STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells

Tetsuya Nosaka, Toshiyuki Kawashima, Kazuhide Misawa, Koichi Ikuta[1], Alice L.-F.Mui[2] and Toshio Kitamura[3]

Department of Hematopoietic Factors, The Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, [1]Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan and [2]Department of Surgery, University of British Columbia, Jack Bell Research Centre, Vancouver Hospital and Health Sciences Centre, 2660 Oak Street, Vancouver, BC, Canada V6H 3Z6

[3]Corresponding author
e-mail: kitamura@ims.u-tokyo.ac.jp

Signal transducers and activators of transcription (STATs) play key roles in growth factor-mediated intracellular signal transduction. In the present study using a constitutively active STAT5 mutant, we show that STAT5 has pleiotropic functions regulating cell proliferation, differentiation and apoptosis in an IL-3-dependent Ba/F3 cell line. The mutant STAT5 possessed constitutive tyrosine phosphorylation and DNA binding activity, induced expression of *bcl-xL* and *pim-1* in the absence of IL-3 in Ba/F3 cells, and rendered Ba/F3 cells factor-independent. Unexpectedly, IL-3 treatment of the factor-independent Ba/F3 cells expressing the constitutively active STAT5 resulted in apoptosis within 24 h, or differentiation followed by cell death. In these cells, mRNA expression of growth inhibitory genes downstream of STAT5 such as CIS, JAB/SOCS-1/SSI-1, and p21$^{WAF1/Cip1}$ was highly induced, correlating with prolonged hyper-phosphorylation of the mutant STAT5 after IL-3 stimulation. Of the STAT5-regulated genes, we found that constitutive expression of JAB/SOCS-1/SSI-1 was sufficient to induce apoptosis of Ba/F3 cells, while p21$^{WAF1/Cip1}$ could induce differentiation of these cells. In contrast, constitutive expression of *pim-1* was sufficient to induce IL-3-independent growth of Ba/F3 cells. These findings suggest that a single transcription factor regulates cell fate by varying the intensity and duration of the expression of a set of target genes.
Keywords: apoptosis/JAB/*pim-1*/p21$^{WAF1/Cip1}$/STAT5

Introduction

Signal transducers and activators of transcription (STATs) are transcription factors indispensable for intracellular signaling after stimulation with cytokines, growth factors and hormones (Darnell *et al*., 1994; Ihle, 1995, 1996; O'Shea, 1997). STAT proteins form homo- or heterodimers upon phosphorylation of tyrosine residues, usually by Janus kinases (JAKs). Dimerized STAT proteins immediately enter the nucleus and bind to the specific DNA sequences in the promoter regions of various genes, resulting in gene activation or repression. Recent gene-targeted mice experiments revealed important biological functions of STATs (Kaplan *et al*., 1996a,b; Meraz *et al*., 1996; Shimoda *et al*., 1996; Takeda *et al*., 1996, 1997; Thierfelder *et al*., 1996; Liu *et al*., 1997; Udy *et al*., 1997; Teglund *et al*., 1998). Among seven members of the STAT family, STAT5A and STAT5B are known to be activated by a wide variety of cytokines (O'Shea, 1997). STAT5A-deficient mice showed incomplete mammopoiesis and failure of lactogenesis based on defective signaling to prolactin stimulation (Liu *et al*., 1997), and decreased proliferation of splenocytes to interleukin (IL)-2 stimulation, which was reported to result from defective induction of IL-2 receptor α chain (Nakajima *et al*., 1997). On the other hand, STAT5B-deficient mice showed a phenotype similar to that of Laron-type dwarfism, which is a human growth hormone (GH)-resistance disease generally associated with a defective GH receptor, and STAT5B was suggested to play a major role in mediating the sexually dimorphic effects of GH pulses in the liver (Udy *et al*., 1997). Recently, STAT5A and -B doubly disrupted mice have been generated (Teglund *et al*., 1998). In addition to the phenotypes of each STAT5-deficient mice, the mice showed mild lymphocytopenia, profound deficiency in peripheral T cell proliferation, and a detectable reduction of colony forming efficiency of bone marrow cells in response to IL-3, IL-5, IL-7 and granulocyte/macrophage colony-stimulating factor (GM-CSF). Interestingly, profound deficiency in peripheral T cell proliferation was not rescued by high concentrations of IL-2, which would bypass the requirement for the expression of the IL-2 receptor α chain, suggesting a direct role of STAT5 for IL-2-induced cell cycle progression of peripheral T cells (Moriggl *et al*., 1999). However, development of thymus and hematopoiesis of myeloid and erythroid lineages were not significantly impaired in the STAT5A and -B doubly deficient mice. This is in sharp contrast to the phenotypes of JAK1-, JAK2- or JAK3-deficient mice in which hematopoiesis was severely affected (Nosaka *et al*., 1995; Park *et al*., 1995; Thomis *et al*., 1995; Neubauer *et al*., 1998; Parganas *et al*., 1998; Rodig *et al*., 1998). On the contrary, *in vitro* experiments suggested that STAT5 is involved in cell proliferation in hematopoietic cell lines (Damen *et al*., 1995; Mui *et al*., 1996), and more than half the cases of freshly isolated human lymphoid leukemic cells were found to show constitutive activation of STAT5 (Weber-Nordt *et al*., 1996). It is, therefore, still controversial whether STAT5 plays critical roles in proliferation and differentiation of hematopoietic cells (Fujii *et al*., 1995; Quelle *et al*., 1996; Matsumura *et al*., 1997; Welte *et al*., 1999). Although generation of null mutation is a powerful approach to study the biological function of the molecule, phenotype is occasionally masked if

biologically alternative pathways complement the functional defects. Another approach to unveiling the function of a particular molecule is to activate a single molecule and examine events directly downstream. The results of this strategy are less affected by the other molecules or pathways compared with the analyses by gene disruption or expression of dominant-negative mutants.

We have recently identified a constitutively active STAT5A which renders IL-3-dependent cell lines IL-3-independent by screening randomly mutated STAT5As (Onishi *et al.*, 1998). Here we report that in addition to inducing IL-3-independent proliferation, the constitutively active STAT5 induces apoptosis and differentiation in the same cell line after IL-3 stimulation.

Results

Constitutive activation of STAT5 in the absence of JAK2 activation

The mutant STAT5A, designated 1*6, which harbors two mutations of H^{298} to R and S^{710} to F (Figure 1A), showed constitutive tyrosine phosphorylation, nuclear localization and DNA binding activity in the absence of IL-3 (Onishi *et al.*, 1998). The 1*6 mutant exhibited prolonged hyper-tyrosylphosphorylation after IL-3 treatment (Onishi *et al.*, 1998), which is a probable molecular basis of its constitutive activity. While stable transfectants expressing high levels of the wild-type STAT5A and those of the single point mutants were easily obtained and maintained, we frequently failed to maintain those of the 1*6 mutant in the presence of IL-3. We therefore suspected a cytotoxic effect of the 1*6 mutant. Interestingly, the 1*6 mutant was cytotoxic in the presence of IL-3, and Ba/F3 cells expressing high levels of the 1*6 mutant could be maintained for a long period only in the absence of IL-3. To obtain the clones expressing higher levels of the mutant STAT5A than those isolated in the presence of IL-3, we selected the Ba/F3 cells by the ability to grow in the absence of IL-3 after retroviral transduction in which the infection efficiency was 30–60%. These cells expressed higher levels of the 1*6 mutant (data not shown) and DNA binding activity (Figure 1B) compared with the cells selected in the presence of IL-3 (Onishi *et al.*, 1998). We do not think that particular clones with secondary mutations were chosen in the absence of IL-3 because 20–50% of the original cell population grew within 48 h of IL-3 deprivation (data not shown). JAK2 was not activated beyond the basal level in the 1*6 cells in the absence of IL-3 (Figure 1C), suggesting that basal level of JAK activity is sufficient for accumulation of phosphorylated form of the mutant STAT5A, or that a previously unrecognized tyrosine kinase is involved in activating the mutant STAT5A.

IL-3-induced apoptosis or differentiation in the cells expressing the mutant STAT5A

Stable transfectants of Ba/F3 cells expressing high levels of the 1*6 mutant grew well without IL-3, albeit more slowly than IL-3-driven parental Ba/F3 cells (Figure 2A and B; Table II). While the transfectants expressing the wild-type STAT5A grew well even in the presence of 10 μg/ml of IL-3, most of the 1*6 cells that express comparable levels of the mutant STAT5A to the levels of
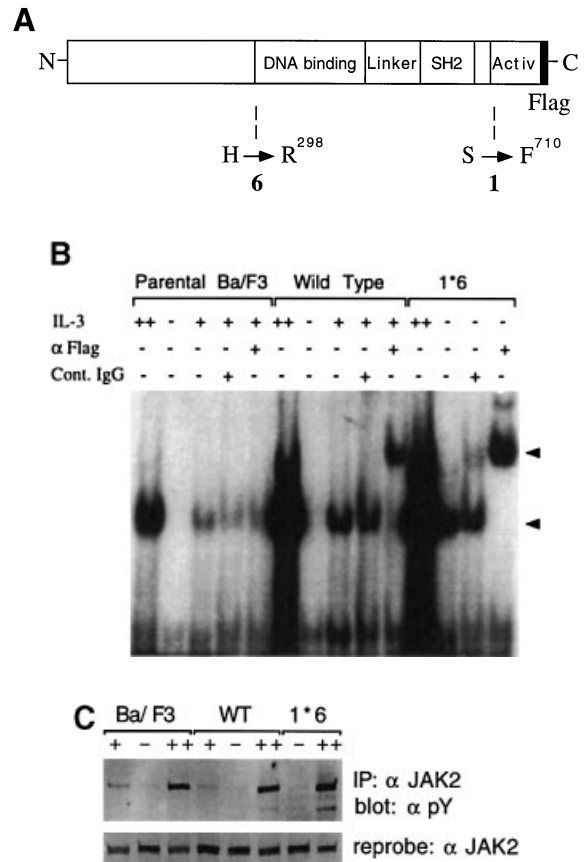

Fig. 1. The mutant STAT5 induces IL-3-independent DNA binding activity without activation of JAK2. (**A**) Structure of the constitutively active mutant STAT5. The 1*6 mutant STAT5 has two mutations; one in the activation domain (designated mutation 1) and the other in the DNA binding domain (mutation 6). Flag was added as an epitope tag to the C-terminal end of the mouse STAT5A to distinguish it from endogenous STAT5A (Wang *et al.*, 1996). (**B**) Electrophoresis mobility shift analysis (EMSA) with STAT5A binding sequence in Ba/F3 stable transfectants. Cell lysates of Ba/F3 cells, Ba/F3 expressing wild-type STAT5A-Flag, and Ba/F3 expressing the 1*6 mutant STAT5A-Flag before and after IL-3 stimulation were subjected to EMSA. –, removal of IL-3 for 11 h (parental Ba/F3 cells and the wild-type transfectant) and continuous culture without IL-3 (1*6 transfectant); +, cultured with 2 ng/ml of IL-3; ++, stimulated with 10 ng/ml of IL-3 for 30 min after IL-3 deprivation. Lower arrowhead, STAT5A–DNA complex; upper arrowhead, supershifted complex with an anti-Flag antibody. (**C**) Immunoprecipitation followed by Western blot analysis for phosphotyrosine of JAK2. – and +, same as in (B); ++, stimulated with 20 ng/ml of IL-3 for 10 min after IL-3 deprivation. WT, wild type.

the transduced STAT5A in the wild-type expressor died within 24 h, showing apoptotic appearance after addition of 2–3 ng/ml of IL-3 (Figures 2B, 3B and 4B), and DNA ladder was observed in the 1*6 cells with IL-3 stimulation (Figure 4A). The 1*6 cells which escaped apoptosis were larger in size than the cells before IL-3 treatment and exhibited round morphology (Figure 3B and D). These cells did not divide further and died within 2 weeks. We also characterized four single clones of the 1*6 cells, and found that two out of four clones underwent morphological differentiation into macrophage-like cells as well as apoptosis upon IL-3 stimulation (Figures 3D and 5C). In these cells, IL-3 stimulation induced expression of CD11b, a macrophage differentiation marker, while the same clone in the absence of IL-3 as well as the parental Ba/F3 cells
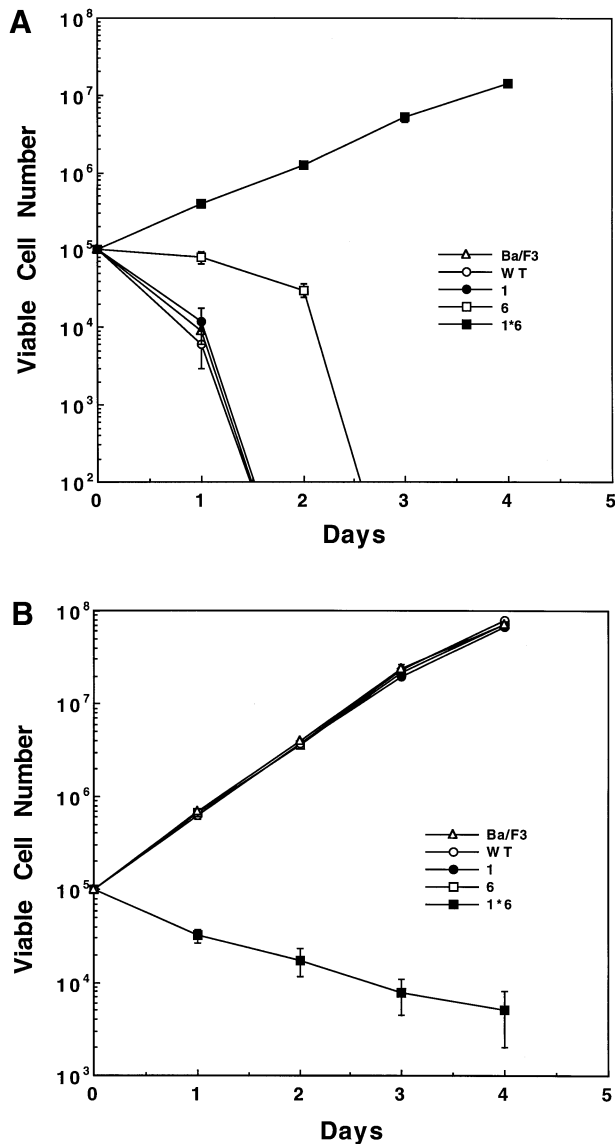
Fig. 2. IL-3 induces growth inhibition of the cells expressing the 1*6 mutant STAT5A-Flag. Growth curves of stable transfectants of Ba/F3 cells expressing the wild-type or the mutant STAT5A-Flag construct either without (**A**) or with (**B**) IL-3 are shown. Live cells of triplicate cultures were counted and mean values together with standard errors were plotted against time. WT, wild type; 1, STAT5 with mutation 1 alone; 6, mutation 6 alone; 1*6, double mutations. IL-3 concentration was 2 ng/ml. Cells were diluted appropriately before reaching confluence.

type in the presence and absence of IL-3, respectively, in a transient transfection assay using Ba/F3 cells (Onishi *et al.*, 1998). We tested whether the wild-type and the mutant STAT5As can induce expression of known STAT5-target genes including *oncostatin M* (Yoshimura *et al.*, 1996), *pim-1* (Selten *et al.*, 1986), *bcl-x* (Boise *et al.*, 1993), c-*fos*, JAB/SOCS-1/SSI-1 (a CIS family gene) (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997), CIS (Yoshimura *et al.*, 1995), and cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ (El-Deiry *et al.*, 1993; Harper *et al.*, 1993) after IL-3 stimulation. Northern blot analysis demonstrated that the same set of target genes was induced in response to IL-3 stimulation in parental Ba/F3 cells, Ba/F3 transfectant expressing the wild-type STAT5A, and that expressing the 1*6 mutant STAT5A (Figure 8A). It is, however, noticeable that induction of all these genes except for c-*fos* was much stronger and sustained for a longer time period in the 1*6 cells than in the wild-type transfectant or parental cells. *pim-1*, *bcl-xL* and c-*myc*, which are positive regulators for cell survival or proliferation, were expressed in the 1*6 cells in the absence of IL-3, while the wild-type transfectant or parental cells showed little or no expression of these genes in the absence of IL-3. *bcl-2* was not up-regulated in the 1*6 cells in the absence of IL-3 (data not shown). Reverse transcription (RT)–PCR analysis confirmed that virtually all the mRNAs hybridized with the *bcl-x* probe in Northern blot analysis were *bcl-xL*, whose product is an inhibitor of apoptosis, and an alternatively spliced *bcl-xS* transcript encoding a promoter of apoptosis, was not detected (Figure 8B). Induction of *bcl-xL* and c-*myc* in 1*6 cells is reminiscent of the previous study showing that activation of any two of the three distinct pathways inducing c-*fos*/c-*jun*, c-*myc* and *bcl-2* is sufficient to confer cytokine-independent growth of Ba/F3 cells (Miyazaki *et al.*, 1995). The mechanism of c-*myc* induction in the 1*6 cells is currently unknown. *pim*-1 was also shown to cooperate with c-*myc* to induce lymphoma (Breuer *et al.*, 1989; van Lohuizen *et al.*, 1989). In addition, it was reported that enforced expression of *pim-1* in mouse myeloid cells resulted in enhancement of factor-independent survival and inhibition of apoptosis (Lilly and Kraft, 1997). Expression of *cyclin D1* and p27^{Kip1} was under the level of detection in these cells (data not shown). Other genes, *cyclin A*, *D2*, *E*, and interleukin-1β converting enzyme (ICE), were not transactivated in the 1*6 cells.

The mutant STAT5A alone is sufficient to induce autonomous cell growth
To identify STAT5-regulated genes that are responsible for autonomous growth of the 1*6 cells, we carried out retrovirus-mediated cDNA expression screening by transduction of a cDNA library from the 1*6 cells maintained in the absence of IL-3, into the parental Ba/F3 cell line. In this strategy, the downstream genes can be isolated only if expression of the single gene was sufficient to confer IL-3-independency on Ba/F3 cells. After the cDNA library transduction, 22 Ba/F3 clones which became IL-3-independent were isolated, and all the clones were found to harbor the cDNA of the 1*6 mutant. This result indicates that the 1*6 gene itself is a prerequisite and sufficient to confer IL-3 independency, and suggests that the 1*6 mutant up-regulated the expression of at least two genes
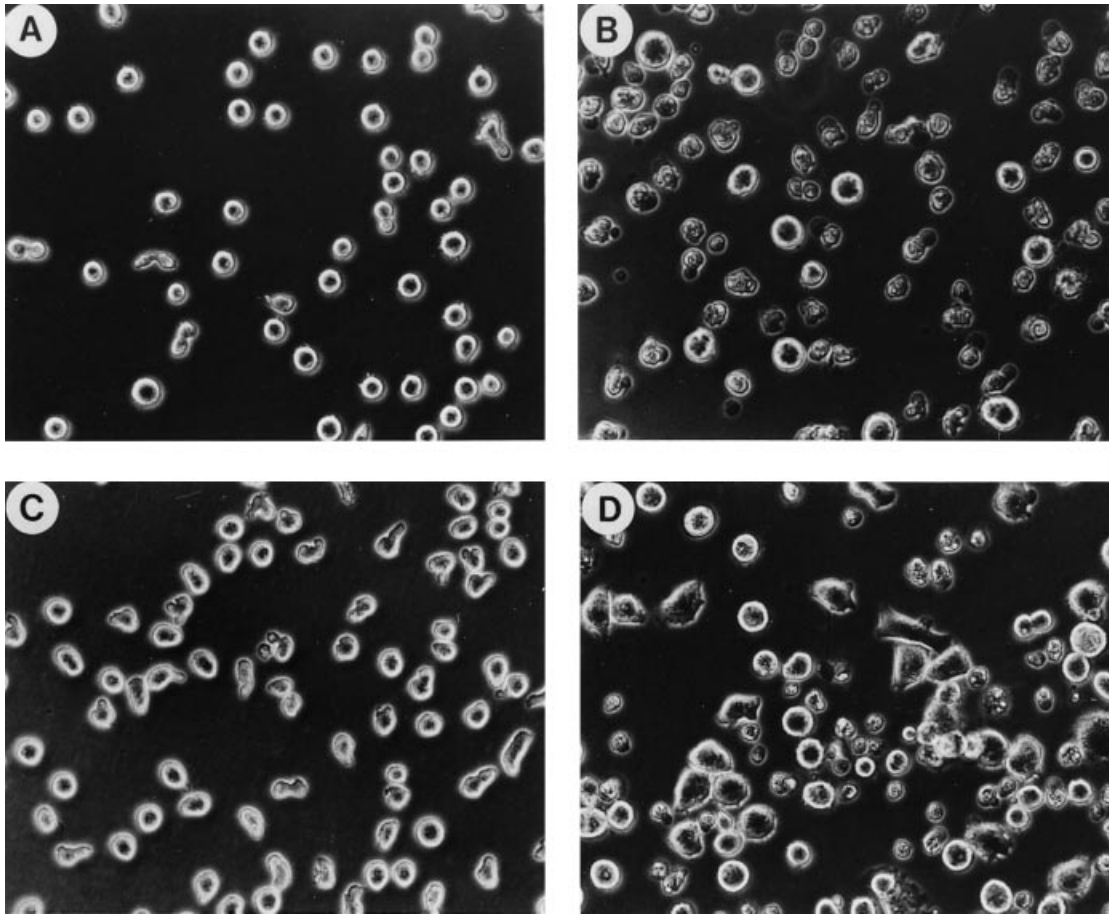
did not express CD11b (Figure 6). Differentiated cells died within 1 week. As a whole population, IL-3-treated 1*6 cells never gave rise to long-term growth (data not shown). Cell cycle analysis of the 1*6 cells after IL-3 stimulation revealed G$_1$ arrest and apoptosis with profound reduction of the number of the cells in S phase, which is similar to the cell cycle profile of the parental Ba/F3 cells after IL-3 deprivation (Figure 7; Table I).

Up-regulation of positive regulators for cell growth in the absence of IL-3 and of negative regulators in the presence of IL-3
The 1*6 mutant of STAT5A transactivated the gene expression from the β-casein promoter containing STAT5 binding sites by 20- and 50-fold compared with the wild

Fig. 3. Phase contrast microscopy of the cells expressing the 1*6 mutant STAT5A-Flag. (**A**) (no IL-3) and (**B**) (after stimulation with 3 ng/ml of IL-3 for 48 h), bulk culture; (**C**) (no IL-3) and (**D**) (3 ng/ml of IL-3 for 41 h), one of the clones showing apoptosis and differentiation followed by cell death upon IL-3 stimulation.

to induce IL-3-independent growth. Interestingly, 11 out of 22 factor-independent clones were found to possess the cDNAs encoding truncated 1*6 STAT5A which is devoid of N-terminal 137 amino acids. The other 11 clones harbored the full-length 1*6 STAT5A. Both forms of 1*6 STAT5A conferred IL-3-independency after re-introduction into Ba/F3 cells and possessed transactivational ability on the β-casein promoter in Ba/F3 cells although the truncated form had less transactivation activity than the full-length form (data not shown). These findings suggest that N-terminal 137 amino acids of the 1*6 STAT5A is not necessary either to confer factor-independent cell growth or to the basal mechanism of transactivation.

IL-3 induced expression of p21$^{WAF1/Cip1}$, but not of p53 and any soluble factors inducing apoptosis/differentiation in cells expressing the mutant STAT5A

High expression of p21 protein induced by IL-3 in the 1*6 cells (Figure 8C) may explain G$_1$ arrest, differentiation (Matsumura *et al*., 1997) and escape from apoptosis (Polyak *et al*., 1996) in some of the 1*6 cells. p53 was not overexpressed in the 1*6 cells undergoing apoptosis (Figure 8C). To examine whether IL-3-induced apoptosis and differentiation in 1*6 cells were mediated by soluble factors produced by the 1*6 cells, supernatant of the bulk culture of the 1*6 cells undergoing apoptosis and

differentiation in the presence of IL-3 was tested for the ability to induce the same phenotypic changes in parental Ba/F3 cells. Ba/F3 cells grew well in the supernatant of the IL-3-treated 1*6 cells without undergoing apoptosis and differentiation, suggesting that no soluble factor was involved in IL-3-induced apoptosis and differentiation of 1*6 cells (data not shown). Although Fas was inducibly expressed on fluorescence-activated cell sorting (FACS) analysis, anti-mouse Fas ligand neutralizing antibody (gift from Dr T.Suda) did not block the IL-3-induced apoptosis of the 1*6 cells, and Fas ligand was not induced in Northern blotting and FACS analyses in 1*6 cells with IL-3 treatment (data not shown), indicating that the Fas/Fas ligand system is not involved in the process.

Enforced expression of pim-1 induces factor-independent growth of Ba/F3 cells

Since activation of at least two genes was thought to be required for IL-3-independent cell growth, we tested combinations of several known genes for the ability to induce cell proliferation. Of the up-regulated genes in the 1*6 cells, c-*myc*, *pim*-1 and *bcl-xL* have been implicated in cell proliferation or survival. Therefore, Ba/F3 cells were retrovirally transduced with these genes in various combinations. Unexpectedly, *pim-1* alone was sufficient to induce IL-3-independency, and co-expression of c-*myc* or *bcl-xL* enhanced the phenotype (Table II), suggesting
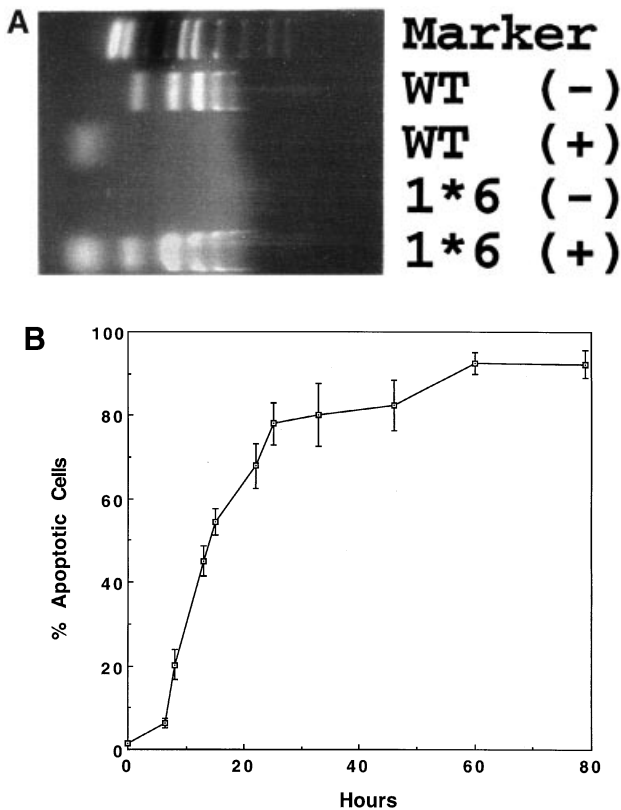
Fig. 4. IL-3-induced apoptosis in Ba/F3 cells expressing the 1*6 mutant STAT5A-Flag. (**A**) DNA ladder formation in Ba/F3 cells expressing the 1*6 mutant STAT5A after IL-3 treatment. The cells expressing the wild-type STAT5A-Flag was deprived of IL-3 for 40.5 h and those expressing the 1*6 mutant STAT5A-Flag were treated with 3 ng/ml of IL-3 for 24 h. –, no IL-3; +, culture with IL-3; Marker, DNA ladder marker (Bio-Rad, 50, 100, 200, 300, 400, 500, 700, 1000, 1500, 2000 bp). (**B**) The rate of apoptotic events in Ba/F3 cells expressing the mutant STAT5A-Flag after stimulation with IL-3. Bulk culture of the stable transfectant expressing the 1*6 mutant STAT5A-Flag was treated with 3 ng/ml of IL-3, and morphologically apoptotic cells were enumerated by trypan blue exclusion test in triplicate.

that 1*6 STAT5A conferred IL-3-independency on Ba/F3 cells through up-regulation of *pim-1*. This finding is somehow inconsistent with our former results in which no gene other than 1*6 STAT5A was identified as an inducer of IL-3-independency after transduction with a cDNA expression library derived from the 1*6 cells. However, a previous study disclosed the dose-dependency of *pim-1* function as an oncogene (van der Houven van Oordt *et al.*, 1998). Transduction of *pim-1* gene via retrovirus infection would result in multiple integrations of the *pim-1* retrovirus in each cell, while in the case of cDNA library trasnduction, each cell should not harbor more than one integration of *pim-1*. Thus, the difference in the expression level may explain this inconsistency. Ability of *bcl-xL* together with c-*myc* to confer IL-3-independency on Ba/F3 cells is consistent with a previous study in which co-expression of *bcl-2* and c-*myc* induced IL-3-independent proliferation of Ba/F3 cells (Miyazaki *et al.*, 1995).

JAB is responsible for inducing apoptosis and p21 for differentiation

In an attempt to identify the genes to induce apoptosis or differentiation, JAB, CIS, and p21 were transduced
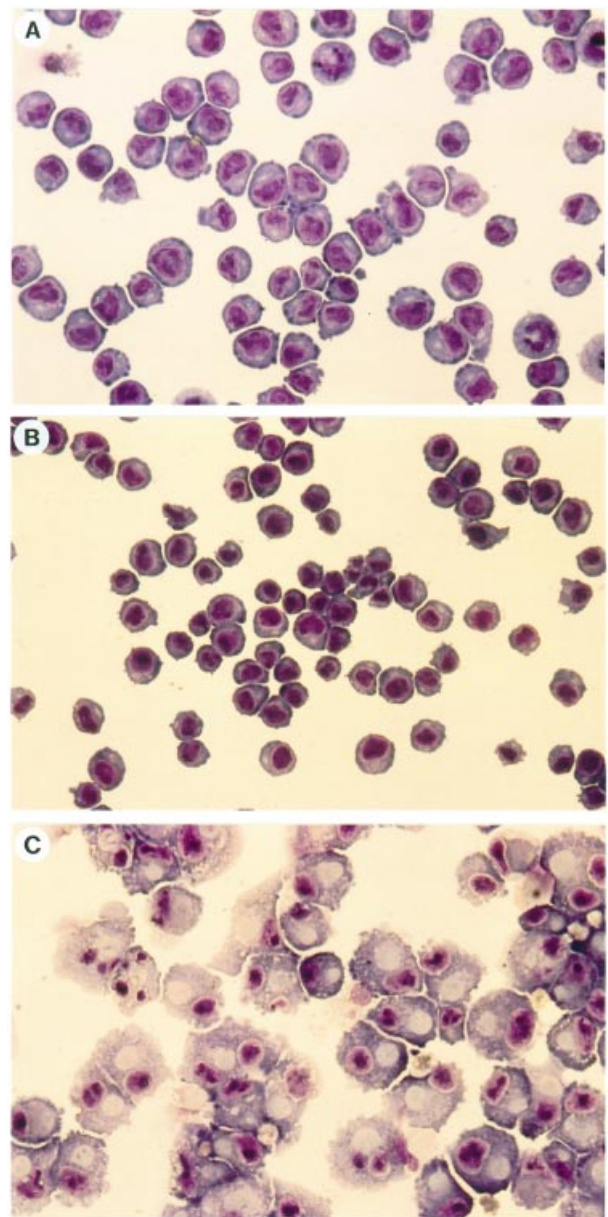

Fig. 5. IL-3-induced differentiation in Ba/F3 cells expressing the 1*6 mutant STAT5A-Flag. May–Giemsa staining of cytospins of parental Ba/F3 cells with IL-3 (**A**) and of one of the clones expressing the mutant STAT5A without (**B**) and with (**C**) IL-3 (3 ng/ml, 45 h) are shown. Original magnification was ×400.

individually into Ba/F3 cells together with an enhanced green fluorescent protein (EGFP) using a bicistronic retro-virus vector pMX-IRES-EGFP. After selecting green fluorescent cells by FACS, the fate of those cells was monitored in the presence of IL-3.

Within 24 h after sorting, virtually all of the cells expressing JAB were found to have undergone apoptosis (Figure 9B), while control cells expressing EGFP alone and the cells expressing CIS continued to grow (Figure 9A and C). Identical results to those in Ba/F3 cells were also obtained in the 1*6 cells in the absence of IL-3 (data not shown). These findings indicate that JAB is one of the genes responsible for IL-3-driven apoptosis in the 1*6 cells. In similar experiments performed with p21 in Ba/ F3 cells, the total number of the sorted cells expressing
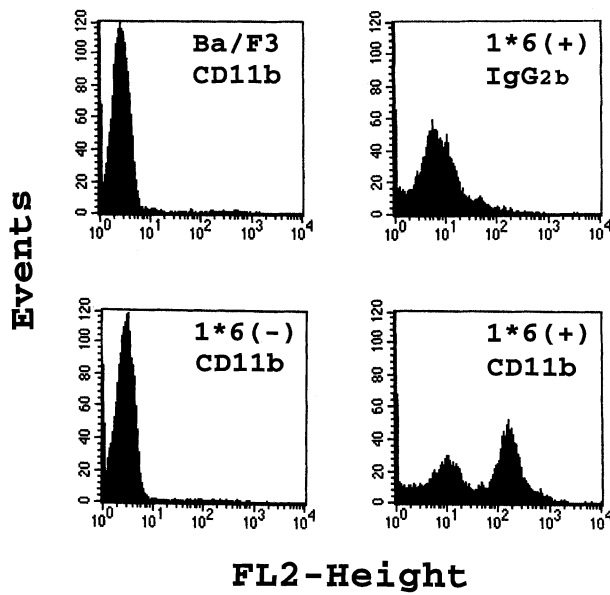
Fig. 6. FACS analysis of the same samples as in Figure 5. The cells were stained with phycoerythrin (PE)-conjugated rat anti-mouse CD11b antibody or PE-conjugated isotype-matched control rat IgG$_{2b}$. −, no IL-3; +, with IL-3.
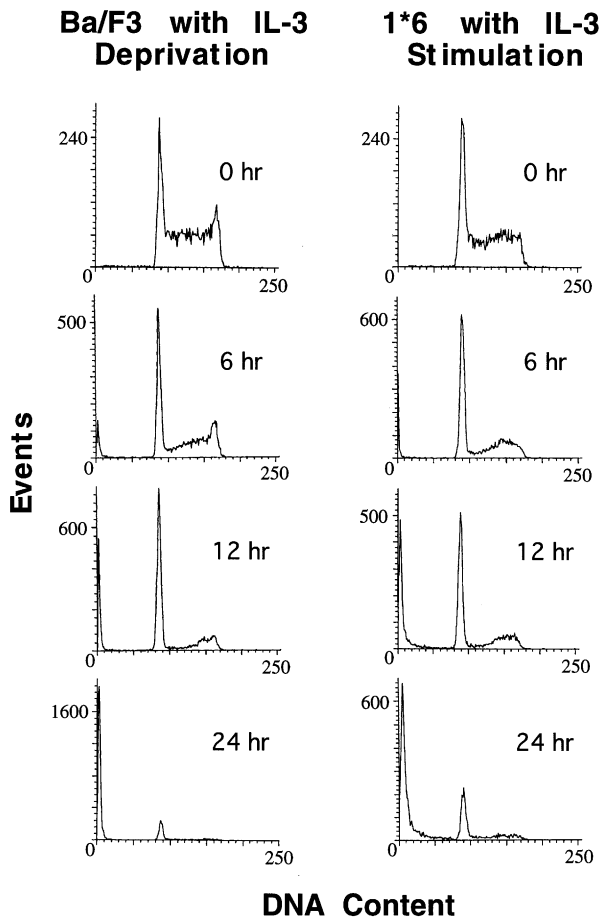

Fig. 7. Cell cycle analysis of the cells expressing the 1*6 mutant STAT5A-Flag. Parental Ba/F3 cells were deprived of IL-3 and the 1*6 cells were treated with 3 ng/ml of IL-3, followed by DNA staining at the indicated time points.

Table I. Cell cycle analysis of the cells expressing the 1*6 mutant STAT5A-Flag

Time	Ba/F3 with IL-3 deprivation				1*6 with IL-3 stimulation			
	G$_0$–G$_1$	S	G$_2$–M	apoptosis	G$_0$–G$_1$	S	G$_2$–M	apoptosis
0 h	32.5	52.6	14.9	1.0	37.6	50.2	12.2	1.1
6 h	46.7	39.0	14.3	5.7	55.7	37.0	7.3	7.2
12 h	74.8	20.0	5.2	21.4	67.2	24.6	8.2	33.2
24 h	72.6	22.6	4.8	82.8	66.7	24.8	8.5	72.4

Percentages of each phase of cell cycle in Figure 7 are shown.

Table II. Ability to confer IL-3-independence on Ba/F3 cells and doubling time of Ba/F3 cells in the absence of IL-3 after transduction

	IL-3-independence	Doubling time (h)
Vector	−	NA
pim-1	+	21.3 ± 1.1
c-*myc*	−	NA
bcl-xL	−	NA
pim-1 and c-*myc*	+	18.0 ± 0.9
pim-1 and *bcl-xL*	+	17.7 ± 1.1
c-*myc* and *bcl-xL*	+	14.6 ± 0.7
pim-1, c-*myc* and *bcl-xL*	+	15.5 ± 0.8
1*6STAT5A-Flag	+	14.0 ± 0.6
Parental Ba/F3 cells	−	9.3 ± 0.3[a]

[a]Doubling time in the presence of IL-3.
NA, not applicable.

p21 was one-tenth of the control cells expressing EGFP alone 5 days after sorting (Figure 10). Ten to 20% of the cells expressing p21 showed large and round morphology and some of them became adherent (Figure 11B), while only 1–2% of the control cells showed such morphology and never became adherent (Figure 11A). This finding indicates that p21 is one of the genes involved in differentiation and escape from apoptosis in Ba/F3 cells.

Expression of pim-1 is elevated in factor-independent human leukemic cells harboring constitutively activated STAT5

Finally, to see whether Pim-1 is involved in autonomous growth of leukemic cell lines, we carried out Northern blot analysis on K562 and HEL cells, both of which show constitutive activation of STAT5 (Carlesso *et al.*, 1996; Liu *et al.*, 1999). A factor-dependent human hematopoietic cell line, TF-1 (Kitamura *et al.*, 1989), was used as a control in this experiment. *pim-1* was constitutively expressed in factor-independent leukemic cell lines K562 and HEL, while *pim-1* expression was induced by GM-CSF in a GM-CSF-dependent cell line, TF-1 (Figure 12). Furthermore, *bcl-x* expression was also elevated in K562 and HEL cells.

Discussion

In this paper, we have shown that the constitutively active STAT5A provokes IL-3-independent cell proliferation as well as IL-3-dependent apoptosis and differentiation in the same cells. The same results as with the 1*6 mutant STAT5A were obtained with the 1*6 mutant STAT5B (Onishi *et al.*, 1998; data not shown) and the other
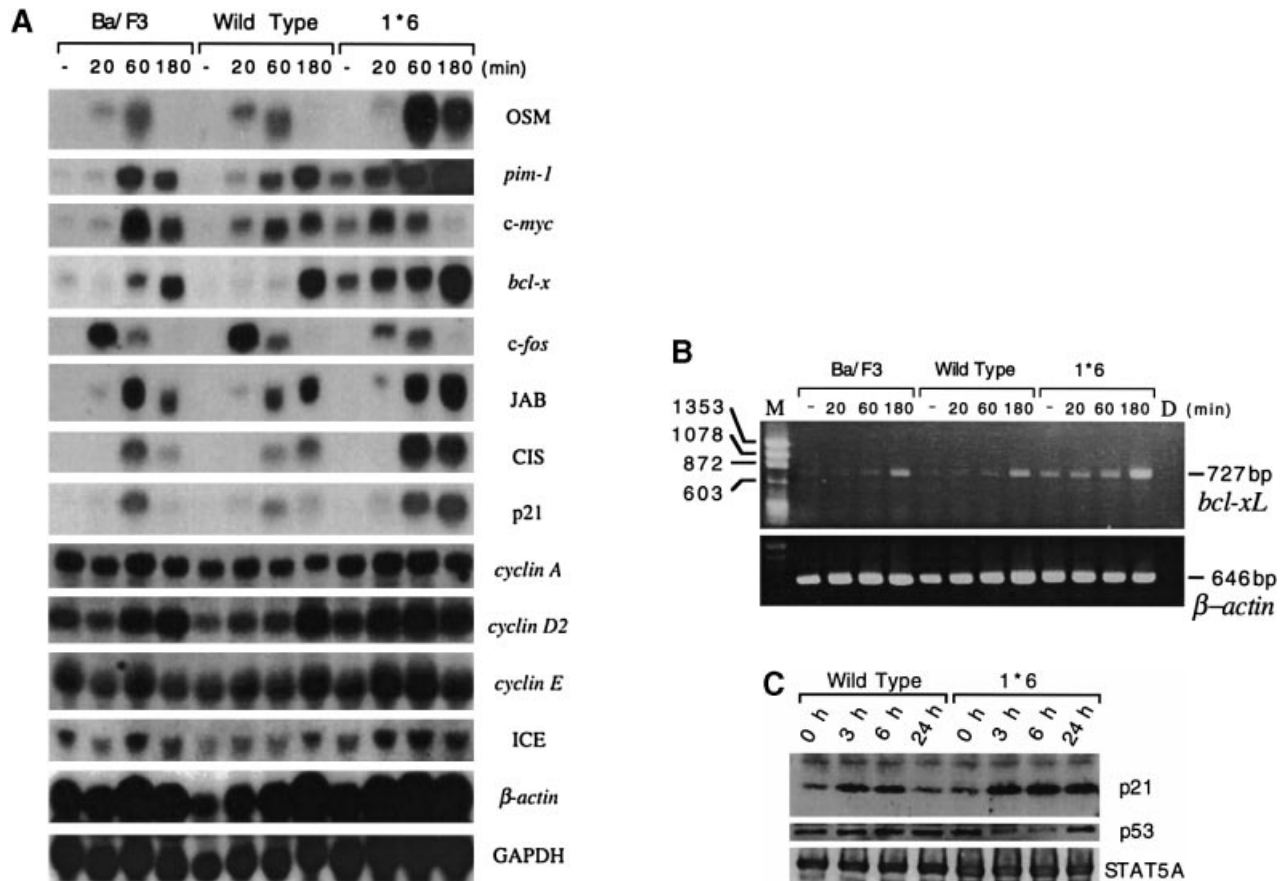
Fig. 8. Analysis of the downstream gene expression in Ba/F3 cells stably expressing the wild type or the 1*6 mutant STAT5A-Flag. (**A**) Northern blot analysis. Ba/F3 cells and a Ba/F3 transfectant with the wild-type STAT5A-Flag, which had been starved of IL-3 for 6.3 h, and that with the 1*6 mutant STAT5A-Flag which had been maintained without IL-3, were stimulated with 10 ng/ml of IL-3 for the indicated periods. OSM, *oncostatin M*; ICE, interleukin-1β converting enzyme (caspase 1); GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**B**) RT–PCR analysis. Total RNAs which were used for the Northern blot analysis were subjected to RT–PCR analysis to distinguish between *bcl-xL* and *bcl-xS*. M, *Hae*III-digested φX174 DNA marker; D, PCR with genomic DNA extracted from Ba/F3 cells. (**C**) p21, but not p53 was overexpressed in the cells expressing the mutant STAT5A. A Ba/F3 transfectant with the wild-type STAT5A-Flag starved of IL-3 for 8.5 h and that with the 1*6 mutant STAT5A-Flag which had been maintained without IL-3 were treated with 3 ng/ml of IL-3, followed by sequential immunoprecipitation (IP) and Western blot analysis with the same antibody as in IP, except that anti-Flag antibody was used for IP and anti-STAT5A antibody for Western blot analysis to detect transduced STAT5A.

STAT5A mutant with double mutations at the positions of E[150] and S[710] (data not shown), or that with a single mutation at the position of N[642] (K.Ariyoshi, T.Nosaka, K.Yamada, M.Onishi, A.Miyajima and T.Kitamura, in preparation), indicating that the biological activities of the mutant STAT5A are not specific to the 1*6 mutant of STAT5A and will reflect physiological functions of STAT5. Thus, although the functions of a mutant molecule do not necessarily reflect those of the wild-type molecule, the constitutively active STAT5 will give us some insights into the function of STAT5 and signal transduction.

Under physiological conditions, cytokine stimulation first results in an activation phase, which is then quickly quenched by negative regulators such as CIS, JAB, p21[WAF1/Cip1] and tyrosine phosphatases. Interestingly, both phases are at least in part regulated by STAT5. The present results suggest that the mutant STAT5A can elicit cell proliferation, differentiation or apoptosis through induction of a defined set of genes with different intensity and duration. It is possible that the affinity of STAT5 to the promoter regions of the target genes is affected by the degree of phosphorylation or other post-translational modification of STAT5 and interaction with other molecules
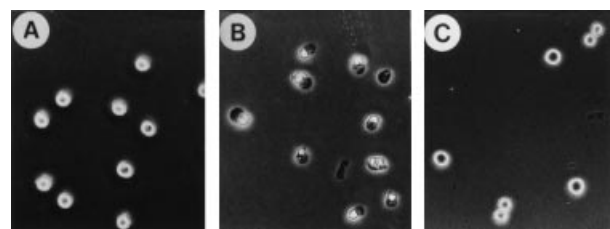

Fig. 9. Enforced expression of JAB induces apoptosis of Ba/F3 cells. Ba/F3 cells were transduced with the bicistronic retroviral vector pMX-IRES-EGFP (**A**), pMX-JAB-IRES-EGFP (**B**) or pMX-CIS-IRES-EGFP (**C**), followed by sorting based on EGFP expression 24 h after infection. Phase-contrast microscopies of the sorted cells 46 h after infection which were cultured in a medium containing 2 ng/ml of IL-3 are shown.

such as CBP/p300. Currently, at least eight members of CIS family genes have been identified (Masuhara *et al.*, 1997; Hilton *et al.*, 1998) and are thought to be target genes of STATs, which inhibit cytokine signaling. Among CIS family members, we examined expression of JAB and CIS in the 1*6 cells, and found that IL-3 strongly induced expression of these genes. JAB inhibits IL-3 signaling by inactivating JAK through direct binding.

Therefore, strong and prolonged induction of JAB will result in suppression of JAK activities below the basal level, leading to growth inhibition and apoptosis of the 1*6 cells. In fact, enforced JAB expression via a bicistronic retroviral vector pMX-IRES-EGFP induced apoptosis of both Ba/F3 (Figure 9B) and the 1*6 cells, indicating that prolonged expression of JAB is one of the molecular bases for the IL-3-induced apoptosis in the 1*6 cells. It is also possible that JAB induces apoptosis through
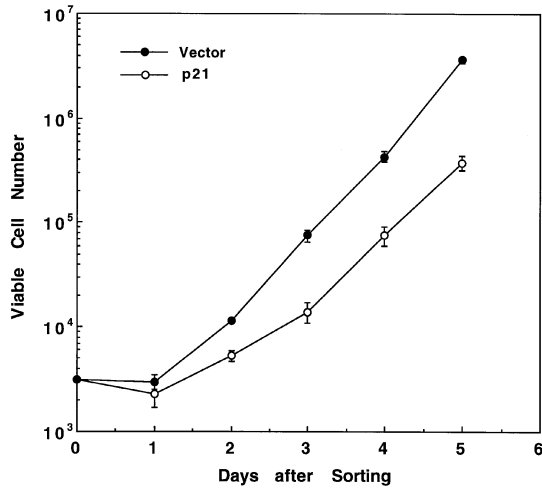
unknown mechanisms such that JAB may function as a scavenger of signaling molecules. In this case, the target molecule which contains phosphotyrosines and is essential for cell survival would become a substrate of the proteolytic machinery after association with JAB. On the other hand, CIS, another member of this family, had no effects on cell viability of both Ba/F3 (Figure 9C) and the 1*6 cells after retrovirus-mediated transduction, indicating that CIS was not involved in IL-3-induced apoptosis of the 1*6 cells. In a similar experiment, p21 was shown to induce differentiation of parental Ba/F3 cells to some extent (Figure 11). However, the differentiation phenotype was weaker than the IL-3-induced apoptosis of the 1*6 cells, suggesting that other genes are also involved in differentiation of the 1*6 cells. Moreover, a serine/threonine kinase Pim-1 expression was found to be sufficient to confer IL-3-independence on Ba/F3 cells. It should be noted that recently Pim-1 was found to cooperate with p100 to enhance the activity of c-Myb which plays a role in both normal hematopoiesis and tumorigenesis (Leverson *et al.*, 1998). Thus, we have shown that the constitutively active STAT5 has pluripotent biological functions as a molecular inducer of proliferation, differentiation and apoptosis. The cell line we established can be used as a model system in which only a single transcription factor determines cell fate in concert with cytokine stimulation. It is possible that physiological STAT5 activation could also result in pleiotropic phenotypes.

Phenomena similar to IL-3-induced apoptosis were described previously in several physiological and pathological situations. Antigen re-stimulation of T cells under the influence of IL-2 causes apoptosis (Lenardo, 1991).
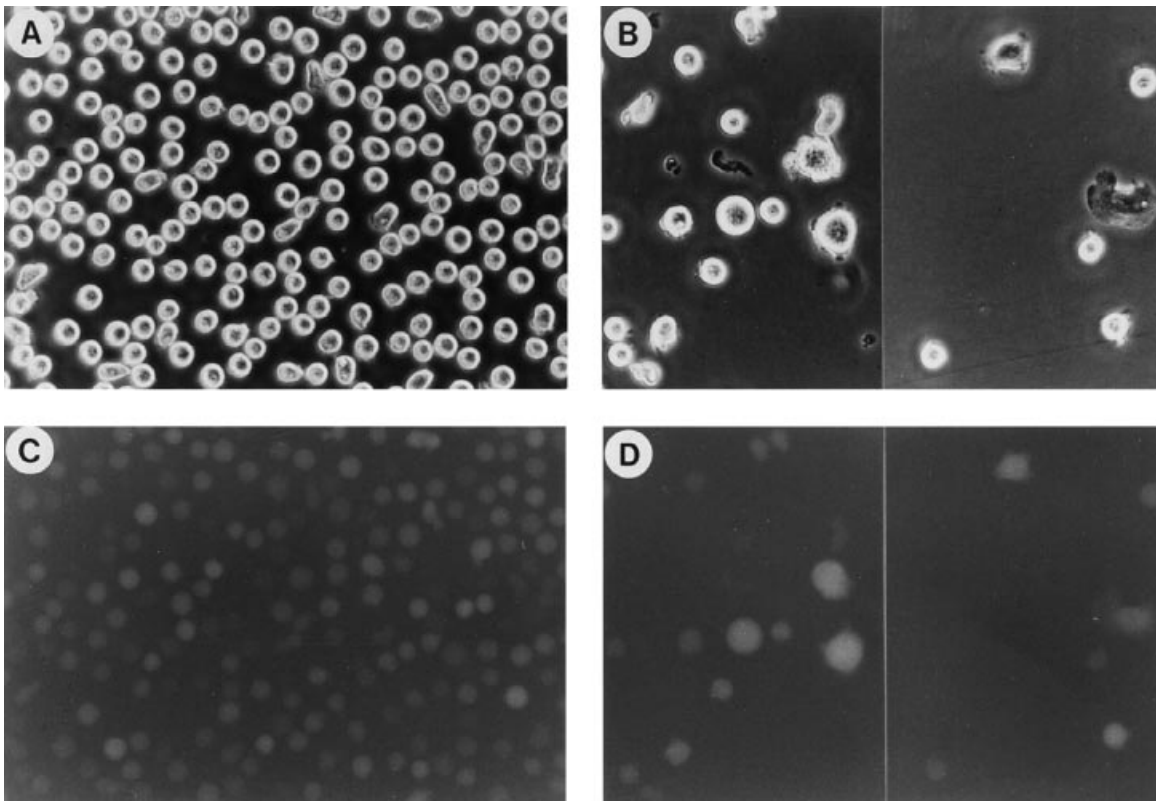

Fig. 10. Enforced expression of p21$^{WAF1/Cip1}$ results in slower rate of cell growth. Growth curves of Ba/F3 cells transduced with either pMX-IRES-EGFP (Vector) or pMX-p21-IRES-EGFP (p21) are shown. The cells were sorted into a medium containing 2 ng/ml of IL-3, based on EGFP expression 24 h after infection. Average values of two independent experiments are shown.

Fig. 11. Enforced expression of p21$^{WAF1/Cip1}$ induces morphological differentiation of Ba/F3 cells. Ba/F3 cells were transduced with pMX-IRES-EGFP (**A** and **C**) or pMX-p21-IRES-EGFP (**B** and **D**), sorted and cultured as in Figure 10. Phase contrast (A, B) and fluorescent (C, D) microscopies 5 days after sorting are shown.
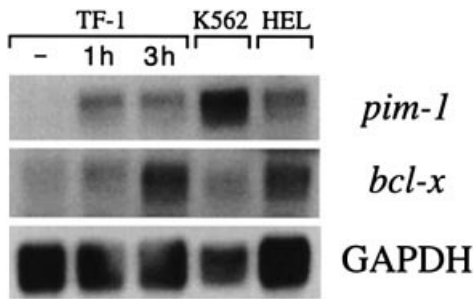
Fig. 12. Up-regulation of *pim-1* expression in the leukemic cells having constitutively activated STAT5. TF-1 cells were deprived of GM-CSF for 11 h (–) and stimulated with 20 ng/ml of human GM-SCF for 1 h (1h) and 3 h (3h). K562 and HEL cells are growth facor-independent cells. PolyA(+) RNAs were extracted from these cells and subjected to Northern blot analysis.

IL-4 induces apoptosis of some human leukemic cells (Manabe *et al.*, 1994). Epidermal growth factor (EGF) treatment inhibits proliferation of epidermoid carcinoma cell line A431 cells (Gill and Lazar, 1981), or induces apoptosis in a breast cancer cell line (Armstrong *et al.*, 1994). In these cells, caspase 1 (ICE) is induced through STAT1 activation upon stimulation with EGF (Chin *et al.*, 1997). IL-3 also induces growth inhibition of follicular small-cleaved-cell lymphoma cells *in vitro* (Younes *et al.*, 1994). Interestingly, it was reported in a patient with follicular lymphoma that autologous bone marrow transplantation and high-dose IL-3 treatment induced terminal differentiation of the lymphoma cells into plasma cells followed by a long-lasting remission (Kramer *et al.*, 1995). It should be noted that the serum level of IL-6 was elevated in this patient after IL-3 treatment, suggesting that multiple factors including p21$^{WAF1/Cip1}$ and cytokines induced by IL-3 (Yoshimura *et al.*, 1996) were involved in differentiation of the lymphoma cells *in vivo*. A similar phenomenon was observed in mouse M1 leukemic cells which differentiated into macrophage after transduction of the 1*6 STAT5A (T.Kawashima, K.Murata, A.Kaneko, T.Nosaka and T.Kitamura, in preparation). In M1 cells expressing the 1*6 STAT5A, IL-6 gene expression was up-regulated, resulting in differentiation of the cells, and addition of anti-IL-6 antibody abolished this effect. However, in a clone of the 1*6 Ba/F3 cells we studied, which showed the strongest phenotype of differentiation in response to IL-3 stimulation, addition of anti-IL-6 antibody did not block differentiation, suggesting that IL-6 secretion was not involved in IL-3-induced differentiation in the 1*6 Ba/F3 cells (data not shown).

Our present results show that sustained STAT5 activation preferentially induces apoptosis and differentiation of cells. Similar observations have been made in other signaling molecules. Low levels of Raf activity induce cell cycle progression, whereas stronger activation of Raf results in cell cycle arrest associated with induction of p21$^{WAF1/Cip1}$ (Woods *et al.*, 1997). c-Myc induces cell proliferation or apoptosis, depending on the concentration of serum (Evan *et al.*, 1992). This finding suggests that activation of an additional signaling pathway by serum stimulation is required for proliferation in collaboration with c-Myc. Similarly, oncogenic Ras provokes premature cell senescence in primary rodent and human cells in association with the accumulation of p53 and p16^{INK4a},
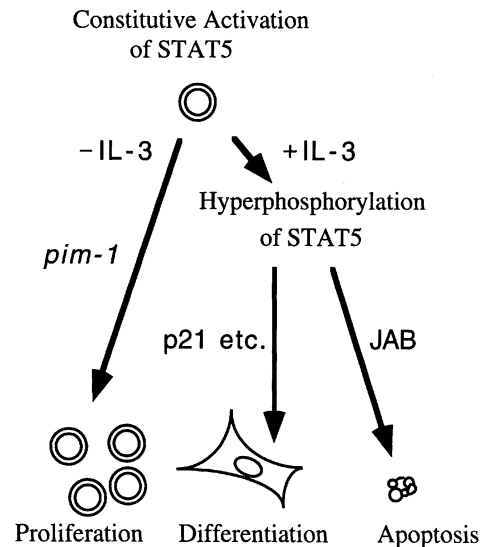

Fig. 13. Model for multiple functions of constitutively active STAT5.

while oncogenic Ras transforms primary cells where another cooperating oncogene is activated or tumor suppressor genes are inactivated (Serrano *et al.*, 1997). Taken together, it is conceivable that overstimulation of a particular signaling pathway leads to cell cycle arrest, senescence or apoptosis of the cells, which may be an important step in eliminating cells with an oncogenic mutation.

Recent discovery of the leukemogenic TEL-JAK2 fusion molecule in human leukemic patients (Lacronique *et al.*, 1997; Peeters *et al.*, 1997), which confers IL-3-independent cell growth on Ba/F3 cells (Lacronique *et al.*, 1997), and our finding of the constitutively active mitogenic STAT5 mutant raise a possibility of leukemogenesis caused by mutations in the STAT5 molecule. Screening for activating mutations of STAT5A and 5B in human leukemic patients is now in progress. It will also be of interest to test IL-3 or IL-2 for growth inhibitory effects on the leukemic cells showing abnormal STAT activation (Migone *et al.*, 1995; Gouilleux-Gruart *et al.*, 1996; Weber-Nordt *et al.*, 1996). Our present study suggested that among a diverse set of genes activated by the mutant STAT5, *pim-1* expression plays a pivotal role in inducing autonomous cell growth.

In summary, we have shown, using a constitutively active form of STAT5, the pleiotropic potential of STAT5 to determine cell fate (Figure 13).

Materials and methods

Cells

A mouse pro-B cell line Ba/F3 was maintained in RPMI 1640 medium containig 10% fetal calf serum (FCS) and 2 ng/ml of murine IL-3 (R&D Systems). Stable transfectants were established as described (Onishi *et al.*, 1998) using pMX retrovirus expression system (Kitamura *et al.*, 1995; Onishi *et al.*, 1996) and the BOSC23 packaging cell line (Pear *et al.*, 1993), except that IL-3 was deprived 18–24 h after infection to obtain the clones expressing higher levels of the mutant STAT5 than the clones which had been selected in the presence of IL-3 as described in the previous paper (Onishi *et al.*, 1998). TF-1 cells were maintained in RPMI 1640 medium containing 10% FCS and 3 ng/ml of human GM-CSF (R&D Systems). Chronic myelogenous leukemic cell line K562 and erythroid/megakaryocytic leukemic cell line HEL were maintained in RPMI 1640 medium with 10% FCS.

Electrophoresis mobility shift analysis

The cells were lyzed in a binding buffer (2×10^7 cells/ml) containing 0.5% Nonidet P-40, 50 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 150 mM NaCl, 100 µM Na$_3$VO$_4$, 50 mM NaF, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 3 µg/ml of aprotinin, 2 µg/ml of pepstatin A, 1 µg/ml of leupeptin and 10% glycerol, and centrifuged at 12 000 *g* for 30 min to remove insoluble materials. The cell extracts (4 µl for each reaction) were incubated with 2 µg of poly(dI-dC) for 15 min on ice, followed by 15 min of incubation with 1 ng of Klenow-labeled DNA harboring the STAT5 optimal binding sequence shown below; 5′-GATCCGAATTCCAGGAATTCA-3′ 3′-GCTTAAGGTCCT-TAAGTCTAG-5′. For supershift analysis, the cell extract in the binding buffer was incubated with anti-Flag M2 monoclonal antibody (Eastman Chemical Company) or mouse IgG as a control for 30 min on ice prior to addition of poly(dI-dC). Samples were separated on a 4.5% polyacrylamide gel in 2.2× TBE (1×TBE=50 mM Tris–borate, 1 mM EDTA) and autoradiographed.

Immunoprecipitation and Western blot analysis

Cells were lyzed in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.2 mM Na$_3$Vo$_4$, 2 mM PMSF). Lysates were cleared of debris by centrifugation at 12 000 *g* for 30 min, and the supernatants were incubated with antibodies at 4°C for 2 h. Immune complexes were precipitated with protein A–Sepharose (Pharmacia), washed with the lysis buffer, and proteins were eluted with sample buffer [62.5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue] for SDS–PAGE. Eluted proteins were electrophoresed on 5–15% gradient gels (Bio-Rad Laboratories) to detect JAK2 and STAT5A, 15% gel for p21 and 8.8% gel for p53, and transferred to nitrocellulose. Membranes were probed with antibodies and visualized with the enhanced chemi-luminescence detection system (Amersham) as described by the manufacturer. The antibodies used were anti-JAK2 antibody C-20 (Santa Cruz Biotechnology), anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Inc.), anti-p21 antibody (PharMingen), anti-p53 mono-clonal antibody Ab-1 (Calbiochem), anti-Flag M2 monoclonal antibody and anti-STAT5A antibody (R&D Systems).

Detection of DNA fragmentation on agarose gels

Fragmented DNA but not intact chromatin was isolated according to the method described previously (Ishida *et al.*, 1992) with minor modifications. Cells were lyzed in a buffer containing 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5% Triton X-100. After 10 min on ice, the lysate was centrifuged (13 000 *g*) for 20 min, and the supernatant was treated with 0.4 mg/ml of RNase A followed by 0.4 mg/ml of Proteinase K for 1 h each at 37°C, and precipitated with 2-propanol. The pellet was dissolved in TE buffer, electrophoresed on a 2% agarose gel, and DNA was visualized by ethidium bromide staining.

FACS analysis

The cells were stained with a phycoerythrin(PE)-conjugated rat anti-mouse CD11b monoclonal antibody (PharMingen) on ice for 30 min after blocking with 100-fold excess of mouse IgG, and analyzed on a FACS Calibur flow cytometer (Becton Dickinson). PE-conjugated rat IgG$_{2b}$ was used as an isotype-matched negative control.

Cell cycle analysis

Cells were fixed with 90% ethanol at 4°C for 4 h, washed with phosphate buffered saline (PBS), incubated in a buffer composed of 24 parts of 0.2 M Na$_2$HPO$_4$ and 1 part of 0.1 M citric acid at room temperature for 30 min, resuspended in PBS, and treated with 0.5 mg/ml of RNase A at 37°C for 30 min. DNA contents of the cells were quantitated by using ModFit LT software (Verity Software House) after staining with propidium iodide and analysis on FACScan (Becton Dickinson).

Northern blot analysis

In analyzing Ba/F3 transfectants, total RNA was isolated from cells by using RNeasy kit (Qiagen). Thirty micrograms of total RNA was denatured in 50% formamide at 60°C, electrophoresed on 1% agarose with 6% formaldehyde gel, and blotted to Hybond-N (Amersham). The filter was incubated at 42°C in 50% formamide, 3× Denhardt's reagent (0.06% polyvinylpyrrolidone, 0.06% bovine serum albumin, 0.06% Ficoll), 5× SSC (standard saline citrate; 1×SSC=0.15 M NaCl, 0.015 M trisodium citrate), 1% SDS, 200 µg/ml of denatured salmon sperm DNA, and randomly primed (Stratagene) ^{32}P-labeled cDNA probe. After hybridization, the filter was washed in 0.1× SSC, 0.1% SDS at room temperature, and autoradiographed. Reprobing was carried out after

washing the filter in a stripping buffer of 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.1% SDS and 0.3× Denhardt's reagent at 90°C for 20 min. The probes used were 1.3 kb fragment of the mouse *oncostatin M*, 0.9 kb fragment of the mouse *pim-1*, 1.1 kb fragment of the mouse c-*myc*, 0.8 kb fragment of the mouse *bcl-x*, 0.8 kb fragment of the mouse c-*fos*, 1.2 kb fragment of the mouse JAB, 1.3 kb fragment of the mouse CIS, 0.9 kb fragment of the mouse p21$^{WAF1/Cip1}$, 1.2 kb fragment of the human *cyclin A*, 1.2 kb fragment of the mouse *cyclin D2*, 1.1 kb fragment of the human *cyclin E*, 1.2 kb fragment of the mouse ICE, 0.6 kb fragment of the mouse β-*actin*, and 0.6 kb fragment of the human glyceraldehyde-3-phosphate dehydrogenase. In analyzing the human leukemic cells, polyA(+) RNA was isolated from cells by using FastTrack 2.0 kit (Invitrogen), and 5 µg of polyA(+) RNA was used for Northern blot experiments.

RT–PCR analysis

Seven micrograms of total RNA were reverse transcribed with random hexamers by using first-strand cDNA synthesis kit (Pharmacia), and one ninth of the reaction mixture was subjected to 20 cycles of PCR with Ex Taq (Takara) at 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min to amplify *bcl-x* cDNAs. The primers used are as follows: 5′bcl-x, 5′-GAAAGAATTCACCATGTCTCAGAGCAACCGG-3′; 3′bcl-x, 5′-GAAAGCGGCCGCTCACTTCCGACTGAAGAGTG-3′.

An *Eco*RI and a *Not*I site were included for further cloning experiments in the 5′ and 3′ pimers, respectively. The *bcl-x* specific sequences (Fang *et al.*, 1994) were underlined. Expected products are 727 bp and 538 bp for *bcl-xL* and *bcl-xS* transcripts, respectively. β-*actin* cDNA was amplified by PCR in the same condition as for *bcl-x* except that the annealing temperature was 56°C. The primers used are as follows: 5′β-actin, 5′-CCTAAGGCCAACCGTGAAAAG-3′; 3′β-actin, 5′-TCT-TCATGGTGCTAGGAGCCA-3′.

Assay to identify which gene renders Ba/F3 cells IL-3-independent

PolyA(+) RNA was isolated from the 1*6 Ba/F3 cells by using FastTrack 2.0 kit and RT–PCR was performed to isolate mouse p34 *pim-1* gene with Pfu DNA polymerase (Stratagene). The primers used were: 5′pim-1, 5′-GAAAGAATTCACCGACACCCTGGAGGT-3′; 3′pim-1, 5′-GAAAGCGGCCGCGCTACTTGCTGGATCCCGG-3′.

An *Eco*RI and a *Not*I site were included on the 5′ end of the primers. The *pim-1* specific sequences (Selten *et al.*, 1986) are underlined. The PCR product was digested with *Eco*RI and *Not*I, subcloned in pMX vector (Onishi *et al.*, 1996), and designated as pMX-pim-1. pMX-bcl-xL was constructed by isolating a 0.7 kb *Eco*RI–*Not*I fragment with the primers 5′bcl-x and 3′bcl-x in the same way, except that Pyrobest DNA polymerase (Takara) was used. pMX-c-myc was constructed by inserting a 1.7 kb *Bam*HI–*Sal*I fragment of the mouse c-*myc* cDNA into pMX. Ba/F3 cells were retrovirally transduced by using these vectors and IL-3 was deprived 28 h after infection.

Analysis of function of JAB, CIS and p21

A bicistronic retroviral vector pMX-IRES-EGFP was constructed by inserting a 1.3 kb *Eco*RI–*Not*I fragment consisting of multi-cloning sites, internal ribosomal entry site (IRES) and EGFP sequences from the plasmid LZRSpBMN-linker-IRES-EGFP (gift from Dr H.Spits) into *Eco*RI and *Not*I sites of pMX. The *Not*I site downstream of EGFP was disrupted so that the *Not*I site in the multi-cloning sites is available. The IRES sequence is derived from the encephalomyocarditis virus, and bicistronic expression was demonstrated (Staal *et al.*, 1996). pMX-JAB-IRES-EGFP was constructed by inserting a 1.2 kb *Bam*HI–*Sal*I fragment of the mouse full-length JAB gene into *Bam*HI and *Xho*I sites of pMX-IRES-EGFP. pMX-CIS-IRES-EGFP was constructed by inserting a 2.1 kb *Eco*RI–*Not*I fragment of the mouse full-length CIS gene into the same sites of pMX-IRES-EGFP. pMX-p21-IRES-EGFP was constructed by inserting a 0.9 kb *Xho*I fragment of the mouse p21 gene into an *Xho*I site of pMX-IRES-EGFP. These vectors and pMX-IRES-EGFP as a negative control were lipofected into BOSC23 cells to obtain the viruses. Ba/F3 cells were infected with the viruses harboring JAB and EGFP, CIS and EGFP, p21 and EGFP, or EGFP alone, and the cells expressing EGFP were sorted into the medium containing 2 ng/ml of IL-3 by using FACS Vantage (Becton Dickinson) 24 h after infection. Ba/F3 cells expressing the 1*6 STAT5A-Flag were also transduced with pMX-JAB-IRES-EGFP, pMX-CIS-IRES-EGFP or pMX-IRES-EGFP, and the cells expressing EGFP were sorted into the medium without IL-3.

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