing cells (lane 7) but not in the TEL-ABL-transformed Ba/F3 cells (lane 8). Constitutive Socs1 expression was also seen in RNA from Ba/F3 cells transformed by the TEL-Jak1, TEL-Jak3 and TEL-Tyk2 fusion proteins (data not shown).

The expression of the Socs1 gene is induced by a variety of cytokines known to activate members of the

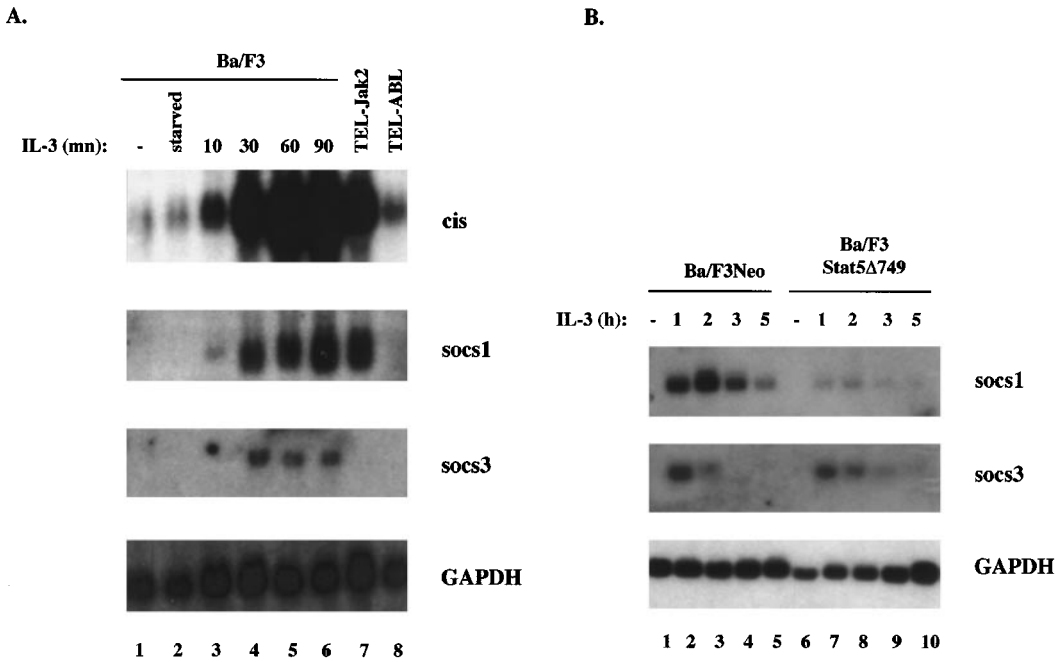


Figure 1 Constitutive activation of Cis/Socs genes in TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells. (**a**) Northern blot analyses of total RNA extracted from the indicated cell lines for Cis, Socs1 and Socs3 expression. RNA from parental Ba/F3 cells cultured in the presence of 5% WEHI conditioned medium (−), starved and stimulated or not by IL-3 for the indicated times were loaded as controls. Note that the blot was overexposed to show the accumulation of Cis transcripts in the TEL-ABL-expressing Ba/F3 cells. (**b**) RNA from the Stat5AΔ749-expressing Ba/F3 cell line and from their Ba/F3Neo control cells were isolated at indicated time points following IL-3 stimulation

Stat family. In an attempt to assess the contribution of Stat5 factors in Socs1 regulation, we examined by Northern blot the accumulation of Socs1 transcripts in IL-3-stimulated Ba/F3 cells that constitutively express a truncated form of Stat5A. The Stat5AΔ749 variant has been previously reported to exert a dominant negative effect on Stat5-responsive gene transcription (Dumon *et al*., 1999). Stat5AΔ749-expressing cells and their respective Ba/F3Neo control cells were starved and then stimulated with recombinant IL-3 and RNA was isolated at different time points following cytokine treatment (Figure 1b). While a prominent induction of Socs1 was detected in the control Ba/F3Neo cells upon IL-3 stimulation (lanes 1–5), accumulation of Socs1 transcripts was dramatically reduced in Stat5AΔ749-expressing cells (lanes 6–10), supporting a Stat5-mediated induction of Socs1. Accordingly, Socs1 expression was also detectable in Ba/F3 cells expressing constitutively active Stat5 mutants (unpublished and Nosaka *et al*., 1999).

We also examined the expression of other Socs family members, Socs2 and Socs3. Socs2 transcripts were barely detectable in all dependent and independent BaF3 cell lines analysed here, and no obvious changes were observed upon IL3 stimulation (data not shown). Socs3 induction was detected upon IL-3 stimulation in the parental Ba/F3 cells (Figure 1a) and levels of induction were found unchanged between control Ba/F3Neo and Stat5AΔ749-expressing Ba/F3 cells (Figure 1b), suggesting thus a minor contribution of Stat5 factors in its expression. We did not observe accumulation of Socs3 transcripts neither in TEL-Jak2- nor in TEL-ABL-transformed cells (Figure 1a), nor in other TEL-Jak-expressing cells (data not shown). These experiments also showed that although Cis, Socs1 and Socs3 are induced by IL-3 in Ba/F3 cells, their time range of induction are clearly different. While both Cis and Socs1 inductions were rapid and maintained as long as the cytokine was present, accumulation of Socs3 transcripts upon IL-3 stimulation was delayed and rapidly returned to basal levels.

Socs1 functionally interacts with the TEL-Jak2 fusion protein

The inhibitory effect of the Socs1 protein on Jak2 signaling is described to occur through its interaction with a phosphorylated tyrosine residue (Y1007) within the JH1 domain, thus interfering with Jak2 catalytic activity and substrate binding (Yasukawa *et al*., 1999). We analysed the capacity of Socs1 to physically interact with the TEL-Jak2 chimera and Socs3 protein, which is the most closely related to Socs1 among the Socs family, was used as control. Socs3 has been reported to bind to Jak2, although with a lower affinity than Socs1 (Cohney *et al*., 1999).

Since available anti-Socs1 antibodies were unsuccessful in detecting endogenous Socs1 protein, we studied the interaction between Myc-tagged Socs1 and HA-tagged TEL-Jak2 proteins by immunoprecipitation of

transiently transfected proteins in the mammalian 293T cell line. As shown in Figure 2, both the anti-Myc and anti-HA antibodies immunoprecipitated the Socs1 and TEL-Jak2 proteins from extracts of 293T cells transfected with the respective expression vectors. The specificity of co-immunoprecipitation experiments was confirmed by the inability of the anti-Myc and the anti-HA antibodies to precipitate TEL-Jak2 and Socs1, respectively. The Socs1-Jak2 interaction has been reported to rely on a phosphorylated (i.e. activated) state of Jak2. Consistently, we found that a kinase inactive mutant of TEL-Jak2 (TEL-Jak2 K882E) did not co-immunoprecipitate with Socs1 in cotransfection assays in 293T cells (data not shown). These results indicate that Socs1 can interact with the JH1 of Jak2 in the context of the TEL-Jak2 fusion. In contrast, when Socs3 was transiently expressed, we were unsuccessful in co-immunoprecipitating Socs3 and TEL-Jak2 using an anti-HA antibody, while both proteins co-immunoprecipitated when using the anti-Myc antibody. Therefore, since immunoprecipitations were performed with the same set of antibodies, the Socs3-TEL-Jak2 interaction appears to be weaker than the Socs1-TEL-Jak2 interaction.

We next examined the ability of Socs1 to alter the TEL-Jak2 kinase activity and the subsequent activation of Stat5. For this purpose, Myc-tagged Socs1 and HA-tagged TEL-Jak2 were transiently expressed together with Stat5A into COS7 cells, known to express little amounts of endogenous Stat5. The phosphorylation status of TEL-Jak2 and Stat5A, which reflects the activated state of the proteins, was studied by anti-phosphotyrosine immunoblotting. As shown in Figure 3a upper panel, co-expression of increasing amounts of Socs1 reduced the phosphotyrosine content of both TEL-Jak2 and Stat5A proteins, as judged by the decreasing intensity of their respective tyrosine phosphorylation state (Figure 3a, lanes 3–5). Controls using anti-Myc (detecting Socs1), anti-Stat5 and anti-N-TEL antibodies showed constant expression levels of Stat5A, whereas Socs1 and TEL-Jak2 protein levels varied inversely when co-expressed (Figure 3a, lower panels).

The same extracts were analysed for Stat5A DNA binding activity by EMSA using the β-casein probe. The co-transfection of TEL-Jak2 and Stat5A expression vectors allowed the formation of a specific Stat5A-β-casein shifted complex (Figure 3b, lane 3) that was not observed in COS7 cells transfected with the TEL-Jak2 expression vector alone (Figure 3b, lane 2). Expression of increasing amounts of Socs1 markedly diminished the DNA binding activity of transfected Stat5A (Figure 3b, compare lane 3 to lanes 4–6) while this inhibition was not observed upon co-expression of Socs3 (Figure 3b, compare lane 3 to lanes 7–9). Similar observations were made for endogenous Stat1 factors, activated by the overexpression of TEL-Jak2 in these experiments. Taken together, these results indicate that Socs1 impaired the TEL-Jak2-induced phosphorylation and activation of Stat5A. In contrast, overexpression of Socs3 had no obvious effect on the phosphorylation state of both TEL-Jak2 and Stat5A (data not shown) and accordingly, did not affect the Stat5A DNA binding activity as assessed by EMSA analysis (Figure 3b). Collectively, our results indicate that although Socs3 can interact with the TEL-Jak2 chimera, it is unable to detectably interfere with Stat5 activation.

Since, co-expression of Socs1 repeatedly altered the TEL-Jak2 expression levels when co-expressed (Figure 3a), we wondered if Socs1 could regulate the stability of the fusion protein. Several studies argue for an ubiquitin-proteasome-dependent regulation of both Socs1 and interacting partners protein levels (Kamura *et al*., 1998; Zhang *et al*., 1999; De Sepulveda *et al*., 2000). Thus, COS7 cells were transfected with the same combination of expression vectors than depicted in Figure 3a, and treated with the specific proteasome inhibitor lactacystin or left untreated before lysis. As shown in Figure 3c, while reduced TEL-Jak2 protein levels were observed in untreated transfected cells when co-expressed with increasing amounts of Socs1 (lanes 1–3), in extracts from lactacystin-treated cells, TEL-Jak2 protein levels were identical whatever amounts of Socs1 expressed (lanes 4–6). A compar-

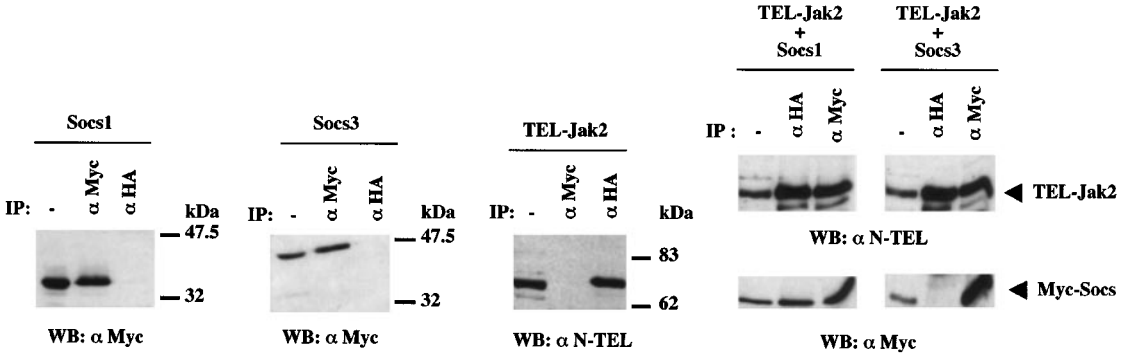


Figure 2 The TEL-Jak2 fusion protein physically interacts with Socs1. Total lysates from 293T cells expressing the transiently transfected Myc-tagged Socs1, Myc-tagged Socs3 or HA-tagged TEL-Jak2 proteins were immunoprecipitated (IP) with the anti-Myc (α Myc) and anti-HA (α HA) antibodies, separated by SDS–PAGE and immunoblotted (WB) with the indicated antibodies. αN-TEL corresponds to a rabbit antiserum directed to the amino-terminal domain of TEL present in the TEL-Jak2 chimera. No signal was observed using mock transfected cells (not shown)

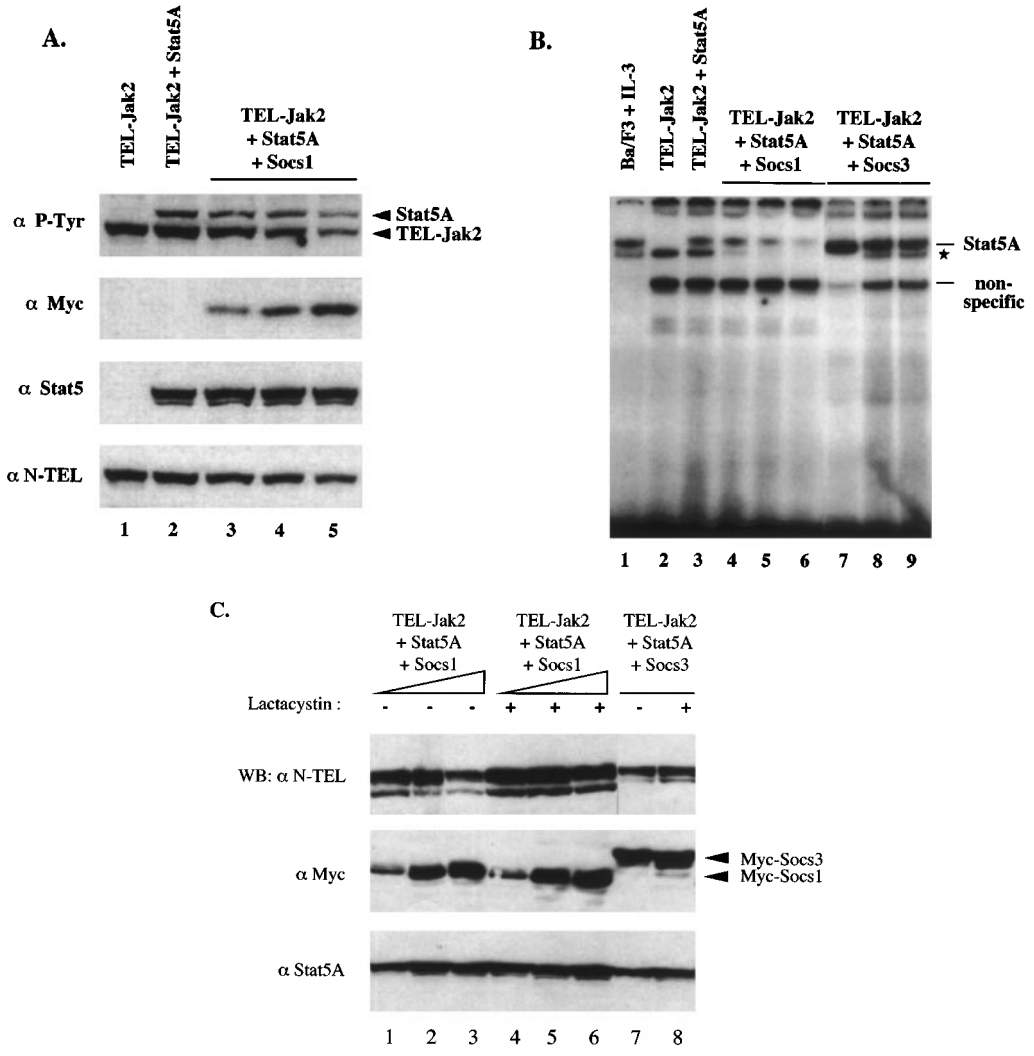


Figure 3 The overexpression of Socs1 interferes with the tyrosine kinase activity of TEL-Jak2 and the subsequent Stat5A activation, and induces the proteasome-mediated degradation of the fusion protein. (**a**) COS7 cells were transiently transfected with increasing amounts (0.2, 0.5 and 2 μg, lanes 3, 4 and 5 respectively) of Myc-tagged Socs1 expression vector together with 0.2 μg of HA-tagged TEL-Jak2 and 0.2 μg of Stat5A vectors. Total cell lysates were analysed by immunoblot using an anti-phosphotyrosine antibody (α P-Tyr) to check for the phosphorylation state of both Stat5A (upper species) and TEL-Jak2 (lower species). Protein expression levels were analysed by stripping and reprobing the membranes with anti-Stat5 and anti-Myc antibodies and with a rabbit antiserum α N-TEL. (**b**) To test for the DNA binding activity of transfected Stat5A, aliquots of total extracts from COS7 cells transfected with the indicated combination of expression vectors (0.2, 0.5 and 2 μg of Myc-tagged Socs3 expression vector, lanes 8, 9 and 10 respectively) were assayed in EMSA with the β-casein probe. As a control for functional Stat5 DNA binding activity, nuclear extracts from IL-3-stimulated parental Ba/F3 cells were loaded (lane 1). Note that a specific shifted complex corresponding to the TEL-Jak2-mediated activation of endogenous Stat1 factors (denoted by a star) was observed in these experiments. Non-specific complexes are indicated. (**c**) To evaluate the possible Soc1-mediated proteasome-dependent degradation of TEL-Jak2, COS7 cells were transiently transfected with the indicated combination of expression vectors and incubated in the presence of 25 μM of lactacystin (+) or control buffer (−) 6 h before lyse. Total cell lysates were resolved in SDS–PAGE and immunoblotted with the indicated antibodies

able analysis of TEL-Jak2 expression co-transfected with a Socs3 expression vector, in similar amounts than those used for the highest Socs1 expression levels (lanes 3 and 6), did not evidence variations of TEL-Jak2 protein levels in the presence or in the absence of lactacystin (lanes 7 and 8, respectively). Reprobing the membrane with an anti-Stat5A antibody showed roughly unchanged expression levels of transfected Stat5A proteins, although a faint increase was observed with extracts of lactacystin-treated cells (compare lanes 1–3 to 4–6). These latter observations may be related to the reported stabilization of phosphorylated forms of Stat5 factors by proteasome inhibitors (Wang *et al*., 2000).

Collectively, our results strongly argue for the direct involvement of Socs1 in the degradation of the TEL-Jak2 fusion protein via the ubiquitin-proteasome pathway.

The IFN-γR signaling pathway is impaired in the TEL-Jak2-transformed Ba/F3 cells

Since we could not directly document the expression of the Socs1 protein in the TEL-Jak2-transformed Ba/F3 cells, we decided to investigate a potential effect of Socs1 on other signaling pathways. As several studies have shown that Socs1 exerts a central and potent inhibitory role on IFN-γR signaling, we examined the activity of some effectors of this pathway in the TEL-Jak2-expressing Ba/F3 cells. IFN-γR stimulation results in the activation of the receptor-associated Jak1 and Jak2 kinases, and subsequently of Stat1 which undergoes homodimerization to form GAF (INF-γ Activated Factor) and participates to the transcriptional induction of a series of IFN-γ-responsive genes.

We thus analysed the ability of IFN-γR to activate Stat1 in the TEL-Jak2-transformed Ba/F3 cells. For this purpose, parental and TEL-Jak2-expressing Ba/F3 cells were starved and either left untreated or were treated with increasing concentrations of murine IFN-γ for 10 min. Nuclear extracts were prepared and the subsequent DNA binding activity of Stat1 was analysed by EMSA by using the specific m67SIE probe (Figure 4a). As a control, the TEL-ABL-transformed Ba/F3 cells, which do not exhibit accumulation of Socs1 transcripts, were treated with IFN-γ in similar conditions. Nuclear extracts from IFN-γ-stimulated parental Ba/F3 cells showed a major shifted protein-DNA complex corresponding to Stat1 homodimers (Figure 4a, lane 2). This activation was found to be dose-dependent, since increasing amounts of activated Stat1 were observed upon treatment with increasing concentrations of cytokine (Figure 4a, lanes 2–5). In a striking contrast, IFN-γ-stimulation of TEL-Jak2-transformed Ba/F3 cells resulted in a very weak activation of Stat1 compared to parental cells, even in the presence of high concentrations of cytokine (Figure 4a, compare lanes 2–5 to 6–9). This impaired activation was not observed in TEL-ABL-transformed Ba/F3 cells, in which Stat1 DNA binding activity could

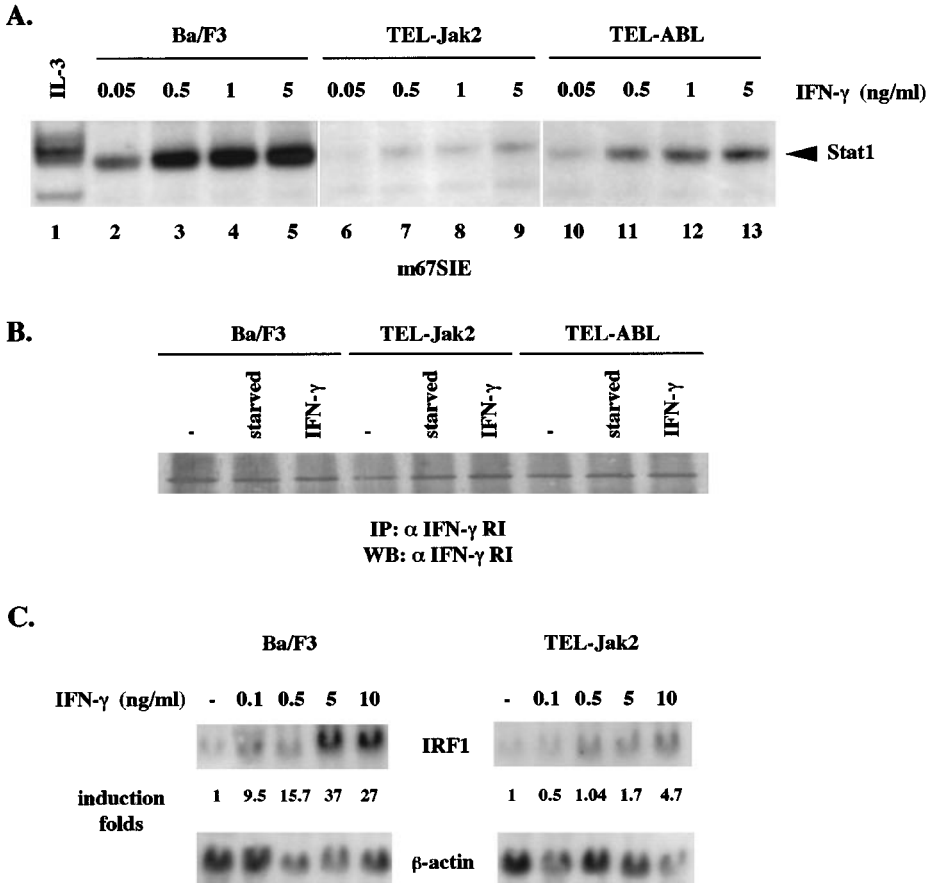


Figure 4 The IFN-γR signaling pathway is specifically impaired in the TEL-Jak2-expressing Ba/F3 cells. (**a**) Analyses of Stat1 DNA binding activity in parental, TEL-Jak2- and TEL-ABL-transformed Ba/F3 cells upon IFN-γ stimulation. The concentrations of IFN-γ used are indicated. Equal amounts of nuclear extracts (2 μg) were submitted to EMSA using the m67SIE probe. (**b**) The IFN-γ receptor chain 1 (IFN-γR1) was immunoprecipitated (IP) from total cell lysates of the indicated Ba/F3 cell lines and analysed by immunoblotting (WB) with the same antibody. Lysates were made from TEL-Jak2-, TEL-ABL-transformed and parental Ba/F3 cells non stimulated (−), starved and stimulated or not with 0.5 ng/ml of IFN-γ. (**c**) Northern blot analysis for IRF1 expression of total RNA extracted from the TEL-Jak2-transformed and parental Ba/F3 cells, untreated (−) or treated with the indicated concentrations of IFN-γ. Folds of IRF1 transcriptional induction were estimated by comparison with β-actin signals

be induced by IFN-γ (Figure 4a, lanes 10–13). As shown in Figure 4b, this lack of activation did not apparently rely on a defective expression of the IFN-γR in the TEL-Jak2-expressing cells, since similar amounts of IFN-γ receptor chain 1 (IFN-γR1) were immunoprecipitated from all the IFN-γ-treated or untreated Ba/F3 cell lines studied. To ensure that the defect in Stat1 activation has consequences on transcriptional regulation, we investigated the transcription of the IRF1 gene (Figure 4c). This gene is expressed at low levels in normally growing cells and its IFN-γ-induced expression relies on binding of GAF complexes in its promoter region. In agreement with our EMSA data, IRF1 expression was not stimulated upon INF-γ treatment in TEL-Jak2-transformed Ba/F3 cells when compared to parental Ba/F3 cells, while its transcriptional induction was not affected in the IFNγ-stimulated TEL-ABL-expressing Ba/F3 cells (data not shown). This indicates that activation of Stat1 which is central in the IFN-γ-Jak-Stat1/IRF pathway is specifically altered in TEL-Jak2-transformed Ba/F3 cells.

To analyse this defect at the cellular level, we compared the effect of IFN-γ treatment on Ba/F3 control cells and their TEL-Jak2-transfected counterparts. Parental and TEL-Jak2-transformed Ba/F3 cells were treated with 0.2 or 20 ng/ml of IFN-γ and the number of viable cells scored over a period of 4 days (Figure 5). While IFN-γ treatment of parental Ba/F3 cells resulted in a strong inhibition of cellular growth at the two concentrations used, the proliferation rate of IFN-γ-treated TEL-Jak2-expressing cells was found to be the same as that observed in absence of IFN-γ. TEL-Jak2-expressing Ba/F3 cells were found resistant to the antiproliferative activity of IFN-γ at all concentrations tested (ranging from 0.2–100 ng/ml, Figure 5 and data not shown).

Collectively, these observations demonstrate a specific defect of the IFN-γ signaling pathway and antiproliferative properties of IFN-γ in Ba/F3 cells transformed by the leukemic TEL-Jak2 fusion protein.

Discussion

Several levels of negative regulation of the Jak-Stat signaling pathway have been characterized, including direct dephosphorylation of Jak kinases by the SH2-domain containing Hematopoietic Phosphatase 1 and 2 (SHP1/PTP1C, SHP2/PTP1D), expression of the Protein Inhibitor of Activated Stats (PIAS) which interact and specifically inhibit the DNA binding of activated Stats, and interaction of the proteins of the Cis/Socs family with activated Jaks. Unlike SHP1 or PIAS which are expressed in non stimulated cells, the expression of Cis/Socs genes is induced upon cytokine stimulation.

Herein, we analysed the expression of members of the Socs family in Ba/F3 cells transformed by the leukemia-associated TEL-Jak2 and TEL-ABL fusion proteins. The transcription of the Socs1 gene is markedly upregulated in the TEL-Jak2-transformed Ba/F3 cells, but not following the TEL-ABL transformation of Ba/F3 cells. Both oncogenic fusion proteins constitutively activate Stat5 in the Ba/F3 cellular context and induced the expression of several known Stat-5 target genes, including Cis (Figure 1a). Con-

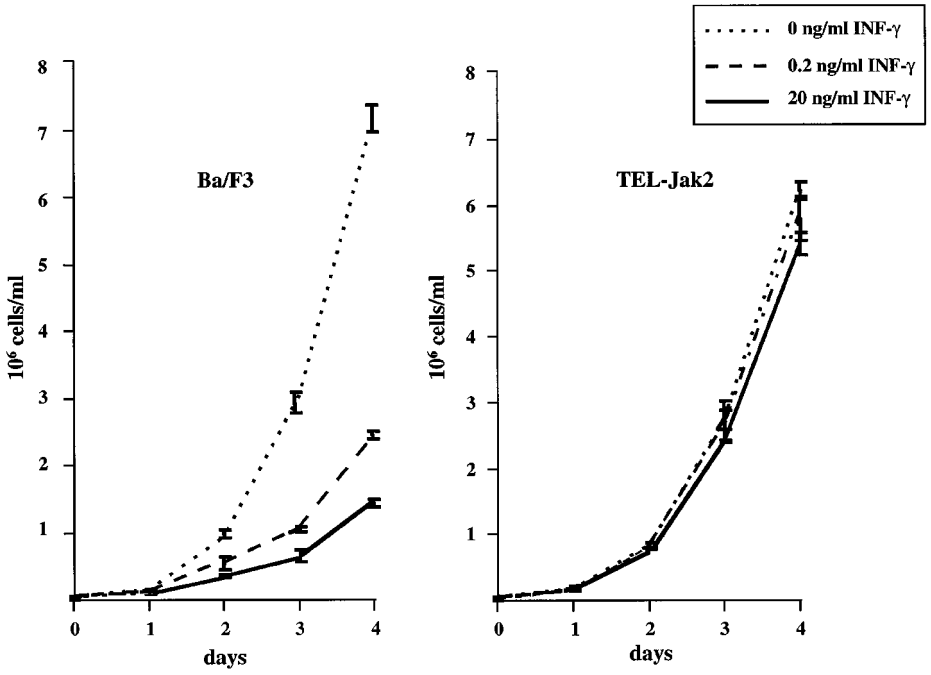


Figure 5 TEL-Jak2-expressing Ba/F3 cells are resistant to the antiproliferative activity of IFN-γ. Tel-Jak2 and parental Ba/F3 cells were plated in the absence or presence of the indicated concentrations of IFN-γ and numbers of viable cells were scored daily. The average numbers of viable cells were from two independent experiments

sistently, Ba/F3 cells expressing constitutively active forms of Stat5 show an upregulation of Cis and Socs1 (Nosaka *et al*., 1999). Unlike Cis, little is known concerning the Stat-dependent transcriptional induction of Socs genes, except that Stat binding elements are present in their promoter regions, and that a dominant negative form of Stat3 inhibits the expression of Socs1 in response to IL-6 (Naka *et al*., 1997). A dramatic drop in IL3-mediated Socs1 activation was observed in our Ba/F3 cell line expressing a dominant negative form of Stat5A, suggesting a role of Stat5 in the IL-3-induced Socs1 expression. It is of note that accumulation of Socs1 transcripts is highly variable upon stimulation by a given cytokine and according to the cell line (for review see Gisselbrecht, 1999), thus suggesting that regulatory factors other than Stat5 may participate to its transcriptional regulation.

Our transient transfection experiments show that the Socs1 protein physically interacts with the TEL-Jak2 fusion protein and interferes with its activity by affecting its expression level. With regard to the interaction between the Socs box and the elonginBC complex, it has been proposed that Socs protein levels may be controlled, in part, through their degradation via a proteasome-dependent pathway (Kamura *et al*., 1998; Zhang *et al*., 1999). The founding member of the Socs protein family, Cis, may mediate the proteasome-dependent inactivation of the erythropoietin receptor (Verdier *et al*., 1998). Recently, Socs1-mediated polyubiquitination has been reported for the Socs1-interacting vav proteins (De Sepulveda *et al*., 2000). To date, there is no evidence of such a contribution of Socs proteins in the regulation of Jak kinases stability. It has been postulated that Socs1 inhibits Jak2 kinase activity by binding the activation loop of the phosphorylated kinase, thus preventing substrates to access (Narazaki *et al*., 1998; Yasukawa *et al*., 1999). Interestingly, we evidenced in transient transfection experiments a specific control of Socs1 in TEL-Jak2 protein expression levels, proteasome-dependentwise. We did not observe a similar contribution of Socs3, that can also interact to a lesser extent with the TEL-Jak2 chimera, even though Socs3 shares the greatest number of features with Socs1 among the Socs family members. Along these lines, we hypothesize that Socs1 proteins may also inhibit Jak2 activity by targeting it to the ubiquitin-proteasome pathway.

Despite the negative input of Socs1 on TEL-Jak2 expression level and activity, TEL-Jak2 exhibits powerful transforming properties in the Ba/F3 cellular model, linked to Stat5 activation (Lacronique *et al*., 1997, 2000). The specific impairment of IFN-γR signaling in the TEL-Jak2-expressing Ba/F3 cells suggests that, at least in this cellular context, Socs1 proteins are expressed and functional. From these observations, one can hypothesize that Socs1 proteins are not expressed at high enough levels to fully inhibit the TEL-Jak2-induced pathways essential to its oncogenic activity. Alternatively, TEL-Jak2 fusion proteins form oligomers of ill-characterized stoichiometry, some of which could be repressed by Socs1 proteins, while some could not be repressed. Escape from the Socs1 negative feedback could also be due to the constitutive nature of the kinase activity of the TEL-Jak2 fusion or to conformational changes within the Jak2 moiety with respect to the wild type Jak2. In addition, based on reports indicating that Socs1 could behave as a signaling adaptor (Ohya *et al*., 1997; De Sepulveda *et al*., 1999), one can infer that Socs1 might link TEL-Jak2 to other signaling pathways such as those arising from the Pyk2 (Takaoka *et al*., 1999), Tec (Yamashita *et al*., 1998) or c-*kit* (Weiler *et al*., 1996) tyrosine kinases.

Interestingly, we observed that TEL-Jak2-transformed Ba/F3 cells are resistant to the antiproliferative activity of IFN-γ, seemingly as a consequence of Socs1 expression, and suggesting that Socs1 could have an effect on the leucemic process by altering the cytokine responses of the leukemic cells. Of interest, it was recently reported that Socs1 constitutive expression protects against the p38 MAP kinase-mediated TNF-α-induced apoptosis (Morita *et al*., 2000). The overexpression of Socs1 has also been shown to correlate with a down-regulation of the phosphorylated state of the SHP2 phosphatase in a prolactine-stimulated cellular model, suggesting synergistic mechanisms in inhibiting cytokine signaling (Tomic *et al*., 1999). The resistance to the antiproliferative activity of IFN-γ of the TEL-Jak2-expressing Ba/F3 cells may also provide some molecular basis of the clinical resistance to IFN, widely used as therapeutic agents in the treatment of hematological malignancies. One can suppose that the limited IFN therapy efficacy may be, in part, explained by sustained Socs1 expression in the leukemic cells, consistent with the recurrent constitutive Stat activities observed in hematopoietic disorders. The availability of TEL-Jak2 transgenic mice will allow analysis of IFN sensibility *in vivo*.

Materials and methods

Plasmids

The pBabe TEL-ABL and HA-tagged pBabe Tel-Jak2 retroviral vectors have been previously described (Lacronique *et al*., 2000). The HA-tagged TEL-Jak2 cDNA was cloned into the pCDNA3 expression vector (Invitrogene) for transient transfections experiments. The pXM-Stat5A expression vector has been reported (Liu *et al*., 1995). Myc-epitope tagged murine Socs1 and human Socs3 cDNAs were gifts from A Yoshimura (Institute of Life Science, Kurume University, Japan). The coding region of the murine Socs2 was cloned by PCR amplification from WEHI mouse genomic DNA, with primers designed according to the published sequence (Starr *et al*., 1997).

Cell culture and transfections

Stable IL-3-independent TEL-Jak2- and TEL-ABL-expressing Ba/F3 cell lines have been reported previously (Lacronique *et al*., 2000). The Ba/F3 cells constitutively expressing the dominant negative form of Stat5A (Stat5AΔ749) and their respective control Ba/F3Neo cells

have been already described (Dumon *et al*., 1999). Parental Ba/F3 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 5% WEHI conditioned medium as a source of murine IL-3. Stimulation with recombinant murine IL-3 and IFN-γ (R&D Systems Europe Ltd, UK) were performed on TEL-Jak2-, TEL-ABL-transformed and parental Ba/F3 cells starved for 5 h in Iscove modification of Dulbecco's minimum essential medium containing 0.4% bovine serum albumin and 20 μg/ml iron saturated transferrin. Cells were stimulated with 15 ng/ml of IL-3 or 0.05–5 ng/ml of IFN-γ for 10 min. The 293 T cells used for transient transfection experiments were cultured in a mixture (1vol/1vol) of Dulbecco's modified Eagle's medium (DMEM) and HAMF12 (Sigma), containing 10% FBS. COS7 cells were maintained in DMEM containing 10% FBS and 2 mM glutamine. Transfections were carried out using the DMRIE-C reagent according to the manufacturer's instructions (Gibco–BRL).

Northern blots

Northern blotting analysis was performed by using 10 μg of total RNA from the different Ba/F3 cell lines and probes corresponding to cDNA fragments for murine Cis, Socs1, Socs2, Socs3 and human IRF1 labeled with ^{32}P-αdCTP by random-priming. Northern blots were normalized by hybridization with GAPDH or β-actin probes.

Cell lysates, immunoprecipitates and Western blots analyses

Transfected COS7 cells were lysed in a buffer containing 1% Triton, 25 mM Tris/Hcl pH 7.4, 8 mM $MgCl_2$, 15% glycerol, 1 mM DTT and complemented with protease and phosphatase inhibitors, then centrifuged at 12 000 *g* for 15 min to remove insoluble materials. Cell extracts were electrophoresed on a 8% SDS-polyacrylamide gel and transferred to nitrocellulose. The antibodies used were the 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.) the 9E10 anti-Myc antibody (Santa Cruz Biotechnology, Inc.) and an anti-TEL rabbit immunserum (Poirel *et al*., 1997). In transient transfection experiments using the lactacystin proteasome inhibitor (Calbiochem), transfected COS7 were incubated for 6 h in the presence of 25 μM of lactacystin or control buffer (DMSO) before to be lysed in the above buffer. For Socs1 and TEL-Jak2 co-immunoprecipitation analysis, transfected 293 T were lysed in 0.1% Nonidet P-40, 50 mM Tris/Hcl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, containing protease and phosphatase inhibitors. Total cellular extracts were made 24 h post-transfection. Cleared whole-cells lysates were incubated 2 h at 4°C with anti-Myc or anti-HA antibodies (Santa Cruz Biotechnology, Inc). Immunoprecipitates were separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose and blotted with the anti-Myc antibody and with the anti-TEL rabbit immunserum. For IFN-γ receptor chain 1 (IFN-γR1) immunoprecipitation analysis, 20×10^6 cells of the different Ba/F3 cell lines were lysed in 1% Nonidet P-40, 50 mM Tris/Hcl pH 7.4, 150 mM NaCl, 2 mM EGTA containing protease inhibitors and cleared supernatants were incubated 2 h at 4°C with a rabbit polyclonal anti- IFN-γR1 antibody (PBL Biomedical Laboratories). Immunoprecipitates were separated by 8% SDS-polyacrylamide gels, transferred to nitrocellulose and blotted with the same antibody. Immunocomplexes were detected using the ECL detection kit (Amersham Pharmacia Biotechnology) and stripping and probing were performed following the manufacturer's instructions.

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays (EMSA), equal amounts of total cellular extracts from transfected COS7 cells were incubated with the ^{32}P-labeled bovine β-casein (5′-AGATTTCTAGGAATTCAAATC-3′) Stat5 binding site. Eight microliters of nuclear extracts from IL-3-stimulated parental Ba/F3, made as reported previously (Lacronique *et al*., 2000), were used as control. In IFN-γ-stimulation experiments, EMSA were performed in the presence of 2 μg of nuclear extracts from the different treated Ba/F3 cell lines by using the radiolabeled m67SIE (5′-CATTTCCCG-TAAATC-3′) probe.

Cell proliferation analysis

5×10^5 TEL-Jak2-expressing Ba/F3 cells and parental Ba/F3 cells were plated in the presence or absence of murine IFNγ (0.2 and 20 ng/ml) and numbers of viable cells were scored daily by blue Trypan exclusion. Parental cells were maintained in 5% WEHI conditioned medium during the experiments.

Acknowledgments
The authors are grateful to A Yoshimura (Institute of Life Science, Kurume University, Japan) for providing the Socs expression vectors, to M Chelbi Alix (Institut Universitaire d'Hématologie, Hôpital St Louis, Paris, France) for the IRF1 probe and the LPH (Hôpital Saint-Louis, Paris, France) for art work. This work was supported in part by grants from the Association contre le Cancer (ARC) and from the Fondation de France. R Monni is supported by a Ministère de l'Education Nationale, de la Recherche et des Technologies (MENRT) fellowship and SCR Santos is supported by Praxis XXI.

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