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Regulation of STAT protein synthesis by c-Cbl

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Many cytokines and growth factors induce transcription of immediate early response genes by activating members of the Signal Transducers and Activators of Transcription (STAT) family. Although significant progress has been made in understanding the events that lead to the activation of STAT proteins, less is known about the regulation of their expression. Here we report that murine embryonic fibroblasts derived from c-Cbl-deficient mice display significantly increased levels of STAT1 and STAT5 protein. In contrast, STAT2 and STAT3 expression, as well as the levels of the tyrosine kinases Jak1 and Tyk2, appear to be regulated independently of c-Cbl. Interestingly, the half-life of STAT1 was unaffected by the presence of c-Cbl, indicating that c-Cbl acts independently of STAT1 degradation. Further analysis revealed similar levels of STAT1 mRNA, however, a dramatically increased rate of STAT1 protein synthesis was observed in c-Cbl-deficient cells. Thus, our findings demonstrate an additional control mechanism over STAT1 function, and also provide a novel biological effect of the Cbl protein family. *Oncogene* (2001) **20,** 7326 – 7333.

Keywords: Interferon; c-Cbl; STAT; translation

Introduction

Signal transducers and activators of transcription (STATs) represent a family of transcription factors that mediate cytokine and growth factor-induced activation of immediate early response genes in the absence of de-novo protein synthesis (Darnell *et al.*, 1994; Larner *et al.*, 1993). Seven genetically distinct mammalian STAT proteins have been described (Fu, 1992; Lin *et al.*, 1996; Liu *et al.*, 1995; Quelle *et al.*, 1995; Yamamoto *et al.*, 1994; Zhong *et al.*, 1994a,b), and related signaling molecules have been found in *Drosophila* (Hou *et al.*, 1996; Yan *et al.*, 1996) and *Dictyostelium* (Kawata *et al.*, 1997). All STAT proteins are activated through rapid tyrosine phosphorylation (Gupta *et al.*, 1996; Heim *et al.*, 1995), which is required for dimerization (Shuai *et al.*, 1994), nuclear translocation (Mowen and David, 1998) and DNA binding (David *et al.*, 1993; Fu, 1992). Tyrosine phosphorylation of STAT proteins in response to cytokines requires the activity of one or more Janus tyrosine kinases (JAKs), whereas STAT phosphorylation via growth factor receptors such as the epidermal growth factor receptor depends on the intrinsic kinase activity of the receptor. In addition to phosphorylation of STAT1 and STAT3 on Tyr701 or Tyr705, respectively, further phosphorylation on Ser727 is essential to maximize their transactivation capabilities (Wen *et al.*, 1995). Although more emphasis had been placed on STAT activation than on inactivation, several negative regulatory components of the Jak/STAT pathway have recently been identified. These proteins target either the activating Jak kinase, or affect the phosphorylated STAT protein directly. However, in either case, it is the activation state or the functionality of the STAT proteins that is affected rather than their protein levels.

The proto-oncogene c-Cbl was originally identified as the mammalian ortholog of the v-Cbl oncogene isolated from the Cas NS-1 murine leukemia virus (Blake *et al.*, 1991; Langdon *et al.*, 1989). Homologs of c-Cbl have been found in *Drosophila* (D-Cbl) (Meisner *et al.*, 1997) and in *C. elegans* (sli-1) (Yoon *et al.*, 1995). The 120 kDa cytoplasmic gene product of c-Cbl, which lacks any detectable enzymatic activity, contains in its amino-terminus a unique SH2-domain, an EF hand domain, and a highly basic region. The COOH-terminal half contains a proline-rich domain, several tyrosine phosphorylation sites, and a ubiquitin-associated (UBA) domain. A centrally located ring-finger domain has been demonstrated to be essential for c-Cbl's function as a ubiquitination-promoting or E3 factor (Joazeiro *et al.*, 1999). Two oncogenic forms of c-Cbl lack this critical feature. The v-Cbl protein represents a carboxy-terminal deletion, lacking the ring-finger and the proline-rich domain. 70Z-Cbl, which was isolated from a pre-B cell line, contains an internal deletion within the ring-finger domain (amino acids 366 – 382), illustrating that mutations or deletions of the ring finger domain can be responsible for the oncogenic potential of c-Cbl (Blake *et al.*, 1991).

Much of the effort in understanding the function of c-Cbl has been focused on its role in the ubiquitin-

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dependent degradation of growth factor receptors such as the epidermal growth factor receptor or the CSF-1 receptor (Lee *et al*., 1999; Levkowitz *et al*., 1998, 1999; Wang *et al*., 1999; Waterman *et al*., 1999). In these cases, c-Cbl facilitates the multi-ubiquitination of activated, tyrosine phosphorylated receptor tyrosine kinases, thereby downregulating an activated signal transduction pathway. Similarly, c-Cbl negatively regulates antigen receptor-mediated signaling events by decreasing the levels of activated, tyrosine phosphorylated ZAP70 or Syk (Andoniou *et al*., 2000; Ota and Samelson, 1997; Thien *et al*., 1999). Consistent with these biochemical findings, c-Cbl-deficient mice display lymphoid hyperplasia and increased antigen receptor signaling (Murphy *et al*., 1998; Naramura *et al*., 1998).

c-Cbl was found to be tyrosine phosphorylated in response to interferons (Uddin *et al*., 1996) and interleukins (Odai *et al*., 1995), however, the role of c-Cbl in the Jak/STAT signaling cascade remains largely unexplored. Here we show that c-Cbl exerts a negative regulatory function on interferon signaling by controlling STAT1 proteins levels. Intriguingly, c-Cbl decreased STAT1 levels in a ligand-independent manner, and acted by suppressing protein synthesis rather than by promoting protein degradation.

Results

c-Cbl−/− cells display increased antiproliferation in response to IFNβ

c-Cbl has been previously implicated in the regulation of the cellular response to growth factors such as EGF, presumably by targeting internalized receptors for ubiquitin-dependent degradation (Levkowitz *et al*., 1998; 1999). We were interested in determining whether c-Cbl would play a similar role in regulating signal transduction through cytokine receptors such as the IFNα/β receptor. We therefore isolated murine embryonic fibroblasts from either wild-type or c-Cbl−/− mice to test their responsiveness towards the antiproliferative effects of murine IFNβ. In addition, two reconstituted clones, R1 and R2, were independently isolated after retroviral introduction of c-Cbl into the c-Cbl deficient MEFs. Expression of c-Cbl in these four cell lines was determined by Western blotting of resolved whole cell lysate with a monoclonal anti-c-Cbl antibody. As shown in Figure 1a, the wild-type MEFs, and the two reconstituted clones express comparable levels of c-Cbl (lanes 1, 3 and 4), whereas the MEFs established from the c-Cbl−/− mice lack detectable c-Cbl protein (lane 2). The faint band with slightly faster mobility has been observed before, and represents a non-functional protein originating from the mutant allele (Murphy *et al*., 1998).

In order to determine whether c-Cbl−/− cells displayed an altered sensitivity towards the antiproliferative effects of IFNβ, we stimulated these cells with 100 U/ml IFNβ, and analysed cell growth over a 5 day period. Indeed, c-Cbl−/− cells were significantly more inhibited in their proliferation by IFNβ than wild-type MEFs or the reconstituted clones R1 and R2 (Figure 1b). It is important to note that all cell lines displayed similar growth rates in the absence of IFNβ (data not shown). To further explore the observed increase in antiproliferative effects of IFNβ in the absence of c-Cbl, we performed a dose-response analysis by determining inhibition of cell growth at 10, 100 or 1000 U/ml IFNβ over a 3 day period. As expected, all concentrations of IFNβ triggered a stronger antiproliferative response in c-Cbl−/− cells than in the wild-type MEFs or in the reconstituted clones R1 and R2 (Figure 1c). Similar results were obtained when murine IFNα was used for stimulation (data not shown). These results demonstrate that c-Cbl not only plays an essential role in the cellular response to growth factors, but also modulates the biological response to antiproliferative cytokines such as IFNβ.

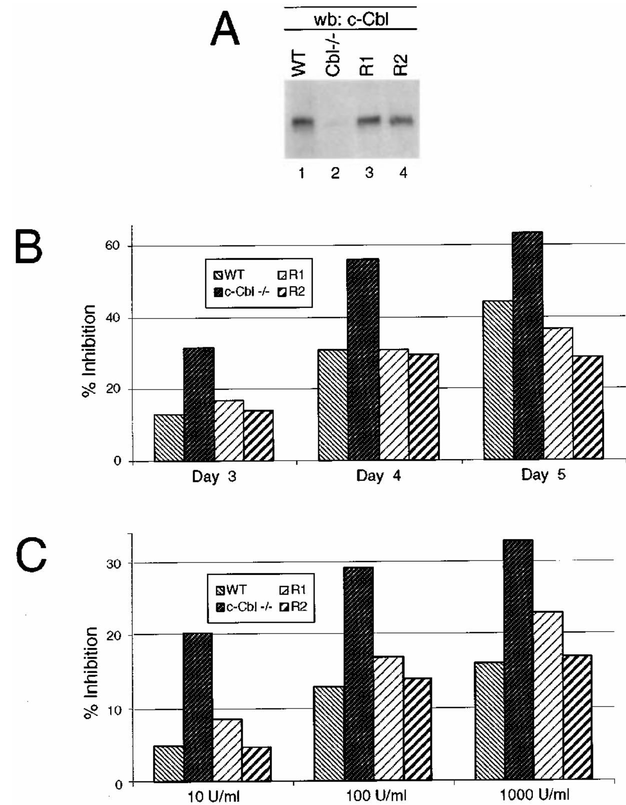

Figure 1 Increased IFNβ-induced antiproliferation in c-Cbl-deficient MEFs. (**a**) Immunoblot analysis of WT, c-Cbl−/−, and reconstituted MEFs: Cell lysates from wild-type (lane 1), c-Cbl −/− (lane 2), R1 (lane 3) and R2 (lane 4) reconstituted cells were resolved and probed for the presence of c-Cbl. (**b**) Increased IFNβ-mediated antiproliferation in the absence of c-Cbl: MEFs were left untreated or stimulated with 100 U/ml IFNβ for the indicated times. Antiproliferative effects of IFNβ are expressed as percentage of growth inhibition relative to untreated cells (a representative of three experiments with similar results is shown). (**c**) Same as **b**, but cells were treated with 10, 100 or 1000 U/ml IFNβ for 3 days

Increased STAT1 phosphorylation and proteins levels in c-Cbl−/− cells

Since the antiproliferative effect of IFNβ requires the activation of the Jak–STAT signal transduction pathway, we compared the levels of STAT1 tyrosine phosphorylation in wild-type, c-Cbl−/−, and the two reconstituted cell lines after IFNβ stimulation. As shown in Figure 2a, the absence of c-Cbl results in dramatically increased phosphorylation of STAT1 in response to IFNβ (compare upper panel, lane 4 with lanes 2, 6, and 8), however, the half-life of tyrosine phosphorylated STAT1 appeared to be similar in all cells (data not shown). Surprisingly, when the blot was reprobed for STAT1, we found that the c-Cbl−/− cells contained significantly higher levels of STAT1 protein (lower panel, lanes 3, 4) when compared to cells expressing c-Cbl (lower panel, lanes 1, 2; 5, 6; 7, 8).

Next, we analysed the protein levels of other STAT family members to test whether the effect of c-Cbl−/− was specific for STAT1, or would globally alter the expression of STATs. Immunoblot analysis of lysates derived from wild-type, c-Cbl−/−, R1 and R2 cells for the presence of STAT1, 2, 3 and 5 revealed that the absence of c-Cbl−/− causes an increase in the amounts of STAT1 and STAT5 protein, whereas the levels of STAT2 and STAT3 protein remained unaffected (Figure 2b).

The protein expression levels of the two Janus tyrosine kinases involved in IFN$\alpha\beta$ signaling, Jak1 and Tyk2, also appear to be regulated independently of c-Cbl (Figure 2c). Interestingly, c-Cbl−/− MEFs displayed a higher level of basal tyrosine phosphorylation of Tyk2 and Jak1 when compared to the reconstituted cell lines. However, no difference in the extent of Jak1 and Tyk2 tyrosine phosphorylation was observed between the c-Cbl−/− and reconstituted cells after stimulation of with IFNβ for 15 min. It is also noteworthy that the increased basal tyrosine phosphorylation of Jak1 and Tyk2 in c-Cbl−/− cells does not translate into a constitutive tyrosine phosphorylation of STAT1 (Figure 2a,c) or STAT2 (data not shown). Interestingly, Erk1/2 phosphorylation, although constitutive, was found to be similar in all cell lines tested (data not shown), indicating that the loss of c-Cbl does not result in a general dysregulation of signaling pathways.

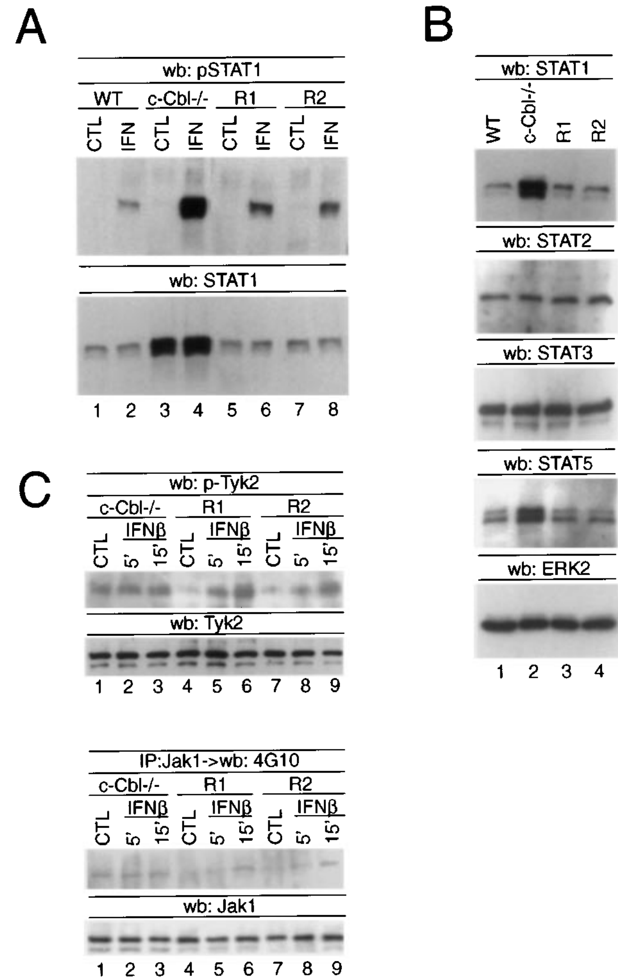

Figure 2 Increased STAT phosphorylation and protein levels in c-Cbl−/− cells. (**a**) STAT1-Y701 hyperphosphorylation in c-Cbl−/− cells: WT, c-Cbl−/−, R1 and R2 cells were left untreated (lanes 1, 3, 5, 7) or treated with 1000 U/ml IFNβ for 30 min. (lanes 2, 4, 6, 8). Proteins were immunoblotted with phospho-(Y701)-specific STAT1 antibody (upper panel). Subsequently, the blot was reprobed with STAT1 antibody (lower panel). (**b**) Increased levels of STAT1 and STAT5, but not STAT2 or STAT3, in c-Cbl−/− cells. Cell lysates from WT (lane 1), c-Cbl−/− (lane 2), R1 (lane 3), and R2 (lane 4) cells were immunoblotted for the presence of STATs 1, 2, 3 and 5. The blot was also blotted for ERK2 to show equal loading. (**c**) C-Cbl does not affect IFNβ induced tyrosine phosphorylation of Jak1 or Tyk2. C-Cbl−/−, R1, and R2 cells were stimulated with 1000 U/ml IFNβ for the indicated times, and cell lysates were immunoblotted with phospho-Tyk2 specific antibody (upper panel). The remaining lysate was subject to immunoprecipitation with Jak1 antisera, and isolated proteins were immunoblotted with 4G10 phosphotyrosine antibody (upper panel). Both blots were reprobed for total Tyk2 and Jak1 protein, respectively (lower panels)

STAT1 mRNA levels are unchanged in c-Cbl-deficient MEF cells

The observed increase in STAT1 protein observed in c-Cbl−/− cells could be due to changes in transcription or mRNA stability, or could be regulated on a posttranscriptional level. To address this question, we performed RNAse protection assays using a radiolabeled STAT1 antisense RNA probe in order to determine the levels of STAT1 mRNA in the wild-type, c-Cbl−/−, and the two reconstituted cell lines. A GAPDH antisense probe was used as an internal control for RNA isolation and processing. No significant difference in the amount of STAT1 mRNA between the cell lines could be detected (Figure 3 upper panel, compare lane 2 with lanes 1, 3 and 4). This was further confirmed by densitometric analysis and normalization to GAPDH (Figure 3, lower panel). Thus, the increased amount of STAT1 protein in the

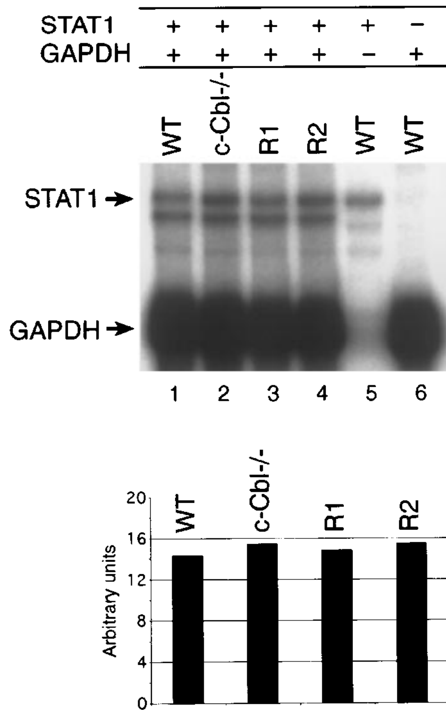

Figure 3 c-Cbl expression does not alter STAT1 mRNA levels. Total RNA from WT (lane 1), c-Cbl−/− (lane 2), R1 (lane 3) and R2 (lane 4) cells was analysed by RNase protection assay using a probe corresponding to the human STAT1 gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for loading. The bar graph depicts the quantitated results, normalized for GAPDH

absence of c-Cbl appears to originate at a posttranscriptional level, either by increased STAT1 protein synthesis, or by decreased STAT1 protein turnover.

c-Cbl Regulates STAT1 synthesis, but not degradation

c-Cbl has been shown to participate in the down-regulation of other signaling events, notably the EGF receptor pathway, by promoting the ubiquitination and proteasome-dependent degradation of components of the signaling cascade. Since it had been previously suggested that phosphorylated STAT1 protein might be subject to proteasome-mediated degradation (Kim and Maniatis, 1996), we reasoned that c-Cbl functions by facilitating efficient degradation of STAT1 protein, even in the absence of its phosphorylation. To test whether c-Cbl represses STAT1 protein levels by ubiquitin-mediated proteolysis, c-Cbl−/−, R1 and R2 cells were treated for either 1 or 6 h with 40 mM MG132, a potent proteasome inhibitor. The resulting cell lysates were then analysed for STAT1 expression levels. Unexpectedly, inhibition of proteasome-mediated protein degradation by MG132 was ineffective in elevating STAT1 levels in the c-Cbl-expressing R1 and R2 cells (Figure 4a, lanes 4–9). Furthermore, MG132 treatment was also unable to cause an additional increase in STAT1 levels in the c-Cbl−/− cells (lanes 1–3). TNFα-mediated IκB degradation in

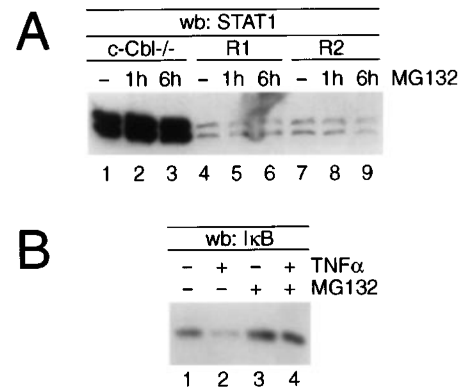

Figure 4 Proteasome inhibitor MG132 does not affect STAT1 protein levels: (**a**) c-Cbl−/− and reconstituted MEFs R1 and R2 were left untreated (lanes 1, 4 and 7) or treated for the indicated times with 40 mM MG132 (lanes 2–3, 5–6 and 8–9). Proteins were immunoblotted with anti-STAT1 antibody. (**b**) R1 cell were treated with TNFα for 30 min in the presence or absence of 40 mM MG132, and lysates were probed for the presence of IκB

untreated or MG132 pretreated cells was used to confirm the inhibitory effects of MG132 on proteasome-mediated degradation in these MEFs (Figure 4b). These results suggested that c-Cbl exerts its negative regulatory effects on STAT1 expression by suppressing the translation of STAT1 rather than promoting proteasome-mediated degradation.

To investigate this hypothesis, we performed metabolic pulse-labeling experiments on c-Cbl−/− and the reconstituted MEFs. Since these cell lines express different levels of STAT1 protein, we decided to use a limiting amount of STAT1 antibody in order to immunoprecipitate equal amounts of STAT1 protein from each of the cell lines. Immunoprecipitates of STAT1 and STAT3 derived from ^{35}S-Met/Cys-labeled cells were resolved by SDS–PAGE, transferred to membranes, and the radiolabeled proteins detected by autoradiography. Strikingly, we found that STAT1 protein synthesis occurs at a dramatically faster rate in c-Cbl−/− cells than in their reconstituted counterparts (Figure 5a, lane 1 versus 2 and 3). In striking contrast, the rate of STAT3 protein synthesis was unaffected by the absence of c-Cbl (lanes 4–6). As such, the identical incorporation of label into STAT3 does not only function as an internal control to verify efficient labeling of the reconstituted cells, but also reinforces our observations depicted in Figure 2b. To further ensure that equal amounts of immunoprecipitated protein from all three cell lines were loaded, the membranes were subsequently immunoblotted for total STAT1 and STAT3 protein (Figure 5a, lower panel).

To further evaluate whether the half-life of STAT1 was indeed unaffected by the presence of c-Cbl as suggested by the inability of MG132 to increase STAT1 levels in c-Cbl-containing cells, we performed ^{35}S-pulse-chase experiments. C-Cbl−/− and R1 cells were labeled with ^{35}S-Met/Cys for 4 h as described above, the ^{35}S-Met/Cys-containing media replaced with regular culture medium, and the cells were incubated

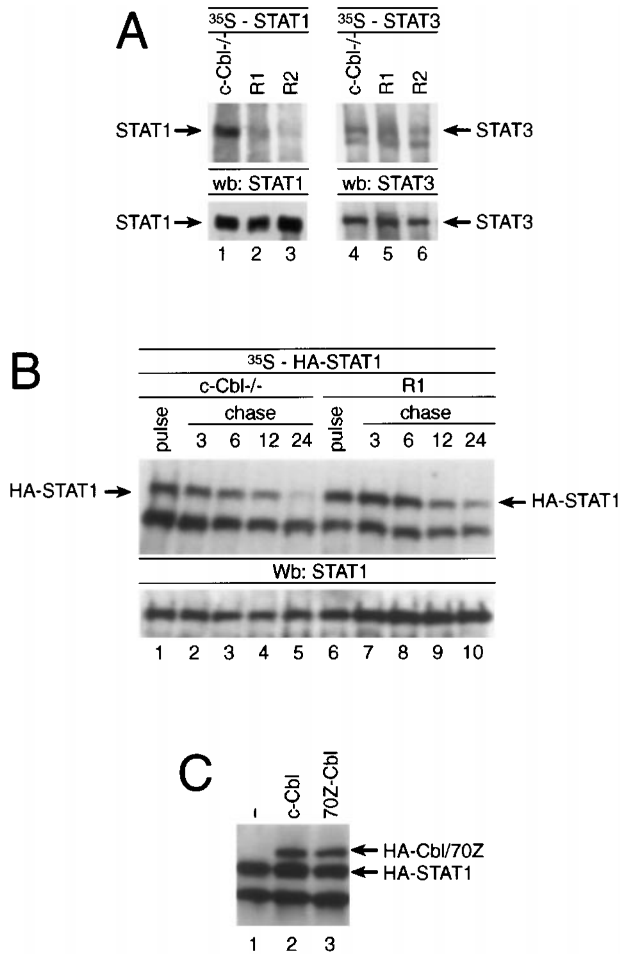

Figure 5 c-Cbl Regulates STAT1 synthesis, but not degradation c-Cbl−/− (lanes 1 and 4), R1 (lanes 2 and 5), and R2 (lanes 3 and 6) MEFs were labeled with ^{35}S-methionine/cysteine for 4 h, and STAT1 and STAT3 were sequentially immunoprecipitated from the resulting cell lysates. Immunoprecipitates were resolved by SDS−PAGE, transferred to PVDF and labeled proteins were visualized by autoradiography (upper panel). The membranes were subsequently immunoblotted with anti-STAT1 and anti-STAT3 monoclonal antibodies (lower panel). (**b**) c-Cbl−/− and R1 cells were transfected with HA-STAT1, labeled for 4 h as in (**a**), and labeled proteins chased for the indicated times. Anti-HA antibody was used for immunoprecipitations. The membrane was subsequently immunoblotted with anti-STAT1 monoclonal antibodies (lower panel). (**c**) C-Cbl−/− cell were transiently transfected either with HA-tagged STAT1 alone (lane 1), or co-transfected with either Ha-tagged wild-type c-Cbl (lane 2) or 70Z-Cbl (lane 3). Whole cell lysates were immunoblotted with anti-HA antibody

for an additional 1, 2, 4, 8 or 24 h. Although the c-Cbl−/− cells showed again a significantly increased rate of protein synthesis, neither one of the cell lines tested displayed an appreciable reduction in the amount of ^{35}S-labeled STAT1 protein over the time periods analysed (data not shown). These results further illustrate that c-Cbl affects protein levels of STAT1 by a mechanism distinct from promoting proteasomal degradation.

The above experiment indicated that the half-life of STAT1 was unaffected by the presence of c-Cbl, and that differences in the translation of the endogenous STAT1 mRNA account for the increased STAT1 levels in c-Cbl−/− cells. To further confirm this hypothesis, the half-life of HA-tagged STAT1, expressed under the control of a CMV promoter, was determined in c-Cbl−/− and R1 cells. In this scenario, equal amounts of radiolabeled STAT1 protein are produced in wild-type and c-Cbl−/− cells (Figure 5b, lanes 1 and 6). As expected, the stability of HA-STAT1 synthesized from the plasmid in c-Cbl−/− cells is essentially identical to that observed in the presence of c-Cbl. Although it is evident that HA-STAT1 is subject to degradation over time, the underlying mechanism appears to be independent of c-Cbl.

Lastly, if the half life of STAT1 would be affected by the ubiquitin ligase activity of c-Cbl, then coexpression c-Cbl, but not the ubiquitin-ligase activity deficient 70Z-Cbl, should reduce the protein levels of HA-tagged STAT1. However, when coexpressed in c-Cbl−/− cells, neither c-Cbl nor 70Z-Cbl effect the expression levels of HA-tagged STAT1 (Figure 5c, lanes 2 and 3). These results further support the notion that c-Cbl does not influence the half-life of STAT1 protein.

Discussion

In recent years, c-Cbl has been extensively studied as a negative regulator of activated growth factor or antigen receptors. Common to the mechanism in each case is the recruitment of c-Cbl to the tyrosine phosphorylated receptors, and the subsequent ubiquitin-mediated degradation of either the receptor itself (Lee *et al*., 1999; Levkowitz *et al*., 1998; 1999), or of associated tyrosine kinases and their substrates (Ota and Samelson, 1997; Thien *et al*., 1999). In contrast, the role of c-Cbl in cytokine signaling remained to be explored in much greater detail. In the present study, we utilized murine embryonic fibroblasts derived from c-Cbl-deficient mice, and two independently reconstituted cell lines to investigate the role of c-Cbl in the biological effects of IFNβ. We demonstrate that the loss of c-Cbl results in an increased antiproliferative response of the cells towards IFNβ. This hyperresponsiveness is caused by increased levels of STAT1 tyrosine phosphorylation, which in turn is due to elevated expression of STAT1 protein. Remarkably, STAT2 and STAT3 protein levels were apparently not affected by the presence or absence of c-Cbl. The increase in STAT1 protein amounts in c-Cbl−/− cells is regulated on a post-transcriptional level, since identical levels of STAT1 mRNA were detected in all cell lines tested.

Thus far, c-Cbl has been investigated predominantly as a component of the protein degradation pathway, where it acts as an E3 ubiquitin ligase in the multi-ubiquitination process of proteins targeted for degradation (Joazeiro *et al*., 1999). Although ubiquitination has been suggested as a mechanism to downregulate activated, tyrosine phosphorylated STAT1 protein after IFNγ treatment (Kim and Maniatis, 1996), we were unable to detect such a modification in

unstimulated MEFs. Incubation of the c-Cbl-containing MEFs with the proteasome inhibitor MG132 was also unable to elevate STAT1 levels in those cells. Furthermore, pulse-chase experiments with the protein synthesis inhibitor cycloheximide revealed that STAT1 protein is very stable, displaying a half-life of greater than 24 h (data not shown). Lastly, it is also noteworthy that the observed differences in STAT1 protein levels caused by the absence of c-Cbl occur in a ligand-independent manner, and therefore have to be regulated independently of STAT1 phosphorylation.

c-Cbl has previously been reported to be an inhibitor of EGF-induced STAT activation (Ueno *et al*., 1997). In these experiments, antisense repression of c-Cbl resulted in an increased phosphorylation of Jak1 and STAT3 in response to EGF, whereas phosphorylation of SOS and Shc was unaffected by the loss of c-Cbl. As c-Cbl clearly regulates degradation of the activated EGF receptor, it is obvious that different requirements exist for EGF to activate distinct signaling cascades. Indeed, a kinase-inactive EGF receptor is still able to mediate Shc phosphorylation and MAP-kinase activation, presumably by dimerizing with other ErbB family members, but fails to induce the tyrosine phosphorylation of Jak1 and STAT proteins (David *et al*., 1996; Wright *et al*., 1995).

Taken together, these results led us to analyse STAT1 protein translation by metabolic labeling of c-Cbl-containing and c-Cbl-deficient cells. Indeed, drastically enhanced translation efficiency of STAT1 was detectable in the absence of c-Cbl. Importantly, this effect was specific for STAT1, as radiolabel incorporation into STAT3 was unaffected by c-Cbl. These results confirm our findings of increased STAT1, but not STAT3, protein levels in c-Cbl−/− cells. It thus appears that c-Cbl is able to exert its negative regulatory function by means other than promoting ubiquitination of phosphoproteins.

Intriguingly, when STAT1 was expressed from a CMV-promoter controlled expression plasmid, no difference between c-Cbl−/− and the reconstituted cells was observed with regard to STAT1 protein levels, or STAT1 metabolic labeling. This further supports the hypothesis that c-Cbl regulates STAT1 levels independently of proteolytic degradation. Since the exogenous STAT1-cDNA encodes only the open-reading frame of STAT1, it is likely that the effects of c-Cbl on STAT1 protein translation from endogenous mRNA are directly or indirectly mediated by sequences in the 3′ and 5′ UTRs. Indeed, the importance of mRNA untranslated regions in translation efficiency has previously been demonstrated (Cok and Morrison, 2001; Gray, 1998; Lokvist *et al*., 2001). However, further research is required to determine whether c-Cbl mediates the tranalational control directly, or if other factors involved in translational regulation are influenced by the functions of c-Cbl.

Although we had expected that c-Cbl might function as a negative regulator of the Jak/STAT pathway, it was surprising to find inhibition of STAT1 protein synthesis as the underlying mechanism. The lack of an MG132 effect, increased STAT1 metabolic labeling in the absence of c-Cbl, and the absence of appreciable differences in STAT1 half-life provide strong evidence for a novel, protein degradation-independent basis by which c-Cbl can negatively regulate expression of certain proteins.

Materials and methods

Cells and reagents

Murine embryonic fibroblasts (MEFs) were derived from d13 embryos of wild-type and c-Cbl−/− mice by trypsinization and homogenization. Two independently reconstituted cells lines, R1 and R2, were obtained by retroviral introduction of c-Cbl into c-Cbl−/− MEFs, and subsequent selection of puromycin-resistant clones. MEFs were cultured in DMEM supplemented with 10% FCS, penicillin, and streptomycin (Irvine Scientific). Murine IFNβ was expressed in CHO cells and purified to homogeneity either as described (Luca *et al*., 1999). The potency of the purified murine IFNβ was determined in an antiviral assay using L929 cells and the encephalomyocarditis virus. In such assays, the protein had a specific activity of $\sim 2 \times 10E8$ Units/mg, where one unit of activity is defined as the concentration of murine IFNβ that gives 50% protection. MG 132 was purchased from Calbiochem (La Jolla, CA, USA).

Western blot analysis

Following treatment, cells were washed with PBS and lysed with lysis buffer (150 ml) containing 20 mM HEPES pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 mM PMSF. Lysates were centrifuged at 15 000*g* for 15 min at 4°C and protein concentration was determined by Bradford (BioRad). Equivalent concentrations of protein were boiled in SDS sample buffer containing β-mercaptoethanol, resolved by SDS–PAGE (BioRad), and electrotransferred onto Immobilon-P membranes (Millipore). After transfer, proteins were detected with specific antibodies against c-Cbl, STAT1, STAT2, STAT3, STAT5, Tyk2, Jak1, ERK2 (Transduction Laboratories), phospho-STAT1, phospho-Tyk2 (New England Biolabs), HA (BabCo) and IκB (Santa Cruz Biotech). All blots were developed with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

RNAse protection assay

Total RNA was isolated with TRIzol (Gibco BRL), and ^{32}P-labeled antisense riboprobes were generated by *in vitro* transcription using T7 or SP6 RNA polymerase (Promega). The $\sim$500 base STAT1 antisense probe was generated via T7 promoter-mediated transcription of *Sma*I-linearized BSKS–STAT1. Labeled probes were incubated with 10 μg total RNA overnight at 56°C before digestion with T1 RNAse (Gibco BRL) for 1 h at 37°C. After phenol extraction, protected fragments were boiled in RNA sample buffer and resolved by electrophoresis on a 4.5% polyacrylamide-urea gel.

Cell proliferation assay

MEFs were plated in 96-well plates and left untreated or treated with 10, 100, 1000 U/ml murine IFNβ. On the indicated days, one plate was subject to a colorimetric MTS

assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega) according to the manufacturers instructions. Unless otherwise indicated, eight samples were averaged, and for each cell line IFNβ-induced antiproliferation was determined as the percentage of growth inhibition relative to untreated cells.

Metabolic labeling and immunoprecipitations

Cell labeling was performed with 80% confluent MEFs in 60-mm tissue culture dishes. Cells were rinsed once with methionine and cysteine-free media, then pulsed for 4 h with 800 mCi/ml ^{35}S-methionine and ^{35}S-cysteine (Tran^{35}S-Label) in the same media. For chase-type experiments, the labeling media was replaced with DMEM + 10% FBS, and the cells further incubated for the indicated times. Cells were lysed as described above, and labeled proteins were immunoprecipitated from pre-cleared cell lysates with previously described antisera (5 ml/mg protein) against STAT1 and STAT3 (David *et al*., 1996). For experiments shown in Figure 5a, 0.5 ml STAT1 antiserum per mg protein was used. Immunoprecipitates of Protein G Sepharose beads (Pharmacia Biotech) were resolved by SDS–PAGE and electrotransferred to Immobilon-P membranes (Millipore). Membranes were then exposed to Biomax-MR film (Kodak) for 24–48 h at -70°C prior to reprobing with anti-STAT1 and anti-STAT3 antibodies.

cDNAs and transient transfections

MEFs were transfected using SuperFect (Qiagen) according to the manufacturer's protocol for 48 h with pSG91-HA-STAT1 prior to metabolic labeling.

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