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Synergistic Roles for Pim-1 and c-Myc in STAT3-Mediated Cell Cycle Progression and Antiapoptosis

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Summary

The activation of STAT3 by the cytokine receptor gp130 is required for both the G1 to S cell cycle transition and antiapoptosis. We found that *Pim-1* and *Pim-2* are targets for the gp130-mediated STAT3 signal. Expression of a kinase-defective Pim-1 mutant attenuated gp130-mediated cell proliferation. Constitutive expression of *Pim-1* together with *c-myc*, another STAT3 target, fully compensated for loss of the STAT3-mediated cell cycle progression, antiapoptosis, and *bcl-2* expression. We also identified valosine-containing protein (*VCP*) as a target gene for the Pim-1-mediated signal. Expression of a mutant VCP led cells to undergo apoptosis. These results indicate that Pim-family proteins play crucial roles in gp130-mediated cell proliferation and explain the synergy between Pim and c-Myc proteins in cell proliferation and lymphomagenesis.

Introduction

gp130 is a subunit that is common to the receptors for the interleukin-6 (IL-6) family of cytokines: IL-6, leukemia inhibitory factor, ciliary neurotrophic factor, IL-11, oncostatin M, and cardiotrophin-1, which are involved in cell proliferation, differentiation, and cell death in various tissues (Hirano et al., 1997). Upon stimulation by these cytokines, gp130 forms a homodimer and activates the gp130-associated Janus tyrosine kinases (JAK1, JAK2, and TYK2) through transphosphorylation, which results in the phosphorylation of tyrosine residues on gp130 and JAKs (reviewed in Hirano et al., 1997). Tyrosine-phosphorylated gp130 recruits signal-transducing molecules that contain Src homology 2 (SH2) domains to itself, where they are phosphorylated on tyrosine. Signal transducer and activator of transcription 3 (STAT3) is an SH2 domain-containing transcription factor. Tyrosine phosphorylation of STAT3 depends on phosphorylated tyrosines in the YXXQ motif (where X is an arbitrary amino acid), which includes Tyr-767, Tyr-814, Tyr-905, and Tyr-915 of human gp130 (Stahl et al., 1995; Fukada et al., 1996; Yamanaka et al., 1996). Tyrosine-phosphorylated STAT3 forms a dimer, enters the nucleus, and regulates a set of genes. SHP-2, the SH2 domain-containing protein tyrosine phosphatase, is also recruited to gp130, where its tyrosine phosphorylation is dependent on binding to phosphorylated Tyr-759 of gp130 (Stahl et al., 1995; Fukada et al., 1996). By using chimeric receptors containing the full-length and mutated cytoplasmic domain of gp130 and by expressing dominant-negative forms of STAT3, we have demonstrated roles for gp130-mediated signals in biological functions in a number of cell lines. STAT3 is required for IL-6-mediated growth arrest and macrophage differentiation in the mouse leukemia M1 cells (Nakajima et al., 1996; Yamanaka et al., 1996). Tyr-759–mediated signals are required for the S to G2/M cell cycle transition in the gp130-mediated cell proliferation of the mouse proB cell line BAF/B03, and the STAT3 signal is required for both antiapoptosis and for the G1 to S cell cycle transition (Fukada et al., 1996, 1998). Although *c-myc* has been identified as one of the target genes of STAT3 (Kiuchi et al., 1999), it remains largely unknown what molecules act downstream of Tyr-759–mediated signals and the STAT3 regulation of cell proliferation.

STAT proteins have been shown to play important roles in cell proliferation induced by cytokines. STAT5 was shown to be involved in IL-3-mediated cell proliferation in an IL-3-dependent cell line (Mui et al., 1996). Targeted disruption of the *stat5a* and *stat5b* genes in mice revealed that STAT5 is needed for IL-2-mediated T cell proliferation and erythropoietin-mediated antiapoptosis in red cell progenitors (Moriggl et al., 1999; Socolovsky et al., 1999). Similarly, targeted disruption of the *stat4* and *stat6* genes showed that STAT4 and STAT6 are required for IL-12- and IL-4-dependent lymphocyte proliferation, respectively (Kaplan et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996; Akira, 1999). Constitutive activation of STAT is observed in cells transformed with human T cell leukemia virus 1 (HTLV-1), *v-src*, *abl*, *bcr-abl*, and *v-eyk*, and some multiple myeloma cells (Besser et al., 1999; Carlesso et al., 1996; Danial et al., 1995; Hilbert et al., 1996; Migone et al., 1995; Turkson et al., 1998; Yu et al., 1995). STAT3 activation is required for transformation with *v-src* and for preventing apoptosis in multiple myeloma cells (Bromberg et al., 1998; Catlett-Falcone et al., 1999). Inactivation of the *stat3* gene in T cells revealed that STAT3 is required for IL-6-mediated antiapoptosis, independent of *bcl-2* induction (Takeda et al., 1998). A constitutively active mutant of *stat3* was recently shown to act as an oncogene and can act alone to induce tumorigenesis (Bromberg et al., 1999).

Pim-1 is a protooncogene that was first identified as a common insertion site in Molony murine leukemia virus (MuLV)–induced T cell lymphomas (Cuypers et al., 1984). The *Pim-1* gene encodes two cytoplasmic serine/threonine protein kinases, which are generated by an alternative translational initiation (Saris et al., 1991). Eμ-*Pim-1*

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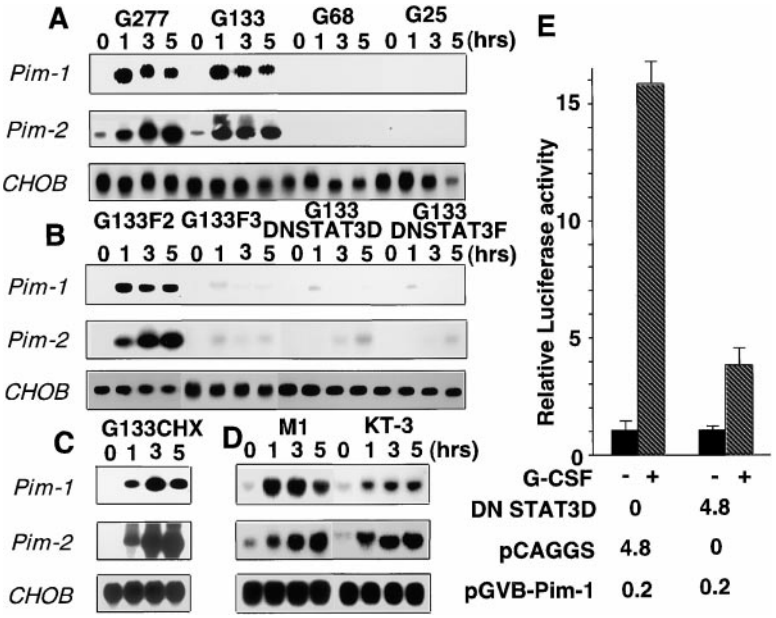

Figure 1. *Pim-1* and *Pim-2* Genes Are Direct Targets for STAT3

(A) Expression of *Pim-1* and *Pim-2* upon stimulation of the truncated chimeric receptors. G277, G133, G68, and G25 cells were stimulated with 100 ng/ml of G-CSF for the indicated periods. Twenty micrograms of total RNA was analyzed by Northern blotting with the *Pim-1*, *Pim-2*, and *CHOB* (loading control) probes.

(B) Expression of *Pim-1* and *Pim-2* depends on the gp130-mediated STAT3 signal. G133F2 cells (in which Tyr-759 was replaced by phenylalanine), G133F3 cells (Tyr-767 replaced by phenylalanine), G133 expressing the dominant-negative STAT3 D (DN STAT3D), and G133 expressing the dominant-negative STAT3F (DN STAT3F), were stimulated with G-CSF for the indicated periods.

(C) Induction of *Pim-1* and *Pim-2* genes does not require de novo protein synthesis. Before stimulation, G133 cells were treated with 10 μg/ml of cycloheximide (CHX) for 1 hr and stimulated with G-CSF for the indicated periods.

(D) *Pim-1* and *Pim-2* induction in M1 and KT-3 cells. M1 cells and KT-3 cells (starved of IL-6 for 12 hr) were stimulated with 100 ng/ml of human IL-6 for the indicated periods.

(E) Luciferase assay for the *Pim-1* promoter. G133 cells (2×10^6) were transfected with the indicated amounts (μg) of pCAGGS-Neo-HASTAT3D (DN STAT3D), pCAGGS-Neo (the control vector, pCAGGS), and pGVB-Pim-1, which contains the 1.8 kb Pim-1 promoter region. The cells were stimulated with G-CSF for 10 hr or left unstimulated. The relative luciferase activities (versus activity in the unstimulated cells) are shown in a bar graph. The error bars indicate the standard deviation (SD) from triplicate experiments.

transgenic mice are highly susceptible to developing T cell lymphoma after MuLV infection and exposure to a chemical carcinogen, and *c-myc* or *N-myc* genes are activated in the resulting lymphomas (Breuer et al., 1989; van Lohuizen et al., 1989). Eμ-*myc* transgenic mice can also develop B cell lymphomas when *Pim-1* has been activated by a secondary proviral insertion (van Lohuizen et al., 1991). These reports suggest a synergistic role for *Pim-1* and *myc* genes in lymphomagenesis. Physiologically, the *Pim-1* gene is induced in response to a number of cytokines such as GM-CSF, G-CSF, IL-3, interferon-α, and IL-6 (Lilly et al., 1992; Sato et al., 1993; Jaster et al., 1999; Matikainen et al., 1999). A homolog of Pim-1, Pim-2, has also been implicated in lymphomagenesis, and its expression is also regulated by a set of cytokines that is similar to those that induce *Pim-1* (van der Lugt et al., 1995; Allen et al., 1997). It remains unclear how the *Pim* gene is regulated by other cytokines, how Pim-1 regulates cell proliferation, and how Pim-1 cooperates with Myc proteins to promote cell proliferation and lymphomagenesis.

Here, we demonstrate that *Pim-1* is a target of the gp130-mediated STAT3 signal and cooperates with *c-myc* to promote cell proliferation through the G1 to S cell cycle transition and prevent apoptosis. Furthermore, we demonstrate that *VCP*, the mammalian homolog of yeast Cdc48p, is a target for Pim-1-mediated signals and is involved in antiapoptosis.

Results

STAT3 Is Required for gp130-Mediated *Pim-1* and *Pim-2* Expression

We previously showed that the membrane-proximal 133-amino-acid region of the cytoplasmic domain of gp130 is sufficient to promote cell proliferation in the mouse IL-3-dependent BAF/B03 proB cells (Fukada et al., 1996). This region of gp130 contains box1 and box2 and Tyr-759 and Tyr-767, which are required for the JAK activation, SHP2/ERK activation, and STAT3 activation, respectively. A BAF/B03 transfected cell line expressing a chimeric receptor composed of the extracellular domain of the human G-CSF receptor and the transmembrane and 133 amino acids of the cytoplasmic region of human gp130 (G133) was used to isolate genes that are activated downstream of gp130 signaling. cDNA libraries were constructed from unstimulated cells and cells in which gp130 signaling was activated through the chimeric receptor by treatment with G-CSF for 2 hr and subjected to a PCR-based subtraction method. In this screen, we identified the *Pim-1* and *Pim-2* protooncogenes, which were induced by gp130 stimulation. To determine which signals from gp130 were necessary for their induction, we performed Northern blot analysis of different cell lines that expressed mutant forms of gp130 (Figure 1). These mutant receptors, which have been described previously, were as follows: G277 contains the entire cytoplasmic domain; G68 contains a minimal region (box1 and box2) for JAK activation but cannot activate the SHP2/ERK and STAT3 pathways; G25 contains box1 alone, which is not sufficient to activate the JAKs; and in G133F2 and G133F3, Tyr-759 and Tyr-767 are replaced by phenylalanines and are unable to activate the SHP2/ERK and STAT3 pathways, respectively (Fukada et al., 1996). Induction of both *Pim-1* and *Pim-2* was detected in cells expressing G277, G133, and G133F2, but not G68, G25, or G133F3, indicating that their expression depended on STAT3 activation (Figures 1A and 1B). The STAT3-dependent expression was further confirmed in cells expressing dominant-negative forms of STAT3 (Figure 1B). IL-6-dependent induction of *Pim-1* and *Pim-2* was also observed in the Lennert's T lymphoma KT-3 cells and M1 cells, suggesting

that their induction is not specific to BAF/B03 cells (Figure 1D). We further performed a reporter assay, in which the luciferase gene was under control of the 1.8 kb promoter region of *Pim-1*. Upon the stimulation of gp130 in the G133 transfectants, a 16-fold induction of the luciferase activity was observed (Figure 1E). Expression of the dominant-negative STAT3 (STAT3D) decreased the induction to about 4-fold, confirming that the *Pim-1* induction was dependent on STAT3. This induction was also observed in cyclohexamide-treated cells, revealing that it did not require protein synthesis (Figure 1C). These results indicate that *Pim-1* and *Pim-2* are likely to be direct targets of the gp130-mediated STAT3 signal.

Pim-1 Is Involved in gp130-Mediated Cell Proliferation

To examine the roles played by Pim proteins in gp130-mediated cell proliferation, we established a G133 cell line that stably expresses a mutated form of Pim-1, K67M Pim-1, in which Lys-67 in the ATP binding domain was replaced by methionine, rendering its kinase domain nonfunctional. We also established a G133 cell line that expresses the K67M Pim-1 in a doxycycline-inducible manner. The G133 cells stably expressing the K67M Pim-1 grew in response to IL-3 but not in response to gp130 stimulation (data not shown). Concordantly, the inducible G133 K67M Pim-1 cells grew as well in response to IL-3 whether or not they were expressing K67M Pim-1, but the gp130-mediated cell proliferation that occurred in the absence of K67M Pim-1 expression was perturbed when K67M Pim-1 expression was induced (Figure 2A). Neither constitutive nor induced expression of wild-type Pim-1 in the G133 cells changed the level of cell proliferation by either IL-3 or gp130 stimulation (data not shown; Figure 2A). These data indicate that Pim-1 is required for gp130-mediated cell proliferation.

Pim-1 Is Not Sufficient to Compensate for Loss of the gp130-Mediated STAT3 Signal

To examine the possibility that Pim-1 could compensate for loss of the gp130-mediated STAT3 signal, we established a G133F3 cell line that was defective in the STAT3 signal but constitutively expressed wild-type Pim-1 (G133F3-Pim-1). Proliferation of the G133F3-Pim-1 cells was not observed in response to gp130 stimulation within a few days, and about 80% of the cells underwent apoptosis. However, about 20% of the cells survived and grew (Figure 2B). We repeated this experiment using the surviving cells, and after factor starvation, similar proportions of the cells died and survived in the presence of gp130 stimulation, indicating that the surviving cells were not transformed into factor-independent cells. These data indicate that Pim-1 partially rescued the defect in the STAT3 signal but that Pim-1 alone was not sufficient to compensate for the loss of the gp130-mediated STAT3 signal.

Pim-1 Cooperates with c-Myc to Promote Cell Cycle Progression and Prevent Apoptosis

Pim-1 seemed likely to cooperate with other targets that act downstream of the STAT3 signal. *c-myc* was a strong candidate for such a role, since it is regulated by the

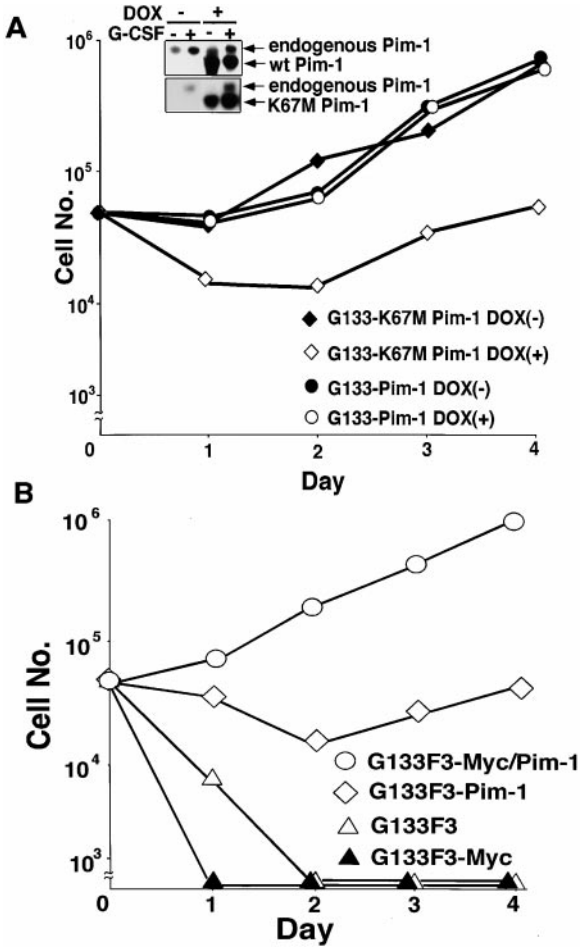

Figure 2. Pim-1 and c-Myc Are Required for gp130-Mediated Cell Proliferation

(A) Cell proliferation of G133 cells expressing K67M Pim-1. G133-wtPim-1 DOX and G133-K67Mpim-1 DOX cells expressed the wild-type Pim-1 and K67M Pim-1, respectively, in a doxycycline-inducible manner (Experimental Procedures). The cells were cultured in the presence of 1 μg/ml of doxycycline for 10 hr before the stimulation and during the stimulation (+) or in the absence of doxycycline (−). Cells (5×10^4 cells) were stimulated with G-CSF for the indicated periods, and the numbers of variable cells were counted. Expression of endogenous *Pim-1* and transfected wild-type (wt) and K67M mutant *Pim-1* are shown.

(B) Cell proliferation of G133F3, G133F3-Pim-1, G133F3-Myc, and G133F3-Myc/Pim-1. Cells (5×10^4) were cultured in the presence of G-CSF for the indicated periods. The numbers of viable cells were counted. One representative clone out of at least five independent cell lines for each transfection condition is shown.

gp130-mediated STAT3 signal (Kiuchi et al., 1999) and cooperates with *Pim-1* to promote lymphomagenesis (Breuer et al., 1989; van Lohuizen et al., 1989). To address this issue, we established G133F3 cell lines that expressed c-Myc alone (G133F3-Myc) or both c-Myc and Pim-1 (G133F3-Myc/Pim-1). The G133F3-Myc cells did not grow in response to gp130 stimulation and underwent apoptosis in response to the withdrawal of IL-3 faster than did the G133F3 cells (Figures 2B, 3A, and 3B), consistent with there being a role for c-Myc in apoptosis, as reported (Askew et al., 1991; Evan et al., 1992). In contrast, the G133F3-Myc/Pim-1 cells grew as well as the G133 cells in a manner dependent on gp130

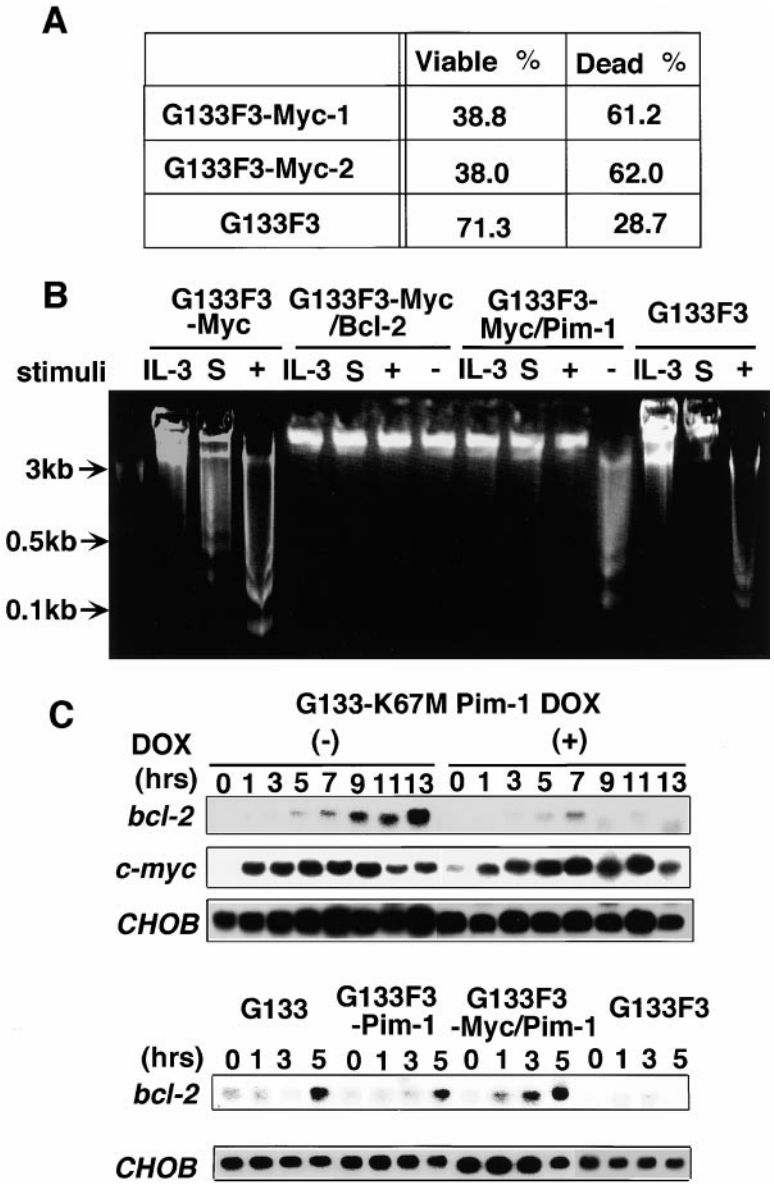

Figure 3. Pim-1 Cooperates with c-Myc in gp130-Mediated Antiapoptosis

(A) Overexpression of c-Myc enhances apoptosis induced by cytokine starvation. 5×10^4 G133F3-Myc (two each of clones 1 and 2) and G133F3 cells were starved of IL-3 and G-CSF for 12 hr. The numbers of viable and dead cells were counted, and the percentages are shown.

(B) DNA fragmentation. 1×10^6 G133F3, G133F3-Myc, G133F3-Myc/Pim-1, and G133-Myc/Pim-1 cells were starved of IL-3 for 12 hr (S) and cultured in the presence of G-CSF (+) or IL-3 (IL-3) or in the absence of the factors (−) for additional 10 hr. The cells were lysed, and the chromosomal DNA was fractionated on 2% agarose gels.

(C) Expression of *bcl-2*. G133-K67Mpim-1 DOX cells were stimulated with G-CSF in the presence (+) or absence (−) of the doxycycline treatment (upper panel). G133, G133F3-Pim-1, G133F3-Myc/Pim-1, and G133F3 cells were stimulated with G-CSF after starving them of IL-3 (lower panel). Expression of *bcl-2*, *c-myc*, and *CHOB* was analyzed by Northern blotting.

stimulation (Figure 2B). These data indicate that coexpression of *Pim-1* with *c-myc* prevents apoptosis and/or promotes cell cycle progression. We previously observed that expression of the antiapoptotic gene *bcl-2* is regulated by the gp130-mediated STAT3 signal (Fukada et al., 1996). The induction of *bcl-2* in response to gp130 stimulation was diminished in the G133 cells expressing K67M Pim-1 (Figure 3C). Although the induction of *bcl-2* was not observed in the G133F3 cells, it was detected in the G133F3-Pim-1 cells, and its enhanced induction was observed in the G133F3-Myc/Pim-1 cells (Figure 3C). These results suggest that Pim-1 is primarily involved in the *bcl-2* induction and c-Myc cooperates with Pim-1 to induce *bcl-2* expression. Consistent with this, IL-3 deprivation–induced apoptosis in G133F3-Myc/Pim-1 was prevented by gp130 stimulation (Figure 3B). The *bcl-2* induction was observed only in the stimulated G133F3-Myc/Pim-1 cells, suggesting that other signals from gp130 are also involved in *bcl-2* expression.

To examine whether Pim-1 is required solely to prevent apoptosis or whether it is also needed for cell cycle progression, we established a line of G133F3-Myc cells expressing *bcl-2* (G133F3-Myc/Bcl-2) and compared them with the G133F3-Myc/Pim-1 cells. It was reported previously that the constitutive expression of *c-myc* and *bcl-2* enables BAF-B03 cells to grow independent of factor availability (Miyazaki et al., 1995). However, in our hands, five cell lines derived from separate clones of G133F3-Myc/Bcl-2 cells, which expressed *c-myc* and *bcl-2* at levels comparable to those seen in stimulated G133 cells, survived but did not grow in response to gp130 stimulation (Figures 3B and 4). Our data indicate that neither the expression of *c-myc* alone nor of *c-myc* and *bcl-2* led to the complete activation of cell cycle progression. In contrast, coexpression of *Pim-1* with *c-myc* was sufficient to promote the cell cycle in a manner dependent on gp130 stimulation without STAT3 activation, indicating that Pim-1 is also required for cell

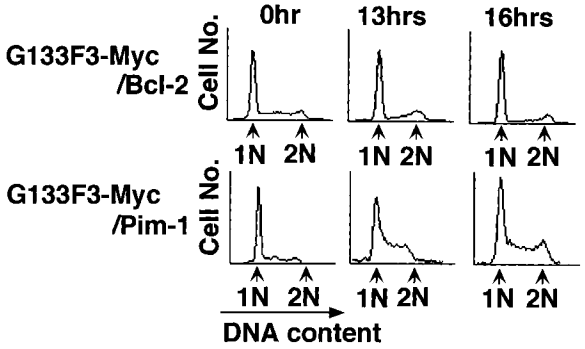

Figure 4. Pim-1 Cooperates with c-Myc in gp130-Mediated Cell Cycle Progression

G133F3-Myc/Bcl-2 and G133F3-Myc/Pim-1 cells were stimulated with G-CSF, fixed at the indicated time, and stained with propidium iodide. Their status in the cell cycle was analyzed by FACS. 1N and 2N indicate the G0/G1 phase and G2/M phase of the cell cycle, respectively.

cycle progression (Figure 4B). These results indicate that both Pim-1 and c-Myc are required and sufficient for effecting the STAT3-mediated antiapoptosis function and cell cycle progression.

VCP (Cdc48p) Is a Target for the Pim-1 Signal

To understand the mechanism(s) by which Pim-1 regulates antiapoptosis and cell cycle progression, we performed a cDNA subtraction using the G133F3-Pim-1 and G133F3 cells stimulated with G-CSF to isolate genes expressed only in the G133F3-Pim-1 cells but not in the G133F3 cells. We identified *VCP*, the mammalian homolog of yeast Cdc48p, in this screen. Expression of *VCP* was detected after 3 hr of stimulation (Figure 5A) in G133 cells, which was slightly later than the expression of Pim-1. Its expression was observed in G133F2 cells, but the level of expression was quite reduced in G133F3 cells. Greatly reduced levels of expression were also seen in G133 cells expressing the dominant-negative STAT3s (DNSTAT3F and DNSTAT3D) and in G133 cells expressing K67M Pim-1 (Figure 5A). Expression of *VCP* was also observed in IL-6-stimulated KT-3 and HepG2 cells, IL-3-stimulated BAF/B03 cells, and IL-2-stimulated KT-3 cells, suggesting that the induction of *VCP* is not specific to BAF/B03 cells or to gp130 signaling (Figure 5A). We further performed a reporter gene assay, in which the luciferase gene was driven by the 400 bp promoter region in the 5′ flanking region of the mouse *VCP* gene. 5-fold induction of luciferase activity was observed upon gp130 stimulation in the G133 cells, and 2.5- to 3-fold induction was observed upon transfection with the Pim-1 expression vector (Figures 5B and 5C). These data indicate that *VCP* is a target gene for the Pim-1-dependent signal. Expression of *VCP* in the G133F3-Pim-1 cells was further enhanced by gp130 stimulation, suggesting that *VCP* may also be regulated by other signals (Figure 5A).

VCP Is Involved in gp130-Mediated Cell Proliferation

To reveal the role(s) of VCP in gp130-mediated cell proliferation, we established a G133F3 cell line expressing VCP (G133F3-VCP) or both VCP and c-Myc (G133F3-Myc/VCP) (Figure 6A). Most of the G133F3-VCP cells underwent apoptosis, even upon the stimulation of gp130 with G-CSF, but a small population (<10%) of cells survived and grew in a manner dependent on gp130 stimulation. This situation was similar to that in the G133F3-Pim1 cells, but a smaller proportion of the G133F3-VCP cells survived. Coexpression of c-Myc with VCP increased the proportion of the cells that initially survived, but it enhanced apoptosis after the third day (Figure 6A). These results suggest that VCP is able to compensate for part of the Pim-1 function.

To examine whether VCP is required for gp130-mediated cell proliferation, we constructed a VCP mutant and established a G133 cell line expressing the wild-type or the mutant VCP molecules in a doxycycline-inducible manner. VCP is an ATPase protein that belongs to the superfamily of AAA (ATPases associated with a variety of cellular activities) (Frohlich et al., 1995). VCP contains two ATPase domains, which consist of a Walker A cassette for ATP binding and a Walker B cassette for ATP hydrolysis (Egerton et al., 1992). The K251/524A-VCP mutant was constructed by replacing Lys-251 and Lys-524 in the Walker A cassettes by alanines (Figure 6B). Expression of the K251/524A-VCP mutant in G133 cells strongly inhibited gp130-dependent cell proliferation, indicating that the K251/524A-VCP mutant acts as a dominant-negative molecule for VCP function (Figure 6C). The cells expressing the K251/524A-VCP mutant underwent apoptosis, as assessed by observing DNA fragmentation (Figure 6D). Furthermore, *bcl-2* induction was suppressed in the K251/524A-VCP-expressing cells (Figure 6E). These data indicate that VCP, a target for Pim-1, is required for both the prevention of apoptosis and the expression of *bcl-2* in gp130-mediated cell proliferation.

Discussion

Pim-1, *Pim-2*, and *c-myc* Are Targets for the gp130-Mediated STAT3 Signal

We demonstrated that *Pim-1* and *Pim-2* are direct targets for the gp130-mediated STAT3 signal (Figure 1). *Pim-1*, *Pim-2*, and *c-myc* are immediate early genes that are all induced by the same cytokines. In the IL-3 signaling pathway, the membrane-proximal region of the common β-chain for the IL-3/IL-5/GM-CSF receptors, which contains box1 and box2, is responsible for the signals that induce the expression of both *Pim-1* and *c-myc* (Sato et al., 1993; Mui et al., 1996). However, expression of *Pim-1* depends on STAT5 but expression of *c-myc* does not (Mui et al., 1996). In contrast, the gp130-dependent expression of the *Pim* genes and *c-myc* mainly depended on the STAT3 signal (Figure 1), indicating that the *Pim* genes and *c-myc* are differently regulated in the signaling of the IL-3 receptor and gp130. This is consistent with the observation that dominant-negative STAT3s inhibit the gp130-mediated but not IL-3-dependent cell proliferation (Fukada et al., 1996), which probably involves the functions of the Pims and c-Myc. The 1.8 kb 5′-flanking region of the *Pim-1* gene was sufficient to induce *Pim-1* in response to gp130 stimulation (Figure 1E). This region does not contain

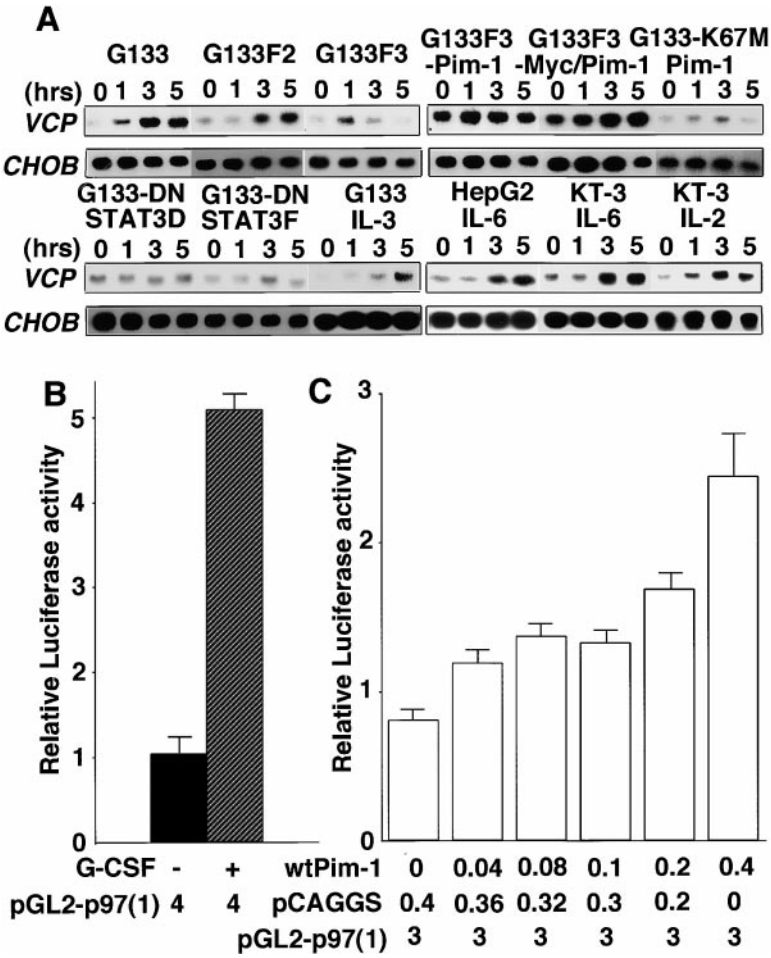

Figure 5. *VCP* Is a Direct Target for the Pim-1-Mediated Signal

(A) Expression of *VCP*. The transfected cell lines were stimulated with 100 ng/ml G-CSF. G133 cells were also stimulated with 50 ng/ml mouse IL-3. HepG2 cells were stimulated with 100 ng/ml human IL-6. KT-3 cells were stimulated with 50 ng/ml human IL-2 or 100 ng/ml human IL-6. Expression of *VCP* and *CHOB* are shown.

(B) Expression of *VCP* in response to the gp130 stimulation. G133 cells were transfected with 4 μg pGL2-p97(1), the *VCP* promoter-containing reporter gene, and stimulated with G-CSF for 10 hr (+) or left unstimulated (−). The relative luciferase activity (versus activity in the unstimulated cells) is shown. The error bars indicate SD.

(C) Pim-1-dependent activation of the *VCP* promoter. G133 cells were transfected with the indicated amounts of pCAGGS-Neo-Pim-1 (wtPim-1), pCAGGS-Neo (pCAGGS), and pGL2-p97(1). The cells were harvested 10 hr after transfection, and their luciferase activities were determined.

the consensus sequence for STAT binding. However, cooperative binding of STAT to DNA modifies the specificity of STAT binding (Xu et al., 1996). Consistent with this, the *Pim-1* promoter contains a DNA sequence that potentially binds STAT oligomers (data not shown). Alternatively, STAT3 may cooperate with other transcription factors to bind the promoter region to regulate *Pim-1* expression.

Synergistic Roles of Pim-1 and c-Myc in Cell Proliferation

A line of evidence shows that activation of the *Pim-1* and *c-myc* genes cooperatively promotes oncogenesis in T and B lymphocytes (Breuer et al., 1989; van Lohuizen et al., 1989), implying that Pim-1 and c-Myc also play physiological roles in the cell proliferation induced by cytokines. In this report, we found that enforced expression of either *Pim-1* or *c-myc* alone was not sufficient to complement the gp130-mediated STAT3 signal for cell proliferation (Figure 2B). However, expression of both genes completely rescued the defect in the STAT3 signal, revealing that *Pim-1* and *c-myc* are major targets for the STAT3 signal that transduce the gp130-mediated cell proliferation. Furthermore, expression of a Pim-1 mutant that lacked a functional kinase domain partially attenuated the gp130-mediated cell proliferation (Figure 2A), implicating Pim-1 in that function as well. The incomplete inhibition may be caused by insufficient expression of the mutant Pim-1, or Pim-2 may rescue its effect. Mice deficient for the *Pim-1* gene display a subtle phenotype, with a decreased response to IL-3 and IL-7 in bone marrow–derived mast cells and early B cell progenitors, respectively (Domen et al., 1993a, 1993b, 1993c). *Pim-2* and/or the recently identified *Pim-3* (Konietzko et al., 1999) may largely compensate for the *Pim-1* deficiency in these mice.

The constitutive expression of *Pim-1* and *c-myc* did not lead to factor-independent proliferation in BAF/B03 cells, indicating that other signals from gp130 than the STAT3 signal are likely to be involved in the cell proliferation. These results contradict a previous report showing that overexpression of *Pim-1* induced IL-3-independent cell proliferation in BAF-B03 cells (Nosaka et al., 1999). We established more than five independent lines of G133F3-Pim-1 and G133F3-Myc/Pim-1, which expressed *Pim-1* and *c-myc* at levels similar to those in stimulated G133 cells, and none of the lines displayed factor-independent cell proliferation (Figure 2B). Our data imply that *Pim-1* and *c-myc* induced by the gp130-mediated STAT3 signal are not sufficient to elicit factor-independent cell proliferation. Studies of lymphomagenesis and leukemiagenesis suggest that, in cooperation with *Pim-1* and *c-myc*, the activation of other genes

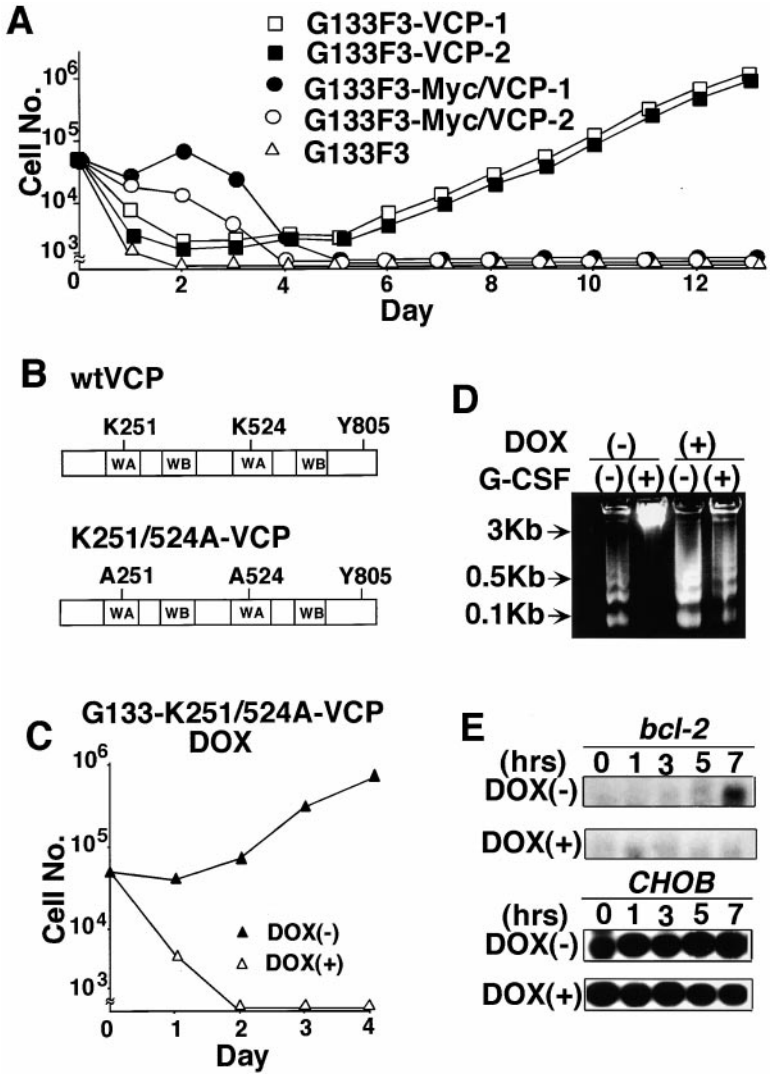

Figure 6. VCP Is Involved in gp130-Mediated Cell Proliferation and Antiapoptosis

(A) Cell proliferation of G133F3, G133F3-VCP, and G133F3-Myc/VCP. The transfectant cells were cultured in the presence of G-CSF, and the numbers of viable cells were counted. Data from two representative clones for G133F3-VCP and G133F3-VCP/Myc are shown.

(B) Wild-type VCP (wtVCP) and K251/254A-VCP mutant. Lys-251 and Lys-524 in the Walker A cassettes of the ATP binding domains were replaced by alanines. WA; Walker A cassette, WB; Walker B cassette.

(C) Cell proliferation in G133 cells expressing the K251/254A-VCP mutant. G133 cells that expressed K251/524A-VCP in a doxycycline-inducible manner (G133-K251/524-VCP cells) were cultured in the presence of G-CSF with (+) or without (−) doxycycline. The numbers of the viable cells were counted on the day indicated.

(D) Apoptosis mediated by K251/524A-VCP. 2×10^5 G133-K251/524A-VCP DOX cells were stimulated with G-CSF for 20 hr (+) or left unstimulated (−) in the presence (+) or absence (−) of doxycycline. The DNA fragmentation assay was performed.

(E) *Bcl-2* expression in G133-K251/524-VCP cells. Cells were stimulated with 100 ng/ml G-CSF for the indicated periods in the presence(+) or absence(−) of doxycycline. Expression of *bcl-2* and *CHOB* was analyzed by Northern blotting analysis.

such as *bmi-1* and *gfi-1* promotes oncogenesis (van Lohuizen et al., 1991; Schmidt et al., 1998). These genes may act downstream of gp130 and support STAT3-mediated cell proliferation.

Pim-1 was also induced upon the gp130 stimulation in M1 cells (Figure 1D), which stop cell proliferation and differentiate to macrophages. The roles of Pim-1 in M1 cells may be different from those in BAF-B03 cells, as the STAT3-mediated signals exert the opposite actions between these cell lines, and gp130 stimulation down-regulates *c-myc* expression in M1 cells (Nakajima et al., 1996; Yamanaka et al., 1996). It would be important to reveal the differences in function of Pim proteins in different cell types.

Roles for Pim-1 and c-Myc in Cell Cycle Progression

We previously demonstrated that STAT3 is required for the gp130-mediated G1 to S cell cycle transition (Fukada et al., 1998). c-Myc is known to be a major regulator for the G1 to S transition and is induced by the gp130-mediated STAT3 signal (Kiuchi et al., 1999), suggesting that the expression of c-Myc alone might be sufficient to promote the G1 to S transition and that Pim-1's role might be restricted to preventing c-Myc-mediated apoptosis. However, this was not the case. Coexpression of *bcl-2* prevented c-Myc-mediated apoptosis but did not rescue the G1 to S cell cycle transition (Figures 3B and 4). In contrast, coexpression of *c-Myc* and Pim-1 promoted the G1 to S cell cycle transition, indicating that Pim-1 is not only involved in preventing apoptosis but is also involved in the G1 to S transition (Figure 4). *cdc25A* is a direct target gene of c-Myc. Its product regulates the activity of cyclin-dependent kinases (CDKs) through the dephosphorylation of threonine and tyrosine residues (Galaktionov et al., 1996). It was recently reported that Pim-1 phosphorylates Cdc25A and activates its phosphatase activity (Mochizuki et al., 1999). Thus, Pim-1 may control the G1 to S transition by activating Cdc25A.

Roles for Pim-1 and c-Myc in Inhibiting Apoptosis

Forced expression of *Pim-1* was shown to enhance factor-independent survival and inhibit apoptosis in certain cell lines (Lilly and Kraft, 1997). The induction of *Pim-1* correlates with *bcl-2* induction in a number of cell lines

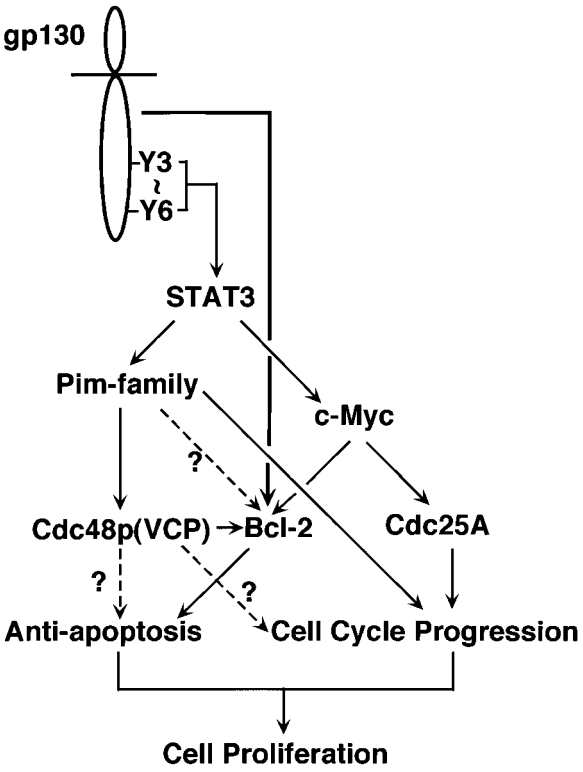

Figure 7. Model for Synergistic Roles Played by Pim-1 and c-Myc Downstream of the gp130-Mediated STAT3 Signal

For details, see the Discussion.

and by various stimuli (Sakai and Kraft, 1997; Krumenacker et al., 1998). Overexpression of *Bcl-2* prevents *c-myc*-induced apoptosis (Bissonnette et al., 1992; Wagner et al., 1993). These data imply a connection between *Pim-1* and *bcl-2*. In this report, we demonstrated that Pim-1 was required for *bcl-2* induction, and forced expression of Pim-1 partly compensated for loss of the STAT3-mediated *bcl-2* expression (Figure 3C). Furthermore, coexpression of Pim-1 and c-Myc enhanced *bcl-2* induction and prevented apoptosis in cells with a defective STAT3 signal (Figure 3C). These data suggest that Pim-1 plays a primary role in *bcl-2* induction, and c-Myc cooperates with Pim-1 for *bcl-2* induction and antiapoptosis. Furthermore, the expression of a mutant VCP inhibited *bcl-2* expression (Figure 6E) and resulted in apoptosis (Figure 6D), indicating that VCP mediates the Pim-1-mediated antiapoptotic signals.

Role of VCP in Cell Proliferation

We identified *VCP* as a target gene for Pim-1. Forced expression of VCP, or VCP and c-Myc, partially rescued cells with a defect in STAT3 activation (Figure 6A). Induced expression of a mutant form of VCP inhibited gp130-mediated antiapoptotic signals (Figures 6D and 6E). These data indicate that VCP participates in the gp130-mediated antiapoptosis function and likely in cell cycle progression as well. However, constitutive expression of VCP did not compensate fully for loss of the Pim-1 signal, suggesting that genes other than *VCP* are required for transmitting the Pim-1 signal. Intriguingly, *VCP* expression was induced not only by gp130 stimulation but also by IL-2 and IL-3, suggesting that regulation of *VCP* expression is inductive rather than constitutive in certain cells. This is consistent with a previous report showing that the promoter region of *VCP* contains DNA elements responsive to a variety of stimuli (Muller et al., 1999).

VCP and Cdc48p exhibit a variety of biological functions. In budding yeast, Cdc48p plays a role in the segregation of cells during the mitosis phase (Frohlich et al., 1991). Yeast cells with a specific mutant allele of Cdc48p display an apoptotic phenotype (Madeo et al., 1997). Similarly, it was reported that MAC-1, a *Caenorhabditis elegans* homolog of VCP/Cdc48p, interacts with CED3, CED4, and CED9 and inhibits apoptosis in the nematode (Wu et al., 1999). VCP may prevent apoptosis through modifying the functions of caspases, Apaf-1, and Bcl-2, which are the mammalian homologs of CED3, CED4, and CED9, instead of through regulating *bcl-2* expression.

In summary (Figure 7), Pim-1 and c-Myc, downstream of the gp130-mediated STAT3 signal, cooperatively regulate cell cycle progression and the prevention of apoptosis. VCP is a key regulator that links the Pim-1 signal to various biological functions.

Experimental Procedures

Isolation of *Pim-1*, *Pim-2*, and *VCP* Genes

The cDNA subtraction was carried out by a PCR-based method using the PCR-Select cDNA subtraction Kit (Clontech) according to the manufacturer's protocol. To isolate genes induced upon gp130 stimulation, poly A^+ RNA was prepared from 1×10^8 G133 cells stimulated with 100 ng/ml G-CSF for 2 hr and from unstimulated G133 cells. The positive clones were sequenced by an ALF Red automated DNA sequencer (Amersham Pharmacia Biotech), and two positive clones were found to be the mouse *Pim-1* and *Pim-2* genes.

Similarly, to isolate genes downstream of the Pim-1 signal, poly A^+ RNA was isolated from 1×10^8 G133F3-Pim-1 and G133F3 cells stimulated with 100 ng/ml G-CSF for 3 hr. Only one positive clone was also found to be positive, and it was identified as the mouse *VCP* gene.

Plasmid Construction

Expression vectors for Pim-1 were constructed from pSP72-Pim-1 (a gift from Dr. A. Berns) (Selten et al., 1986). To construct the K67M Pim-1cDNA, Lys-67 in the ATP binding domain was replaced by a methionine using PCR-mediated mutagenesis. Myc-tagged Pim-1 and K67M Pim-1 cDNA were inserted to pcDNA3 (Invitrogen), pCAGGS-Neo, and pUHD10-3 (Kistner et al., 1996).

To construct a reporter gene for the promoter of *Pim-1*, the 1.8 kb SacI–SacII fragment (from −1705 to 155, 1 defined as the transcriptional initiation site) was excised from the mouse *Pim-1* genomic DNA (a gift from Dr. A. Berns) (Selten et al., 1986) and was inserted into pGVB (Toyo inki, pGVB-Pim-1).

To construct an expression vector for murine VCP, the VCP cDNA fragment, which was fused to a Myc epitope at the carboxyl terminus, was excised from pSXSRα-VCP-myc (a gift from Dr. L. E. Samelson; Egerton et al., 1992) and subcloned into pTrace-EF (Invitrogen, pTrace-EF-VCP). The VCP cDNA fragment was inserted into pUHD10-3 (pUHD10-3 VCP). To construct the K251/524A-VCP mutant, Lys-251 and Lys-524 in the Walker A cassettes in the ATP binding domain were replaced by alanines using PCR-mediated mutagenesis (pUHD10-3 K251/524A-VCP). Details of the construction are available upon request.

pcDNA3-c-Myc and pUC-CAGGS-bcl2 were previously described and were gifts from Drs. Y. Kuchino (Kagaya et al., 1997) and Y. Tujimoto (Shimizu et al., 1996), respectively. pCAGGS-Neo,

pCAGGS-Neo-HASTAT3D, and pGL2-p97(1) were also previously described (Nakajima et al., 1996; Muller et al., 1999).

Cell Culture, Transfection, and Recombinant Cytokines

BAF-B03, KT-3, M1, and HepG2 cells were maintained as described previously (Fukada et al., 1996; Nakajima et al., 1996; Yamanaka et al., 1996; Takahashi-Tezuka et al., 1998; Nishida et al., 1999). The BAF-B03 transfectant cells expressing the chimeric receptors and the cells expressing both G133 and DN-STAT3D or DN-STAT3F were previously described (Fukada et al., 1996). The cells expressing G133, G133F2, and G133F3 were established by an electroporation-mediated transfection with pEF-BOS-G133, G133F2, or G133F3, and pMIK-Hyg.

G133F3-Pim-1 and G133F3-Myc transfectants were established by transfecting G133F3 cells with pcDNA3-Pim-1 and pcDNA3-c-Myc, respectively. G133F3-Myc/Pim1 and G133F3-Myc/Bcl2 transfectants were established by transfecting G133F3-Myc cells with pcDNA3-Pim1 and the Zeocin resistance gene and pUC-CAGGS-bcl2 and the Zeocin resistance gene, respectively. The G133F3-VCP cell line was established by transfecting G133F3 cells with pTrace-EF-VCP. The G133F3-Myc/VCP line was established by transfecting G133F3-VCP cells with pcDNA3-c-Myc and the Zeocin-resistance gene. All the transfected cells were checked by Northern blotting analysis.

To construct a doxycycline-inducible system (Tet/On-inducible system), G133 cells were transfected first with pUHD172-1Neo, which encodes the reverse-Tet-transactivator gene (rtTA), and selected with hygromycin and G418 (rtTA-G133). RtTA-G133 cells were further transfected with pUHD10-3 Pim-1, pUHD10-3 K67M Pim-1, or pUHD10-3 K251/524A-VCP, and the Zeocin resistance gene. To induce the proteins, the transfectant cells were stimulated with 1 μg/ml doxycycline for 10 hr prior to G-CSF stimulation.

To stimulate BAF-B03 transfectants, the cells were starved of IL-3 for 12 hr, and stimulated with 100 ng/ml of human G-CSF in RPMI 1640, 10% fetal calf serum, antibiotics, and selection reagents (hygromycin, G418, and Zeocin) for the time periods indicated in the figure legends.

Recombinant human G-CSF, human IL-6, mouse IL-3, and human IL-2 were obtained from Chugai Pharmaceutics, Ajimomoto Research Institute, Peprotech, and Otsuka Pharmaceutical.

Luciferase Assay

BAF-B03 cells (2×10^6) were starved of IL-3 for 5 hr and transfected with the luciferase reporter plasmid [pGVB-Pim-1 and pGL2-p97(1)], pEF-LacZ (1 μg), and the expression plasmids, as indicated in the figure legends, by a DEAE-dextran method. The cells were washed and suspended in Tris-buffered saline supplemented with Ca^{2+} and Mg^{2+} (TBS++: 25 mM Tris, 137 mM NaCl, 0.5 mM Na_2HPO_4, 0.49 mM $MgCl_2$, and 0.68 mM $CaCl_2$). The DNA mixture was prepared in 0.72 mg/ml DEAE-dextran and TBS++. Five hundred and fifty microliters of the cells and the DNA-DEAE-dextran mixture were mixed and kept at room temperature for 30 min. The cells were washed with TBS++ and resuspended in RPMI 1640, 10% FCS with antibiotics. The transfected cells were stimulated with 100 ng/ml of human G-CSF for 10 hr or left unstimulated. Luciferase activities were determined, as described previously (Nakajima et al., 1996).

Northern Blotting Analysis, DNA Fragmentation Assay, and Cell Cycle Analysis

Northern blotting analysis, the DNA fragmentation assay, and cell cycle analysis were performed as described previously (Fukada et al., 1996). The probes used here were *CHO-B* (0.6 kb, EcoRI–BamHI fragment), *Pim-1* cDNA (1.0 kb, EcoRI–XhoI fragment), *Pim-2* cDNA (2.1 kb, ClaI–EcoRI fragment) (van der Lugt et al., 1995), *bcl-2* genome (2.7 kb, HindIII fragment), *c-myc* cDNA (0.75 kb, HindIII–PvuII fragment) (Keath et al., 1984), and *VCP* cDNA (2.3 kb, XbaI fragment) (Egerton et al., 1992).

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