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Two Signals Are Necessary for Cell Proliferation Induced by a Cytokine Receptor gp130: Involvement of STAT3 in Anti-Apoptosis

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Summary

gp130 is a common signal transducer for the interleukin-6-related cytokines. To delineate the gp130-mediated growth signal, we established a series of pro-B cell lines expressing chimeric receptors composed of the extracellular domain of the granulocyte colony-stimulating factor receptor and the transmembrane and cytoplasmic domains of gp130. The second tyrosine (from the membrane) of gp130, which was required for the tyrosine phosphorylation of SHP-2, its association with GRB2, and activation of a MAP kinase, was essential for mitogenesis, but not for anti-apoptosis. On the other hand, the tyrosine in the YXXQ motifs essential for STAT3 activation was required for *bcl-2* induction and anti-apoptosis. Furthermore, dominant-negative STAT3 inhibited anti-apoptosis. These data demonstrate that two distinct signals, mitogenesis and anti-apoptosis, are required for gp130-induced cell growth and that STAT3 is involved in anti-apoptosis.

Introduction

Cytokines play crucial roles in the immune system, hematopoiesis, inflammation, embryogenesis, and morphogenesis, and the abnormal expression of cytokines is involved in a variety of diseases. Their signals are transmitted through specific receptors on the cell membrane. The receptors for cytokines such as the interleukins (interleukin-2 [IL-2] through IL-7, IL-9, IL-11), the colony-stimulating factors (colony-stimulating factor [CSF], granulocyte CSF [G-CSF], granulocyte/macrophage CSF [GM-CSF]), hormones (growth hormone and prolactin), and other biological mediators (cardiotrophin 1 and leptin, the gene product of *obese*) have amino acid sequence homology that is characterized by conserved cysteines and WSXWS motifs in the extracellular domain. Thus, these receptors are designated as members of the type I cytokine receptor superfamily (Bazan, 1990). These receptors also have weak homology in the membrane-proximal region of their cytoplasmic domains, termed box1 and box2. (Fukunaga et al., 1991; Murakami et al., 1991). This region has been shown to associate with the tyrosine kinase janus kinases (JAKs), which are believed to play a major role in signal transduction through the cytokine receptors (Ihle, 1995).

IL-6 is a typical cytokine that exhibits various functions in the immune system, hepatocytes, and the nervous system (Haegeman et al., 1986; Zilberstein et al., 1986; Hirano et al., 1986; Sehgal et al., 1989; Van Snick, 1990; Hirano, 1994). The receptor for IL-6 is composed of a ligand-binding α subunit and a signal-transducing component, gp130, which is also referred to as the β subunit (Yamasaki et al., 1988; Taga et al., 1989; Hibi et al., 1990). Both subunits of the IL-6 receptor belong to the type I cytokine receptor superfamily, but only gp130 has box1 and box2 in its cytoplasmic domain and is capable of transmitting signals. gp130 is not only involved in IL-6 signal transduction, but is also shared by the receptors for leukemia inhibitory factor, ciliary neurotrophic factor, IL-11, oncostatin M, and cardiotrophin-1 (Ip et al., 1992; Yin et al., 1993; Hirano et al., 1994; Pennica et al., 1995; Hibi et al., 1996). JAK1, JAK2, and TYK2 have been shown to associate with gp130 through its membrane-proximal region (Lutticken et al., 1994; Stahl et al., 1994). The binding of IL-6 to the α subunit induces the homodimerization of gp130 and subsequently activates gp130-associated JAKs, possibly by transphosphorylation (Murakami et al., 1993; Lutticken et al., 1994; Matsuda et al., 1994; Narazaki et al., 1994; Stahl et al., 1994). JAKs are thought to phosphorylate tyrosine residues on gp130 and on JAKs themselves. Phosphotyrosines on cytokine receptors have been shown to recruit various src homology (SH2) domain–containing signal-transducing molecules to them, resulting in their tyrosine phosphorylation and activation. Two such molecules have been found to be adaptors that bind the tyrosine-phosphorylated gp130. One is the protein tyrosine phosphatase SHP-2, and the other is the transcriptional factor STAT3 (signal transducer and activator of transcription 3) (Sadowski et al., 1993; Akira et al., 1994; Stahl et al., 1995; Boulton et al., 1994). gp130 has six tyrosine residues in its cytoplasmic domain, and the tyrosine phosphorylation of SHP-2 and that of STAT3 have been shown to depend on the second (from the membrane) and any one of the four tyrosines (from third to sixth) in the carboxyl terminus (Stahl et al., 1995; Yamanaka et al., 1996), respectively.

SHP-2 (also referred as PTP1D, SHPTP-2, PTP2C, and Syp) is a member of the protein tyrosine phosphatase family containing two SH2 domains (Adachi et al., 1996). Among the members of this family, SHP-1 (also named SHPTP-1, SHP, HCP, and PTP1C) is thought to negatively regulate receptor-mediated signals (Shen et al., 1991; Plutzky et al., 1992; Yi et al., 1993; Matthews et al., 1992). In the case of the erythropoietin receptor (EpoR), SHP-1 binds phosphotyrosine (Y429) in the cytoplasmic domain of EpoR and inactivates the receptor-associated JAK2 (Klingmuller et al., 1995). In contrast, SHP-2 has a higher homology with the Drosophila tyrosine phosphatase, Corkscrew (CSW) (Freeman et al., 1992), which

acts downstream of the Drosophila tyrosine kinase Torso (Perkins et al., 1992). Like CSW, SHP-2 has been shown to act downstream of tyrosine kinases such as the epidermal growth factor (EGF) receptor, the fibroblast growth factor (FGF) receptor, and the insulin receptor (Noguchi et al., 1994; Li et al., 1994; Tang et al., 1995; Bennett et al., 1996). In the type I cytokine receptor superfamily, SHP-2 has been reported to associate with the prolactin receptor-JAK2 complex, and its SH2 domain and phosphatase activity are involved in the expression of the β-casein gene in response to prolactin (Ali et al., 1996). gp130 has a YSTV motif (known to be recognized by SHP-2) at the second tyrosine, and upon ligand binding SHP-2 has been shown to be tyrosine phosphorylated in a manner dependent on the second tyrosine of gp130 (Stahl et al., 1995).

STATs were originally identified as transcriptional factors responsible for interferon-α- and interferon-γ-dependent gene expression (Darnell et al., 1994). Experiments employing molecularly cloned STAT proteins revealed that STAT is generally involved in cytokine signal transduction (Ihle, 1996). All STAT molecules have an SH2 domain, which recognize phosphotyrosine in a specific peptide motif. Among the STAT family members, IL-6 has been shown to induce the tyrosine phosphorylation of STAT1 and STAT3, their nuclear translocation, and their binding to a specific DNA element (Akira et al., 1994; Fujitani et al., 1994; Nakajima et al., 1995). The tyrosine phosphorylation of STAT3 has been reported to require any one of four tyrosines in the carboxy-terminal region of gp130. These tyrosines all have a glutamine at the third position away (YXXQ motifs) (Stahl et al., 1995). Since each cytokine activates a relatively specific species of STAT family proteins, STATs may have a role in determining the specificity of the signal transduction through a given receptor. In fact, each STAT contributes to a specific biological activity. Marrele/D-STAT has been shown to play a pivotal role in the regulation of the pair rule gene expression in Drosophila early development (Yan et al., 1996). STAT1 is known to be involved in the innate immunity elicited by interferon-α and interferon-γ (Meraz et al., 1996; Durbin et al., 1996). STAT4 is involved in the development of natural killer and T helper type 1 (Th1) cells in response to IL-12 (Kaplan et al., 1996b; Thierfelder et al., 1996). STAT6 is involved in the transition of T lymphocytes to Th2 and in immunoglobulin E (IgE) production and lymphocyte proliferation in response to IL-4 (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). In IL-6 signaling, the activation of STAT3 has been found to be essential for the differentiation of mouse M1 leukemia cells (Yamanaka et al., 1996; Nakajima et al., 1996). However, the role of STAT3 in the growth signal is unknown.

In this study, we used chimeric receptors to show that in addition to the box1 and box2 regions, the tyrosine residues in the carboxyl terminus were necessary for gp130-mediated growth in BAF-B03 cells. Furthermore, we demonstrated that the signal from the second tyrosine and the STAT3 pathway from YXXQ motifs are both essential for the gp130-mediated cell growth. Moreover, STAT3 activation was shown to be linked with anti-apoptotic signals through the induction of *bcl-2*.

Results

The Membrane-Proximal 68 Amino Acid Region Is Sufficient for c-*myc* Induction, but Not for Cell Proliferation

It has been reported previously that when human gp130 is expressed in the mouse pro-B cell line BAF-B03, IL-6 and the soluble α receptor bind gp130 and induce thymidine incorporation in the cells (Murakami et al., 1991). In that system it was concluded that the membrane-proximal 61 amino acid region, which contains the box1 and box2 regions, is sufficient to induce transient growth. To address the question of whether this region is sufficient to induce long-term cell proliferation, we constructed a series of chimeric receptors that consist of the extracellular domain of the human G-CSF receptor (G-CSFR) and the transmembrane and cytoplasmic domains of human gp130 (Figure 1) and transfected BAF-B03 cells, which do not express endogenous G-CSFRs (Fukunaga et al., 1991). The chimeric receptor system has been proven to generate a gp130-mediated signal in the mouse leukemia cell line M1 by inducing cell differentiation and growth arrest (Yamanaka et al., 1996). We detected their expression by fluorescence-activated cell sorting (FACS) using an anti–human G-CSFR antibody. All the transfectants established and used in the present study had comparable numbers of receptor molecules expressed on the cell surface (Figure 1). The protein levels of the various chimeric receptors in their transfectants were also comparable, as determined by immunoblotting with anti-G-CSFR antibodies (data not shown).

BAF-B03 cells are IL-3 dependent and were maintained in the presence of 10% conditioned medium from WEHI3B as a source for IL-3. Without the starvation of IL-3 (Figure 2A), cells expressing the G-68 chimeric receptor, which contains 68 amino acids from the membrane, incorporated [^{3}H]thymidine to levels as high as those incorporated by cells expressing the full-length chimeric receptor G-277 (for further explanation of this terminology, see the legend to Figure 1). This result was consistent with the previous report (Murakami et al., 1991). However, when the transfectant cells were starved of IL-3, G-68 transfectant cells no longer incorporated thymidine in response to G-CSF, whereas G-277 transfectants did (Figure 2B). Although the data showed that the G-68 chimeric receptor is not sufficient to complete the cell growth signal, we observed c-*myc* mRNA induction in G-68 transfectants in response to G-CSF (Figure 2C).

The Amino Acid 69–133 Region Is Necessary to Complete the Growth Signal and Is Required for the Activation of SHP-2 and STAT3

To find out which portions of gp130 are necessary to complete the growth signal, we established BAF-B03 transfectants expressing carboxy-terminal truncation mutants of the chimeric receptor and assessed their long-term growth. As shown in Figure 3A, truncation at the amino acid 133 residues from the membrane did not significantly affect cell growth. However, truncation at amino acid 68 completely abolished G-CSF-dependent

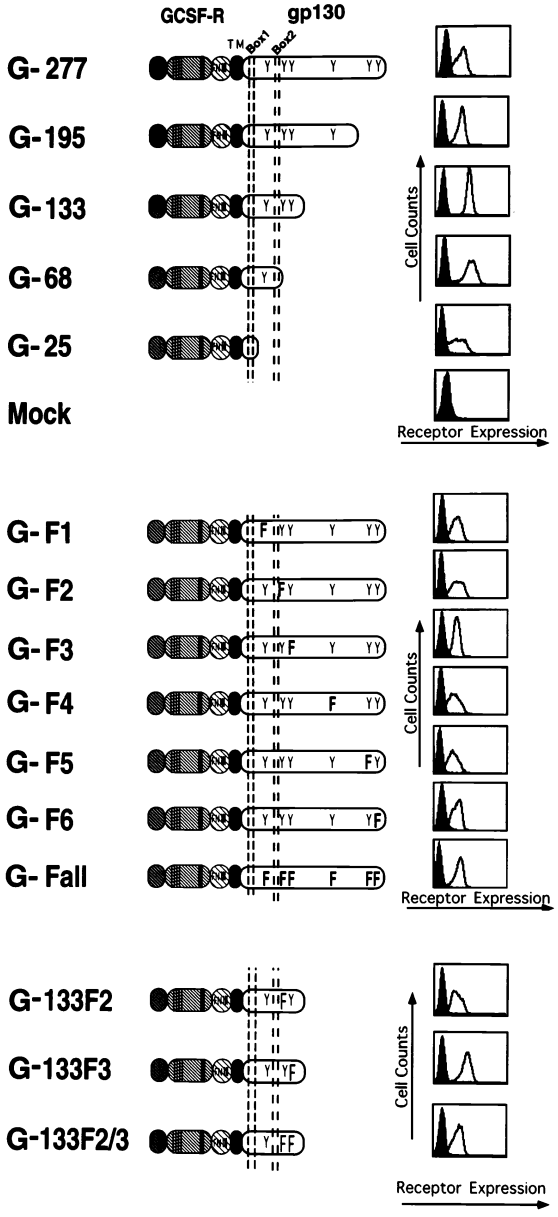

Figure 1. Structure and Expression of the G-CSFR–gp130 Chimeric Receptors in BAF-B03 Cells

(Left) Structures of full-length, truncated, and tyrosine-mutated chimeric receptors. Chimeric receptors are composed of the extracellular domain of G-CSFR (hatched boxes) and the transmembrane and cytoplasmic domains of human gp130 (open boxes). The transmembrane domain is indicated (TM). Vertical broken lines indicate box1 and box2 from the transmembrane domain. The chimeric receptors are named according to the last amino acid position from the membrane: full-length cytoplasmic domain (277 amino acids, G-277) and receptors truncated at 195 (G-195), 133 (G-133), 68 (G-68), and 25 (G-25) amino acids from the membrane. Human gp130 has six tyrosines (amino acids 42, 118, 126, 173, 262, and 274 from the membrane; Y) in its cytoplasmic domain. In the G-CSFR-F1 (G-F1) mutant, the first tyrosine in the cytoplasmic domain is replaced by phenylalanine (F). In the G-F2, G-F3, G-F4, G-F5, and G-F6 mutants, the second, third, fourth, fifth, and sixth tyrosines are replaced by phenylalanines, respectively. In the G-133F2, G-133F3, and G-133F2/3 mutants, the second, third, or both second and third tyrosines are replaced by phenylalanines.

(Right) Expression of the chimeric receptors on BAF-B03 transfectants. Cells were incubated with the anti-human G-CSFR (against the extracellular domain of G-CSFR) antibody and then with fluorescein isothiocyanate (FITC)-conjugated goat anti–mouse IgG (open area). Closed areas indicate the fluorescence profile of the cells stained with FITC-conjugated goat anti–mouse IgG alone (as the control).

cell proliferation, suggesting that the amino acid 69–133 region is critical for full cell growth signal. This region includes the second and third tyrosine residues (from the membrane), which are known to be essential for the tyrosine phosphorylation of SHP-2 and the activation of STAT3. We evaluated the tyrosine phosphorylation of STAT3 and SHP-2 in transfectants expressing truncation mutants of the chimeric receptor (Figure 3B). Consistent with previous reports (Stahl et al., 1995; Yamanaka et al., 1996), gp130 truncated at amino acid 133 sustained the tyrosine phosphorylation of both SHP-2 and STAT3 upon stimulation in BAF-B03 cells, but truncation to amino acid 68 abolished their tyrosine phosphorylation.

The Signal from the Second Tyrosine Is Necessary for the gp130-Mediated Mitogenesis

To assess the role of signals from the tyrosine residues of gp130 in the growth signal, we constructed point mutants in the context of the full-length chimeric receptor (Figure 1) and established BAF-B03 transfectant cells expressing them. As shown in Figure 4A, cells expressing G-Fall, in which all the tyrosine residues of gp130 were mutated to phenylalanines, did not proliferate in response to G-CSF, but rather died within a few days. The mutation of the second tyrosine (G-F2) also abolished cell proliferation. However, G-F2 transfectants survived for at least 4 days in the presence of G-CSF, indicating that G-F2 could not generate a mitogenic signal but could generate a survival signal in the cells. The other single point mutations did not affect cell growth or survival, indicating either that the other tyrosine residues could complement each other or that they were not involved in the growth signal. The surrounding amino acid sequence of the second tyrosine is YSTV, which could be a docking site for SHP-2, so we examined whether the phosphorylated second tyrosine, but not the other tyrosines, binds SHP-2. We prepared peptides 15 amino acids in length containing phosphorylated or nonphosphorylated tyrosines (Y6 and P-Y6 contain only 11 amino acid lengths because they are on the carboxyl terminus) and mixed them with the lysates of BAF-B03 G-277 transfectants. The peptide with a phosphorylated second tyrosine (P-Y2 in Figure 4B) precipitated SHP-2 in the lysate of the cells, but neither the peptide with a nonphosphorylated second tyrosine, the peptides phosphorylated on other tyrosines, nor the other nonphosphorylated peptides could precipitate SHP-2 from the cell lysates. None of the peptides could precipitate SHP-1, another SH2-containing tyrosine phosphatase (Figure 4B). These results indicate that the second tyrosine is a unique tyrosine that binds SHP-2 when phosphorylated and that its function cannot be complemented by the other tyrosines on gp130. The interaction between gp130 and SHP-2 in cells was observed when they were stimulated (Figure 4C), suggesting that SHP-2 associates with gp130 upon stimulation. SHP-2 was reported to bind the adaptor molecule

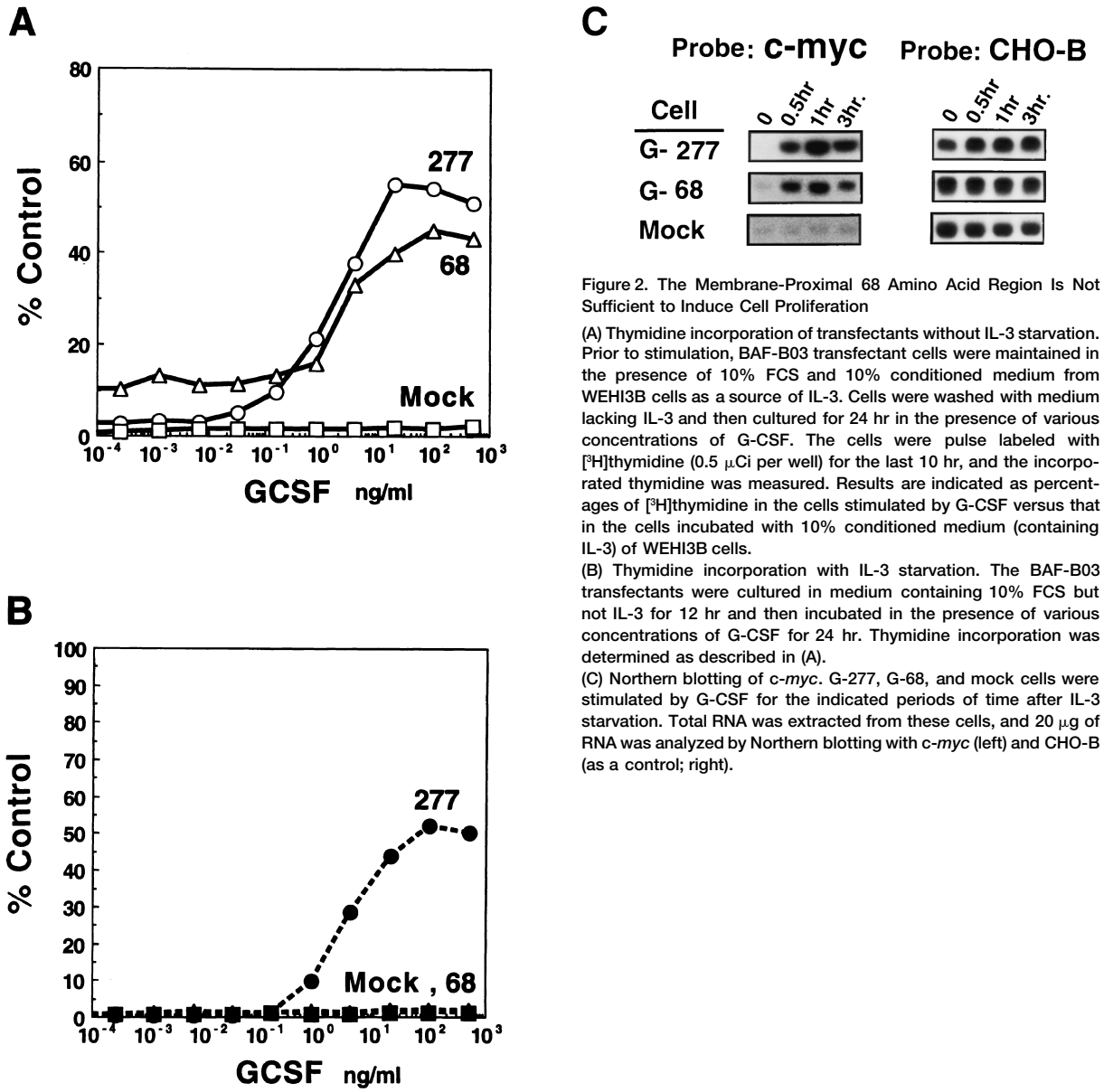

Figure 2. The Membrane-Proximal 68 Amino Acid Region Is Not Sufficient to Induce Cell Proliferation

(A) Thymidine incorporation of transfectants without IL-3 starvation. Prior to stimulation, BAF-B03 transfectant cells were maintained in the presence of 10% FCS and 10% conditioned medium from WEHI3B cells as a source of IL-3. Cells were washed with medium lacking IL-3 and then cultured for 24 hr in the presence of various concentrations of G-CSF. The cells were pulse labeled with [^{3}H]thymidine (0.5 μCi per well) for the last 10 hr, and the incorporated thymidine was measured. Results are indicated as percentages of [^{3}H]thymidine in the cells stimulated by G-CSF versus that in the cells incubated with 10% conditioned medium (containing IL-3) of WEHI3B cells.

(B) Thymidine incorporation with IL-3 starvation. The BAF-B03 transfectants were cultured in medium containing 10% FCS but not IL-3 for 12 hr and then incubated in the presence of various concentrations of G-CSF for 24 hr. Thymidine incorporation was determined as described in (A).

(C) Northern blotting of c-*myc*. G-277, G-68, and mock cells were stimulated by G-CSF for the indicated periods of time after IL-3 starvation. Total RNA was extracted from these cells, and 20 μg of RNA was analyzed by Northern blotting with c-*myc* (left) and CHO-B (as a control; right).

GRB2 through the phosphotyrosine(s) on SHP-2 upon platelet-derived growth factor (PDGF) stimulation. We assessed the interaction between SHP-2 and GRB2 upon gp130 stimulation. In G-277 transfectant cells, G-CSF induced the association between SHP-2 and GRB2, as well as the tyrosine phosphorylation of SHP-2. The mutation of the second tyrosine abolished either the tyrosine phosphorylation of SHP-2 or the association between SHP-2 and GRB2 (Figure 4D). Intriguingly, another tyrosine-phosphorylated protein whose molecular mass is approximately 100 kDa was coprecipitated with SHP-2, and it disappeared with the mutation of the second tyrosine. GRB2 constitutively associated with Sos (Figure 4E), a GDP/GTP exchanger for Ras, as observed in other cell lines. We further examined the activation of the Ras pathway by measuring the activity of the mitogen-activated protein (MAP) kinase ERK2, a major target induced downstream of the Ras pathway. G-CSF induced MAP kinase activity as IL-3 did, and the mutation of the second tyrosine in gp130 abolished the induction of this enzyme activity (Figure 4F). These data indicate that the signal from the second tyrosine is necessary for gp130-mediated mitogenesis and suggest that SHP-2, ERKs, or the cascade from SHP-2 to ERKs may play a role in mitogenic signaling.

STAT3 Is Necessary for the gp130-Mediated Survival Signal

It has already been established that any one of the four tyrosines in the carboxyl terminus of gp130 can induce the tyrosine phosphorylation and activation of STAT3. Therefore, single mutations in the context of the full-length chimeric receptor could not abolish its tyrosine phosphorylation (data not shown). The region containing 133 amino acids from the membrane was sufficient to induce cell proliferation, and this region contains three

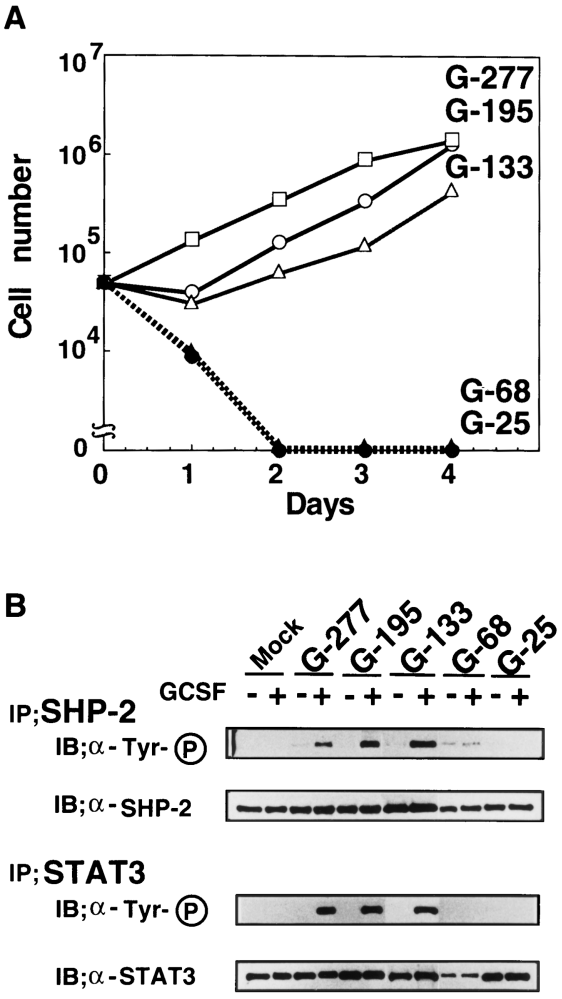

Figure 3. The Region Containing Amino Acids 69–133 Is Essential for Long-Term Growth

(A) Cell proliferation of transfectants expressing the truncated chimeric receptors. Transfectant cells (5×10^4) were cultured in 1 ml of medium containing 100 ng/ml of G-CSF for 4 days. The number of viable cells was then counted on the days indicated.

(B) Tyrosine phosphorylation of SHP-2 and STAT3. After cells were starved of IL-3 for 12 hr, 1×10^7 cells were either stimulated (plus) with G-CSF (100 ng/ml) for 15 min or left unstimulated (minus). SHP-2 or STAT3 was immunoprecipitated with anti-SHP-2 antibody or anti-STAT3 antibody, respectively. Immunoprecipitates were separated on a 4%–20% gradient SDS–polyacrylamide gel and transferred to membranes. The tyrosine phosphorylation and expression were analyzed by blotting with anti-phosphotyrosine antibody and anti-SHP-2 antibody or anti-STAT3 antibody.

tyrosine residues, with only the third being a docking site for STAT3. To determine the role of STAT3 in cell proliferation, we constructed point mutations of the second, third, or both tyrosine residues in the context of G-133. The mutation of either the second (Figure 1, G-133F2) or the third (Figure 1, G-133F3) tyrosine residue selectively abolished the tyrosine phosphorylation of either SHP-2 or STAT3 (Figure 5A). The G-133F2 transfectants did not proliferate (Figure 5B) and were arrested at the S phase of the cell cycle without transition to the G2/M phase (Figure 5C), but survived for more than 4 days, like the G-F2 transfectants in which the second tyrosine was mutated in the context of the full-length construct (Figure 4A). On the other hand, both G-133F3 and G-133F2/3 transfectant cells died within a few days, suggesting that STAT3 activated through the third tyrosine is critical for the cell survival signal. We assayed DNA fragmentation (Figure 5D), which is an indicator for apoptosis. In the presence of G-CSF, G-277, G-133, G-133F2, or G-F2 (data not shown), transfectants were not subject to apoptosis. In contrast, G-133F3 and G-133F2/3 transfectants underwent apoptosis in 24 hr, and cell cycle analysis showed that they died in 1 day with the loss of DNA content in the nucleus. These data clearly demonstrate that a signal through the third tyrosine was critical for preventing cell death. To show that a survival signal from the third tyrosine does in fact involve STAT3, we utilized a dominant-negative *Stat3* gene that inhibits the activity of endogenous STAT3 function. We previously demonstrated that the STAT3F and STAT3D mutants, in which Tyr-705, a phosphoacceptor site of STAT3, was mutated to phenylalanine (STAT3F) and Glu-434 and Glu-435 of the DNA-binding domain were mutated to alanines (STAT3D), act specifically in a dominant-negative manner (Nakajima et al., 1996). We transfected the G-277 transfectants with the expression vector for STAT3F or STAT3D. Cells expressing either of the dominant-negative STAT3 proteins did not proliferate (Figure 6A) and underwent cell death accompanied by DNA fragmentation (Figure 6B). We further examined the expression of *bcl-2*, whose gene product is known to be involved in preventing apoptosis, in cells expressing the series of G-133 chimeric receptors or dominant-negative STAT3 proteins. As shown in Figure 7, the expression of *bcl-2* was induced in G-277, G-133, and G-133F2 transfectants, but was abolished in G-133F3, G-133F2/3, and G-277 transfectants expressing STAT3F or STAT3D, correlating with their susceptibility. These data show that the activation of STAT3 is critical for the anti-apoptotic signal.

Discussion

Multiple Signals Are Generated from gp130 for Cell Growth

In the first part of this investigation, we identified the regions of gp130 essential for cell growth. Using G-CSFR–gp130 chimeric receptors, we determined that the membrane-proximal 68 amino acid region, containing box1 and box2, could induce thymidine incorporation in BAF-B03 cells without prior IL-3 starvation, but with starvation of IL-3 thymidine incorporation was not induced at all, although c-*myc* induction could still occur. These data suggest the possibility that certain signals involved in the G1 to S transition are generated by the 68 amino acid region. However, since the cells were not synchronized at the G0/G1 phase without the IL-3 starvation, it is difficult to interpret the results simply under this complicated condition. Under the synchronized condition, it is clear that the signals generated from the membrane-proximal 133 amino acids of gp130 are necessary and sufficient for full cell growth. We demonstrated that at least two signals generated from the

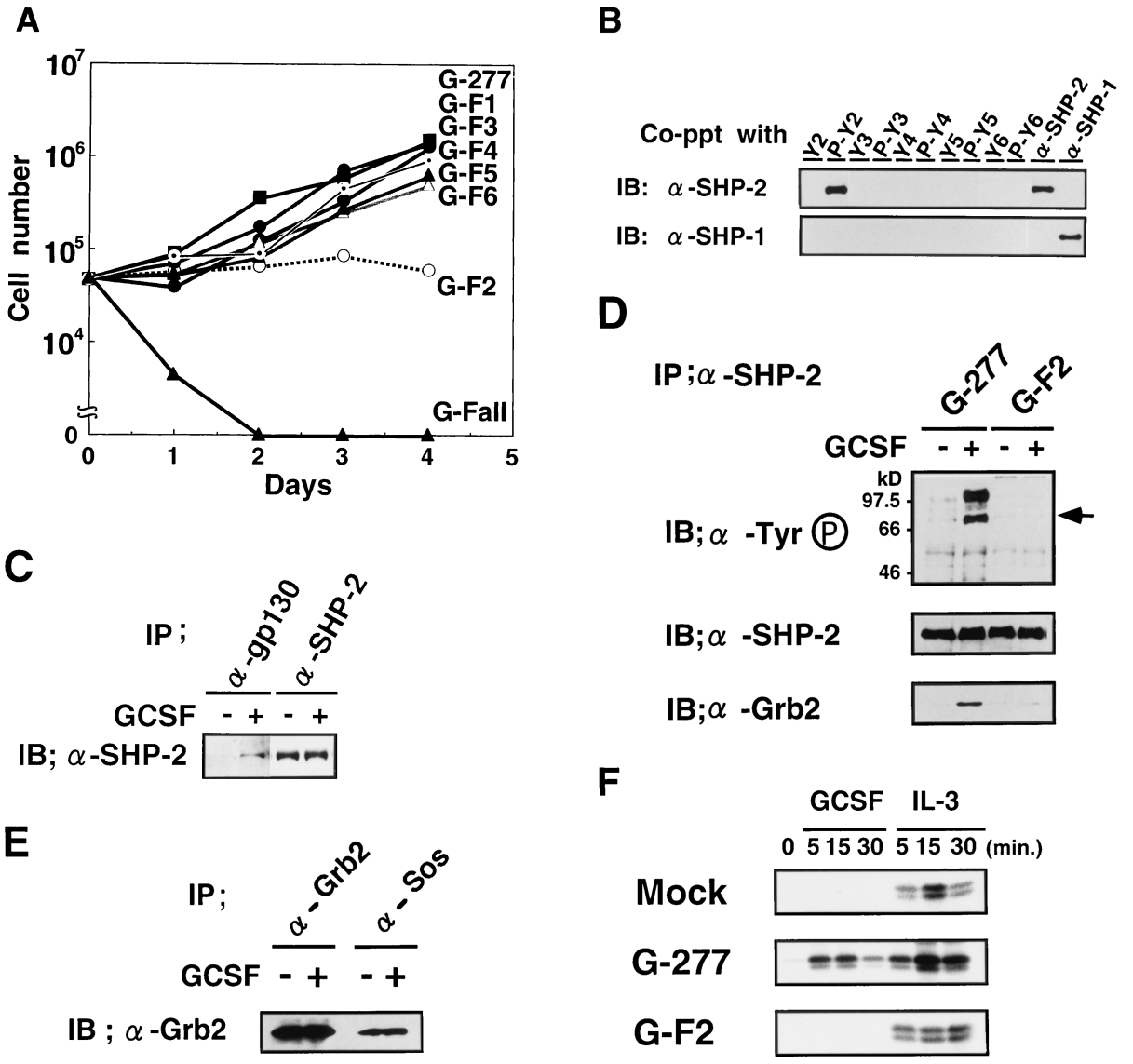

Figure 4. The Signal from the Second Tyrosine Is Necessary for gp130-Mediated Mitogenesis

(A) Cell proliferation of transfectants expressing the point-mutated chimeric receptors. Long-term growth of cells expressing point mutations in the chimeric receptor was determined as described in Figure 3.

(B) The phosphorylated second tyrosine–containing peptide binds SHP-2. We mixed 1×10^7 cell lysates with the following biotinylated peptides: Y2, NTSSTVQY*STVVHSG; Y3, STVVHSGY*RHQVPSV; Y4, GILPRQQY*FKQNCSQ; Y5, DEGMPKSY*LPQTVRQ; Y6, QTVRQGGY*MPQ; or their tyrosine-phosphorylated peptides P-Y2, P-Y3, P-Y4, P-Y5, and P-Y6 (tyrosines marked with an asterisk are replaced by phosphotyrosines). The peptide-bound fractions were recovered with streptavidin–agarose, and the immunoprecipitates of anti-SHP-2 or anti-SHP-1 antibody (for control) were separated by SDS–PAGE. The transferred membranes were blotted with anti-SHP-2 or anti-SHP-1 antibody.

(C) SHP-2 interacts with gp130 in cells. HepG2 cells (1×10^7) were cultured in FCS-free medium for 6 hr and then stimulated with human IL-6 (100 ng/ml) for 15 min (plus) or left unstimulated (minus). gp130 or SHP-2 was immunoprecipitated with anti-gp130 antibody or anti-SHP-2 antibody, respectively. Immunoprecipitates were separated by SDS–PAGE and analyzed with anti-SHP-2 antibody.

(D) Tyrosine phosphorylation of SHP-2 correlates with the association of SHP-2 and GRB2. SHP-2 was immunoprecipitated from stimulated (plus) or unstimulated cells (minus) with anti-SHP-2 antibody and blotted with anti-phosphotyrosine (top), anti-SHP-2 (middle), or anti-GRB2 antibodies (bottom). The arrow indicates the location of SHP-2 (top), and another tyrosine-phosphorylated protein (p100) was observed in the SHP-2 immunoprecipitate of stimulated G-277 transfectants.

(E) The constitutive association of GRB2 and Sos. GRB2 and Sos were immunoprecipitated and blotted with anti-GRB2 antibody.

(F) The activation of ERK2 depends on the signal from the second tyrosine. Cells (1×10^7) were stimulated with either G-CSF (100 ng/ml) or 10% conditioned medium of WEHI3B cells or were incubated with medium alone (as a control) for 5, 15, and 30 min. The MAP kinase ERK2 was immunoprecipitated with anti-ERK2-specific antibody. The kinase reaction was carried out by incubating the precipitated fractions with [γ-^{32}P]ATP and myelin basic protein (as a substrate) in the kinase buffer. The phosphorylated myelin basic protein was separated by SDS–PAGE and subjected to autoradiography.

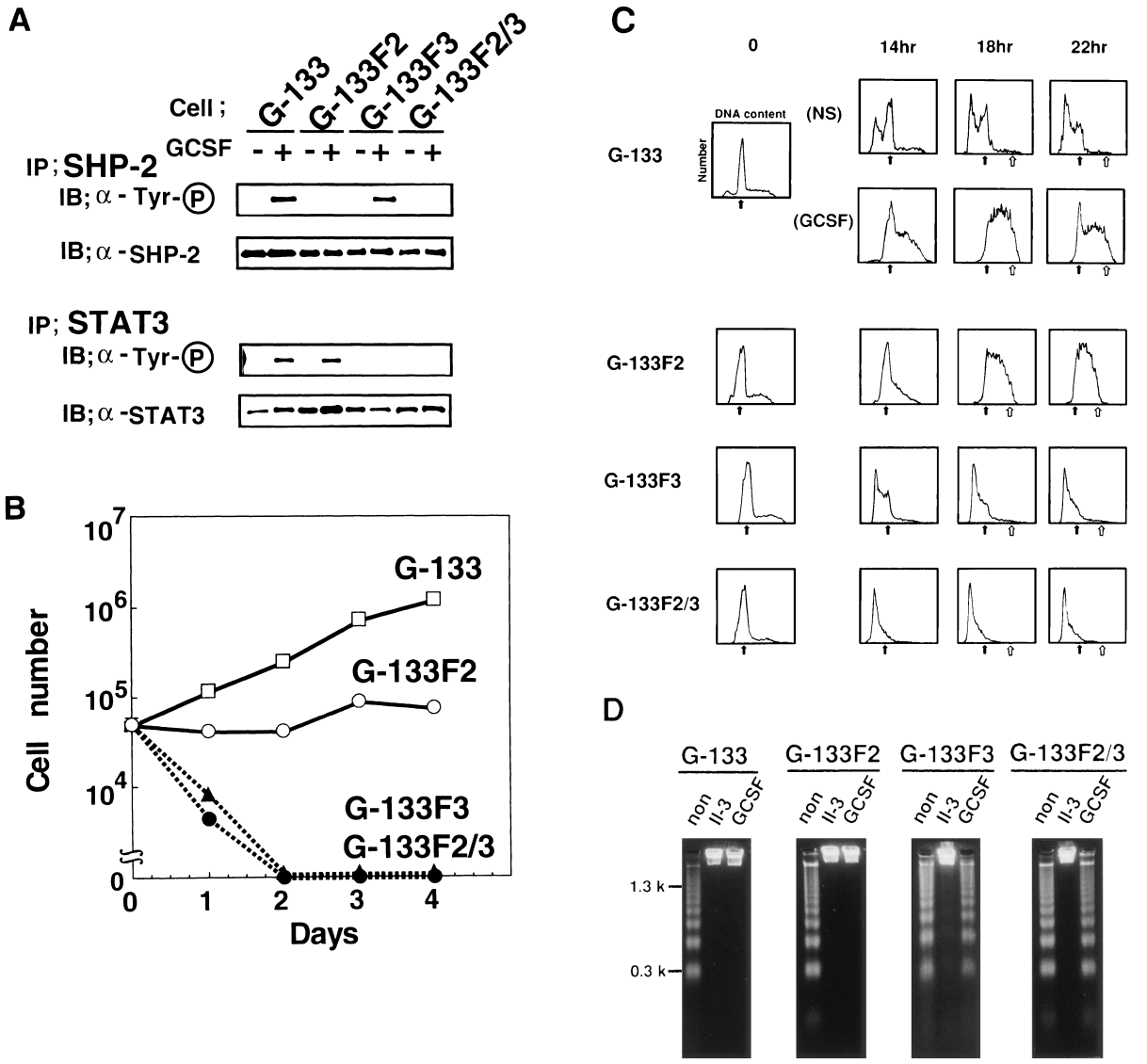

Figure 5. The Second and Third Tyrosines Mediate Distinct Signals on the Basis of the G-133 Chimeric Constructs

(A) Tyrosine phosphorylation of SHP-2 and STAT3. The procedure was carried out as described in Figure 4.

(B) Cell proliferation. The procedure was carried out as described in Figure 3.

(C) Cell cycle analysis. Cells were synchronized by IL-3 starvation for 12 hr and then stimulated with either G-CSF (100 ng/ml) or left unstimulated (NS). Cells were fixed at the indicated times and stained with propidium iodine, and their status in the cell cycle was analyzed by FACS. The closed arrows indicate the G1/G0 phase and the open arrows indicate the G2/M phase of the cell cycle.

(D) DNA fragmentation. After IL-3 starvation, cells were stimulated with either G-CSF (100 ng/ml) or 10% conditioned medium from WEHI3B cells (IL-3) or left unstimulated (NS) for 24 hr. Cells were then lysed, and chromosomal DNA was separated by TBE 2% agarose gel electrophoresis as described in Experimental Procedures.

second and third tyrosines are necessary to complete the growth signal. The chimeric receptors with the mutation of the second tyrosine could exhibit a G1 to S phase transition, but not an S to G2/M transition, indicating that a signal(s) from the second tyrosine is necessary for the G2/M transition. The induction of a transition of G1 to S, but not of S to G2/M, when BAF-B03 cells expressing the EGF receptor were stimulated by EGF has been reported (Shibuya et al., 1992). Our present results support the notion that multiple signals are involved in the growth signal mediated through the type I cytokine receptors, such as the IL-3 receptor and the IL-2 receptor (Kinoshita et al., 1995; Miyazaki et al., 1995; Friedmann et al., 1996). This study of the cytokine receptor family, however, establishes that two distinct tyrosine modules display independent signals necessary for cell growth.

The Signal Elicited from the Second Tyrosine Is Essential for Mitogenesis, and This Process May Involve the SHP-2–MAP Kinase Pathway

BAF-B03 cells expressing the chimeric receptor with the mutation of the second tyrosine exhibited a transition of

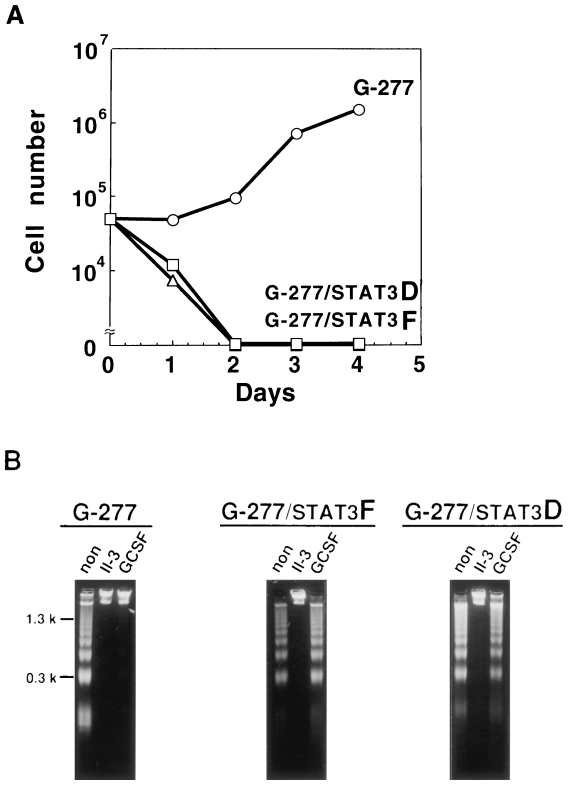

Figure 6. Dominant-Negative STAT3 Proteins Inhibit the Anti-Apoptotic Signal

G-277 cells were transfected with the expression vectors for STAT3F and STAT3D, dominant-negative STAT3s in which either Tyr-705, a major phosphoacceptor site on STAT3, was mutated to phenylalanine (G-277/STAT3F) or Glu-434 and Glu-435 of STAT3 in the DNA-binding domain were mutated to alanines (G-277/STAT3D), respectively.

(A) Cell proliferation. One representative clone for each construct was subjected to cell proliferation determination as described in Figure 3.

(B) DNA fragmentation was analyzed as described in Figure 5.

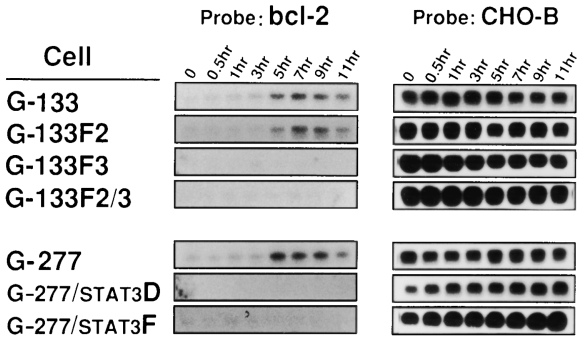

Figure 7. Northern Blotting of *bcl-2*

G-133, G-133F2, G-133F3, G-133F2/F3, G-277, G-277/STAT3F, and G-277/STAT3D cells were stimulated by G-CSF for the indicated periods of time following IL-3 starvation. Total RNA was extracted from these cells, and 20 μg of RNA was analyzed by Northern blotting with *bcl-2* (left) and CHO-B (as a control; right).

G1 to S phase without further progression in the cell cycle, but survived for at least 4 days in the presence of the ligand. These results are consistent with our previous observation that the second tyrosine is essential for the gp130-mediated growth-enhancing signal in M1 leukemia cells (Nakajima et al., 1996). The same mutation abolished either the tyrosine phosphorylation of SHP-2 or the association between SHP-2 and the GRB2–Sos complex, correlating with the loss of the activation of the MAP kinase ERK2, a major target of the Ras pathway. Upon the stimulation of PDGF receptor, SHP-2 has been reported to bind phosphorylated Tyr-1009 on the receptor, resulting in its phosphorylation at Tyr-542 by the receptor kinase (Bennett et al., 1994). Then, GRB2 can bind to SHP-2 through the phosphorylated Tyr-542. In BAF-B03 transfectants, we observed that SHP-2 associated with gp130 upon gp130 stimulation (Figure 4C). Furthermore, we detected the association between SHP-2 and GRB2 only when SHP-2 was tyrosine phosphorylated, and we observed that GRB2 constitutively associated with Sos, suggesting that SHP-2 plays a role in transmitting a signal from the second tyrosine to the Ras pathway. In addition to GRB2, another tyrosine-phosphorylated protein (p100) was shown to associate with SHP-2 when SHP-2 was tyrosine phosphorylated (Figure 4D), and it might be involved in other signal transduction cascades. The expression of a mutant SHP-2 lacking the phosphatase activity has been shown to suppress EGF, insulin, and FGF signaling (Noguchi et al., 1994; Tang et al., 1995; Bennett et al., 1996), but a mutation of Tyr-542 and Tyr-580 in the carboxyl terminus, including a GRB2-binding site, did not suppress the MAP kinase activation induced by EGF or PDGF. SHP-2 has two more YXNX motifs in addition to Tyr-542, the consensus sequence for GRB2 binding. These sites may participate in transmitting signal to the Ras pathway through GRB2. Recent genetic study of CSW, a homolog of SHP-2, in Drosophila revealed that CSW acts downstream of the tyrosine kinases Torso and Sevenless, but did not determine whether it acts upstream of or parallel to Ras (Perkins et al., 1992; Herbst et al., 1996; Raabe et al., 1996). To reveal the true role of SHP-2 in gp130 signaling, it will be necessary to examine the effect of expression of a phosphatase inactive mutant or a mutant lacking all of the GRB2-binding sites on the cell growth.

In addition to SHP-2, Shc, another adaptor molecule in the Ras pathway, has been reported to be involved in the gp130-mediated growth signal in plasmacytoma cell lines (Kumar et al., 1994). We did not observe tyrosine phosphorylation of Shc in BAF-B03 transfectants upon stimulation (data not shown). Thus, there might be cell type–specific adaptor molecules for gp130.

In the case of IL-3 and GM-CSF, which share the common β chain for the receptor with IL-5, truncation of the carboxy-terminal region of β chain required for Ras activation could still elicit a signal inducing DNA synthesis, but cells with the truncation underwent apoptosis (Sato et al., 1993; Kinoshita et al., 1995). In response to EGF activation, however, the SHP-2–MAP kinase pathway, which might involve Ras activation, was shown to be necessary for cell cycle progression in NIH 3T3 cells (Bennett et al., 1996). The activation of Ras by GRB2 was also shown to induce DNA synthesis and

transformation (Lowenstein et al., 1992). In gp130 signaling, the SHP-2–GRB2–Ras pathway may be involved in mitogenesis, although this remains to be elucidated.

STAT3 Transmits an Anti-Apoptotic Signal

Various STAT family proteins are reported to be activated in response to cytokines. Their involvement in growth signals remains unclear. In the case of the IL-2 receptor, a deletion of a region containing Tyr-392 and Tyr-510 of the common β chain of the IL-2 receptor abolished the tyrosine phosphorylation and activation of STAT5, but cells with the mutated receptor could still grow in response to IL-2 (Fujii et al., 1995). However, for maximal proliferation it was shown that the activation of STAT5 through these tyrosines was necessary (Friedmann et al., 1996). A mutant erythropoietin (Epo) receptor that lacks Tyr-343 and Tyr-401 has been shown to reduce the activation of STAT5 as well as its mediated cell growth (Damen et al., 1995; Gobert et al., 1996). In the case of the IL-3/GM-CSFR, a correlation between the activation of STAT5 and the mitogenic signal was observed. Furthermore, the expression of a STAT5 mutant truncated in its carboxyl terminus partially blocked the mitogenic signal (Mui et al., 1996). In addition, there are several reports suggesting that STAT proteins are involved in the cell growth signal. STAT3 or STAT5 and STAT6 have been reported to be activated in v-*src*- or v-*abl*-transformed cells, respectively (Yu et al., 1995; Daniel et al., 1995). STAT3 and STAT5 have also been shown to be constitutively activated in acute T cell leukemia cells that had lost IL-2 dependency (Migone et al., 1995). The targeted disruption of *Stat4* and *Stat6* genes in mice revealed that STAT4 and STAT6 are involved in growth signals in response to IL-12 and IL-4, respectively (Kaplan et al., 1996a, 1996b; Shimoda et al., 1996; Takeda et al., 1996; Thierfelder et al., 1996). Here, we have definitively demonstrated that STAT3 is involved in the growth signal by preventing apoptosis. These findings were verified by using chimeric receptors defective in the generation of STAT3 activation and the dominant-negative *Stat3* genes. The study reveals how STAT3 is involved in the process of cell proliferation. We further demonstrated that STAT3 is involved in the expression of *bcl-2*, an anti-apoptotic gene. We do not know if the expression of *bcl-2* is activated directly by STAT3 or whether there are intermediate processes. Further promoter analysis using transfectants of the chimeric receptors or the dominant-negative STAT3 will reveal the precise mechanisms. In any case, STAT3 is essential for gp130-mediated anti-apoptotic signals. In addition to anti-apoptotic signals, we do not exclude the possible role of STAT3 in mitogenesis, including DNA synthesis and progression of the cell cycle, although at the moment we cannot evaluate this possibility because cells with either the chimeric receptor lacking STAT3 activation or the dominant-negative STAT3 immediately underwent apoptosis before proceeding to mitogenesis, even in the presence of the cytokines.

We previously demonstrated that STAT3 is involved in the differentiation and growth arrest of mouse leukemia M1 cells, but not in cell growth (Yamanaka et al., 1996; Nakajima et al., 1996). M1 cells can be maintained in media containing horse serum without any other soluble factors, suggesting that they constitutively generate an anti-apoptotic signal under these conditions and do not need STAT3 for their survival but, rather, need it for their differentiation. Alternatively, STAT3 may mediate different biological actions in synergy with unknown pathways or may induce the expression of a different set of genes depending on the cell type involved.

Cell proliferation seems to involve a variety of complicated processes. In this report, we showed that at least two signals, a mitogenic signal and an anti-apoptotic signal, are critical for the cell proliferation induced by the cytokine receptor gp130. This model may be applied to the signal transduction of other cytokine receptors.

Experimental Procedures

Cell Culture, Transfection, and Biological Reagents

BAF-B03 cells were maintained in RPMI medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 10% conditioned medium from WEHI3B cells as a source of IL-3, 100 U/ml penicillin, and 100 mg/ml streptomycin. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% FCS and antibiotics. Transfection was performed by the electroporation method using a Gene Pulser (Bio-Rad). We cotransfected 50 μg of the expression vectors for either the chimeric receptors or the dominant-negative *Stat3* genes (*Stat3D* and *Stat3F*) with 5 μg of pSV2-neo or pMIK-Hyg, respectively. Transfectants were selected with 1 mg/ml G418 or 200 μg/ml hygromycin for pSV2-neo or pMIK-Hyg, respectively, and transfectant-independent clones were established using a limiting dilution procedure. Expression of the chimeric receptors was detected using a cell sorter (FACScan, Becton Dickinson), and the expression levels of *Stat3F* and *Stat3D* were analyzed by Northern blotting.

Plasmid Construction

To construct the chimeric receptor, we incorporated an EcoRI site into the amino-terminal side of the transmembrane domain of the human G-CSFR cDNA in pEFBOS (Fukunaga et al., 1991; a gift from Dr. Nagata) by PCR. The XbaI (derived from pEFBOS)–EcoRI fragment was subcloned into pBluescript SK(+) (Stratagene). The transmembrane and cytoplasmic domains of human wild-type or mutated gp130 were excised from the pSP72 containing them (Yamanaka et al., 1996) by EcoRI and HindIII. The fragment was subcloned into pBluescript containing the extracellular domain of the G-CSFR. The fragments of chimeric receptors were excised by XbaI and cloned into the pEFBOS expression vector. The construction of the dominant-negative STAT3F and STAT3D has been previously described (Nakajima et al., 1996).

Immunoprecipitation, Immunoblotting, Peptide Binding Assay, and Immunological Reagents

Cells (1×10^7) were harvested after stimulation and suspended with 1 ml of lysis buffer (1% NP-40, 20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM sodium vanadate, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Lysates were cleared by a 10,000 × g ultracentrifugation at 4°C for 30 min and mixed with 10 μl of protein A–Sepharose (Pharmacia) and 3 μl of anti-SHP-2, anti-SHP-1, anti-gp130, or anti-STAT3 antibodies, followed by an 8 hr incubation at 4°C. The beads were washed five times with 1 ml of the lysis buffer in the absence of protease inhibitors. The immunoprecipitates were eluted with Laemmli's SDS loading buffer, separated on a 4%–20% SDS–polyacrylamide gradient gel, and transferred to a PVDF membrane Immobilon P (Millipore). The membranes were incubated with either 1:2000 diluted anti-SHP-2 antibody, 1:2000 diluted anti-SHP-1 antibody, 1:2000 diluted anti-STAT3 antibody, or 0.1 μg/ml anti-phosphotyrosine antibody (4G10) for 1 hr at room temperature, washed three times with TBST (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20), and then incubated with 1:2000 diluted horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse antibodies

(TAGO). The immune complex was visualized using a chemiluminescence system (Renaissance, DuPont New England Nuclear). The peptide binding assay was performed by mixing 5 μg of biotinylated peptides and lysates derived from 1×10^7 transfectant cells. The bound fraction was recovered with 10 μl of streptavidin–agarose (Calbiochem) and analyzed by immunoblotting with anti-SHP-2 or anti-SHP-1 antibodies. Rabbit antibodies for the extracellular domain of the human G-CSFR were raised by immunizing a rabbit with the extracellular domain of the G-CSFR fused to the Fc region of human IgG, previously expressed in 293T cells. The anti-STAT3 antibody (Nakajima et al., 1996) and the anti-gp130 antibody (Hibi et al., 1990) have been described previously. Anti-SHP-2, anti-SHP-1, and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology, and the anti-phosphotyrosine monoclonal antibody, 4G10, was purchased from UBI.

MAP Kinase Assay

Cells (1×10^7) were harvested after stimulation and suspended with 1 ml of lysis buffer (0.5% NP-40, 50 mM Tris–HCl [pH 7.4], 250 mM NaCl, 1 mM sodium vanadate, 5 mM β-glycerol phosphate, 3 mM EDTA, and protease inhibitors). Lysates were cleared by ultracentrifugation and mixed with 3 μl of anti-ERK2 antibody and 10 μl of protein A–Sepharose. After 3 hr of incubation at 4°C, the beads were washed three times with 1 ml of the lysis buffer in the absence of protease inhibitors and twice with 1 ml of kinase buffer (20 mM Tris–HCl [pH 7.4], 20 mM $MgCl_2$, and 5 mM β-glycerol phosphate). The beads were finally suspended with 20 μl of the kinase buffer containing 10 μM cold ATP, 5 μCi of [γ-^{32}P]ATP, and 1 μg of myelin basic protein, and incubated at 30°C for 20 min. The reactions were stopped by the addition of Laemmli's SDS loading buffer, and phosphorylated myelin basic protein was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by the image analyzer BAS2000 (Fuji Film Co.).

Cell Cycle Analysis

After stimulation, 2×10^5 cells were washed once with ice-cold saline, suspended with 100 μl of Dulbecco's phosphate-buffered saline (DPBS), and fixed by the addition of 900 μl of ethanol. Cells were incubated at −20°C for 20 min, pelleted, resuspended with 300 μl of staining buffer (1 mg/ml RNase, 20 μg/ml propidium iodine, 0.01% NP-40 in DPBS), and incubated at 37°C for 10 min. The DNA contents in the nucleus of the cells were analyzed using a cell sorter (FACSort, Becton Dickinson).

DNA Fragmentation Assay

After stimulation, 1×10^6 cells were harvested and suspended with a lysis buffer (0.5% sodium N-lauroylsarcosinate, 50 mM Tris–HCl [pH 7.8], 10 mM EDTA). Lysates were incubated with 0.5 mg/ml RNase A at 50°C for 30 min and with 0.5 mg/ml proteinase K at 50°C for 60 min. DNA-containing lysates were cleared by centrifugation at 5000 × g for 10 min and separated by TBE 2% agarose.

Northern Blotting Analysis

The extraction of total RNA was performed by using the TRIzol reagent (GIBCO BRL) according to the procedures recommended by the manufacturer. We separated 20 μg of total RNA from cells in each condition by 1% agarose–formaldehyde gels and transferred them to Hybond N+ (Amersham) nylon filters. Filters were hybridized in a hybridization buffer (0.5 M $NaPO_4$ [pH 7.0], 1 mM EDTA, 7% SDS, 1% BSA) at 65°C for 12–16 hr and washed with 2× SSC, 0.1% SDS for 10 min and with 0.1× SSC, 0.1% SDS for 40 min at 65°C and subjected to autoradiography. The probes used here were mouse *Stat3* (Nakajima et al., 1996), mouse *bcl-2* (2.5 kb, HindIII fragment; a gift from Dr. Tsujimoto), mouse *c-myc* (1.75 kb, PvuII fragment containing the second and third exons), and CHO-B (0.6 kb, EcoRI–BamHI fragment).

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References

Adachi, M., Fischer, E.H., Ihle, J., Imai, K., Jirik, F., Neel, B., Pawson, T., Shen, S.-H., Thomas, M., Ullrich, A., and Zhao, Z. (1996). Mammalian SH2-containing protein tyrosine phosphatases. Cell *85*, 15.

Akira, S., Nishio, Y., Inoue, M., Wang, X.J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994). Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell *77*, 63–71.

Ali, S., Chen, Z., Lebrun, J.-J., Vogel, W., Kharitonenkov, A., Kelly, P.A., and Ullrich, A. (1996). PTP1D is a positive regulator of the prolactin signal leading to β-casein promoter activation. EMBO J. *15*, 135–142.

Bazan, J.F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA *87*, 6934–6938.

Bennett, A.M., Tang, T.L., Sugimoto, S., Walsh, C.T., and Neel, B.G. (1994). Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor β to Ras. Proc. Natl. Acad. Sci. USA *91*, 7335–7339.

Bennett, A.M., Housdorff, S.F., O'Reilly, A.M., Freeman, R.O., Jr., and Neel, B.G. (1996). Multiple requirements for SHPTP2 in epidermal growth factor-mediated cell cycle progression. Mol. Cell. Biol. *16*, 1189–1202.

Boulton, T.G., Stahl, N., and Yancopoulos, G.D. (1994). Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J. Biol. Chem. *269*, 11648–11655.

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.

Daniel, N.N., Pernis, A., and Rothman, P.B. (1995). Jak-STAT signaling induced by the v-abl oncogene. Science *269*, 1875–1877.

Darnell, J.J., 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.

Durbin, J.E., Hachenmiller, 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.

Freeman, R.M., Jr., Plutzky, J., and Neel, B.G. (1992). Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. Proc. Natl. Acad. Sci. USA *89*, 11239–11243.

Friedmann, M.C., Migone, T.S., Russel, S.M., and Leonard, W.J. (1996). Different interleukin 2 receptor β-chain tyrosines couple to at least two signaling pathways and synergistically mediate interleukin 2-induced proliferation. Proc. Natl. Acad. Sci. USA *93*, 2077–2082.

Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J.N., Minami, Y., Miyazaki, T., and Taniguchi, T. (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.

Fujitani, Y., Nakajima, K., Kojima, H., Nakae, K., Takeda, T., and Hirano, T. (1994). Transcriptional activation of the IL-6 response element in the junB promoter is mediated by multiple Stat family proteins. Biochem. Biophys. Res. Commun. *202*, 1181–1187.

Fukunaga, R., Ishizaka Ikeda, E., Pan, C.X., Seto, Y., and Nagata, S. (1991). Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. *10*, 2855–2865.

Gobert, S., Chretein, S., Gouilleux, F., Muller, O., Pallard, C., Dusanter-Fourt, I., Groner, B., Lacombe, C., Gisselbrecht, S., and Mayeux, P. (1996). Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5. EMBO J. *15*, 2434–2441.

Haegeman, G., Content, J., Vorckaert, G., Derynck, R., Tavernier, J., and Fiers, W. (1986). Structural analysis of the sequence encoding for an inducible 26-kDa protein in human fibroblasts. Eur. J. Biochem. *159*, 625–632.

Herbst, R., Carroll, R.M., Allard, J.D., Schilling, J., Raabe, T., and Simon, M.A. (1996). Daughter of Sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during Sevenless signaling. Cell *85*, 899–909.

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990). Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell *63*, 1149–1157.

Hibi, M., Nakajima, K., and Hirano, T. (1996). Il-6 cytokine family and signal transduction: a model of cytokine system. J. Mol. Med. *74*, 11–12.

Hirano, T. (1994). Interleukin-6. In The Cytokine Handbook, A. Thomson, ed. (London: Academic Press), pp. 145–168.

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwamura, S., Nakajima, K., Koyama, K., Iwamatsu, A., Tsunasawa, S., Matsui, H., Takahara, Y., Taniguchi, T., and Kishimoto, T. (1986). Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature *324*, 73–76.

Hirano, T., Matsuda, T., and Nakajima, K. (1994). Signal transduction through gp130 that is shared among the receptors for the interleukin 6 related cytokine subfamily. Stem Cells *12*, 262–277.

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

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

Ip, N.Y., Nye, S.H., Boulton, T.G., Davis, S., Taga, T., Li, Y., Birren, S.J., Yasukawa, K., Kishimoto, T., Anderson, D.J., Stahl, N., and Yancopoulos, G.D. (1992). CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell *69*, 1121–1132.

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

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

Kinoshita, T., Yokota, T., Arai, K., and Miyajima, A. (1995). Suppression of apoptotic death in hematopoietic cells by signaling through the IL-3/GM-CSF receptors. EMBO J. *14*, 266–275.

Klingmuller, U., Lorenz, U., Cantley, L.C., Neel, B.G., and Lodish, H.F. (1995). Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell *80*, 729–738.

Kumar, G., Gupta, S., Wang, S., and Nel, A.E. (1994). Involvement of Janus kinases, p52shc, Raf-1, and MEK-1 in the IL-6-induced mitogen-activated protein kinase cascade of a growth-responsive B cell line. J. Immunol. *153*, 4436–4447.

Li, W., Nishimura, R., Kashishian, A., Batzer, A.G., Kim, W.J., Cooper, J.A., and Schlessinger, J. (1994). A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase. Mol. Cell. Biol. *14*, 509–517.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*, 431–442.

Lutticken, C., Wegenka, U.M., Yuan, J., Buschmann, J., Schindler, C., Ziemiecki, A., Harpur, A.G., Wilks, A.F., Yasukawa, K., Taga, T., Kishimoto, T., Barbirri, G., Pellegrini, S., Sendtner, M., Heinrich, P.C., and Horn, F. (1994). Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. Science *263*, 89–92.

Matsuda, T., Yamanaka, Y., and Hirano, T. (1994). Interleukin-6-induced tyrosine phosphorylation of multiple proteins in murine hematopoietic lineage cells. Biochem. Biophys. Res. Commun. *200*, 821–828.

Matthews, R.J., Bowne, D.B., Flores, E., and Thomas, M.L. (1992). Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. Mol. Cell. Biol. *12*, 2396–2405.

Meraz, M.A., White, J.M., Sheedhan, K.C.F., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Gleenlund, A.C., Campbell, D., Carver-Moore, K., DuBois, R.N., Clark, R., Aguet, M., and Schreiber R.D. (1996). Targeted disruption of the mouse *Stat1* gene in mice reveals unexpected physiologic specificity in the JAK–STAT signaling pathway. Cell *84*, 431–442.

Migone, T.S., Lin, J.X., Cereseto, A., Mulloy, J.C., O'Shea, J.J., Franchini, G., and Leonard, W.J. (1995). Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I. Science *269*, 79–81.

Miyazaki, T., Liu, Z.J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E.L., Permutter, R.M., and Taniguchi, T. (1995). Three distinct IL-2 signaling pathways mediated by *bcl-2*, c-*myc*, and *lck* cooperate in hematopoietic cell proliferation. Cell *81*, 223–231.

Mui, A.L., Wakao, H., Kinoshita, T., Kitamura, T., and Miyajima, A. (1996). Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J. *15*, 2425–2433.

Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991). Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA *88*, 11349–11353.

Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T., and Kishimoto, T. (1993). IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science *260*, 1808–1810.

Nakajima, K., Matsuda, T., Fujitani, Y., Kojima, H., Yamanaka, Y., Nakae, K., Takeda, T., and Hirano, T. (1995). Signal transduction through IL-6 receptor: involvement of multiple protein kinases, stat factors, and a novel H7-sensitive pathway. Ann. NY Acad. Sci. *762*, 55–70.

Nakajima, K., Yamanaka, Y., Nakae, K., Kojima, H., Ichiba, M., Kiuchi, N., Kitaoka, T., Fukada, T., Hibi, M., and Hirano, T. (1996). A central role for Stat3 in IL-6-induced regulation of growth and differentiation in M1 leukemia cells. EMBO J. *15*, 3651–3658.

Narazaki, M., Witthuhn, B.A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J.N., Kishimoto, T., and Taga, T. (1994). Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc. Natl. Acad. Sci. USA *91*, 2285–2289.

Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994). Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. Mol. Cell. Biol. *14*, 6674–6682.

Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paoni, N.F., and Wood, W.I. (1995). Cardiotrophin-1: biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. J. Biol. Chem. *270*, 10915–10922.

Perkins, L.A., Larsen, I., and Perrimon, N. (1992). *corkscrew* encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. Cell *70*, 225–236.

Plutzky, J., Neel, B.G., and Rosenberg, R.D. (1992). Isolation of a src homology 2-containing tyrosine phosphatase. Proc. Natl. Acad. Sci. USA *89*, 1123–1127.

Raabe, T., Riesgo-Escovar, J., Liu, X., Bausenwein, B.S., Deak, P., Maröy, P., and Hafen, E. (1996). DOS, a novel pleckstrin homology domain–containing protein required for signal transduction between Sevenless and Ras1 in Drosophila. Cell *85*, 911–920.

Sadowski, H.B., Shuai, K., Darnell, J.E., Jr., and Gilman, M.Z. (1993). A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science *261*, 1739–1744.

Sato, N., Sakamaki, K., Terada, N., Arai, K., and Miyajima, A. (1993). Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common β subunit responsible for different signaling. EMBO J. *12*, 4181–4189.

Sehgal, P.B., Grieninger, G., and Tosato, G., eds. (1989). Regulation of the acute phase an immune responses: interleukin-6. Ann. NY Acad. Sci. *557*, 1–580.

Shen, S.H., Bastien, L., Posner, B.I., and Chretien, P. (1991). A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases Nature *352*, 736–739. Erratum: Nature *353*, 868.

Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K., and Taniguchi, T. (1992). IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-*myc*. Cell *70*, 57–67.

Shimoda, K., van Deursen, J., Sangster, M.Y., Sarwar, S.R., Carson, R.T., Tripp, R.A., Chu, C., Quelle, F.W., Nosaka, T., Vignali, D.A.A., Doherty, P.C., Grosveld, G., Paul, W., and Ihle, J.N. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature *380*, 630–633.

Stahl, N., Boulton, T.G., Farruggella, T., Ip, N.Y., Davis, S., Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J.N., and Yancopoulos, G.D. (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6β receptor components. Science *263*, 92–95.

Stahl, N., Farruggella, T.J., Boulton, T.G., Zhong, Z., Darnell, J.J., and Yancopoulos, G.D. (1995). Choice of STATs and other substrates specified by modular tyrosine based motifs in cytokine receptors. Science *267*, 1349–1353.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989). Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell *58*, 573–581.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996). Essential role of Stat6 in IL-4 signaling. Nature *380*, 627–630.

Tang, T.L., Freeman, R.M., Jr., O'Reilly, A.M., Neel, B.G., and Sokol, S.Y. (1995). The SH2-containing protein-tyrosine phosphatase SH-PTP2 is required upstream of MAP kinase for early Xenopus development. Cell *80*, 473–483.

Thierfelder, W.E., van Deursen, J.M., Yamamoto, K., Tripp, R.A., Sarawar, S.R., Carson, R.T., Sangster, M.Y., Vignali, D.A.A., Doherty, P.C., Grosveld, G.C., and Ihle, J.N. (1996). Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature *382*, 171–174.

Van Snick, J. (1990). Interleukin-6: an overview. Annu. Rev. Immunol. *8*, 253–278.

Yamanaka, Y., Nakajima, K., Fukada, T., Hibi, M., and Hirano, T. (1996). Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. EMBO J. *15*, 1557–1565.

Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., and Kishimoto, T. (1988). Cloning and expression of the human interleukin-6 (BSF-2/IFNβ2) receptor. Science *241*, 825–828.

Yan, R., Small, S., Desplan, C., Dearolf, C.R., and Darnell, J.E. (1996). Identification of a *Stat* gene that functions in Drosophila development. Cell *84*, 421–430.

Yi, T., Mui, A.L., Krystal, G., and Ihle, J.N. (1993). Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor β chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. Mol. Cell. Biol. *13*, 7577–7586.

Yin, T., Taga, T., Tsang, M.L., Yasukawa, K., Kishimoto, T., and Yang, Y.C. (1993). Involvement of IL-6 signal transducer gp130 in IL-11-mediated signal transduction. J. Immunol. *151*, 2555–2561.

Yu, C.L., Meyer, D.J., Campbell, G.S., Larner, A.C., Carter-Su, C., Schwartz, J., and Jove, R. (1995). Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science *269*, 81–83.

Zilberstein, A., Ruggieri, R., Korn, J.H., and Revel, M. (1986). Structure and expression of cDNA and genes for human interferon-β2, a distinct species inducible by growth-stimulatory cytokines. EMBO J. *5*, 2529–2537.