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Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells

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STAT5 is a member of a family of transcription factors that participate in the signal transduction pathways of many hormones and cytokines. Although STAT5 is suggested to play a crucial role in the biological effects of cytokines, its downstream target(s) associated with cell growth control is largely unknown. In a human interleukin-3 (IL-3)-dependent cell line F-36P-mpl, the induced expression of dominant-negative (dn)-STAT5 and of dn-ras led to inhibition of IL-3-dependent cell growth, accompanying the reduced expression of cyclin D1 mRNA. Also, both constitutively active forms of STAT5A (1*6-STAT5A) and ras (H-ras^{G12V}) enabled F-36P-mpl cells to proliferate without added growth factors. In NIH 3T3 cells, 1*6-STAT5A and H-ras^{G12V} individually and cooperatively transactivated the cyclin D1 promoter in luciferase assays. Both dn-STAT5 and dn-ras suppressed IL-3-induced cyclin D1 promoter activities in F-36P-mpl cells. Using a series of mutant cyclin D1 promoters, 1*6-STAT5A was found to transactivate the cyclin D1 promoter through the potential STAT-binding sequence at –481 bp. In electrophoretic mobility shift assays, STAT5 bound to the element in response to IL-3. Furthermore, the inhibitory effect of dn-STAT5 on IL-3-dependent growth was restored by expression of cyclin D1. Thus STAT5, in addition to ras signaling, appears to mediate transcriptional regulation of cyclin D1, thereby contributing to cytokine-dependent growth of hematopoietic cells.

Keywords: cyclin D1/proliferation/ras/STAT5

Introduction

STAT (signal transducers and activators of transcription) proteins are transcription factors that become activated upon phosphorylation in response to cytokines and hormones. When phosphorylated by the Janus family of protein tyrosine kinases (JAKs), STATs dimerize, translocate to the nucleus and participate in transcriptional regulation by binding to specific DNA sequences in the promoter of target genes (for reviews see Ihle, 1995, 1996; Darnell, 1997). Among seven members of the STAT family, STAT5 originally was identified as a mammary gland factor (MGF) that was activated by prolactin (PRL) (Wakao *et al*., 1994). In addition, STAT5 has been reported to be activated by a variety of cytokines and hormones such as interleukin-2 (IL-2), IL-3, granulocyte–macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO), thrombopoietin (TPO), growth hormone and insulin (Gouileux *et al*., 1995; Mui *et al.*, 1995; Pallard *et al*., 1995; Wakao *et al*., 1995; Chen *et al*., 1997). Subsequently, this protein was renamed STAT5A, because a closely related gene denoted STAT5B was identified in mice and human (Azam *et al*., 1995; X.Liu *et al.*, 1995; Mui *et al.*, 1995; Lin *et al*., 1996).

STAT proteins have been reported to be involved in some aspects of cell growth, differentiation, survival and transformation. For example, STAT1 was reported to induce cell cycle arrest through the induction of p21^{WAF1} (Chin *et al.*, 1996). STAT3 was revealed to play roles in IL-6-induced macrophage differentiation (Nakajima *et al.*, 1996) and granulocyte colony-stimulating factor (G-CSF)-induced granulocytic differentiation (Shimozaki *et al*., 1997). Furthermore, STAT3 was shown to transmit an anti-apoptotic signal from IL-6 (Fukada *et al*., 1996) and to be responsible for anchorage-independent proliferation of v-*src*-transformed NIH 3T3 cells (Bromberg *et al*., 1998; Turkson *et al*., 1998). In the case of STAT5, it was reported to be involved in EPO-induced erythroid differentiation of murine erythroleukemia cell lines ELM-I-1 and SKT6 (Iwatsuki *et al*., 1997; Wakao *et al*., 1997), while an opposite result was shown in a human IL-3-dependent erythrocytic leukemia cell line TF-1 (Chretien *et al*., 1996). Moreover, the previous studies have provided contradictory results regarding the role of STAT5 in cytokine-induced cell growth. By using dominant-negative (dn) forms of STAT5, STAT5 was shown to participate in IL-3- and TPO-dependent proliferation of hematopoietic cells (Mui *et al*., 1996; Matsumura *et al*., 1998). In contrast, mutant forms of EPO and IL-2 receptors, both of which were unable to activate STAT5, were shown to transmit mitogenic signals as efficiently as wild-type receptors (Fujii *et al*., 1995; Quelle *et al.*, 1996). It has been reported recently that, although STAT5A-targeted mice undergo normal development without apparent hematopoietic abnormalities (Lie *et al.*, 1997), macrophages obtained from the bone marrow of STAT5A-targeted mice showed a 33% decreased proliferative response to GM-CSF compared with that of those from normal mice (Feldman *et al.*, 1997). Furthermore, Teglund *et al.* (1998) have reported that STAT5A$^{-/-}$/5B$^{-/-}$ mice

reveal no abnormality in numbers of peripheral hematopoietic cells, but the number and proliferative potential of myeloid progenitor cells assayed by *in vitro* culture with IL-3, IL-5, TPO or GM-CSF were reduced by ~50% in the bone marrow cells of these mice. These lines of evidence suggested that STAT5 is involved, at least partially, in cytokine-dependent proliferation of hematopoietic cells, although its target molecule(s) associated with cell proliferation has not been identified.

It has become increasingly apparent that cell proliferation is dependent on the receipt of appropriate signals to complete transitions through the cell cycle, and that cell cycle progression is tightly regulated by cell cycle regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) (for reviews see Sherr, 1994; Sherr and Roberts, 1995). The mitogenic growth factors act during the G_1 phase of the cell cycle, and drive the cells to the restriction (R) point, after which the factors are no longer required to complete cell division. Progression through G_1 and the G_1/S transition is known to be regulated by cdk4, cdk6 and cdk2. The catalytic activities of cdk4 and cdk6 are up-regulated in mid- to late-G_1 phases dependent on the formation of the complexes with D-type cyclins, whereas they are also regulated negatively by CKIs such as $p15^{INK4A}$, $p16^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. Moreover, cdk2 forms complexes with both cyclins D and E and is activated later in G_1 phase. Among D-type cyclins, cyclin D1 originally was identified as a molecule that links growth factor signaling and cell cycle machinery (Matsushime *et al.*, 1991). Experimental evidence indicates that cyclin D1 is a critical molecule in the regulation of progression through the G_1 phase of the cell cycle, thereby leading to cell proliferation (Baldin *et al.*, 1993; Quelle *et al.*, 1993; Arber *et al.*, 1996). It has been shown that cyclin D1 together with its CDK partners is responsible for the G_1/S transition by phosphorylating the retinoblastoma (Rb) protein, which then releases transcription factors important for the initiation of DNA replication. In the G_1 phase of the cell cycle, the expression of cyclin D1 is induced rapidly by growth factors, and is suggested to be regulated by ras, a small GTP-binding protein that plays a key role in growth factor signaling by transmitting mitogenic signals from cell surface receptor to a protein kinase cascade consisting of Raf, MEK (MAPK-activating enzyme, also called MAPKK) and MAPKs (mitogen-activated protein kinases) (for a review see McCormick, 1994; Marshall, 1995; Downward, 1996, 1997). However, the molecular mechanisms governing the regulation of cell cycle by growth factor signaling are not well understood.

In the present study, we have examined the effect of dn-STAT5 and dn-ras on IL-3-induced proliferation and expression of cell cycle regulatory molecules, and found that dn-STAT5 as well as dn-ras suppressed the induction of cyclin D1 mRNA by IL-3, thereby leading to growth suppression. In accordance with this result, an activated form of STAT5 was found to recognize specifically a STAT-responsive element in the promoter of the cyclin D1 gene, and to regulate the induction of cyclin D1 expression. Furthermore, the inhibitory effect of dn-STAT5 on IL-3-dependent growth was abrogated by the expression of cyclin D1. Thus STAT5 appears to mediate transcriptional regulation of cyclin D1 and to participate in cytokine-dependent growth of hematopoietic cells.

Results

Both STAT5 and ras are involved in cytokine-dependent proliferation of F-36P-mpl cells

In order to examine the mechanism of cytokine-dependent proliferation, we expressed several mutant signaling molecules in F-36P-mpl, a human IL-3-dependent cell line transfected with the TPO receptor c-mpl (Matsumura *et al.*, 1998) by using a Lac-inducible system, in which expression of the target protein is induced by isopropyl-β-D-thiogalactopyranoside (IPTG) treatment. To evaluate the efficiency of this system, we initially prepared a stable clone from F-36P-mpl cells in which dn-JAK2 is inducibly expressed. In this clone, IPTG treatment led to rapid induction of dn-JAK2 in Western blot analysis, and IPTG-induced dn-JAK2 severely inhibited (by ~90%) both IL-3- and TPO-induced proliferation in a [^{3}H]thymidine incorporation assay (data not shown), suggesting that the Lac-inducible system works fairly well in F-36P-mpl cells. To examine the functional roles of two downstream molecules of JAK2, STAT5 and ras, we inducibly expressed hemagglutinin (HA)-tagged dn-STAT5, constitutively active STAT5 1*6-STAT5A, dn-ras and an activated form of H-ras^{G12V} in F-36P-mpl cells, and the clones were designated F-36P-dn-STAT5, F-36P-1*6-5A, F-36P-dn-ras and F-36P-H-ras^{G12V}, respectively. In F-36P-dn-STAT5 cells, the addition of 0.5 mM IPTG to the culture medium led to the rapid induction of dn-STAT5 protein as early as 4 h, and its expression reached a peak at ~24 h and was maintained for up to 48 h in the HA-immunoprecipitated proteins (Figure 1A, upper left panel). Furthermore, Western blot analysis of the whole-cell lysates revealed that the induced dn-STAT5 was far more abundant than endogenous STAT5 (Figure 1A, upper right panel). In F-36P-1*6-5A cells, the treatment with IPTG resulted in rapid induction of 1*6-STAT5A in STAT5-immunoprecipitated proteins (Figure 1A, middle panel). Also, a similar induction of dn-ras and H-ras^{G12V} was observed in F-36P-dn-ras and F-36P-H-ras^{G12V} cells during the IPTG treatment by Western blot analysis of the whole-cell lysates (Figure 1A, lower panel). In a [^{3}H]thymidine incorporation assay, the induced expression of either dn-STAT5 or dn-ras by IPTG treatment led to the suppression (~30%) of recombinant human (rh) IL-3-induced proliferation of F-36P-mpl cells, whereas IPTG treatment did not affect the proliferation of a control cell line which was transfected with a Lac-inducible empty expression vector (Figure 1B). Next, we examined the effect of 1*6-STAT5A and H-ras^{G12V} on cell proliferation in the absence of exogenous growth factors. When rhIL-3 was removed from the culture medium without IPTG treatment, F-36P-1*6-5A and F-36P-H-ras^{G12V} cells ceased to proliferate, and the numbers of total viable cells gradually decreased due to apoptosis (Figure 1C). By contrast, when 1*6-STAT5A expression was induced by IPTG treatment, F-36P-1*6-5A cells were found to proliferate even in the absence of rhIL-3, albeit to a lesser degree than those stimulated with rhIL-3, indicating that 1*6-STAT5A positively contributes to cell growth and can suffice for slow growth (Figure 1C). The induced

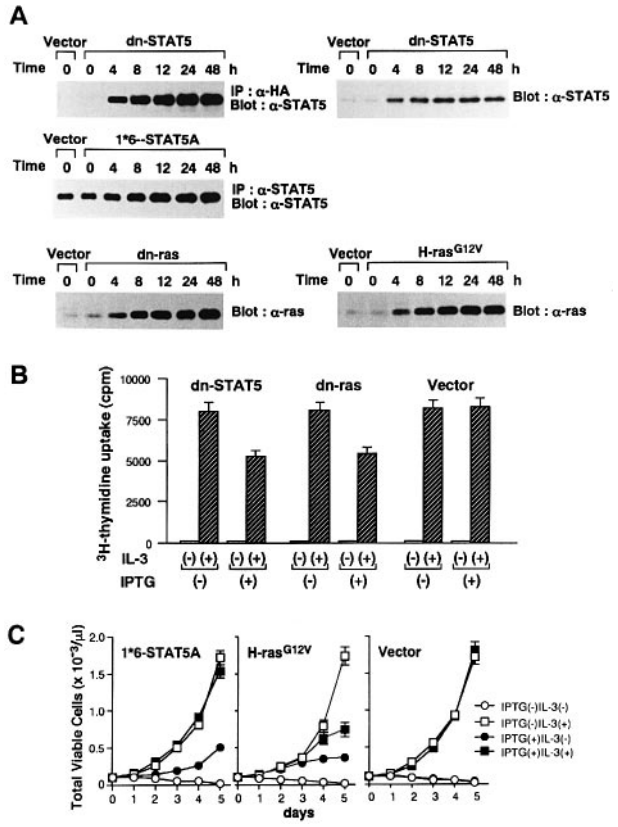

Fig. 1. (**A**) Inducible expression of HA-tagged dn-STAT5, 1*6-STAT5A, dn-ras and H-ras^{G12V}. F-36-dn-STAT5, F-36P-dn-ras, F-36P-1*6-5A and F-36P-H-ras^{G12V} cells were treated with 0.5 mM IPTG for the time indicated. To detect inducible expression of dn-STAT5 and 1*6-STAT5A, the cellular lysates were immunoprecipitated with murine anti-HA mAb (12CA5) or rabbit anti-STAT5b polyclonal Ab (C17), respectively. Immunoprecipitated proteins were subjected to SDS–PAGE, and the blots were probed with anti-STAT5b Ab (C17). Also, inducible expression of dn-STAT5 was examined by Western blot analysis on the total cell lysates. The inducible expression of dn-ras and H-ras^{G12V} was examined by Western blot on the whole-cell lysates by probing with anti-pan-Ras mAb (OP40). (**B**) Effects of dn-STAT5 and dn-ras on rhIL-3-induced proliferation. F-36P-dn-STAT5, F-36P-dn-ras and F-36P/Vector cells were starved of rhIL-3 for 24 h in the presence or absence of 0.5 mM IPTG, then cultured with 10 ng/ml of rhIL-3 with or without 0.5 mM IPTG for 48 h, and subjected to a [^{3}H]thymidine incorporation assay. The results are shown as mean ± SD of triplicate experiments. (**C**) Effects of 1*6-STAT5A and H-ras^{G12V} on proliferation of F36P-1*6-5A and F-36P-H-ras^{G12V} cells. F36P-1*6-5A and F-36P-H-ras^{G12V} cells were cultured with or without IPTG for 24 h, resuspended in 10% FCS RPMI at a cell density of 100/μl in the presence or absence of rhIL-3 and/or IPTG, and then cultured for the period indicated. The total viable cell number was counted by the Trypan blue dye exclusion method [○, IPTG(–), IL-3 (–); □, IPTG(–), IL-3 (+); ●, IPTG(+), IL-3 (–); ■, IPTG(+), IL-3 (+)]. The results are shown as the mean ± SD of triplicate cultures.

expression of H-ras^{G12V} also enabled F-36P-H-ras^{G12V} cells to proliferate without any added growth factors (Figure 1C). However, the growth rate became slow after 4 days, because long-term induction of H-ras^{G12V} gave rise to megakaryocytic differentiation of F-36P-mpl cells (Matsumura *et al.*, 1998). These results suggested that an activated form of either STAT5 or ras could substitute, at least partially, for IL-3 to induce cell cycle progression.

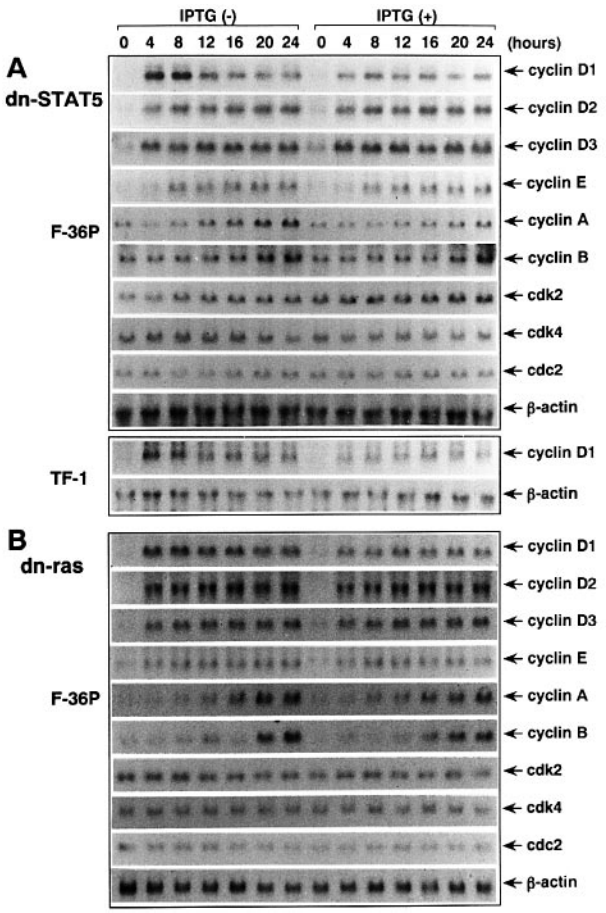

Fig. 2. Effects of dn-STAT5 (**A**) and dn-ras (**B**) on expression of cell cycle regulatory molecules during IL-3-induced proliferation of F-36P-dn-STAT5, TF-1-dn-STAT5 and F36P-dn-ras cells. After 24 h of IL-3 starvation with or without IPTG treatment, F-36P-dn-STAT5, TF-1-dn-STAT5 and F-36P-dn-ras cells were cultured with 10 ng/ml of rhIL-3 in the presence or absence of IPTG for up to 24 h, and expression levels of cell cycle regulatory molecules and β-actin were examined by Northern blot analysis.

Suppression of IL-3-induced cyclin D1 mRNA expression by dn-STAT5 and dn-ras

In an effort to clarify the mechanisms of STAT5- and ras-mediated cell proliferation, we examined the effects of dn-STAT5 and dn-ras on expression of cell cycle regulatory molecules, including cyclins and CDKs. F-36P-dn-STAT5 and F-36P-dn-ras cells were deprived of growth factor for 24 h and then stimulated with rhIL-3 in the presence or absence of IPTG for the times indicated. In F-36P-dn-STAT5 cells, Northern blot analysis showed that the expression of cyclin D1 was up-regulated in response to rhIL-3 as early as 4 h, and sustained for up to 24 h (Figure 2A), nearly to the the same levels as in parental F-36P-mpl cells (data not shown). However, IPTG-induced dn-STAT5 suppressed cyclin D1 mRNA induction 48% at 4 h and 35% at 8 h, by densitometric analysis, while the expression and induction levels of cyclin D2, cyclin D3, cyclin E, cyclin A, cyclin B, cdk2, cdk4 and cdc2 were not affected by dn-STAT5 (Figure 2A). To examine the effects of STAT5 on cyclin D1 expression in a hematopoietic cell line other than F-36P-mpl, we prepared a stable clone from a human IL-3-dependent cell line TF-1 (Kitamura *et al.*, 1989), in which dn-STAT5 is inducibly

expressed (designated TF-1-dn-STAT5). In TF-1-dn-STAT5 cells, IL-3-dependent proliferation was inhibited similarly by IPTG-induced dn-STAT5 by ~30% in a [^{3}H]thymidine incorporation assay (data not shown). In addition, IL-3-induced cyclin D1 mRNA expression at 4–8 h was significantly inhibited (45% suppression at 4 h; 37% suppression at 8 h) by dn-STAT5 in TF-1-dn-STAT5 cells (Figure 2A).

In F36P-dn-ras cells, the induced expression of dn-ras suppressed rhIL-3-induced cyclin D1 mRNA expression 45% at 4 h and 40% at 8 h, as was the case with dn-STAT5 (Figure 2B). In addition, dn-ras showed a minimal inhibitory effect on cyclin D2 mRNA induction at 4–8 h. Like dn-STAT5, dn-ras did not influence the expression or induction levels of cyclin D3, cyclin E, cyclin A, cyclin B, cdk2, cdk4 or cdc2 mRNA (Figure 2B). Furthermore, cell cycle analysis revealed that the rhIL-3-induced G_1/S transition was significantly suppressed by both dn-STAT5 and dn-ras (data not shown). Together, these findings suggested that both STAT5 and ras may be involved in transcriptional regulation of cyclin D1.

Relationship between cyclin D1 overexpression and the growth inhibition by dn-STAT5 and dn-ras

To determine if cyclin D1 was linked to STAT5- and ras-mediated cell growth, the Lac-inducible expression vector of cyclin D1 or cyclin D2 was transfected further into F-36P-dn-STAT5 cells (each designated F-36P-dn-STAT5/cyclin D1 or F-36P-dn-STAT5/cyclin D2) and F-36P-dn-ras cells (each designated F-36P-dn-ras/cyclin D1 or F-36P-dn-ras/cyclin D2). As a control, furthermore, the Lac-inducible empty expression vector was transfected into F-36P-dn-STAT5 and F-36P-dn-ras cells (designated F-36P-dn-STAT5/Vector and F-36P-dn-ras/Vector). After rhIL-3 deprivation for 24 h, these clones were treated with IPTG for the time indicated in the absence of growth factors. Expression of endogenous cyclin D1 and cyclin D2 protein was not observed after 24 h rhIL-3 deprivation and was not induced during 24 h IPTG treatment in F-36P-dn-STAT5/Vector or F-36P-dn-ras/Vector cells (data not shown). In contrast, IPTG treatment led to rapid induction of cyclin D1 or cyclin D2 protein in F-36P-dn-STAT5/cyclin D1, F-36P-dn-ras/cyclin D1, F-36P-dn-STAT5/cyclin D2 and F-36P-dn-STAT5/cyclin D2 cells (Figure 3A). In a [^{3}H]thymidine incorporation assay, IL-3-dependent proliferation of control F-36P-dn-STAT5/Vector and F-36P-dn-ras/Vector cells was partially suppressed (~30%) by IPTG treatment at every concentration of rhIL-3 from 1 to 100 ng/ml, as was the case with the parental F-36P-dn-STAT5 and F-36P-dn-ras cells (Figure 3B). In F-36P-dn-STAT5/cyclin D1 cells, the growth inhibition by dn-STAT5 was reversed by overexpression of cyclin D1 (Figure 3B). However, the induced expression of cyclin D2 led to only minimal cancellation of the growth-inhibitory effects of dn-STAT5 in F-36P-dn-STAT5/cyclin D2 cells (Figure 3B). In addition, overexpression of cyclin D1 or cyclin D2 did not really affect the growth suppression by dn-ras in F-36P-dn-ras/cyclin D1 and F-36P-dn-ras/cyclin D2 cells (Figure 3B). Similar results were observed in four independent clones of each type, in which cyclin D1 or cyclin D2 protein was induced effectively by IPTG treatment (data not shown). These

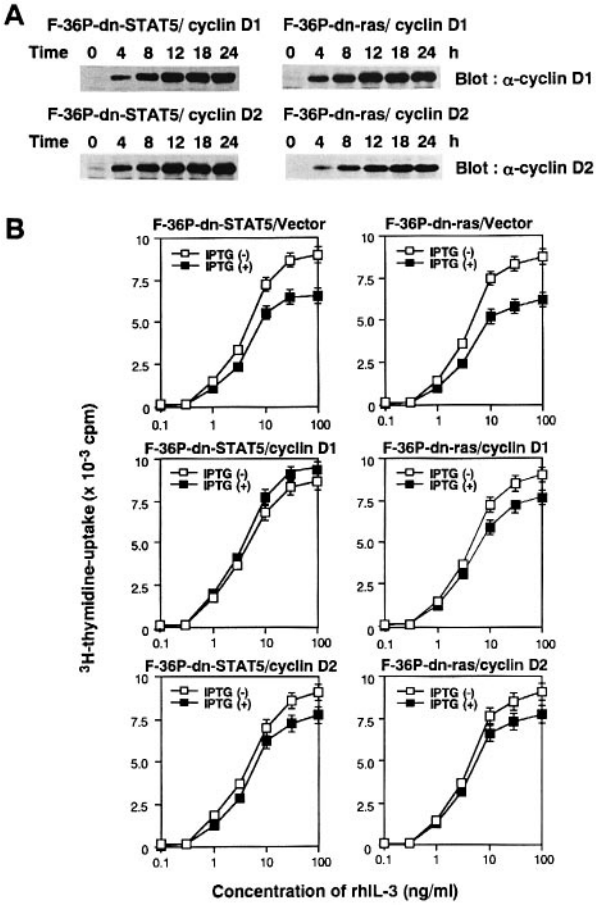

Fig. 3. (**A**) Inducible expression of cyclin D1 and cyclin D2 in F-36P-dn-STAT5/cyclin D1, F-36P-dn-STAT5/cyclin D2, F-36P-dn-ras/cyclin D1 and F-36P-dn-ras/cyclin D2 cells. The cells of each clone were starved of serum and rhIL-3 for 24 h, and then cultured with IPTG for the time indicated. Total cellular lysates were subjected to SDS–PAGE, and the blots were probed with anti-cyclin D1 or anti-cyclin D2 mAb. (**B**) Effects of overexpression of cyclin D1 or cyclin D2 on growth inhibition by dn-STAT5 and dn-ras. The cells of each clone were deprived of rhIL-3 with or without 0.5 mM IPTG for 24 h, then cultured with various concentrations of rhIL-3 in the presence or absence of IPTG for 48 h, and subjected to a [^{3}H]thymidine incorporation assay. The results are shown as mean ± SD of triplicate experiments [□, IPTG (–); ■, IPTG (+)].

results raised the possibility that cyclin D1 might be a crucial target of STAT5 in IL-3-stimulated F-36P cells.

Transactivation of the cyclin D1 promoter by constitutively active forms of STAT5 and ras

To examine the mechanism of transcriptional regulation of the cyclin D1 promoter, we constructed a reporter gene encoding –1745 bp of the human cyclin D1 promoter, linked to a luciferase reporter gene (–1745-CD1-Luc) (the structure is shown in Figure 6). First, we performed luciferase assays using either wild-type (WT)-STAT5A or 1*6-STAT5A as an effector. When NIH 3T3 cells were transfected with an expression vector of WT- or 1*6-STAT5A along with –1745-CD1-Luc, 1*6-STAT5A led to a dose-dependent transactivation (up to 3.8-fold) of –1745-CD1-Luc, whereas WT-STAT5A showed a minimal effect (up to 1.4-fold) in the serum-deprived conditions (Figure 4A). In combination with or without 1*6-STAT5A, we next examined the effects of other signal transducers

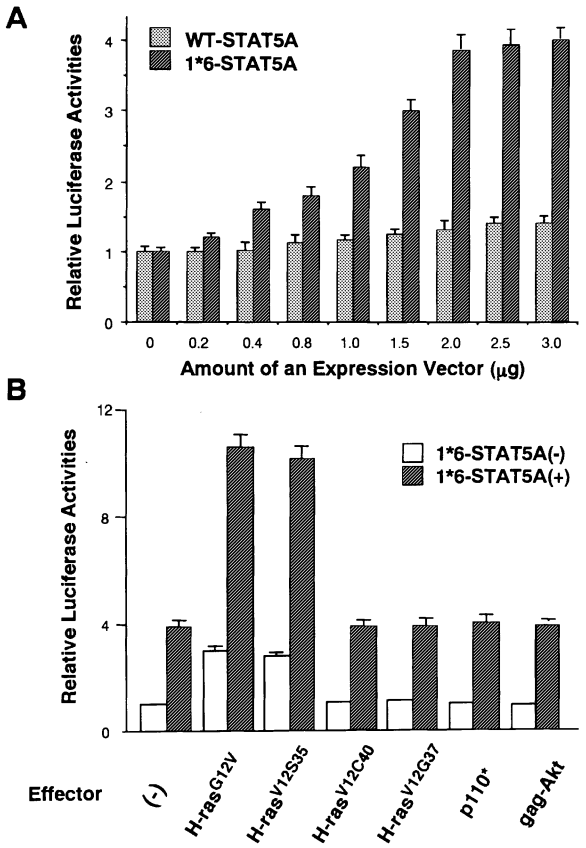

Fig. 4. (**A**) Dose-dependent effects of 1*6-STAT5A and WT-STAT5A on –1745-CD1-Luc activities. NIH 3T3 cells were transfected with various amounts of an expression vector pcDNA3-1*6-STAT5A or pcDNA3-WT-STAT5A along with 3 μg of –1745-CD1-Luc and 10 ng of pRL-CMV-Rluc by the calcium phosphate co-precipitation method. After 12 h, the cells were washed, serum-starved for 24 h and then subjected to luciferase assays. Luciferase assays were performed using a Dual-Luciferase Reporter System, in which relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the renilla luciferase activities. (**B**) Cooperative effects of 1*6-STAT5A with various types of signaling molecules on –1745-CD1-Luc activities in NIH 3T3 cells. NIH 3T3 cells were transfected with 3 μg of –1745-CD1-Luc and 10 ng of pRL-CMV-Rluc together with 2 μg of the expression vector indicated, alone or in combination with 2 μg of pcDNA3-1*6-STAT5A, by the calcium phosphate co-precipitation method. Luciferase assays were performed as described above. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments. The results are shown as the mean ± SD of triplicate cultures.

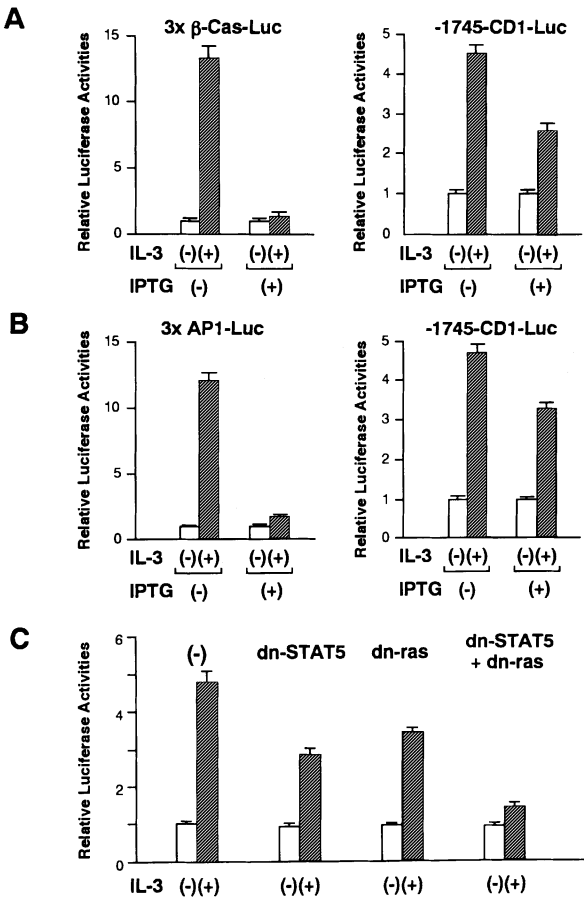

Fig. 5. (**A** and **B**) Effects of the induced expression of dn-STAT5 and dn-ras on IL-3-stimulated –1745-CD1-Luc, 3× β-Cas-Luc and 3× AP1-Luc activities. F-36P-dn-STAT5 and F-36P-dn-ras cells were transfected with 2.8 μg of each reporter gene together with 200 ng of pRL-CMV-Rluc by lipofection. After 24 h culture, the cells were serum and rhIL-3 starved for 12 h, stimulated with 10 ng/ml of rhIL-3 for 5 h, and then subjected to luciferase assays as described in Figure 4. To examine the effects of dn-STAT5 and dn-ras, the cells were pre-treated with 0.5 mM IPTG for 24 h before transfection, and cultured with IPTG during the assays. (**C**) Cooperative effects of the transfected dn-STAT5 and dn-ras on –1745-CD1-Luc activities in F-36P-mpl cells. F-36P-mpl cells were transfected with 1 μg of –1745-CD1-Luc and 0.2 μg of pRL-CMV-Rluc along with 0.9 μg of dn-STAT5 expression vector and/or 0.9 μg of dn-ras expression vector by lipofection. After 24 h culture, the cells were serum- and rhIL-3-starved for 12 h, stimulated with 10 ng/ml of rhIL-3 for 5 h, and then subjected to luciferase assays as described in Figure 4. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments. The results are shown as the mean ± SD of triplicate cultures.

of IL-3 on –1745-CD1-Luc activities, including p110* (constitutively active PI3-kinase), gag-PKB/Akt (constitutively active PKB/Akt), H-ras^{G12V} and three types of H-ras^{G12V}-derived mutants (H-ras^{V12S35}, which exclusively activates the raf/MAPK pathway; H-ras^{V12C40}, which activates only the PI3-kinase pathway; H-ras^{V12G37}, which interacts only with Ral-GDS). NIH 3T3 cells were transfected with –1745-CD1-Luc together with an effector gene alone or in combination with 1*6-STAT5A. As shown in Figure 4B, H-ras^{G12V} and H-ras^{V12S35} were able to transactivate –1745-CD1-Luc by 3.1- and 2.9-fold, respectively, while p110*, gag-PKB/Akt, H-ras^{V12C40} or H-ras^{V12G37} were ineffective. In addition, H-ras^{G12V}- and H-ras^{V12S35}-induced –1745-CD1-Luc activities were augmented further by co-transfected 1*6-STAT5A to 10.9- and 10.4-fold, respectively, suggesting that activated STAT5 and ras (raf/MAPK pathway) can transactivate the cyclin D1 promoter coordinately.

IL-3-activated STAT5 and ras lead to transactivation of the cyclin D1 promoter in F-36P-mpl cells

We examined whether cytokine-activated STAT5 and ras could stimulate cyclin D1 promoter activity as well as 1*6-STAT5A and H-ras^{G12V}. F-36P-dn-STAT5 cells were cultured in the presence or absence of IPTG for 24 h, and then transfected with a reporter gene for STAT5, 3× β-Cas-Luc (Matsumura *et al.*, 1998) or –1745-CD1-Luc. In the absence of IPTG treatment, rhIL-3 induced 3× β-Cas-Luc 13.3-fold, and the IL-3-induced trans-

activation was almost completely abrogated by IPTG induction of dn-STAT5 (Figure 5A, left panel). As shown in Figure 5A (right panel), rhIL-3 stimulated –1745-CD1-Luc 4.7-fold in the absence of IPTG treatment, which was reduced to 2.7-fold by IPTG-induced dn-STAT5; this coincided with the findings on the effects of dn-STAT5 on rhIL-3-induced proliferation (Figures 1B and 3B) and cyclin D1 expression (Figure 2, upper panel). Next, we performed luciferase assays with F-36P-dn-ras cells by using the reporter genes –1745-CD1-Luc and 3× AP1-Luc, which is transactivated by ras-mediated AP1 (Matsumura *et al.*, 1998). Without IPTG treatment, rhIL-3 stimulated 3× AP1-Luc 12.3-fold, which was reduced to 1.5-fold by the induced expression of dn-ras (Figure 5B, left panel). In addition, rhIL-3-stimulated –1745-CD1-Luc activity was reduced from 4.7- to 3.4-fold by IPTG treatment (Figure 5B, right panel). To examine the cooperative effects of dn-STAT5 and dn-ras, F-36P-mpl cells were transfected with dn-STAT5 and/or dn-ras along with a reporter gene –1745-CD-Luc. Consistent with the effects of the induced expression of dn-STAT5 and dn-ras (Figure 5A and B), transiently transfected dn-STAT5 and dn-ras also reduced rhIL-3-induced –1745-CD1-Luc activity from 4.9- to 2.9- and to 3.6-fold, respectively (Figure 5C). In addition, rhIL-3-induced –1745-CD1-Luc activity (4.9-fold) was reduced further to 1.6-fold when dn-STAT5 and dn-ras were co-transfected. These results suggested that both STAT5 and ras are crucial regulators of IL-3-induced cyclin D1 promoter activity.

Identification of the STAT5-binding motif at –481 bp of the cyclin D1 promoter (D1-SIE1)

Because activated ras was reported to transactivate the cyclin D1 promoter through the AP1 response element which is located at –964 bp in the cyclin D1 promoter (Albanese *et al.*, 1995), we tried to determine a responsive element for STAT5 in the cyclin D1 promoter. We performed luciferase assays with a series of cyclin D1 promoter–reporter constructs in NIH 3T3 cells by using 1*6-STAT5A as an effector (Figure 6A, left panel). The reporter gene activities induced by 1*6-STAT5A were observed in –1745-CD1-Luc to –674-CD1-Luc, whereas they were severely reduced for the –261-CD1-Luc and –143-CD1-Luc constructs (Figure 6A, right panel). These results suggested that a response element for STAT5 may be present between –674 and –261 bp in the cyclin D1 promoter. Within the proximal 2 kb of the cyclin D1 gene promoter region, two putative STAT-binding motifs were identified: one motif was at –481 bp (designated D1-SIE1) and another at –247 bp (designated D1-SIE2). To examine the roles of these elements, mutations were introduced into the D1-SIE1 site and/or D1-SIE2 site of –674-CD1-Luc to generate –674-SIE1m-Luc, –674-SIE2m-Luc and –674-SIE1.2m-Luc. Also, reporter genes that contain three tandem repeats of D1-SIE1, D1-SIE2 and D1-SIE1m were constructed (Figure 6A, left panel). 1*6-STAT5A was able to stimulate –674-SIE2m-Luc in nearly the same way as –674-CD1-Luc, while it had little effect on –674-SIE1m-Luc and –674-SIE1.2m-Luc. Furthermore, 1*6-STAT5A induced activation of 3× D1-SIE1-Luc 13.7-fold, but did not induce 3× D1-SIE2-Luc or 3× D1-SIE1m-Luc. In addition, 1*6-STAT5B, a constitutively active form of murine STAT5B, similarly transactivated –1745-CD1-Luc

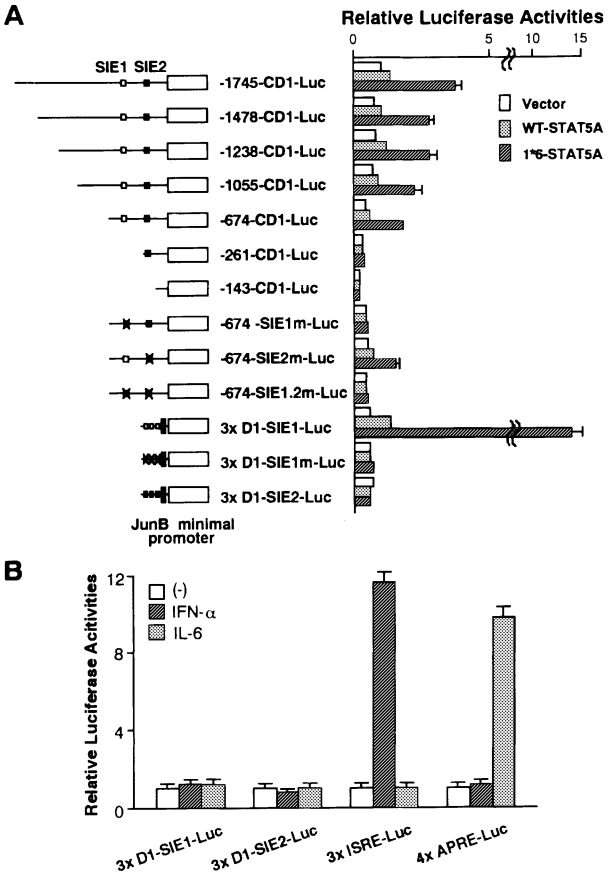

Fig. 6. (**A**) Effects of 1*6-STAT5A and WT-STAT5A on various types of mutant cyclin D1 promoters. NIH 3T3 cells were transfected with 2 μg of an empty pcDNA3, pcDNA3-1*6-STAT5A or pcDNA3-WT-STAT5A, together with 10 ng of pRL-CMV-Rluc and 3 μg of the reporter gene indicated, by the calcium phosphate co-precipitation method, and subjected to luciferase assays as described in Figure 4. (**B**) Effects of rhIFN-α-stimulated STAT1 and rhIL-6-stimulated STAT3 on 3× D1-SIE1-Luc, 3× D1-SIE2-Luc, 3× ISRE-Lu and 4× APRE-Luc activities. NIH 3T3 cells were transfected with 2 μg of each reporter gene and 10 ng of pRL-CMV-Rluc by the calcium phosphate co-precipitation method. After 12 h, the cells were washed, serum starved for 24 h, then either not stimulated or stimulated with rhIFN-α (30 ng/ml) or rhIL-6 (30 ng/ml) for 6 h, and subjected to luciferase assays. The experiments were performed in triplicate and similar results were obtained from at least three independent experiments. The results are shown as the mean ± SD of triplicate cultures.

and 3× D1-SIE1-Luc but not 3× D1-SIE2-Luc or 3× D1-SIE1m-Luc (data not shown). Thus, 1*6-STAT5A and 1*6-STAT5B appeared to transactivate the cyclin D1 promoter through the D1-SIE1 element in a sequence-specific manner.

Specific binding of activated STAT5, but not STAT1 or STAT3, to the D1-SIE-1 sequence

We next examined the roles of other members of the STAT family in the transcriptional regulation of D1-SIE1 and D1-SIE2 elements. NIH 3T3 cells were transfected with 3× D1-SIE1-Luc, 3× D1-SIE2-Luc, 3× ISRE-Luc (a reporter gene for STAT1) or 4× APRE-Luc (a reporter gene for STAT3) (Matsumura *et al.*, 1998), stimulated with recombinant human interferon-α (rhIFN-α) or rhIL-6, and subjected to luciferase assays. As shown in Figure 6B, the treatment with rhIFN-α stimulated 3×

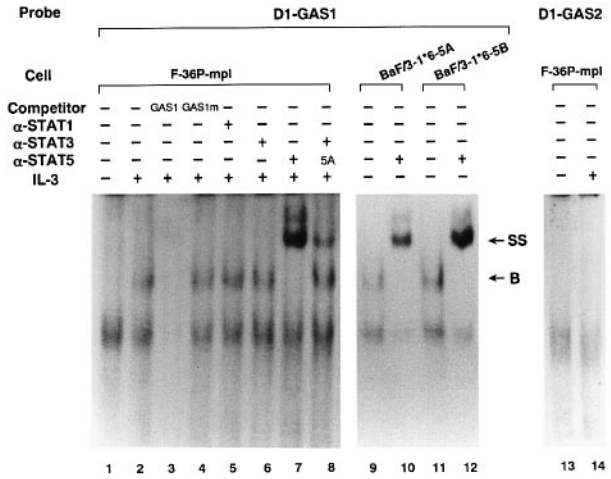

Fig. 7. Identification of the D1-SIE site-binding proteins by EMSA. The nuclear lysates were extracted from F-36P-mpl cells before and after 15 min treatment with rhIL-3 (10 ng/ml) and from serum- and factor-starved Ba/F3-1*6-5A and Ba/F3-1*6-5B cells. Nuclear extract (15 μg of each sample) was incubated with end-labeled D1-SIE1 or D1-SIE2 probe (30 000 c.p.m.) in 20 μl of binding buffer containing 1 μg of poly(dI–dC) at 4°C for 20 min, and subjected to EMSA as previously reported. In competition assays, nuclear extracts were pre-incubated with a 200-fold molar excess of unlabeled competitor oligonucleotide before the binding reaction with labeled probe. In supershift assays, the nuclear proteins were incubated with 1 μg of rabbit anti-STAT1 (C-136), anti-STAT3 (C-20), anti-STAT5B (C-17) (reactive with both STAT5A and STAT5B) or anti-STAT5A (L-20) (specific for STAT5A, not cross-reactive with STAT5B) polyclonal Ab for 30 min at 4°C, and then the binding reaction was performed. B, DNA-binding complex; SS, supershifted DNA-binding complex.

ISRE-Luc by 10.8-fold, but not 3× D1-SIE1-Luc, 3× D1-SIE2-Luc or 4× APRE-Luc. Also, rhIL-6 stimulated 4× APRE-Luc by 9.8-fold, but not 3× D1-SIE1-Luc, 3× D1-SIE2-Luc or 3× ISRE-Luc. These results suggested that neither 3× D1-SIE1-Luc nor 3× D1-SIE2-Luc are stimulated by STAT1 or STAT3.

Because both rhIL-3 and rhTPO induced tyrosine phosphorylation of STAT1 and STAT3 as well (data not shown), we further examined the specific binding of STAT5 to the D1-SIE1 element by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from F-36P-mpl cells before and after stimulation with rhIL-3 or rhTPO, and also from factor-starved Ba/F3-1*6-5A and Ba/F3-1*6-5B cells (a murine IL-3-dependent cell line stably transfected with 1*6-STAT5A and 1*6-STAT5B, respectively). As shown in Figure 7, the rhIL-3-treated nuclear extracts possessed binding activity to D1-SIE1 oligonucleotide (lanes 1 and 2). The binding was competed effectively by the D1-SIE1 competitor (lane 3) but not by equimolar amounts of the D1-SIE1m oligonucleotide (lane 4), suggesting that the rhIL-3-induced DNA-binding complex was formed in a sequence-specific manner. The nuclear extracts from Ba/F3-1*6-5A and Ba/F3-1*6-5B cells also formed a DNA-binding complex with a similar mobility (lanes 9 and 11). Furthermore, the DNA-binding complexes formed from rhIL-3-treated F-36P-mpl cells (lane 2), Ba/F3-1*6-5A (lane 9) and Ba/F3-1*6-5B cells (lane 11) were supershifted completely by pre-incubation with anti-STAT5 antibody (Ab), which recognizes both STAT5A and STAT5B (lanes 7, 10 and 12), but not with anti-STAT1 or anti-STAT3 Ab (lanes 5 and 6). In addition, rhIL-3-induced DNA-binding complex from F-36P-mpl cells was not supershifted completely by pre-incubation with anti-STAT5A-specific Ab (lane 8), suggesting that the D1-SIE1-binding proteins were composed of both STAT5A and STAT5B. In contrast, the nuclear extracts from F-36P-mpl cells did not show any change in binding activity to D1-SIE2 before and after rhIL-3-treatment (lanes 13 and 14). Similar results were observed for the nuclear extracts of rhTPO-treated F-36P-mpl cells (data not shown). These results again suggested that IL-3- or TPO-activated STAT5 (both STAT5A and STAT5B), but not STAT1 or STAT3, bound to the D1-SIE1 sequence.

Discussion

Hematopoiesis is regulated by a number of hematopoietic growth factors through their interaction with receptors of the hematopoietin receptor superfamily, including receptors for IL-3, IL-5, IL-6, GM-CSF, G-CSF, EPO and TPO. Although these receptors do not possess cytoplasmic catalytic domains, ligand binding results in the initiation of a series of biochemical events such as tyrosine phosphorylation and activation of JAK family tyrosine kinases, STATs, phosphatidylinositol 3-kinase (PI3-kinase) and Shc. In this pathway, Jak2 has been shown to play an essential role in EPO-, TPO-, IL-3- and GM-CSF-induced proliferation, because myeloid progenitor cells in the fetal liver of Jak2-targeted mice fail to respond to these cytokine stimuli, and these mice display an embryonic lethality due to the absence of definitive erythropoiesis (Neubauer *et al*., 1998; Parganas *et al*., 1998). Although ras has been proposed to transduce mitogenic signals downstream of Jak2, it was reported that dn-ras did not affect IL-3-dependent proliferation of Ba/F3 cells (Terada *et al*., 1995), suggesting the possibility that signaling molecule(s) other than ras could transmit mitogenic signals. Supporting this hypothesis, IL-2-activated PI3-K recently was shown to induce E2F activity, thereby leading to phosphorylation of Rb, induction of cyclin D3 and degradation of $p27^{Kip1}$ (Brennan *et al*., 1997). In addition, we and others have demonstrated the involvement of STAT5 in IL-3- and TPO-dependent proliferation of hematopoietic cells (Mui *et al*., 1996; Matsumura *et al*., 1998). However, little is known about how STAT5 couples cytokine signals to the cell cycle machinery, although STAT5 has been shown to participate in transcriptional regulation of various genes such as β-casein, $p21^{WAF1}$, CIS, oncostatin M, pim-1, c-fos, Id-1 and IL-2 receptor α chain (Matsumoto *et al*., 1995; Mui *et al*., 1995; Yoshimura *et al*., 1995; Matsumura *et al*., 1997; Nakajima *et al*., 1997).

In the present study, we showed that dn-STAT5 suppressed IL-3-dependent proliferation of F-36P-mpl cell as effectively as dn-ras. In addition, dn-STAT5 suppressed IL-3-induced cyclin D1 mRNA expression with an efficiency almost similar to that of dn-ras, while dn-STAT5 did not affect the expression and induction levels of cyclin D2, cyclin D3, cyclin E, cyclin A, cyclin B, cdk2, cdk4 or cdc2. These results raised the possibility that STAT5, in addition to ras, may participate in cytokine-induced cell growth through transcriptional regulation of cyclin D1 that plays a key role in both G_1 progression and G_1/S transition in the cell cycle. Consistent with this hypothesis, constitutively active murine STAT5 (1*6-STAT5A and 1*6-STAT5B) and IL-3-activated STAT5

were able to transactivate the cyclin D1 promoter, and the effect of IL-3-activated STAT5 was almost equivalent to that of ras in F-36P-mpl cells. Furthermore, the IL-3-induced cyclin D1 promoter activity essentially was suppressed by the combination of dn-STAT5 and dn-ras. These results suggest that the STAT5-binding site (D1-SIE1) and the ras-responsive element (AP1) may be critical targets for the induction of cyclin D1 transcription by IL-3 in F-36P-mpl cells, although several other regulatory elements such as binding sites for Sp1, Ets, CREB and E2F have also been identified in the promoter region of the cyclin D1 gene (Albanese *et al*., 1995; Watanabe *et al.*, 1996a,b, 1998).

Our data also suggest that cyclin D1 may be a crucial target of STAT5 in cytokine-dependent proliferation. In the culture of F-36P-mpl cells with IL-3, the growth-inhibitory effects of dn-STAT5 were cancelled by over-expression of cyclin D1, but not of cyclin D2. In contrast, overexpression of cyclin D1 or cyclin D2 led to only minimal abrogation of growth suppression by dn-ras. This observation is not perfectly consistent with the findings by Aktas *et al.* (1997) showing that overexpression of cyclin D1 could completely overcome the growth inhibition by dn-ras in serum-induced proliferation of NIH 3T3 cells. In the cell cycle machinery, however, ras is reported not only to induce cyclin D1 expression, but also to possess several other activities such as down-regulation of $p27^{Kip1}$ expression (Aktas *et al.*, 1997) and collaboration with myc in inducing accumulation of active cyclin E–cdk2 and E2F (Leone *et al*., 1997). In accord with these findings, ras is shown to act at several distinct phases of the cell cycle including G_0/G_1, early G_1, the G_1/S boundary and at G_2/M (Doborowolski *et al*., 1994; Pan *et al*., 1994; J.J.Liu *et al*., 1995; Winston *et al*., 1995; Peeper *et al.*, 1997). Therefore, the discrepancy might result from the difference in ras activities according to cell types and/or growth factors.

Because STAT5A-, STAT5B-, STAT5A/5B- and cyclin D1-targeted mice were shown to grow without distinct hematopoietic abnormalities (Fantl *et al.*, 1995; Lie *et al.*, 1997; Udy *et al*., 1997; Teglund *et al*., 1998), the functional roles of STAT5 and cyclin D1 in hematopoiesis might be redundant and replaced sufficiently by other signaling molecules and D-type cyclins. However, the number and size of myeloid colonies that developed after culture with IL-3, IL-5, TPO or GM-CSF were reduced to ~50% in the bone marrow cells of $STAT5A^{-/-}5B^{-/-}$ mice (Teglund *et al*., 1998), suggesting that STAT5 contributes to cytokine-dependent proliferation of hematopoietic progenitor cells. Furthermore, it would be of great interest to show that STAT5A-, STAT5A/B- and cyclin D1-targeted mice revealed an apparent defect in mammary gland development. Because PRL is a major growth factor for mammary gland cells and has been shown to activate STAT5 (Wakao *et al*., 1994), it is possible that the STAT5–cyclin D1 pathway may not be redundant and may be indispensable for PRL-induced growth of mammary gland cells.

In summary, we demonstrated here that STAT5 mediates transcriptional regulation of cyclin D1, thereby contributing to cytokine-dependent growth of hematopoietic cells. Since 1*6-STAT5A protein suffices for slow growth in factor-deprived conditions, STAT5 is proposed to participate positively in protection from apoptosis as well as cell cycle progression. Further studies, including analysis of target molecules of STATs that exert cell cycle progression and anti-apoptotic effects, may be important in understanding the mechanisms by which cytokines regulate cell proliferation, differentiation and survival.

Materials and methods

Reagents and antibodies

Highly purified rhTPO, rhIL-3 and rhIL-6 were provided by Kirin Brewery Co. Ltd (Tokyo, Japan). Highly purified rhIFN-α was a gift from the Sumitomo Pharmaceutical Co. Ltd (Tokyo, Japan). Rabbit anti-STAT1 (C-136), STAT3 (C-20), STAT5A (L-20) and STAT5B (C-17) polyclonal Abs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Murine anti-HA monoclonal antibody (mAb) 12CA5, murine anti-pan-Ras mAb (OP40), murine anti-cyclin D1 mAb (C20320) and rat anti-cyclin D2 mAb (34B1-3) were purchased from Boehringer Mannheim (Mannheim, Germany), Oncogene Research Product (Cambridge, MA), Transduction Laboratories (Rockdale, Australia) and Santa Cruz Biotechnology Inc. (CA), respectively.

Cells and cell culture

F-36P, a human IL-3-dependent erythroleukemia cell line originally established by Chiba *et al*. (1991) was obtained from Riken Cell Bank (Tsukuba, Japan). Several stable transformants from F-36P cells were cultured in RPMI 1640 (Nakarai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Flow, North Ryde, Australia) in the presence of 5 ng/ml rhIL-3. NIH 3T3 cells were obtained from Human Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Nakarai) supplemented with 10% FCS.

Plasmid construct and cDNAs

The human cyclin D1 promoter gene and cDNA were generously provided by Dr A.Arnold (Massachusetts General Hospital, Boston). Human cyclin D2 and D3 cDNAs were supplied by Dr G.Peters (Imperial Cancer Research Fund, London, UK). Human cyclin A, cyclin B, cyclin E, cdk2 and cdk4 cDNAs were a gift from Dr H.Kiyokawa (University of Illinois, Cancer Center, Chicago). Human cdc2 cDNA was supplied by Dr Ed Harlow (Massachusetts General Hospital, Boston). A constitutive active form of murine STAT5A cDNA (1*6-STAT5A) harbors substitutions at amino acid residue 711 from serine to phenylalanine (S711F) and at 299 from histidine to arginine (H299R), as previously reported (Onishi *et al*., 1998). To construct expression vectors of murine WT-STAT5A and 1*6-STAT5A, these cDNAs were subcloned into the *Not*I–*Eco*RI site of pcDNA3 (Invitrogen, The Netherlands). An expression vector for c-mpl, Humplpas12, was provided by Kirin Brewery Co. Ltd (Tokyo, Japan). Lac-inducible expression vectors for cyclin D1, cyclin D2 and 1*6-STAT5A were generated by subcloning these cDNAs into the *Kpn*I–*Xba*I site of pOPRSVI/MCS (Stratagene, La Jolla, CA). Lac-inducible expression vectors for dn-STAT5 (pOPRSVI-HA-MGF^{694F}), dn-ras (pOPRSVI-H-ras^{S17N}) and activated ras (pOPRSVI-H-ras^{G12V}) were described previously (Matsumura *et al*., 1998). An expression vector for constitutively active PKB/Akt (pSG5-gagPKB/Akt) was kindly provided by Dr P.Coffer (University Hospital of Utrecht, The Netherlands) (Burgering and Coffer, 1995). Expression vectors for constitutively active PI3-kinase p110* (myc-tagged p110-CAAX in pcDNA3), H-ras^{V12S35} (pSG5-H-ras^{V12S35}), H-ras^{V12C40} (pSG5-H-ras^{V12C40}) and H-ras^{V12G37} (pSG5-H-ras^{V12G37}) were reported previously (White *et al*., 1995; Joneson *et al*., 1996; Khwaja *et al*., 1997).

Lac-inducible system

To express a target cDNA, we used a LacSwitchTMII inducible expression system (Stratagene), in which transcription of a target cDNA is initiated by IPTG treatment. In brief, F-36P cells initially were co-transfected with expression vectors for the Lac repressor, pCMV-LacI and c-mpl, by electroporation (Matsumura *et al.*, 1998). After the culture with 0.5 mg/ml of hygromycin (Sigma, St Louis, MO), one clone (designated F-36P-mpl) was transfected further with a Lac-inducible expression vector for dn-STAT5, 1*6-STAT5A, dn-ras or activated ras. After the selection with G418 (Sigma) at a concentration of 1.5 mg/ml, the induction levels of the target protein were examined before and after 0.5 mM IPTG treatment by Western blot analyses. Four clones designated F-36P-dn-STAT5, F36P-1*6–5A, F-36P-dn-ras and F-36P-H-ras^{G12V} were subjected to further analyses because the target proteins were

induced most efficiently by IPTG treatment in these clones. To prepare clones, that can inducibly express cyclin D1 or cyclin D2, and a mock transfectant, F36P-dn-STAT5 and F-36P-dn-ras cells were co-transfected further with pOPRSVI-cyclin D1, pOPRSVI-cyclin D2 or an empty pOPRSVI/MCS together with pSV2bsr, an expression vector for blasticidin S deaminase (Kaken Pharma. Co., Tokyo, Japan). After the selection with 30 μg/ml of blastcidin S hydrochloride (Funakoshi, Tokyo, Japan), several blasticidin-resistant cells were cloned and subjected to further analyses.

Northern blot analysis

The isolation of total cellular RNA and the method for Northern blot analysis were described previously (Matsumura *et al*., 1995).

Cell proliferation assay

To quantitate proliferation of the cells, a [^{3}H]thymidine incorporation assay was used as previously described (Sugahara *et al*., 1994). In brief, after 24 h serum and rhIL-3 starvation, triplicate aliquots of cells [3.0×10^4 cells resuspended in 100 μl of serum-free Cos 004 medium (Cosmobio, Tokyo, Japan)] were cultured in 96-well flat-bottomed microtiter plates for 48 h at 37°C in the presence or absence of the growth factor(s). [^{3}H]Thymidine was added for the final 4 h of the culture, and incorporated [^{3}H]thymidine was measured with a scintillation counter. To examine the effects of the IPTG-induced proteins (dn-STAT5, dn-ras, cyclin D1 or cyclin D2), the cells were treated with 0.5 mM IPTG during 24 h starvation and following a 48 h culture period.

Flow cytometry

The DNA content of cultured cells was quantitated by staining with propidium iodide (PI) and analyzed on FACSort (Beckon Dickinson, Oxnard, CA) as previously reported (Matsumura *et al*., 1997). Cell cycle analysis was performed with the program Modfit LT2.0 (Beckon Dickinson).

Immunoprecipitation and immunoblotting

Preparation of cell lysates, immunoprecipitation, gel electrophoresis and immunoblotting were performed according to the methods described previously (Matsumura *et al*., 1996). Briefly, the cultured cells were lysed in lysis buffer containing 1% Nonidet P-40 (NP-40) and protease inhibitors, and insoluble materials were removed by centrifugation. The whole-cell lysates (15 μg per each lane) or immunoprecipitated proteins were subjected to SDS–PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA). After blocking the residual binding sites on the filter, immunoblotting was performed with an appropriate Ab. Immunoreactive proteins were visualized with the enhanced chemiluminescence detection system (DuPont NEN, Boston, MA).

Construction of reporter genes that include mutant cyclin D1 promoter

An 1878 bp *Pvu*II fragment (–1743 to +133, numbered from a transcription initiation site) was subcloned into the plasmid, PSP72-Luciferase (Nakajima *et al*., 1993), to construct a reporter gene –1745-CD1-Luc. Reporter genes that include a series of deletion mutants of the cyclin D1 promoter were generated by using restriction enzyme recognition sites as follows: –1478 (*Eco*NI); –1238 (*Afl*II); –1055 (*Acc*I); –674 (*Bst*EII); –261 (*Pst*I); –143 (*Nar*I). To construct –674-SIE1m-Luc, –674-SIE2m-Luc and –674-SIE1.2m-Luc, point mutations were introduced into the D1-SIE1 site and/or the D1-SIE2 site of –674-CD1-Luc by PCR, and the sequences are as follows: D1-SIE1m, 5′-GTCTTGGTA-3′; and D1-SIE2m, 5′-GTCTATGTA-3′ (mutated sites are underlined). To generate 3× D1-SIE1-Luc, 3× D1-SIE2-Luc and 3× D1-SIE1m-Luc, three types of double-stranded oligonucleotides were subcloned into Jun B-PSP72-Luc at the *Kpn*I–*Sal*I site just upstream of the murine minimal JunB promoter (–42 to +136) linked to the firefly luciferase gene (Nakajima *et al*., 1993), and their sequences were as follows: 3× D1-SIE1-Luc, 5′-CTCGTGGCGTTCTTGGAAATGCGC-CC-3′, recognition site is underlined; 3× D1-SIE2-Luc, 5′-GATTT-GCATTTCTATGAAAACCGGAC-3′, recognition site is underlined; 3× D1-SIE1m-Luc, 5′-CTCGTGGCGGTCTTGGTAATGCGCCC-3′, mutated recognition site is underlined.

Luciferase assays

NIH 3T3 cells (2×10^5 cells) were seeded in a 60 mm dish, cultured for 24 h and transfected with various amounts of an expression vector of pcDNA3-1*6-STAT5A or pcDNA3-WT-STAT5A along with 3 μg of an appropriate reporter gene and 10 ng of pRL-CMV-Rluc, an expression vector of renilla luciferase, by the calcium phosphate co-precipitation method. After 12 h culture, the cells were washed with phosphate-buffered saline (PBS), serum starved for 24 h and then subjected to luciferase assays. In addition, 2 μg of various types of expression vectors including pcDNA3-1*6-STAT5A, pSG5-gagPKB/Akt, myc-tagged p110-CAAX in pcDNA3, RSV-H-ras^{G12V}, pSG5-H-ras^{V12S35}, pSG5-H-ras^{V12C40} and pSG5-H-ras^{V12G37} were transfected as an effector gene either alone or in combination with 2 μg of pcDNA3-1*6-STAT5A. To examine the involvement of STAT1 and STAT3 in transcriptional regulation of D1-SIE1 and D1-SIE2 elements, NIH 3T3 cells were transfected with 2 μg of 3× D1-SIE1-Luc, 3× D1-SIE2-Luc, 3× ISRE-Luc (a reporter gene for STAT1) or 4× APRE-Luc (a reporter gene for STAT3) (Matsumura *et al*., 1998) along with 10 ng of pRL-CMV-Rluc by the calcium phosphate co-precipitation method. After 12 h, the cells were serum starved for 24 h, then either left stimulated or stimulated with rhIFN-α (30 ng/ml) or rhIL-6 (30 ng/ml) for 6 h, and subjected to luciferase assays. Luciferase assays with F-36P-dn-STAT5, F-36P-dn-ras and F-36P-mpl cells were performed by lipofection. F-36P-dn-STAT5 and F-36P-dn-ras cells were co-transfected with 2.8 μg of a reporter gene (–1745-CD1-Luc, 3× β-Cas-Luc or 3× AP1-Luc) (Matsumura *et al*., 1998) and 0.2 μg of pRL-CMV-Rluc by lipofectin (Gibco-BRL, Gaithburg, MD). Also, F-36P-mpl cells were transfected with 0.9 μg of dn-ras and/or 0.9 μg of dn-STAT5 together with 1 μg of –1745-CD1-Luc and 0.2 μg of pRL-CMV-Rluc by lipofectin according to the manufacturer's instructions. Briefly, the mixture of 3 μg of DNA (total amounts of transfected DNA were adjusted to 3 μg by an appropriate empty expression vector) and 20 μl of lipofectin were suspended in 200 μl of Opti-MEM (Gibco-BRL) for 30 min at room temperature, and added to 1×10^6 cells resuspended in 800 μl of Opti-MEM in a 35 mm dish. After 5 h incubation, the cells were washed and resuspended in the culture medium. At 24 h post-transfection, the cells were serum and factor deprived for 12 h, stimulated with 10 ng/ml of rhIL-3 for 5 h, and then subjected to luciferase assays. To examine the effects of the IPTG-induced dn-STAT5 or dn-ras, the cells were pre-treated with 0.5 mM IPTG for 24 h and cultured with IPTG during the assay. Luciferase assays were performed using the Dual-Luciferase Reporter System (Promega, Madison, WI), in which relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the renilla luciferase activities. The cells were lysed in lysis buffer supplied by the manufacturer, followed by the measurement of the firefly and renilla luciferase activities on a luminometer LB96P (Berthold Japan, Tokyo, Japan). The relative firefly luciferase activities were calculated by normalizing transfection efficiency according to the renilla luciferase activities. The experiments were performed in triplicate, and similar results were obtained from at least three independent experiments.

Electrophoretic mobility shift assay

The isolation of nuclear extracts was performed as previously described (Matsumura *et al*., 1997). Three types of double-stranded oligonucleotides (D1-SIE1, D1-SIE2 and D1-SIE1m) were used as a probe or a competitor, and their sequences are described above. EMSA was performed as previously reported (Matsumura *et al*., 1997). Briefly, nuclear extract (15 μg of each sample) was incubated in 20 μl of binding buffer containing 1 μg of poly(dI–dC) (Pharmacia Biotech., Uppsala, Sweden) and labeled probe (30 000 c.p.m.) for 20 min at 4°C. The reaction mixture was electrophoresed, dried and subjected to autoradiography. In competition assays, nuclear extracts were pre-incubated with a 200-fold molar excess of unlabeled competitor oligonucleotide, and the binding reaction to the labeled probe was performed. In supershift assays, the nuclear proteins were incubated with 1 μg of rabbit anti-STAT1, anti-STAT3, anti-STAT5B or anti-STAT5A polyclonal Ab for 30 min at 4°C, and then the binding reaction was performed.

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