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A small amphipathic α-helical region is required for transcriptional activities and proteasome-dependent turnover of the tyrosine-phosphorylated Stat5

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Cytokines induce the tyrosine phosphorylation and associated activation of signal transducers and activators of transcription (Stat). The mechanisms by which this response is terminated are largely unknown. Among a variety of inhibitors examined, the proteasome inhibitors MG132 and lactacystin affected Stat4, Stat5 and Stat6 turnover by significantly stabilizing the tyrosine-phosphorylated form. However, these proteasome inhibitors did not affect downregulation of the tyrosine-phosphorylated Stat1, Stat2 and Stat3. With Stat5 isoforms, we have observed that tyrosine-phosphorylated carboxyl-truncated forms of Stat5 proteins were considerably more stable than phosphorylated wild-type forms of the protein. Also, the C-terminal region of Stat5 could confer proteasome-dependent downregulation to Stat1. With a series of C-terminal deletion mutants, we have defined a relatively small, potentially amphipathic α-helical region that is required for the rapid turnover of the phosphorylated Stat5 proteins. The region is also required for transcriptional activation, suggesting that the functions are linked. The results are consistent with a model in which the transcriptional activation domain of activated Stat5 is required for its transcriptional activity and downregulation through a proteasome-dependent pathway.

Keywords: downregulation/proteasome/Stat proteins/transcription

Introduction

Cytokines regulate a variety of cellular functions through their interaction with receptors of the cytokine receptor superfamily (Darnell *et al*., 1994; Ihle, 1995; Taniguchi, 1995). This family of receptors function through their ability to associate with and, as a result of receptor aggregation, to mediate the activation of members of the *Janus* family of protein tyrosine kinases (Jaks). Once activated, the Jaks tyrosine-phosphorylate a number of proteins involved in signal transduction that are recruited to the receptor complex through their ability to recognize specific sites of tyrosine phosphorylation on the receptor chains. Among the substrates of the Jaks are members of the signal transducers and activators of transcription (Stat) family of transcription factors. Once phosphorylated on a C-terminal tyrosine, the Stats dimerize, translocate to the nucleus and are responsible for the activation of a variety of genes (Darnell *et al*., 1994; Schindler and Darnell, 1995; Ihle, 1996).

To date, seven mammalian Stat members have been identified, and each member functions in a remarkably restricted biological system. Stat1 is critical for interferon (IFN)-induced viral resistance (Durbin *et al*., 1996; Meraz *et al*., 1996). Stat4 is critical for interleukin IL-12 signaling (Kaplan *et al*., 1996a; Thierfelder *et al*., 1996), while Stat6 specifically mediates the effects of IL-4 and IL-13 on B or T cells (Kaplan *et al*., 1996b; Shimoda *et al*., 1996). However, Stat3 deficiency results in very early embryonic lethality, for unknown reasons (Takeda *et al*., 1997). The Stat5 proteins are activated in the response to a variety of cytokines including IL-3, erythropoietin (Epo), growth hormone (GH), prolactin and IL-2 (Wakao *et al*., 1994, 1995; Damen *et al*., 1995; Fujii *et al*., 1995; Gaffen *et al*., 1995; Gouilleux *et al*., 1995; Hou *et al*., 1995; Mui *et al*., 1996; Quelle *et al*., 1996). A number of genes have been identified that are under the transcriptional regulation of Stat5, including CIS (Matsumoto *et al*., 1997), oncostatin M (OSM; Yoshimura *et al*., 1996) and the IL-2 receptor α-chain (John *et al*., 1996; Lecine *et al*., 1996). Among the two highly related Stat5 proteins, Stat5a plays a critical role in prolactin signaling in lactating mammary gland, where it is highly expressed relative to Stat5b (Liu *et al*., 1997; Teglund *et al*., 1998). In contrast, Stat5b functions in GH signaling in the liver, where this isoform is highly expressed (Udy *et al*., 1997; Teglund *et al*., 1998). In addition, the Stat5a/5b nullizygous mice illustrate that Stat5a and Stat5b play a key role in prolactin regulation of ovarian function (Teglund *et al*., 1998) and IL-2-induced T cell proliferation (Moriggl *et al*., 1999).

Although much is known about the initial recruitment of Stat proteins to the cytokine receptor complex and their subsequent activation, little is known concerning the mechanisms involved in Stat translocation to the nucleus and Stat downregulation. Initial experiments suggested that a nuclear tyrosine phosphatase downregulates Statl function, based on the effects of the phosphatase inhibitor vanadate (David *et al*., 1993). Subsequent studies provided evidence that a ubiquitin-dependent proteasome pathway mediated Statl turnover (Kim and Maniatis, 1996). It was demonstrated both that Statl was ubiquitylated and that

its turnover could be blocked by proteasome inhibitors. However, another study demonstrated that the effects of the proteasome inhibitors were largely on the turnover of the receptor, and that the apparent stability of phosphorylated Statl was due to sustained signaling and not to a direct effect on Statl turnover (Haspel *et al*., 1996). These studies concluded that the turnover of phosphorylated, activated Statl was probably mediated by a phosphatase.

Here, we have found that the downregulation of phosphorylated Stat4, Stat5 and Stat6 is inhibited by the proteasome inhibitors MG132 and lactacystin, while the downregulation of phosphorylated Stat1, Stat2 and Stat3 is not sensitive to these inhibitors. These data suggest that there are different mechanisms regulating the inactivation of the Stat proteins. In addition, we have localized a region within the C-terminus of Stat5 and Stat1 that appears to be involved in the regulation of the phosphorylated form of the molecules. The presence of the Stat5a carboxyl-domain on Stat1 resulted in a stabilization of phosphorylated Stat1 by the proteasome inhibitor MG132. Likewise, the presence of the Stat1 C-terminal domain on Stat5a ablated the stability of the phosphorylated chimera in the presence of MG132. With a series of C-terminal deletion mutants, we have defined more precisely the region within the Stat5 protein that is required for turnover of the phosphorylated wild-type protein. Strikingly, the region that conferred rapid turnover was also the domain that was required for transcriptional activation. Our results suggest that the transcriptional activation domain of Stat5 also contains a proteasome-sensitive component for downregulation of the phosphorylated, activated molecule.

Results

Proteasome inhibitors stabilize the activated Stat4, Stat5 and Stat6, but not the activated Stat1, Stat2 and Stat3

The turnover of the tyrosine-phosphorylated form of Stats could be due to either a tyrosine phosphatase or proteolytic cleavage. The latter possibility is of interest since previous studies have suggested that tyrosine-phosphorylated Stat1 turnover is mediated by a proteasome-dependent pathway (Kim and Maniatis, 1996). We initially examined the effects of various inhibitors on Stat5 tyrosine phosphorylation. For these experiments, IL-3-dependent myeloid cells 32Dcl(Epo1 wt) were treated with both IL-3 and different inhibitors, the cytokine was removed and the level of tyrosine-phosphorylated Stat5a was assessed at various time points following cytokine removal. As illustrated in Figure 1, there were no detectable effects on the rate of disappearance of the tyrosine-phosphorylated form of Stat5 with the protein synthesis inhibitor, cycloheximide. The phosphatase inhibitor, sodium orthovanadate, at high concentrations partially stabilized the tyrosine-phosphorylated form. In contrast, the proteasome inhibitors MG132 and lactacystin significantly stabilized the tyrosine-phosphorylated form of Stat5. Because we followed the disappearance of the tyrosine-phosphorylated form of Stat5 after the removal of the cytokine, we know that the protein stabilization is not due to factors that contribute to the generation of the tyrosine-phosphorylated form of Stat5. Nonetheless, to assess this type of contribution, we also examined the effects of tyrosine kinase inhibitors on the

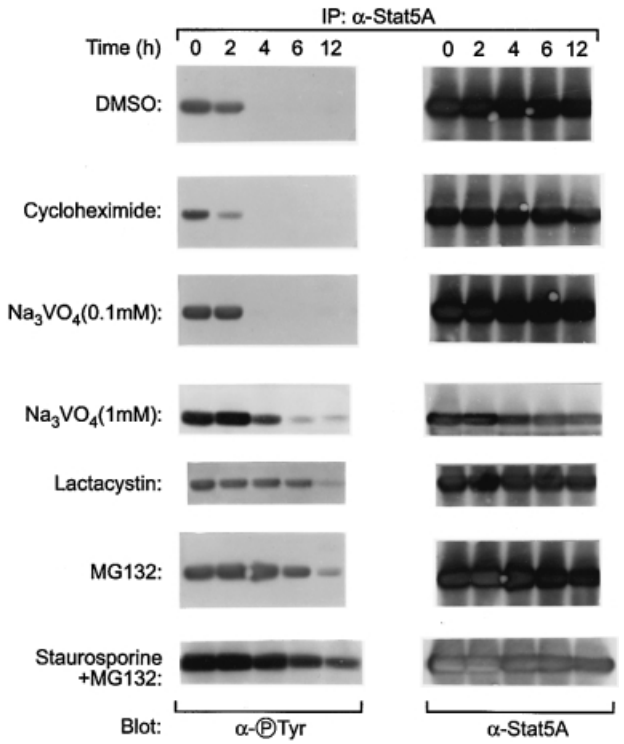

Fig. 1. Proteasome inhibitors stabilize phosphorylated Stat5. 32Dcl(Epol wt) cells cultured in IL-3 media were treated with DMSO, cycloheximide, Na_3VO_4, lactacystin, MG132, or staurosporin + MG132 for 1 h. Cells were then removed from IL-3 and lysed at the times indicated. Cell lysates were immunoprecipitated (IP) with the polyclonal antibody against Stat5a. Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose, and blotted with an antibody against phosphotyrosine (α-ⓅTyr) or to the Stat5a.

protein stabilization observed in the presence of MG132. As illustrated in Figure 1, staurosporin (or genestein; data not shown) did not eliminate the stabilization seen with MG132.

We next wished to assess whether the effects of proteasome inhibitors on Stat5 were also observed with other Stats. For these experiments, the effect of MG132 on Stat protein stability was assessed utilizing various cell lines. The stability of activated Statl and Stat2 was examined in Kit225 cells following stimulation with IFNα. Comparable results were obtained in 32Dcl cells (data not shown). Similarly, activated Stat4 stability was examined in Kit225 cells following stimulation and removal of IL-12. In contrast, the stability of activated Stat3 was examined in granulocyte colony-stimulating factor (G-CSF) receptor-expressing FDC-P1 cells following exposure of the cells and subsequent removal of G-CSF. Finally, the rate of disappearance of tyrosine-phosphorylated Stat6 was examined in CTLL cells following stimulation and removal of IL-4. As illustrated in Figure 2, two distinct phenotypes were evident. Under the conditions of these experiments, MG132 had no detectable effect on the stability of the tyrosine-phosphorylated forms of Statl, Stat2 or Stat3. In contrast, MG132 dramatically stabilized the tyrosine-phosphorylated forms of Stat4 and Stat6, similar to the stabilization that was evident with the Stat5 protein.

The N-terminus of Stat5 does not control the turnover of activated Stat5

We then sought to identify the region within the Stat5 proteins that determined the stability of the molecule in

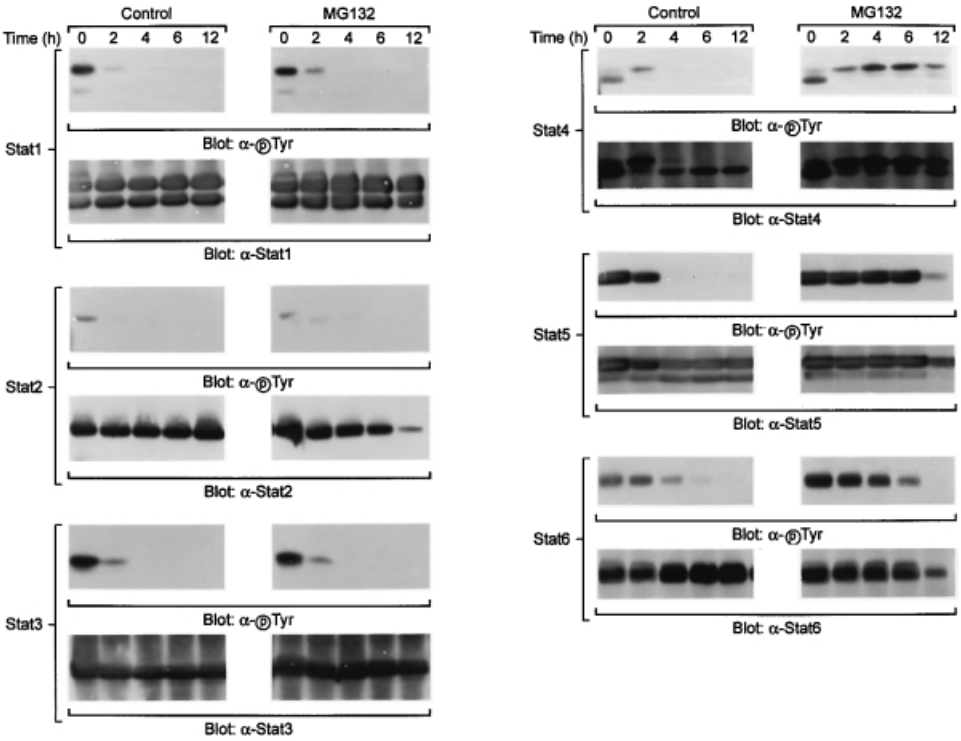

Fig. 2. Proteasome inhibitor does not stabilize phosphorylated Statl, Stat2 or Stat3, but stabilizes phosphorylated Stat4, Stat5 and Stat6. Cells were cultured in the absence of growth factor for 16 h and then stimulated with cytokines for 15 min. Cells were then removed from cytokines and lysed at the times indicated. The lysates from Kit225 cells stimulated with IFNα were immunoprecipitated (IP) with the polyclonal antibody against Statl or Stat2. The lysates from G-CSF receptor-containing FDC-P1 cells stimulated with G-CSF were immunoprecipitated with the polyclonal antibody against Stat3. The lysates from Kit225 cells stimulated with IL-12 were immunoprecipitated with the polyclonal antibody against Stat4. The lysates from 32Dcl(Epol wt) cells stimulated with IL-3 were immunoprecipitated with the polyclonal antibody against Stat5. The lysates from CTLL cells stimulated with IL-4 were immunoprecipitated with the polyclonal antibody against Stat6. Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose, and blotted with an antibody against phosphotyrosine (α-ⓅTyr) or to the corresponding Stat proteins.

the presence of MG132. In a previous study, we demonstrated that naturally occurring, carboxyl-truncated, dominant-negative forms of Stat5a or Stat5b were significantly more stable than their full-length counterparts (Wang *et al*., 1996). However, studies have indicated that the N-terminal domain of Statl controlled the stability of activated Stat1 (Shuai *et al*., 1996). Therefore, we initially compared the stability of amino- and carboxyl-truncated Stat5a mutants. Clones of IL-3-dependent cells expressing epitope-tagged wild-type Stat5a, Stat5a carboxyl-truncated at Ala-713 ($Stat5A_{C\Delta713}$), or Stat5a amino-truncated at Met-136 ($Stat5A_{N\Delta136}$) were stimulated briefly (15 min) with IL-3 and the amount of tyrosine-phosphorylated Stat5a was assessed at various times following cytokine removal. As illustrated in Figure 3A, loss of the tyrosine-phosphorylated, amino-truncated Stat5a was equivalent to that of the wild-type, full-length protein. In contrast, carboxyl-truncation of Stat5a resulted in a dramatic stabilization of the tyrosine-phosphorylated, activated form of the molecule following cytokine removal. This suggests that the C-terminus of Stat5 controls the turnover of activated Stat5.

DNA binding is not essential for the rapid turnover of the tyrosine-phosphorylated Stat5

The observation that a region of the carboxyl-domain of Stat5 is required for both transcriptional activation and for stability of the tyrosine-phosphorylated protein suggests that the turnover of Stat5 might require its participation in a transcriptional complex. For this reason, it was of interest to determine whether the ability to bind DNA was essential for targeting the turnover of activated Stat5 protein. To address this question we utilized a mutant Stat5 containing an EE to AA mutation in the DNA-binding domain (Horvath *et al*., 1995). This mutant is tyrosine-phosphorylated in response to cytokines and translocates to the nucleus, but lacks the ability to bind DNA (unpublished data). As illustrated in Figure 3B, the turnover of the tyrosine-phosphorylated DNA-binding mutant was comparable to that of the wild-type protein. Therefore, the ability to bind DNA is not essential for the rapid turnover of the tyrosine-phosphorylated form.

The C-terminus of Stat5 stabilizes activated Stat1 in the presence of MG132

We next assessed the ability of the Stat5a carboxyl region to confer to Stat1 stabilization by proteasome inhibitors (Figure 4). Chimeric molecules were constructed in which the carboxyl-domain of Stat5 was placed at the appropriate position on the Stat1 protein, and the C-terminus of the Stat1 protein substituted for the terminal 84 amino acids of the Stat5a protein. Constructs encoding for the chimeric proteins were introduced into 32Dcl(Epol wt) cells, and the Statl–Stat5a chimera was activated by stimulation of the cells with IFNγ while the Stat5a–Statl chimera was activated by stimulation of the cells with IL-3. In each case, the rate of disappearance over time of the tyrosine-phosphorylated form of the Stat chimera was examined

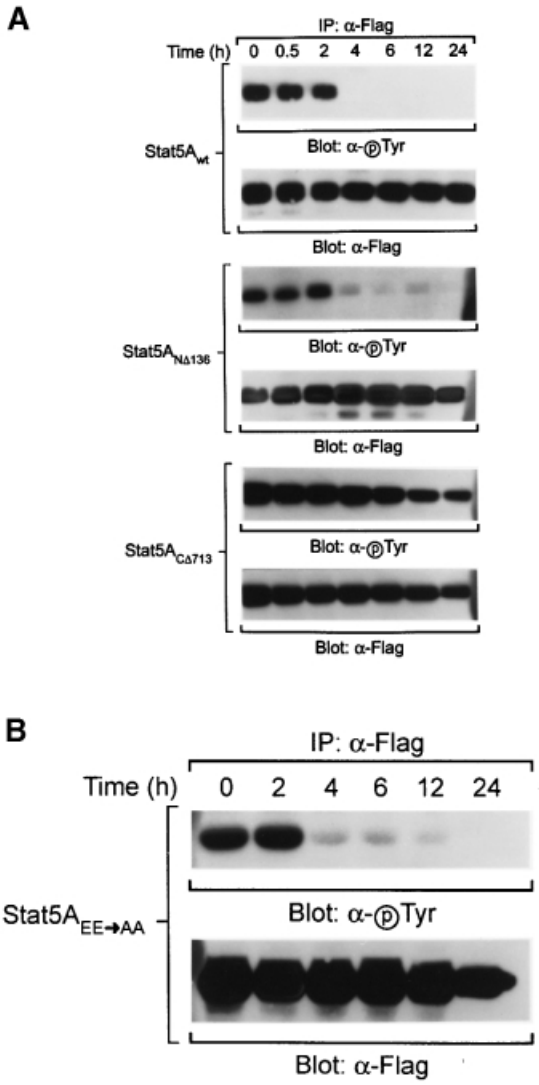

Fig. 3. (**A**) A carboxyl-truncated Stat5a stabilizes the tyrosine-phosphorylated form. 32Dcl(Epol wt) cells expressing tagged full-length, amino- or carboxyl-truncated Stat5a were removed from IL-3 and lysed at the indicated times. Cell lysates were immunoprecipitated (IP) with the monoclonal antibody against the epitope tag (α-Flag). Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with an antibody against phosphotyrosine (α-ⓟTyr) or to the epitope tag (α-Flag). (**B**) The ability to bind DNA is not essential for the turnover of the tyrosine-phosphorylated Stat5. 32Dcl(Epol wt) cells expressing tagged Stat5a DNA-binding mutant were removed from IL-3 and lysed at the times indicated. Cell lysates were immunoprecipitated (IP) with the monoclonal antibody against the epitope tag (α-Flag). Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with an antibody against phosphotyrosine (α-ⓟTyr) or against the epitope tag (α-Flag).

in the presence or absence of MG132 following removal of cytokine. On the one hand, the Stat1–Stat5 chimera had a prolonged state of activation in the presence of MG132 similar to wild-type Stat5 protein (Figure 4). However, on the other hand, the stability of activated Stat5–Stat1 chimera following growth factor withdrawal in the presence of MG132 was similar to wild-type Stat1 protein. These data imply that the carboxyl region of Stat protein determines the turnover mechanism of activated Stat proteins.

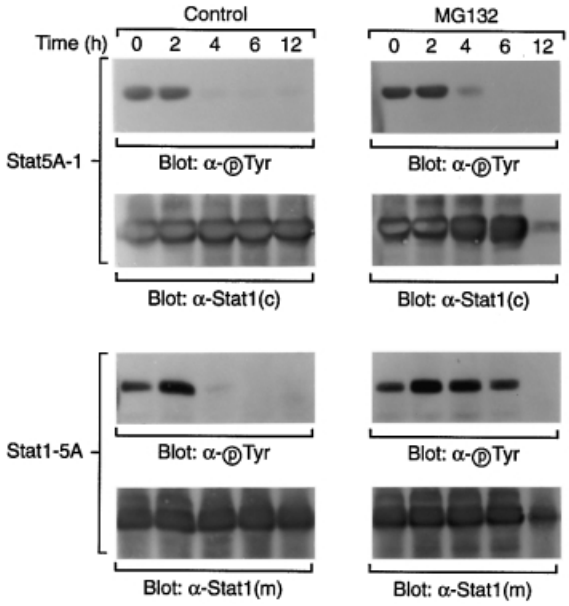

Fig. 4. The carboxyl-domain of Stat5 confers stabilization in response to proteasome inhibitor (MG132) on Statl. 32Dcl(Epol wt) cells expressing Statl–Stat5a or Stat5a–Statl chimera were starved in the absence of growth factor for 16 h and then stimulated with IFNγ or IL-3, respectively, for 15 min. Then cells were removed from cytokines and lysed at the times indicated. Lysates from 32Dcl(Epol wt) cells expressing Statl–Stat5a were immunoprecipitated (IP) with the polyclonal antibody against the C-terminal region of Stat5a. Lysates from 32Dcl(Epol wt) cells expressing Stat5a–Statl were immunoprecipitated with the polyclonal antibody against the C-terminal region of Statl. Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with an antibody against phosphotyrosine (α-ⓟTyr), to the C-terminal region of Statl, or against the middle region of Statl.

A potential amphipathic, α-helical region within the Stat5 C-terminal domain contains the signal for turnover

To define the region involved in turnover, IL-3-dependent cell lines were obtained that expressed a series of progressively carboxyl-truncated Stat5a genes (Figure 5A). The cells were induced with IL-3, the cytokine removed and the disappearance of activated, tyrosine-phosphorylated Stat5a was assessed. As illustrated in Figure 5B, Stat5a proteins with deletions to amino acid 773 or 762 displayed a stability of tyrosine phosphorylation comparable to that seen with the full-length, wild-type Stat5. However, Stat5a proteins with deletions to amino acid 751 or 740 displayed a rate of turnover comparable to that of the truncated Stat5. These results suggest that the region between amino acids 751 and 762 is important in controlling the turnover of Stat5. This region is a potential amphipathic, α-helical region, according to secondary structure predictions (Garnier *et al*., 1978; Moriggl *et al*., 1996).

The same potential amphipathic, α-helical region within the Stat5a C-terminal domain is sufficient for transcriptional activation

The carboxyl domain of Stat proteins is required for transcriptional activation and proteins that lack the carboxyl domain can dominantly suppress wild-type protein function. We therefore sought to determine whether there was a correlation between the dominant-negative ability of Stat5 C-terminal truncations and the turnover rate

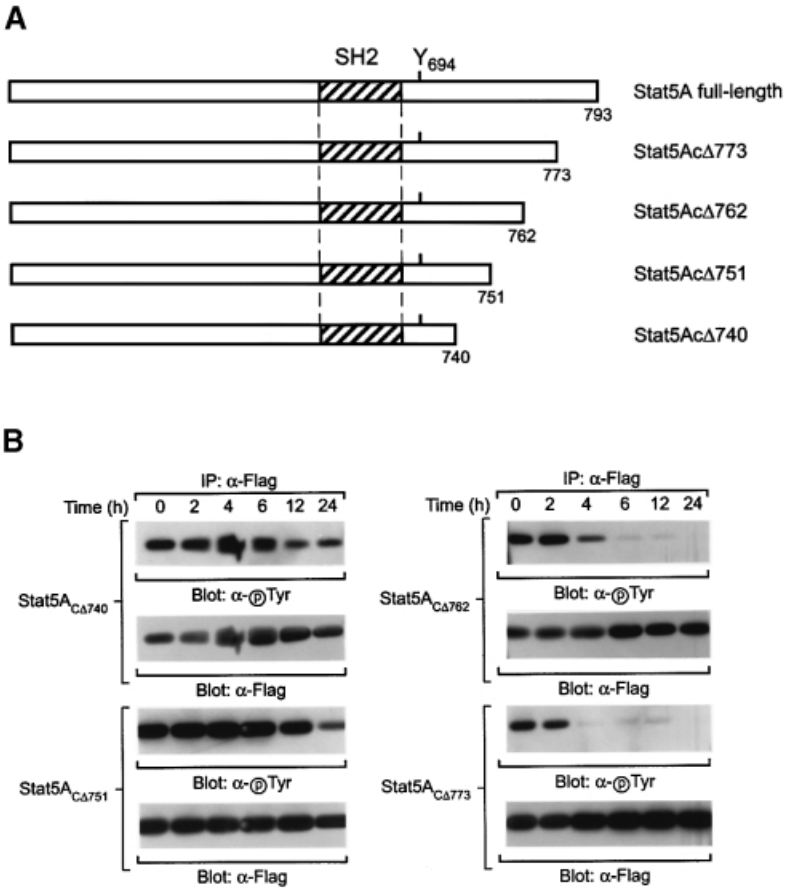

Fig. 5. The amphipathic region of amino acids 751–762 controls the stabilization of the tyrosine-phosphorylated Stat5. (**A**) Schematic representation of the full-length, carboxyl-truncated Stat5a genes at amino acids 773, 762, 751 and 740. The SH2 domain and the phosphorylated tyrosine are indicated. (**B**) 32Dcl(Epol wt) cells expressing tagged carboxyl-truncated Stat5a at amino acids 773, 762, 751 or 740 were removed from IL-3 and lysed at the times indicated. Cell lysates were immunoprecipitated (IP) with the monoclonal antibody against the epitope tag (α-Flag). Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with an antibody against phosphotyrosine (α-ⓅTyr) or against the epitope tag (α-Flag).

controlled by the C-terminal domain. To assess this, first the ability of IL-3 to induce the expression of various Stat5-responsive genes was examined in cell lines expressing different carboxyl-truncated proteins; and secondly, the ability of Stat5a proteins to rescue the proliferation defect in Stat5ab-deficient peripheral T cells was assessed. As demonstrated in Figure 6A, we observed a correlation between the transcriptional activity of Stat5 and the turnover rate. Specifically, the Stat5a molecule in which the C-terminus was truncated at amino acids 740 or 751 effectively blocked the induction of both CIS and OSM, whereas the C-terminal truncations at amino acids 762 or 773 had no effect on the induction of either gene. These results suggest that the region between amino acids 751 and 762 is also required for transcriptional activation.

As a second approach, we attempted to rescue the proliferation defect of Stat5ab-deficient peripheral T cells with different truncation forms of the Stat5 molecule. Previously, we have shown that Stat5ab-deficient T cells fail to enter the cell cycle due to an inability to upregulate cyclin D2, cyclin D3 and cdk6. Stat5ab$^{-/-}$ peripheral T cells regain the ability to proliferate upon introduction of full-length wild-type Stat5a (Figure 6B). To assess directly whether the defined region is sufficient for transcriptional activation, the Stat5a truncated mutants at 749, 764 and 771 were introduced back in Stat5ab-deficient peripheral T cells through a murine stem cell virus (MSCV)-based retrovirus capable of expressing green fluorescent protein (GFP). After 12 days in culture, cells grew out from Stat5Awt-, Stat5A$_{C\Delta764}$- or Stat5A$_{C\Delta771}$-transduced Stat5ab-deficient T cells with >95% GFP positivity, while few cells grew out from Stat5A$_{C\Delta749}$ or control GFP virus-transduced Stat5ab-deficient T cells with <5% GFP positivity (data not shown). In addition, the thymidine incorporation analysis of viable cells showed that the Stat5A$_{C\Delta764}$ and Stat5A$_{C\Delta771}$ deletion mutants could fully restore proliferative ability to Stat5ab-deficient peripheral T cells similarly to wild-type Stat5a, while the Stat5A$_{C\Delta749}$ mutant failed to rescue the defect of Stat5ab-deficient peripheral T cells (Figure 6B). These data demonstrated that the region between amino acids 749 and 764 had full transcriptional activities.

Pulse–chase experiments to assess the potential functions of proteasomes in the turnover of activated Stat5

The above studies illustrate the importance of a small carboxyl-domain of Stat5 in transcriptional activation and in controlling the turnover through a proteasome-dependent pathway. To try to define further the function of proteasome degradation in the turnover, pulse–chase experiments were carried out (Figure 7). Cells were labeled

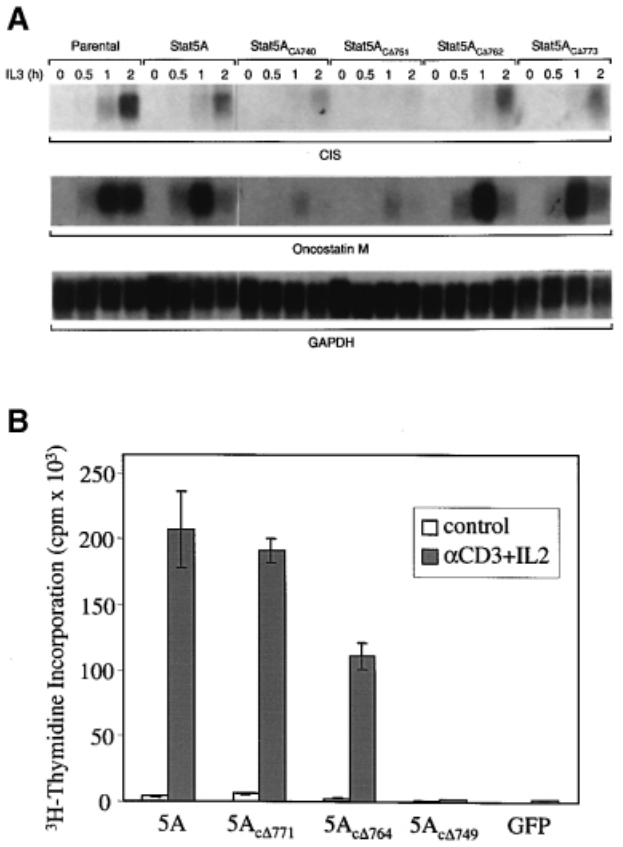

Fig. 6. The amphipathic region of amino acids 751–762 is required for transcriptional activation. (**A**) Parental 32Dcl(Epol wt) cells and 32Dcl(Epol wt) cells expressing tagged full-length, or one of the carboxyl-truncated Stat5a proteins were cultured in the absence of growth factor for 16 h and then left untreated (0) or stimulated with IL-3 for 0.5, 1 or 2 h. Total RNA was extracted from cells and subjected to Northern analysis with the indicated probes as described in Materials and methods. (**B**) The amphipathic region is required for rescue of Stat5-deficient peripheral T cell function. Stat5ab-deficient splenic lymphocytes were incubated with an MSCV-based retrovirus capable of expressing GFP and wild-type Stat5a or one of the carboxyl-truncated Stat5a proteins for 2 days in the presence of anti-CD3 and IL-2. A virus expressing GFP only was used as a negative control. After being expanded for 7 days in IL-2, viable cells were examined for proliferation by [^{3}H]thymidine incorporation in the absence or presence of anti-CD3 and IL-2. The viral constructs utilized are indicated at the bottom. A representative of three independent experiments is shown.

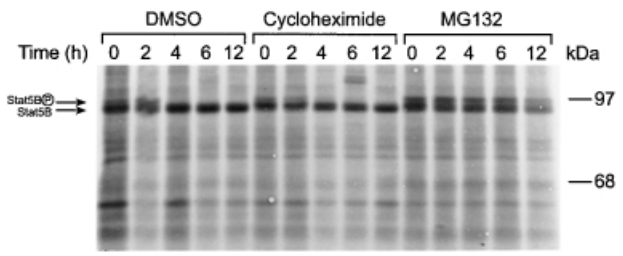

Fig. 7. Analysis of tyrosine-phosphorylated Stat5b turnover with [^{35}S]methionine labeling. 32Dcl(Epol wt) cells were pulse-labeled with methionine for 3 h and then treated with DMSO, cycloheximide or MG132 for an additional 1 h. Cells were removed from IL-3 and lysed at the times indicated. Cell lysates were immunoprecipitated (IP) with the polyclonal antibody against Stat5b. Precipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose and exposed to film.

with [^{35}S]methionine, stimulated with cytokine and then removed from cytokine in media containing a vast excess of unlabeled methionine. For these studies, analysis of Stat5b was particularly important, since following phosphorylation the protein migrates slightly more slowly on gels than the non-phosphorylated protein (Figure 7). As shown, cytokine induced a shifted Stat5b band that rapidly disappeared in the absence of cytokines or in the presence of cycloheximide. However, the phosphorylated form is stable when cells are cultured in the presence of MG132. Several conclusions can be drawn from these results. First, the half-life of the tyrosine-phosphorylated Stat5b is dramatically shorter than that of the non-phosphorylated Stat5b. Secondly, from the pool of Stat5b only a small fraction (~10%) is activated in response to cytokine. Thirdly, since treatment with MG132 would cause the accumulation of ubiquitylated forms, the absence of a shift of the phosphorylated form to higher molecular weight forms would suggest that Stat5b is not being ubiquitylated. The critical question, however, is whether the tyrosine-phosphorylated form is returning to the non-phosphorylated pool of protein or is being degraded in a proteasome-dependent but ubiquitylation-independent manner. Unfortunately, because of the small fraction of Stat5b that becomes tyrosine-phosphorylated, this question cannot be answered definitively. In the absence of MG132, the appearance of lower molecular weight forms is not apparent, although they would be expected to be difficult to detect. Similarly, because of the small fraction, it is unlikely that a detectable 'shift' to the non-phosphorylated pool would be detectable. Possible models that take this information into consideration are discussed below.

Discussion

It might have been predicted that the turnover of the tyrosine-phosphorylated Stats would be regulated in a similar manner. The results indicate clearly that this is not the case. For example, the carboxyl-truncation of Statl does not have the same dramatic effect on the stability of the tyrosine-phosphorylated form that is seen with Stat5. Moreover, the proteasome inhibitor MG132 had little effect on the turnover of the tyrosine-phosphorylated forms of Stat1, Stat2 or Stat3, in contrast to the effects it has on the turnover of the tyrosine-phosphorylated forms of Stat4, Stat5 and Stat6. The data from the chimeras indicate that the difference is due to the absence of carboxyl sequences in Statl, since the Stat5 phenotype of stability in the presence of MG132 can be conferred on Statl by simply replacing the C-terminus with that of Stat5. Irrespective of this, what the physiological significance of these differences is has yet to be determined.

The key unanswered question is the mechanism by which the tyrosine-phosphorylated form is turned over; specifically, is a phosphatase or protease involved? The information relating to this question comes from the effects of inhibitors on the turnover. Specifically, high levels of sodium orthovanadate had a small but detectable effect in stabilizing the tyrosine-phosphorylated form. However, this effect might be due to the fact that sodium orthovanadate itself could activate Jak/Stat pathways (Ruff *et al*., 1997). In contrast to the phosphatase inhibitors, the proteasome inhibitors MG132 and lactacystin both had a

dramatic effect on stabilizing the tyrosine-phosphorylated form. It should be noted that all our studies are done under conditions in which cytokine has been removed and decay of the induced state is followed. Thus it would be unlikely that inhibition of phosphatases associated with the receptor, such as SHP-1, will be relevant.

Based on extensive amounts of literature dealing with protein targeting to proteasomes, we anticipated that ubiquitylation would be involved. However, we have been unable to provide any definitive support for ubiquitylation. Specifically there are no shifts in protein sizes in inhibitor-treated cells that would be consistent with ubiquitylation (Figure 7). Nor have we been able to detect the incorporation of ubiquitin into Stat5a when an HA-tagged ubiquitin gene was co-expressed with Jak2 in COS cells (data not shown).

We also used methionine labeling experiments to assess the mechanisms in the turnover. As illustrated, Stat5b was particularly useful since the phosphorylated form can be distinguished from the non-phosphorylated form by electrophoresis. Methionine-labeled, non-phosphorylated Stat5 was stable over the 12 h examined following the methionine pulse both in the presence and absence of MG132. The slower migrating, phosphorylated Stat5b rapidly disappeared in the control cells (dimethylsulfoxide, DMSO) or in cells treated with cycloheximide, while the slower migrating protein was stabilized in cells treated with MG132. However, since only a small fraction of the Stat5b shifts in its migration, it is difficult to conclude that the tyrosine-phosphorylated form is being recycled to the non-phosphorylated form. In addition, there was an apparent absence of lower molecular weight fragments as evidence of degradation, although again the relatively small fraction involved and the possibility that degradation does not generate specific fragments or occurs rapidly precludes any definitive conclusions.

Assuming that the activated Stat5 is dephosphorylated and recycles to the non-phosphorylated pool, an indirect role for proteasomes must be considered. In this regard, a model could be envisaged in which a phosphatase exists in the transcriptional activation complex that is responsible for the dephosphorylation of Stat5. Its presence in the complex would be predicted to be dependent upon functional proteasome activity. For example, the phosphatase may associate with another protein and thereby be inhibited in its function or localization. However, this binding protein is susceptible to proteasome-mediated degradation. This model would be identical to that of the regulation of NF-κB by binding to IκB.

The results demonstrate that a relatively small region of the carboxyl-domain of Stat5 determines whether the tyrosine-phosphorylated form is rapidly turned over; or, conversely, its absence is associated with stabilization of the tyrosine-phosphorylated form. The region is a potential amphipathic α-helical region and fulfills the criteria for a transcriptional activation domain. Analysis of the carboxyl-domains of other Stat proteins, including Stat4 and Stat6, have failed to identify a region with sequence or predicted structural similarity. Previous studies identified a 38 amino acid region of the carboxyl-domain of Stat6 that, when deleted, also resulted in prolonged tyrosine phosphorylation (Moriggl *et al*., 1997). However, this region does not contain a predicted amphipathic α-helical region. Therefore, it does not appear that a shared domain, by sequence or predicted structure, can be associated with a comparable functional property.

Functionally, the region is also a transcriptional activation domain as defined by the correlation of the loss of the region with the ability to dominantly suppress gene induction by the wild-type protein. In addition, reconstitution studies in Stat5-deficient T cells have localized the transcriptional activation domain to this region. Whether the two functions require the same interactions is an intriguing but unanswered question. For example, it is possible that this region recruits a transcriptional complex that contains the components necessary to initiate turnover of the tyrosine-phosphorylated proteins. Alternatively, it is possible that this region independently recruits two protein complexes, one that mediates transcriptional activation and another that mediates turnover. As the proteins that associate with the carboxyl-domain of Stat5 are purified, it will be possible to address this model further.

Materials and methods

Cell culture, cytokines, antibodies and inhibitors

All mammalian cell lines used were grown in RPMI 1640 containing 10% fetal bovine serum and supplemented with appropriate factors. Parental 32Dcl(Epol wt) cells, 32Dcl(Epol wt) cells expressing various Stat5 proteins and FDC-P 1 (GCSF-wt) cells were maintained in media supplemented with murine IL-3 (25 U/ml). Murine CTLL and human Kit225 cells were maintained in media supplemented with human IL-2 (25 ng/ml). Splenic T cells were isolated from 8- to 12-week-old wild-type or Stat5ab-deficient mice and cultured as described previously (Moriggl *et al.*, 1999).

Cycloheximide (Sigma) was dissolved in phosphate-buffered saline (PBS) and used at a final concentration of 20 μg/ml. Staurosporin (Sigma) was dissolved in DMSO and used at a final concentration of 500 nM. MG132 (Peptide Institute, Inc.) was dissolved in DMSO and used at a final concentration of 40 μM. Lactacystin (E.J.Corey, Harvard University) was dissolved in H_2O and used at a final concentration of 10 μM.

Anti-Statl (E23 and C24) and anti-Stat2 (C20) antibodies were purchased from Santa Cruz Biotechnology. Antisera against Stat3, Stat4, Stat5A, Stat5B and Stat6 have been described previously (Witthuhn *et al*., 1993, 1994; Quelle *et al*., 1995; Wang *et al*., 1996). The monoclonal antibody against FLAG epitope was purchased from Eastman Kodak Company.

Constructs, transfectants and retroviral transduction

Construction of FLAG epitope-tagged full-length Stat5a and C-terminally truncated Stat5a at amino acid 713 ($Stat5a_{c\Delta713}$) have been described previously (Wang *et al*., 1996). C-terminally truncated Stat5a proteins, truncated at amino acid 740 ($Stat5a_{c\Delta740}$), 751 ($Stat5a_{c\Delta751}$), 762 ($Stat5a_{c\Delta762}$) and 773 ($Stat5a_{c\Delta773}$), with C-terminal FLAG epitope tags, were constructed by mutagenesis PCR. Chimeric Statl–Stat5a was constructed by replacing amino acids 706–739 of Statl with amino acids 709–793 of Stat5a. Chimeric Stat5a–Stat1 was constructed by replacing amino acids 704–793 of Stat5a with amino acids 692–739 of Statl. The DNA-binding mutant ($Stat5a_{EE\text{-}AA}$) of Stat5a was generated by mutating amino acids EE (437 and 438) to AA. FLAG epitope was added to the C-terminus of $Stat5a_{EE\text{-}AA}$. The constructs in Prk5 vector were co-electroporated into 32Dcl(Epol wt) cells with the selectable marker pGK/hygro. Transfected cells were selected in bulk in media containing hygromycin (1 mg/ml). Individual clones were then assayed for their expression of mutant proteins by Western blotting. C-terminally truncated Stat5a mutants at amino acid 749 ($Stat5a_{c\Delta749}$), 764 ($Stat5a_{c\Delta764}$) and 771 ($Stat5a_{c\Delta771}$) were subcloned into a bicistronic retroviral vector containing GFP. Splenic T cells were co-cultured on irradiated ecotropic producer cells as described previously (Moriggl *et al.*, 1999).

Immunoprecipitation, Western and Northern blotting

Preparation of cell lysate, immunoprecipitation, SDS–PAGE, and Western and Northern blotting were as described previously (Wang *et al*., 1996).

Pulse–chase experiments

Log-phase 32Dcl(Epol wt) cells were washed twice with PBS and cultured in methionine-free Dulbecco's modified Eagle's medium at 2×10^7/ml

containing 10% dialyzed fetal bovine serum with murine IL-3 (25 U/ml) and 100 μCi/ml of [^{35}S]methionine for 3 h. Label was removed and cells were resuspended at 1×10^6/ml in fresh medium without IL-3. At the times indicated, whole-cell lysate was generated and immunoprecipitation and SDS–PAGE was carried out. Then the proteins were transferred to nitrocellulose membrane and the membrane was exposed to film.

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References

Damen,J.E., Wakao,H., Miyajima,A., Krosl,J., Humphries,R.K., Cutler,R.L. and Krystal,G. (1995) Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. *EMBO J.*, **14**, 5557–5568.

Darnell,J.E.,Jr, Kerr,I.M. and Stark,G.R. (1994) Jak–STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, **264**, 1415–1421.

David,M., Grimley,P.M., Finbloom,D.S. and Larner,A.C. (1993) A nuclear tyrosine phosphatase downregulates interferon-induced gene expression. *Mol. Cell. Biol.*, **13**, 7515–7521.

Durbin,J.E., Hackenmiller,R., Simon,M.C. and Levy,D.E. (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*, **84**, 443–450.

Fujii,H. *et al.* (1995) Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor β chain but is not essential for the proliferative signal transmission. *Proc. Natl Acad. Sci. USA*, **92**, 5482–5486.

Gaffen,S.L., Lai,S.Y., Xu,W., Gouilleux,F., Groner,B., Goldsmith,M.A. and Greene,W.C. (1995) Signaling through the interleukin 2 receptor β chain activates a STAT-5-like DNA-binding activity. *Proc. Natl Acad. Sci. USA*, **92**, 7192–7196.

Garnier,J., Osguthorpe,D.J. and Robson,B. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.*, **120**, 97–120.

Gouilleux,F., Pallard,C., Dusanter-Fourt,I., Wakao,H., Haldosen,L.A., Norstedt,G., Levy,D. and Groner,B. (1995) Prolactin, growth hormone, erythropoietin and granulocyte–macrophage colony stimulating factor induce MGF–Stat5 DNA binding activity. *EMBO J.*, **14**, 2005–2013.

Haspel,R.L., Salditt-Georgieff,M. and Darnell,J.E.,Jr (1996) The rapid inactivation of nuclear tyrosine phosphorylated stat1 depends upon a protein tyrosine phosphatase. *EMBO J.*, **15**, 6262–6268.

Horvath,C.M., Wen,Z. and Darnell,J.E.,Jr (1995) A STAT protein domain which determines DNA sequence recognition suggests a novel DNA binding domain. *Genes Dev.*, **9**, 984–994.

Hou,J., Schindler,U., Henzel,W.J., Wong,S.C. and McKnight,S.L. (1995) Identification and purification of human Stat proteins activated in response to interleukin-2. *Immunity*, **2**, 321–329.

Ihle,J.N. (1995) Cytokine receptor signalling. *Nature*, **377**, 591–594.

Ihle,J.N. (1996) STATs: signal tranducers and activators of transcription. *Cell*, **84**, 331–334.

John,S., Robbins,C.M. and Leonard,W.J. (1996) An IL-2 response element in the human IL-2 receptor α chain promoter is a composite element that binds stat5, elf-1, HMG-1(Y) and a GATA family protein. *EMBO J.*, **15**, 5627–5635.

Kaplan,M.H., Sun,Y.-L., Hoey,T. and Grusby,M.J. (1996a) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*, **382**, 174–177.

Kaplan,M.H., Schindler,U., Smiley,S.T. and Grusby,M.J. (1996b) Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. *Immunity*, **4**, 313–319.

Kim,T.K. and Maniatis,T. (1996) Regulation of interferon-γ-activated stat1 by the ubiquitin–proteasome pathway. *Science*, **273**, 1717–1719.

Lecine,P., Algarte,M., Rameil,P., Beadling,C., Bucher,P., Nabholz,M. and Imbert,J. (1996) Elf-1 and stat5 bind to a critical element in the new enhancer of the human interleukin-2 receptor α gene. *Mol. Biol. Cell*, **16**, 6829–6840.

Liu,X., Robinson,G.W., Wagner,K.U., Garrett,L., Wynshaw-Boris,A. and Hennighausen,L. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.*, **11**, 179–186.

Matsumoto,A., Masuhara,M., Mitsui,K., Yokouchi,M., Ohtsubo,M., Misawa,H., Miyajima,A. and Yoshimura,A. (1997) CIS, a cytokine inducible SH2 protein, is a target of the jak–stat5 pathway and modulates stat5 activation. *Blood*, **89**, 3148–3154.

Meraz,M.A. *et al.* (1996) Targeted disruption of the *Stat1* gene in mice reveals unexpected physiologic specificity in the JAK–STAT signaling pathway. *Cell*, **84**, 431–442.

Moriggl,R. *et al.* (1996) Deletion of the carboxyl-terminal transactivation domain of MGF–Stat5 results in sustained DNA binding and a dominant negative phenotype. *Mol. Cell. Biol.*, **16**, 5691–5700.

Moriggl,R. *et al.* (1997) Comparison of the transcriptional activation domains of Stat5 and Stat6 in lymphoid cells and mammary epithelial cells. *Mol. Cell. Biol.*, **17**, 3663–3678.

Moriggl,R. *et al.* (1999) Stat5 is required for IL-2 induced cell cycle progression of peripheral T cells. *Immunity*, **10**, 249–259.

Mui,A.L., Wakao,H., Harada,N., O'Farrell,A.M. and Miyajima,A. (1996) Interleukin-3, granulocyte–macrophage colony-stimulating factor and interleukin-5 transduce signals through two forms of STAT5. *J. Leukoc. Biol.*, **57**, 799–803.

Quelle,F.W., Thierfelder,W., Witthuhn,B.A., Tang,B., Cohen,S. and Ihle,J.N. (1995) Phosphorylation and activation of the DNA binding activity of purified Stat1 by the *Janus* protein tyrosine kinases and epidermal growth factor receptor. *J. Biol. Chem.*, **270**, 20775–20780.

Quelle,F.W., Wang,D., Nosaka,T., Thierfelder,W.E., Stravopodis,D., Weinstein,Y. and Ihle,J.N. (1996) Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. *Mol. Cell. Biol.*, **16**, 1622–1631.

Ruff,S.J., Chen,K. and Cohen,S. (1997) Peroxovanadate induces tyrosine phosphorylation of multiple signaling proteins in mouse liver and kidney. *J. Biol. Chem.*, **272**, 1263–1267.

Schindler,C. and Darnell,J.E.,Jr (1995) Transcriptional responses to polypeptide ligands: the JAK–STAT pathway. *Annu. Rev. Biochem.*, **64**, 621–651.

Shimoda,K. *et al.* (1996) Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted *Stat6* gene. *Nature*, **380**, 630–633.

Shuai,K., Liao,J. and Song,M.M. (1996) Enhancement of antiproliferative activity of γ interferon by the specific inhibition of tyrosine phosphorylation of Stat1. *Mol. Cell. Biol.*, **16**, 4932–4941.

Takeda,K., Noguchi,K., Shi,W., Tanaka,T., Matsumoto,M., Yoshida,N., Kishimoto,T. and Akira,S. (1997) Targeted disruption of the mouse *Stat3* gene leads to early embryonic lethality. *Proc. Natl Acad. Sci. USA*, **94**, 3801–3804.

Taniguchi,T. (1995) Cytokine signaling through nonreceptor protein tyrosine kinases. *Science*, **268**, 251–255.

Teglund,S. *et al.* (1998) Stat5a and Stat5b proteins have essential and non-essential, or redundant, roles in cytokine responses. *Cell*, **93**, 841–850.

Thierfelder,W.E. *et al.* (1996) Stat4 is required for IL-12 mediated responses of NK and T-cells. *Nature*, **382**, 171–174.

Udy,G.B., Snell,R.G., Wilkins,R.J., Park,S.-H., Ram,P.A., Waxman,D.J. and Davey,H.W. (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc. Natl Acad. Sci. USA*, **94**, 7239–7244.

Wakao,H., Gouilleux,F. and Groner,B. (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. *EMBO J.*, **13**, 2182–2191.

Wakao,H., Harada,N., Kitamura,T., Mui,A.L.F. and Miyajima,A. (1995) Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. *EMBO J.*, **14**, 2527–2535.

Wang,D., Stravopodis,D., Teglund,S., Kitazawa,J. and Ihle,J.N. (1996) Naturally occurring dominant negative variants of Stat5. *Mol. Cell. Biol.*, **16**, 6141–6148.

Witthuhn,B., Quelle,F.W., Silvennoinen,O., Yi,T., Tang,B., Miura,O. and Ihle,J.N. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following EPO stimulation. *Cell*, **74**, 227–236.

Witthuhn,B.A., Silvennoinen,O., Miura,O., Lai,K.S., Cwik,C., Liu,E.T. and Ihle,J.N. (1994) Involvement of the JAK3 Janus kinase in IL-2 and IL-4 signalling in lymphoid and myeloid cells. *Nature*, **370**, 153–157.

Yoshimura,A., Ichihara,M., Kinjyo,I., Moriyama,M., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., Hara,T. and Miyajima,A. (1996) Mouse oncostatin M: an immediate early gene induced by multiple cytokines through the JAK–STAT5 pathway. *EMBO J.*, **15**, 1055–1063.

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