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STAT3 orchestrates contradictory signals in cytokine-induced G_1 to S cell-cycle transition

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The signal transducer and activator of transcription molecules (STATs) play key roles in cytokine-induced signal transduction. However, their role in cell growth has not been clear. In the present study, we show that STAT3 plays a key role in the G_1 to S phase cell-cycle transition induced by the cytokine receptor subunit gp130, through the upregulation of cyclins D2, D3 and A, and cdc25A, and the concomitant downregulation of p21 and p27. Furthermore, unexpectedly, we found that gp130 could induce the expression of p21 when STAT3 activation was suppressed. Such contradictory signals regulating cell-cycle progression could be simultaneously delivered from distinct cytoplasmic regions of gp130. We propose an 'orchestrating model' for cytokine and growth factor action in which contradictory signals are orchestrated to produce a specific effect in a target cell.

Keywords: cell cycle/cytokine/gp130/signal transduction/STAT3

Introduction

The signal transducers and activators of transcription (STATs) comprise a family of functionally related proteins that play key roles in a variety of biological activities such as cell differentiation and proliferation. They exert their activities through cytokine and growth factor receptors, and are believed to be involved in determining the biological specificity of specific cytokines on various target tissues (Darnell *et al*., 1994; Ihle and Kerr, 1995; Ihle, 1996; Darnell, 1997). STATs have been suggested to be involved in cell growth: the constitutive activation of STATs has been shown in cells transformed by human T-cell leukaemia virus type 1 (HTLV-1), *v-src*, *abl* and *bcr-abl* (Migone *et al.*, 1995; Yu *et al.*, 1995; Carlesso *et al.*, 1996; Bromberg *et al.*, 1998; Turkson *et al*., 1998); STAT5 has been suggested to be involved in interleukin (IL)-3-induced cell growth (Muli *et al*., 1996); and lymphocytes obtained from STAT6- and STAT4-deficient mice do not grow in response to IL-4 and IL-12, respectively (Kaplan *et al*., 1996; Shimoda *et al*., 1996; Takeda *et al*., 1996; Thierfelder *et al*., 1996). STAT3 is required for gp130-mediated cell-survival signals (Fukada *et al*., 1996). Recently, it was reported that activation of STAT3 is required for cell transformation by Src oncoprotein (Bromberg *et al*., 1998; Turkson *et al*., 1998). These observations suggest that although STATs play essential roles in cell growth, their exact function in cell cycle progression has not been defined.

The molecular events that underlie cell proliferation are tightly regulated in a cell cycle-dependent manner. The cell division cycle is coordinated by the activation and inactivation of the cyclin-dependent kinases (CDKs) (Hunter and Pines, 1994; Sherr, 1996; Hunter, 1997). The G_1 to S phase transition in the cell cycle is thought to be controlled by CDKs that are sequentially regulated by cyclins D, E and A. These CDKs are positively and negatively regulated. They are activated by the dephosphorylation of threonine and tyrosine residues (e.g. Thr14 and Tyr15 of CDK2) by Cdc25s, phosphatases that are upregulated in the G_1 to S transition. On the other hand, CDKs are negatively regulated by two distinct families of CDK inhibitors. One family, known as the INK4-family, which includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, inhibits CDK4 and CDK6. The other family includes $p21^{CIP}$, $p27^{KIP1}$ and $p57^{KIP2}$, which bind and inhibit cyclin D-, E- and A-dependent kinases. However, it has not been clear how the cytokine receptor signals regulate the CDKs and their regulators.

gp130 is a common subunit in the receptors for the IL-6 cytokine family, including leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11 and cardiotrophin-1 (CT-1) (Hirano *et al.*, 1997; Hirano, 1998). Ligand binding to gp130 induces the activation of gp130-associated Janus kinases (JAKs) JAK1, JAK2 and TYK2, resulting in the phosphorylation of specific tyrosine residues in both gp130 and JAKs (Lutticken *et al*., 1994; Stahl *et al*., 1994). This process induces at least three distinct signal transduction pathways: one is generated directly by JAKs, another—the SHP-2/RAS/MAPK pathway—is induced by the phosphorylation of gp130 on Tyr759, and a third is generated via STAT3 activation through the phosphorylation of any one of the four tyrosines (Y767, Y814, Y905, Y915) in the C-terminal region of gp130 (Stahl *et al*., 1995; Fukada *et al*., 1996; Nakajima *et al*., 1996; Yamanaka *et al*., 1996). We previously demonstrated various roles for STAT3 in the gp130-mediated signalling involved in cell differentiation, survival and neurite outgrowth (Fukada *et al*., 1996; Nakajima *et al*., 1996; Yamanaka *et al*., 1996; Ihara *et al.*, 1997; also see review by Hirano *et al.*, 1997).

In the case of gp130-mediated growth signalling, the membrane-proximal region of gp130, consisting of 133 amino acid residues, is required and sufficient for ligand-

mediated cell growth (Fukada *et al*., 1996). This region of gp130 contains Tyr759 and Tyr767, which are required for activation of the SHP-2/RAS/MAPK pathway and STAT3, respectively. We also showed that Tyr759 is required for the gp130-induced S to G_2/M cell-cycle transition and, moreover, that STAT3 is essential for gp130-mediated cell survival, most likely by inducing *bcl2* gene expression (Fukada *et al*., 1996). However, whether STAT3 has any roles for cell-cycle progression remains to be clarified. Here, we establish that STAT3 also plays an important role in the G_1 to S phase transition through the upregulation of cyclins D2, D3 and A, and cdc25A, and the concomitant downregulation of p21 and p27. Furthermore, gp130 could induce gene expression of p21 through the membrane-proximal region when STAT3 activation was suppressed. Our findings suggest that such contradictory signals regulating cell-cycle progression could be simultaneously delivered from distinct cytoplasmic regions of gp130, and a regulator—STAT3 in this case—orchestrates or regulates the balance of contradictory signals through cytokine and growth factor receptors.

Results

STAT3 is necessary for gp130-mediated G_1 to S phase of cell-cycle transition

To study the molecular mechanisms by which gp130 induces cell growth, we used a factor-dependent BAF/B03 pro-B cell line that expresses a chimeric receptor consisting of the extracellular domain of the G-CSF receptor, and the transmembrane and the cytoplasmic domains of gp130. As mentioned above, the membrane-proximal region of gp130, consisting of 133 amino acid residues is required and sufficient for ligand-mediated cell growth (Fukada *et al*., 1996). This region of gp130 contains Tyr767 that is required for the activation of STAT3, and STAT3 is essential for gp130-mediated cell survival, accompanied by inducing *bcl2* gene expression (Fukada *et al*., 1996). As shown in Figure 1A, stable expression of *bcl2* in the cell line expressing the chimeric receptor consisting of the membrane-proximal 68 amino acid residues (G68) or that containing 133 amino acid residues with a mutation at Tyr767 to phenylalanine (G133F3), both of which failed to activate STAT3 (Fukada *et al*., 1996), could substitute the requirement of STAT3 activation for cell-survival signal (G68 versus G68-bcl2 and G133F3 versus G133F3-bcl2). The same was true in the transfectants expressing both dominant-negative forms of STAT3, STAT3D and STAT3F (Figure 1B; G133-STAT3D and G133-STAT3F versus G133-bcl2-STAT3D and G133-bcl2-STAT3F); we therefore investigated the role of STAT3 in cell growth utilizing the stable transfectants expressing both the chimeric receptor consisting of the membrane-proximal 133 amino acid residues and *bcl2* (G133-bcl2). Intriguingly, Tyr767 was still required for cell growth (Figure 1A; G133F3-bcl2), suggesting a role of STAT3 in cell-cycle progression. Actually, both dominant-negative forms of STAT3 inhibited gp130-induced cell growth (Figure 1B). The cells expressing chimeric receptor mutated at Tyr767 (G133F3-bcl2) or that with the membrane-proximal 68 amino acid residues (G68-bcl2) remained in G_1 (Figure 1C) and more importantly, both dominant-negative forms of STAT3 inhibited the entry of the cells to S phase upon the stimulation of gp130 (Figure 1C; G133-bcl2-STAT3D, G133-bcl2-STAT3F). The results clearly indicated that STAT3 activation was essential for G_1 to S cell cycle transition.

STAT3 is involved in the induction of CDK activators; cyclins D and A and cdc25A

The G_1 to S cell-cycle transition is regulated by the activation of a catalytic complex of CDKs, which requires the formation of complexes with G_1 cyclins (Sherr, 1996; Hunter, 1997). In fact, gp130 stimulation induced cyclins D2 and D3, which are both required for the activation of CDK4 and CDK6 and are involved in the early to late G_1 transition, and cyclin A, which binds to CDK2 and is required for the G_1 to S phase transition (Figure 2A–C). These inductions required the membrane-proximal 133 amino acid residues (Figure 2A–C; G133-bcl2 versus G68-bcl2) and Tyr767 (Figure 2A–C; G133-bcl2 versus G133F3-bcl2) of gp130. Furthermore, dominant-negative forms of STAT3 inhibited these inductions (Figure 2A–C; G133-bcl2 versus G133-bcl2-STAT3D and G133-bcl2-STAT3F). These observations showed that the induction of both cyclins D and A required the activation of STAT3. The kinase activity of these complexes is also positively regulated by the phosphatase cdc25A (Hunter, 1997). As shown in Figure 2D, the induction of cdc25A was totally dependent on Tyr767 and STAT3 activation (G133-bcl2 versus G133F3-bcl2, and G133-bcl2 versus G133-bcl2-STAT3D and G133-bcl2-STAT3F). These results show that STAT3 is involved in the upregulation of positive regulators for the G_1 CDKs.

STAT3 is involved in downregulation of CDK inhibitors

Since the activity of cyclin/CDK complexes is negatively regulated by several CDK inhibitors (Sherr, 1996; Hunter, 1997), we next investigated the effect of gp130 and STAT3 on the expression of two such inhibitors, p21 and p27. Stimulation by gp130 scarcely induced p21 expression in G133-bcl2 cells (Figure 3A), consistent with the fact that gp130 induces growth. However, surprisingly, the truncated gp130 containing only the membrane-proximal 68 amino acid residues (G68-bcl2) could induce p21 mRNA. The same was true of cells expressing gp130 mutated at Tyr767 (G133F3-bcl2) or expressing dominant-negative STAT3s (G133-bcl2-STAT3F, G133-bcl2-STAT3D) (Figure 3A). These observations indicate that the region of gp130 consisting of the membrane-proximal 68 amino acid residues can induce p21 expression, and that this induction can be suppressed by activated STAT3, suggesting a dual role for STAT3 in the G_1 to S cell-cycle transition. A similar role for STAT3 was also observed in p27 expression. As shown in Figure 3B, p27 mRNA accumulated in G_1-arrested G133-bcl2 cells, and this accumulation was downregulated by gp130 stimulation. However, the downregulation of p27 did not occur in G68-bcl2 cells. As was seen for p21, the downregulation of p27 was totally dependent on Tyr767 and STAT3 activation (Figure 3B). Consistent with the changes in mRNAs observed, the protein levels of both p21 and p27 were also negatively regulated by STAT3 and this downregulation was also dependent on Tyr767 and inhibited by dominant-negative STAT3s (Figure 3C). These

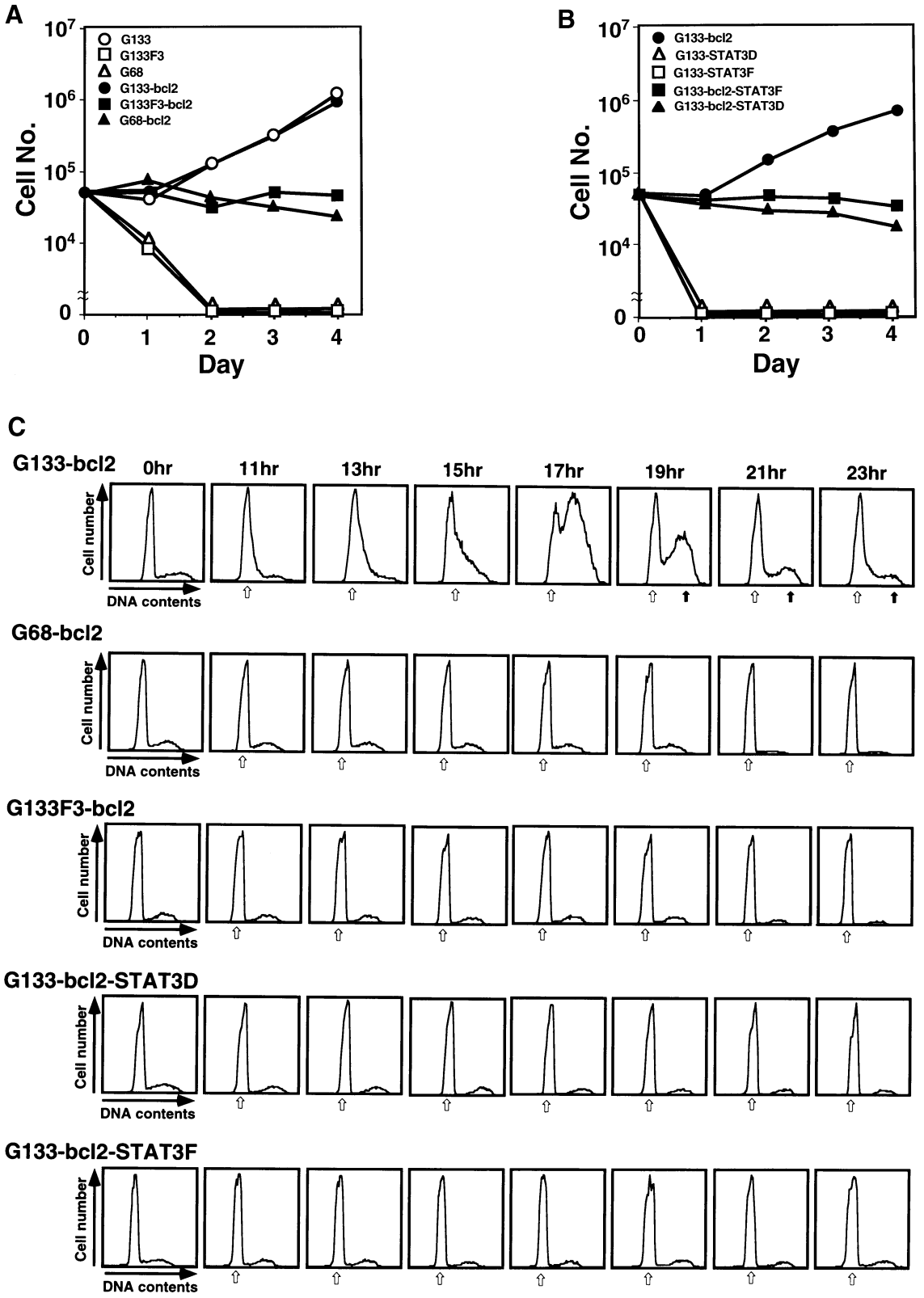

Fig. 1. STAT3 is necessary for the gp130-mediated G_1 to S cell-cycle phase transition. (**A**) Expression of bcl2 substitutes for the cell survival, but not the cell proliferation, signals that are normally elicited through Tyr767 of gp130. 5×10^4 of BAF/B03 transfectant cells expressing the chimeric receptor (G133, ○; G133F3, □; G68, △), or the receptor and bcl2 (G133-bcl2, ●; G133F3-bcl2, ■; G68-bcl2, ▲) were cultured in 1 ml medium containing 100 ng/ml of G-CSF, for 4 days. The number of viable cells was then counted on the day indicated. (**B**) STAT3 is required for cell proliferation. BAF/B03 transfectants expressing G133 and bcl2 (●), G133 and dominant-negative STAT3s (G133-STAT3D, △; G133-STAT3F, □), or G133, bcl2 and dominant-negative STAT3s (G133-bcl2–STAT3D, ▲; G133-bcl2–STAT3F, ■) were cultured in the presence of G-CSF. The cell number was counted on the day indicated. (**C**) The gp130-induced G_1 to S phase cell-cycle phase transition was dependent on STAT3 activation. BAF/B03 transfectants were cultured in the presence of G-CSF for the indicated times and subjected to cell-cycle analysis. The open arrows indicate the G_1/G_0 phase and the closed arrows the G_2/M phase of the cell cycle.

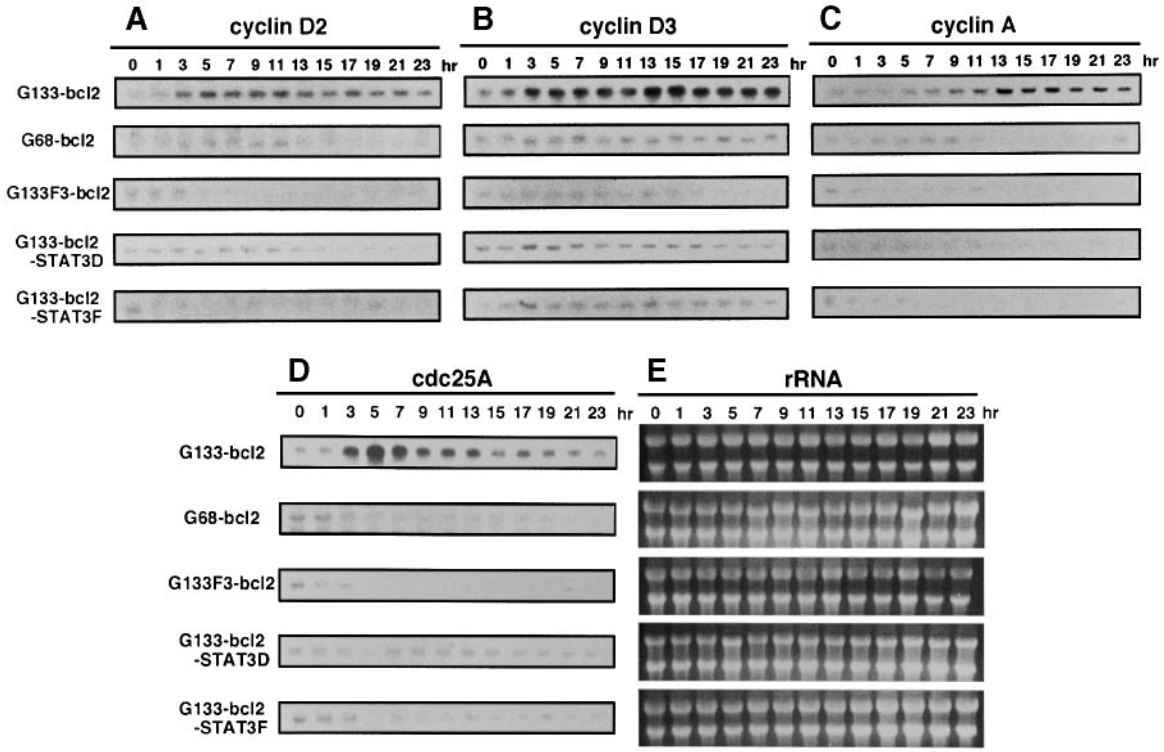

Fig. 2. STAT3 is involved in the induction of cyclins D and A, and cdc25A. BAF/B03 transfectants were cultured with G-CSF for the indicated times. Total RNA (20 μg) was isolated and subjected to Northern blotting with cyclin D2 (**A**), D3 (**B**) and A (**C**) and cdc25A (**D**) cDNA probes. (**E**) Loading controls. The RNA (20 μg for each condition) used for Northern blotting was subjected to electrophoresis and then stained with ethidium bromide. The two major bands represent the 28S and 18S ribosomal RNAs.

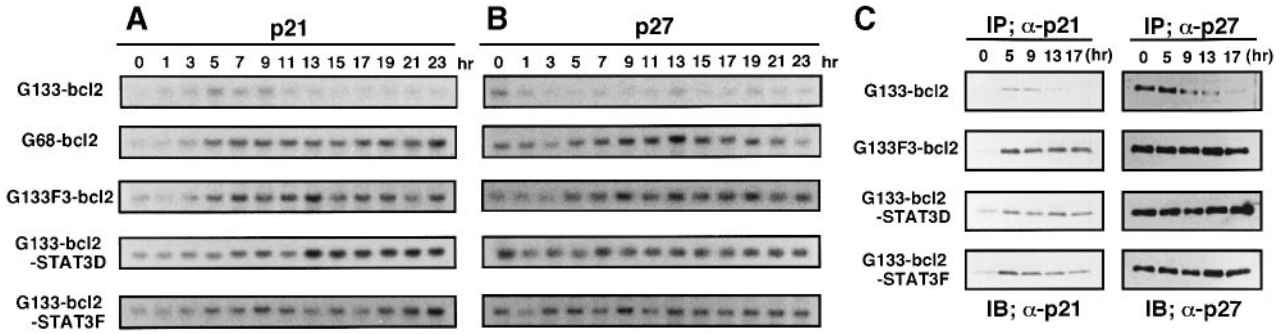

Fig. 3. STAT3 is involved in the downregulation of CDK inhibitors. The expression of p21 and p27 are downregulated by STAT3. The same filters used in Figure 2 were reprobed with (**A**) p21 and (**B**) p27 cDNAs. (**C**) Protein levels of p21 and p27. Transfectants were stimulated with G-CSF for the indicated times. CDK inhibitors were then immunoprecipitated from the cell lysates with anti-p21 (left panel) or p27 (right panel) antibodies, and analysed by immunoblotting using the respective antibodies as probes.

findings suggest that STAT3 is involved in downregulation of CDK inhibitors.

gp130-induced activation of CDK4, CDK6 and CDK2 depends on STAT3 activation

Our results indicate that STAT3 plays a critical role in the G_1 to S phase transition by upregulating activators and downregulating inhibitors for the G_1 cyclin/CDK complexes, suggesting that the induction of catalytic activity of G_1 CDKs by gp130 stimulation is dependent on STAT3 activation. Indeed, as shown in Figure 4A and B, gp130 induced the activation of CDK4, CDK6 and CDK2. In each case, activation required the integrity of the critical Tyr767 residue and was inhibited by both STAT3F and STAT3D.

These data clearly show that the induction by gp130 of the activation of G_1 CDKs is totally dependent on STAT3 activation.

Discussion

We demonstrate that STAT3 plays crucial roles in the G_1 to S cell-cycle transition, and emphasize the following two findings. First, a cytokine receptor gp130 could simultaneously induce contradictory signals regulating cell-cycle progression from distinct cytoplasmic regions; the region containing the membrane-proximal 68 amino acid residues can induce G_1 cyclin/CDK inhibitors, p21, while that containing the 133 amino acid residues elicits cyclin D, A and cdc25A in a manner dependent on STAT3

activation. Second, STAT3 orchestrates contradictory signals in gp130-induced G_1 to S cell-cycle transition through the upregulation of cyclin D, A and cdc25A, and the concomitant downregulation of p21 and p27 (Figure 5). The results were consistent with the data that activation of CDK2, 4 and 6 was dependent on STAT3 activation.

Many growth factors and cytokines have been shown to regulate activators and/or inhibitors for CDKs. However, dissection of signal transduction pathways for regulation of CDKs from receptors has not yet been clarified. In cytokine signalling, it was reported that IL-2 induces expression of cyclin D3 and degradation of p27 through PI-3 kinase and its downstream Akt in certain cell types (Brennan *et al*., 1997). In contrast, our present data indicate that STAT3 in gp130 signalling exhibits a similar role as the PI-3 kinase pathway in IL-2 signalling. This may reflect utilities of distinct signalling pathways by different cytokines for cell proliferation: gp130 needs STAT3 activation for the G_1–S transition, whereas IL-2 dose not require the activation of STAT5, which acts downstream of the IL-2 receptor (Fujii *et al*., 1995). On the other hand, it was shown that cells derived from *Stat6*- and *Stat4*-disrupted mice expressed a high level of p27, while IL-4- and IL-12-induced downregulation of p27 was abrogated, indicating that STAT6 and STAT4 are also involved in the downregulation of p27 (Kaplan *et al*., 1998).

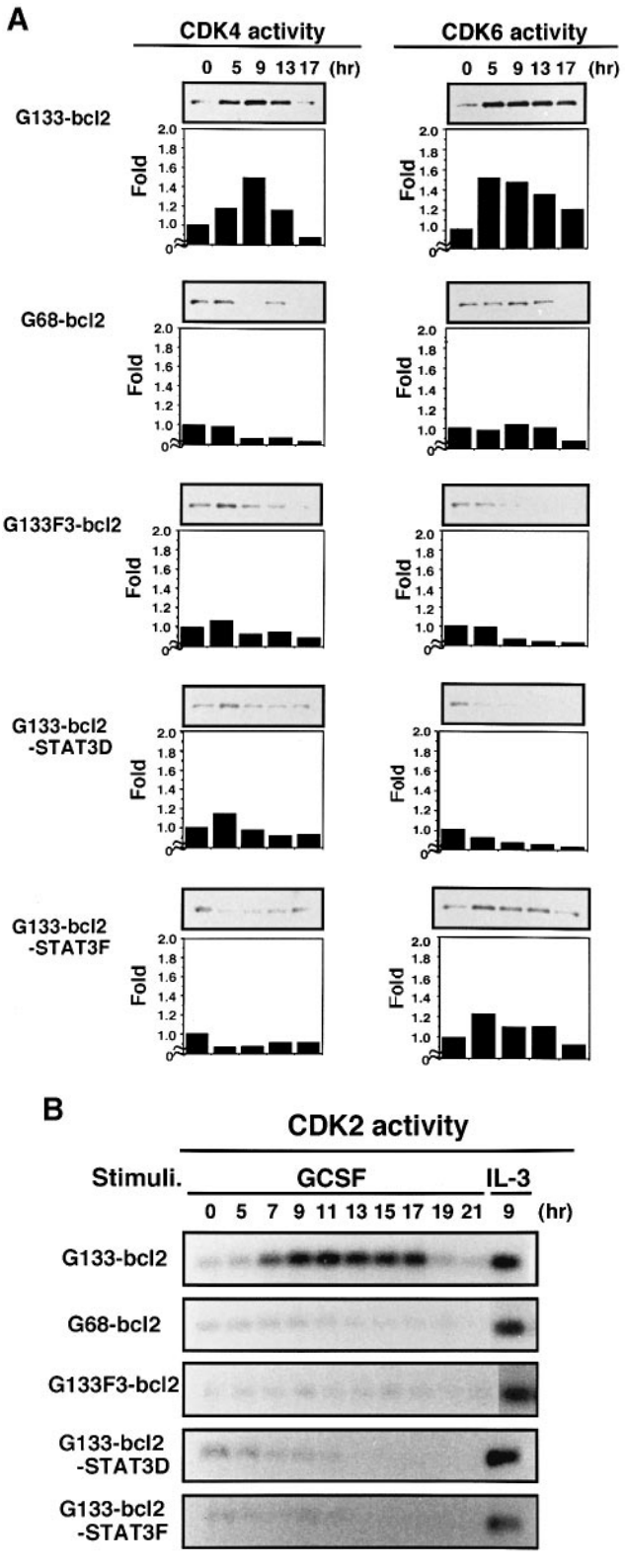

Fig. 4. The gp130-induced activation of CDK4, CDK6 and CDK2 depends on STAT3 activation. (**A**) The activation of CDK4, CDK6 depends on STAT3. BAF/B03 transfectants were stimulated with G-CSF for the indicated times. CDK4, CDK6 were then immunoprecipitated from the cell lysates and their kinase activities were assayed *in vitro* using GST-pRb. The phosphorylated substrates were separated by SDS–PAGE and analysed by autoradiography. The kinase activities shown in the graphs are expressed as described in Materials and methods. (**B**) The activation of CDK2 also depends on STAT3. BAF/B03 transfectants were stimulated with G-CSF or IL-3 for the indicated periods of time. CDK2 was then immunoprecipitated from the cell lysates and their kinase activities were assayed *in vitro* using histone H1 as substrates. The phosphorylated substrates were separated by SDS–PAGE and analysed by autoradiography.

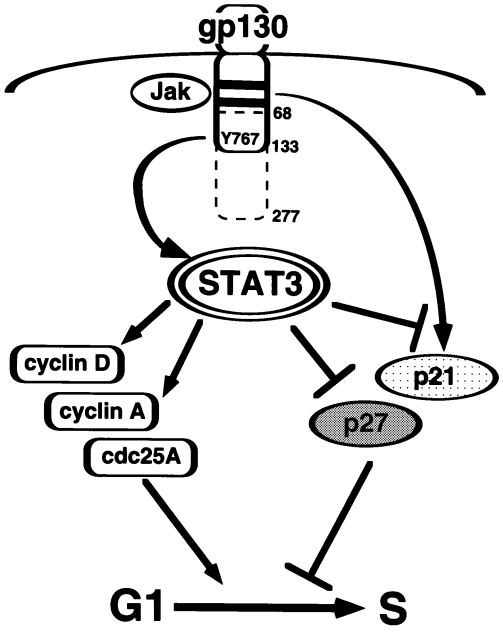

Fig. 5. An 'orchestrating model' for the contradictory signals elicited by cytokines and growth factors. Distinct cytoplasmic regions of a cytokine or growth factor receptor may generate contradictory signals, as in the case of gp130, and the resulting chaotic situation could be orchestrated by a regulator, for example STAT3, to effect a directed biological action, thus bringing order from chaos. Such a mechanism may underlie the functional pleiotrophy that is characteristic of cytokine and growth factor action.

Other STATs may also be involved in coordinated regulation of CDK activators and inhibitors.

Although expressions of cyclin D2, D3 and A, and cdc25A depended strictly on STAT3 activation, their time-courses were different from each other (Figure 2A–D). Expressions of cyclin Ds and cdc25A preceded that of cyclin A. This is consistent with the different roles of these CDK activators in the G_1–S transition (Sherr, 1996). This also suggests that mechanisms by which STAT3 controls the expression of these genes are different. Among these genes, cdc25A was reported to be a direct target of c-Myc protein (Galaktionov *et al*., 1996). In this respect, one of the possible target genes of STAT3 is the c-*myc* proto-oncogene, although this intriguing question remains to be answered. It will be interesting to identify molecules that act downstream of STAT3 and regulate expression of cyclin D2 and D3. On the other hand, the late expression of cyclin A is likely controlled by the preceding events, in which cyclin Ds and cdc25A are likely to be involved. Cyclin A is known to be a target of E2F/DP-family transcription factors (Amati *et al.*, 1998). As shown in Figure 4, CDK4 and 6 were activated prior to the expression of cyclin A, in correlating with the expression of cyclin D2 and D3 and cdc25A. These suggest that CDK4 and 6 induce gene expression of cyclin A through phosphorylating Rb protein and subsequently releasing E2F/DP transcription factors (Amati *et al.*, 1998). Mechanisms by which STAT3 represses p21 and p27 remain to be elucidated. It is not clear whether the repression occurs at the level of transcription or degradation of the mRNAs. However, our preliminary data indicate that the repression does not occur without *de novo* protein synthesis (data not shown). The repression of p21 and p27 are likely controlled by molecules whose expressions are regulated by STAT3.

Intriguingly, p21 was induced by a signal from the membrane-proximal region containing 68 amino acid residues of gp130. Not only gp130 stimulation, but also many cytokines repress cell-cycle progression in certain cells, and induce their differentiation. A variety of CDK inhibitors have been shown to be involved in these signal processes. Upregulation of p21 by interferon-γ and thrombopoietin (Chin *et al*., 1996; Matsumura *et al*., 1997), and that of p18, p19 and p21 by IL-6 (Morse *et al*., 1997; Narimatsu *et al*., 1997), has been suggested to be involved in cell-cycle arrest, linking to cell differentiation. Transcriptional activation of certain CDK inhibitors were reported to be directly controlled by STATs (Chin *et al*., 1996; Matsumura *et al*., 1997). Since the induction of p21 is not inhibited by a protein synthesis inhibitor (data not shown), the regulation of p21 expression is likely a direct target of a signal from the membrane-proximal region. We previously found that STAT5 is activated by direct interaction with JAKs, and the membrane-proximal region is sufficient for its activation (Fujitani *et al*., 1997). Activation of p21 by STAT5 was also reported in thrombopoietin signalling (Matsumura *et al*., 1997). These findings suggest that STAT5 may control the expression of p21, though further investigation will be required to clarify this point.

Although a given cytokine or growth factor can exert distinct biological activities—indeed, even completely opposite activities—on different target cells, it is believed that a cytokine can generate various signals that cooperatively exhibit a unified biological activity on a given target cell. However, our observations support the possibility that contradictory signals can be elicited simultaneously in a cell by a variety of cytokines or growth factors. Consistent with this, we previously reported that gp130-stimulation could simultaneously induce growth-enhancing and -suppressing signals in M1 cells (Nakajima *et al.*, 1996); gp130 can generate at the same time both positive and negative signals affecting neurite outgrowth in PC12 cells (Ihara *et al.*, 1997). Furthermore, TNF stimulation elicits simultaneously both apoptotic and anti-apoptotic signals (Liu *et al.*, 1996). All evidence suggests that the cytokine receptor can simultaneously induce contradictory intracellular signalling pathways, at least in certain cells (Hirano *et al.*, 1997). If this hypothesis were true, there should be a regulator, such as STAT3 in this case, that can orchestrate or regulate the balance of conflicting signals to express a unified output; otherwise, such chaotic situations might lead to a sterile end point (Figure 5). Such a regulator may be a transcription factor, protein kinase or phosphatase, for example. Furthermore, such a mechanism may at least in part underlie the functional pleiotropy that is a characteristic feature of cytokine and growth factor action. Although the generality of such a mechanism remains to be determined, this model is attractive enough to be examined further and may prove to be one of the important molecular mechanisms that govern the complex actions of cytokines and growth factors.

Materials and methods

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.

BAF-B03 transfectants expressing the chimeric receptors G133, G133F3 and G68 were previously described and maintained in the presence of G418 (Fukada *et al.*, 1996). To establish bcl2 transfectants, 50 μg of human bcl2 expression vector (pUC–CAGGS–bcl2, the gift of Dr Y.Tujimoto) was co-transfected with 5 μg of pMIK–Hyg (Fukada *et al.*, 1996) by an electroporation method. To establish transfectants expressing dominant-negative STAT3 genes, 50 μg of pCAGGS–STAT3D or pCAGGS–STAT3F (Nakajima *et al.*, 1996) was co-transfected with 5 μg of a Zeocin-resistance gene expression vector (modified from pVgRXR; Invitrogen). Transfectants were selected by 200 μg/ml of hygromycin for the bcl2 transfectants and/or 150 μg/ml of Zeocin for the dominant-negative STAT3 transfectants. Transfection-independent clones were established using a limiting dilution procedure. Expression levels of bcl2, STAT3D and STAT3F were analysed by Northern blotting.

Immunoprecipitation, immunoblotting and immunological reagents

Cells were starved of IL-3 for 12 h, after which 1×10^7 cells were stimulated with G-CSF (100 ng/ml) for the indicated periods of time, then suspended in 1 ml of lysis buffer (1% NP-40, 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 μg/ml aprotinin, 0.1 μM PMSF). Lysates were cleared by ultracentrifugation (10 000 *g*, 4°C for 20 min) and mixed with 10 μl of protein A–Sepharose (Pharmacia) and 3 μl of either anti-p21 (sc-398; Santa Cruz) or anti-p27 (sc-528; Santa Cruz), followed by an 8-h incubation at 4°C. The beads were washed five times with 1 ml of lysis buffer in the absence of protease inhibitors. The immunoprecipitates were eluted with Laemmli's SDS loading buffer, separated on a 15–25% SDS–polyacrylamide gradient gel, and transferred to a PVDF Immobilon P membrane (Millipore). The membranes were incubated with 2000× diluted anti-p21 or anti-p27 antibody for 2 h at room temperature, washed three times with TBST (20 mM Tris–HCl pH 7.4,

150 mM NaCl, 0.1% Tween20), then incubated with 5000× diluted horseradish peroxidase-conjugated goat anti-rabbit antibodies (TAGO). The immune complex was visualized using a chemiluminescence system (Renaissance; DuPont-NEN).

Cyclin-dependent kinase assay

1×10^7 cells were stimulated with either G-CSF (100 ng/ml) or IL-3 for the time indicated, then cells were harvested and suspended in 1 ml of lysis buffer [0.1% Tween-20, 50 mM HEPES pH 8.0, 150 mM NaCl, 0.1 mM sodium vanadate, 10 mM β-glycerophosphate, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 10% (v/v) glycerol, 5 μg/ml aprotinin, 0.1 μM PMSF]. Lysates were cleared by ultracentrifugation and mixed with 3 μl of anti-CDK4 (sc-260; Santa Cruz), anti-CDK6 (sc-177; Santa Cruz) or anti-CDK2 (sc-163; Santa Cruz) antibody and 10 μl of protein A–Sepharose. After a 6 h incubation at 4°C, the beads were washed twice with 1 ml of lysis buffer in the absence of protease inhibitors and twice with 1 ml of HEPES buffer (50 mM HEPES pH 8.0, 1 mM DTT). The beads were finally suspended in 20 μl of kinase buffer (50 mM HEPES pH 8.0, 10 mM $MgCl_2$, 0.1 mM sodium vanadate, 10 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT) containing 5 μM non-radiolabelled ATP, [γ-^{32}P]ATP (5 μCi) and 1 μg of kinase substrate, then incubated at 30°C for 20 min. GST-fused retinoblastoma protein (GST-pRb; sc-4112; Santa Cruz) was used as a substrate for the CDK4 and CDK6 kinase reactions, and histone H1 (H1; Boehringer Mannheim) as the substrate for CDK2. The reactions were stopped by the addition of Laemmli's SDS loading buffer and the phosphorylated GST-pRb or H1 was detected by fractionating the reaction mixtures by SDS–PAGE and subjecting them to autoradiography. A conditioned medium of WEHI3B cells (10% v/v) was used as a source of IL-3.

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 content of nuclei was analysed using a cell sorter (FACSort; Becton Dickinson, USA).

Northern blotting

The extraction of total RNA was performed by using the TRIzol reagent (Gibco-BRL) according to the manufacturer's protocol. Twenty micrograms of total RNA from cells in each condition was separated by 1% agarose–formaldehyde gels and transferred 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 h 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 cDNA probes used were mouse cyclin D2 (1.2 kb, *Eco*RI fragment), mouse cyclin D3 (1.7 kb, *Eco*RI fragment), mouse cyclin A (1.3 kb, *Xho*I–*Pst*I fragment), human cdc25A (*Bgl*II–*Nde*I fragment), mouse p21 (0.8 kb, *Eco*RV–*Eco*RI fragment) and mouse p27 (0.65 kb, *Eco*RI–*Xho*I fragment).

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