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Flt3 mutation activates p21$^{WAF1/CIP1}$ gene expression through the action of STAT5☆

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Abstract

Flt3 is a type III RTK and approximately 30% of AML patients harbor an internal tandem duplication (ITD) of the juxtamembrane region or a point mutation of the Flt3 protein leading to the constitutive activation of downstream signaling pathways and aberrant cell growth. The cyclin-dependent kinase inhibitor p21 inhibits cell growth when expressed at high levels and induces cell growth when expressed at lower levels. In this study, we have addressed the role of Flt3-ITD in the regulation of p21. Co-transfection of p21 promoter-luciferase constructs with Flt3-ITD plasmid into K562 and BaF3 cells results in the induction of p21 promoter activity and a −692/−684 STAT site is important for the induction. STAT5a binds specifically to this element and Flt3-ITD enhances the protein binding to this site. Overexpression of Flt3-ITD led to the induction of endogenous p21 expression in various cells. These results may implicate p21 in Flt3-ITD induced leukemogenesis.

Keywords: Flt3; p21$^{WAF1/CIP1}$; Promoter; STAT5a; Leukemia

The receptor tyrosine kinase Flt3 plays an important role in proliferation and survival of hematopoietic stem and progenitor cells [1]. Flt3 is the most frequently mutated gene (20–30%) in cases of AML [1,2]. The majority of Flt3 mutations are internal tandem duplications (ITD) in the juxtamembrane domain of Flt3 receptor [1,2]. This mutation results in the constitutive activation of STAT5 and Ras/mitogen-activated protein kinase (MAPK) pathways [3]. Retroviral transduction of Flt3-ITD mutations into primary murine bone marrow cells results in a myeloproliferative phenotype in a bone marrow transplantation assay [4]. Flt3-ITD overexpression leads to factor independent growth of BaF3 and 32Dcl3 cells [3,5]. Flt3-ITD mutations are linked with a poor prognosis in AML [6]. Thus, Flt3-ITD is considered to play a role in leukemogenesis and leukemia progression.

The MAPK pathway is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation, and survival [7]. Constitutive activation of the MAPK pathway drives the oncogenic transformation of normal fibroblasts results from a variety of genetic alterations, including Ras mutations and overexpression of growth factor receptors [7]. Inappropriate MAPK activation may also play a role in the leukemic transformation of myeloid cells [8]. p21$^{WAF1/CIP1}$ (p21) is a cyclin-dependent kinase (CDK) inhibitor whose expression in mammalian tissues is highly induced in response to stress as well as during normal development and differentiation [9]. p21 inhibits cell cycle progression by binding to G1 cyclin–CDK complexes [10]. On the other hand, p21 also serves to promote proliferation following serum stimulation of quiescent cells [11]. p21 promotes the assembly of cyclin–CDK-proliferating cell nuclear antigen complexes and therefore exerts a positive influence on cell cycle progression [9]. It was shown that cells treated with growth factor exhibit low-level induction of

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p21 [12] and p21 at low concentrations can promote the association of cyclin and CDK subunits to initiate cell cycle progression [13]. Additionally, p21 plays a critical role in the normal mitogenic response of human factor-dependent myeloid cells and maintenance of the stem/progenitor cells [14].

It was previously reported that activation of MAPK as well as Ras results in the stimulation of transcription of p21 in a p53-independent mechanism [15,16]. Quite recently, it was reported that Flt3 specific inhibitor, SU5614, downregulates p21 [17]. These facts suggested that the Flt3-ITD, which stimulates downstream kinase pathways, may upregulate the cell growth regulator, p21.

In this study, we examined the transactivation of the p21 promoter by Flt3-ITD. Our results showed that p21 is a target gene for Flt3-ITD and this upregulation differs from the Ras dependent mechanism. Promoter analysis revealed that a STAT binding site at −692 to −684 in p21 promoter is critical for Flt3-ITD inducibility. Furthermore, overexpression of Flt3-ITD was found to induce endogenous p21 expression. These results suggest that p21 may be important in Flt3-ITD induced leukemogenesis.

Materials and methods

Cell culture. BaF3 cells were maintained in RPMI with 10% heat inactivated fetal bovine serum, 100 ng/L recombinant mouse IL-3 (R&D Systems, Minneapolis, MN), 50 μM/L of 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. K562, HEL cells were maintained in RPMI with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. KG1 cells were cultured in Iscove's modified Dulbecco's medium with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmids. MSCV-Flt3-ITD-GFP was provided from Dr. D. G. Gilliland (Harvard Medical School, Boston, MA). The pcDNA-Flt3-ITD was constructed by digesting MSCV-Flt3-ITD-GFP by *Hpa*I and subcloned into *Eco*RV site of pcDNA3.1 (Invitrogen, Carlsbad, CA). Various fragments of the p21 promoter were provided from Dr. L. Freedman (Merck Research, West Point, PA) [18]. The Ha-Ras plasmid was a gift from Dr. R. Kraus (Mount Sinai School of Medicine, New York, NY). –837 STAT-m was generated by Quick change site directed mutagenesis kit XL (Stratagene, La Jolla, CA) using following primers, respectively. −837 STAT-m sense; 5′-GTCTCTCCAATTCC CTCCGCATGTGAC AATCAACAAC-3′, −837 STAT-m anti-sense; 5′-GTTGTTGATTGT CACATGCGGAGGGAATTGGAGAGAC-3′. The sequences of the constructs were confirmed by sequence analysis.

Reporter assays. K562 cells were transfected as follows. As many as 2×10^6 cells/well in six-well plates were transfected with 500 ng promoter constructs, 25 ng pRL-*Renilla* as an internal control, and 2 μg pcDNA-Flt3-ITD or Ha-Ras or pcDNA empty vector by using Fugene 6 (Roche Molecular Biochemicals, Basel, Switzerland). For BaF3 cells, cells were transfected by electroporation. Briefly, 2×10^7 cells were washed twice and suspended 400 μl serum free RPMI, transferred into BTX cuvettes with 8 μg reporter, 16 μg effector, and 400 ng *Renilla* plasmid, and then electroporated by 240 V, 2800 μF, 72 Ω. Transfected cells were harvested after 24 h transfection. For IL-3 starvation, cells were washed twice 24 h after transfection and cultured in IL-3 deprived medium for 6 h. Luciferase and *Renilla* activities were assayed using the Dual-luciferase reporter assay system (Promega, Madison, WI) as described previously [19].

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) was performed as described previously [20]. The following oligomers were used as probes and/or cold-competitors for EMSA: STAT-wild, 5′-CCCTCCTTCCCGGAAGCATG-3′; STAT-mut, 5′-CCCTCCATGCCGCATGCATG-3′. The oligomers were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Nuclear extracts from MSCV-GFP control plasmid or MSCV-Flt3-ITD-GFP plasmid transfected BaF3 cells were prepared as described previously [21] from sorted GFP positive cells 3 days after transfection with 6 h IL-3 deprivation. Five microgram aliquots of nuclear extracts were incubated in reaction mixture including 20 mM Hepes buffer (pH 7.8), 60 mM KCl, 0.2 mM EDTA, 6 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 10% [v/v] glycerol, and 1.5 μg poly(dI–dC) with ^{32}P-labeled oligomer for 15 min on ice. For supershift assays, mouse anti-STAT5a, STAT3, and STAT5b (Zymed Laboratories, South San Francisco, CA) were added to the reaction mixture after addition of the probe and incubated on ice for 30 min. The mixture was then loaded onto a 4% polyacrylamide gel and electrophoretically separated at 150 V at 4 °C.

Real time PCR. Transfections were performed as described above. Three days after transfection, GFP positive cells were sorted, IL-3 starved for 6 h, and total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcriptase was performed by Superscript II (Invitrogen). Real time PCR was performed using Quantitect SYBR green PCR reagent (Qiagen, Miami, FL) following the manufacturer's protocol using Opticon real-time PCR instrument (MJ Research, Waltham, MA). Briefly, thermal cycler conditions for the murine primers were: step 1, 96 °C for 15 min; step 2, 96 °C for 30 s; step 3, 55 °C for 30 s; and step 4, 72 °C for 30 s. For human primers, step 1, 96 °C for 15 min; step 2, 96 °C for 30 s; step 3, 51 °C for 30 s; and step 4, 72 °C for 1 min. Steps 2–4 were repeated for 40 cycles. The sequences of the primers used, each at concentrations of 200 nM, except mouse GAPDH primers (800 nM), were: mouse p21, forward 5′-CCGTGGACAGTGAGCAGTTG-3, reverse 5′-TGGGCACTTCA GGGTTTTCT-3′; mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-GTGGCAAAGTGGAGATTGTTGCC-3′, reverse 5′-GATGATGACCCGTTGGCTCC-3′; human p21, forward 5′-AGTGGACAGCGAGCAGCTGA-3′, reverse 5′-TAGAAATCTG TCATGCTGGTCTG-3′; and human GAPDH, forward 5′-CCAAAA TCAAGTGGGGCGATG-3′, reverse 5′-AAAGGTGGAGGAGTG GGTGTCG-3′. To avoid amplification of contaminating genomic DNA, every primer was placed in different exon. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis. The relative copy number of target sequences in DNA samples was determined using a comparative Ct (ΔΔCt) method [22]. Standard curves for the mouse GAPDH and mouse p21 $y = 22.94 - 3.09x$ ($r = 0.989$) and $y = 30.51 - 3.24x$ ($r = 0.998$), respectively. For human GAPDH and p21 standard curves are $y = 31.00 - 3.86x$ ($r = 0.998$) and $y = 42.31 - 4.01x$ ($r = 0.999$). The relative copy number of the p21 is given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct^{Flt3-ITD} - \Delta Ct^{vec}$ and each $\Delta Ct = Ct^{p21} - Ct^{GAPDH}$.

Results

Upregulation of p21 promoter by Flt3-ITD and importance of STAT site at −692/−684 in Flt3-ITD mediated induction

To determine the effect of the Flt3-ITD mutant on p21 gene regulation, we first co-transfected an expression vector for this protein together with a p21-promoter luciferase construct [18] into K562 (human

chronic myelogenous leukemia) cells and assayed for luciferase activity. We also compared with the effect of Ha-Ras as a positive control, as it was previously reported to potently activate p21 promoter resulting in growth suppression of K562 cells [15]. K562 cells were first chosen as a hematopoietic cell with high transfection efficiency that lacks the expression of p53, a major regulator of p21 [23]. As compared to controls transfected with pcDNA empty vector, Ha-Ras induced 10-fold increase in p21 promoter activity whereas Flt3-ITD yielded a more modest but reproducible 1.8-fold enhancement of transcription (Fig. 1A). In order to determine the site in p21 promoter conferring Flt3-ITD-induced transcriptional activation, a series of 5′ deletion constructs based on −2.3 kb p21 promoter [18] were utilized. Although the basal expression of the promoter was decreased, deletion of up to −837 bp relative to the transcription start site retained similar levels of induction as the full length promoter construct in response to Flt3-ITD. Further deletion of the p21 promoter to −539 bp of the p21 promoter led to loss of response to Flt3-ITD (Fig. 1B). This indicates that Flt3-ITD is capable of transcriptional activation of p21 through response elements between −837 and −539 in the p21 promoter. The human p21 promoter sequence between −837 and −539 bp from the transcription start site carries one complete STAT site (Fig. 2). We focused on this site since STAT proteins are known downstream targets of Flt3 signal transduction [24,25]. To determine the importance of this site, we internally deleted the STAT site in the context of the −837 p21 luciferase reporter. The wild type reporter (−837 STAT-w) retained activation in response to Flt3-ITD, but the STAT deleted construct, (−837 STAT-m), was not activated by Flt3-ITD (Fig. 1C). These results suggest that STAT binding site at −692 to −684 is a major site for Flt3-ITD transactivation for p21 promoter. To see that this effect is relevant to other cells, we next tried the IL-3 dependent hematopoietic progenitor BaF3 cells. As shown in Fig. 3A, in the presence of IL-3, overexpression of Flt3-ITD had no obvious effect on the p21 promoter activity (Fig. 3A, left panel). In contrast, in the absence of IL-3, Flt3-ITD transactivated the p21 promoter as much as 4-fold (Fig. 3A, right panel), suggesting that the Flt3-ITD signal was redundant in the presence of IL-3 stimulation of the cells. Mutation of the STAT site in the context of the −837 construct reduced the ability of Flt3 to activate the reporter by 60% (Fig. 3B), suggesting that the STAT site, at least in part, mediates the activity of the p21 promoter in these cells.

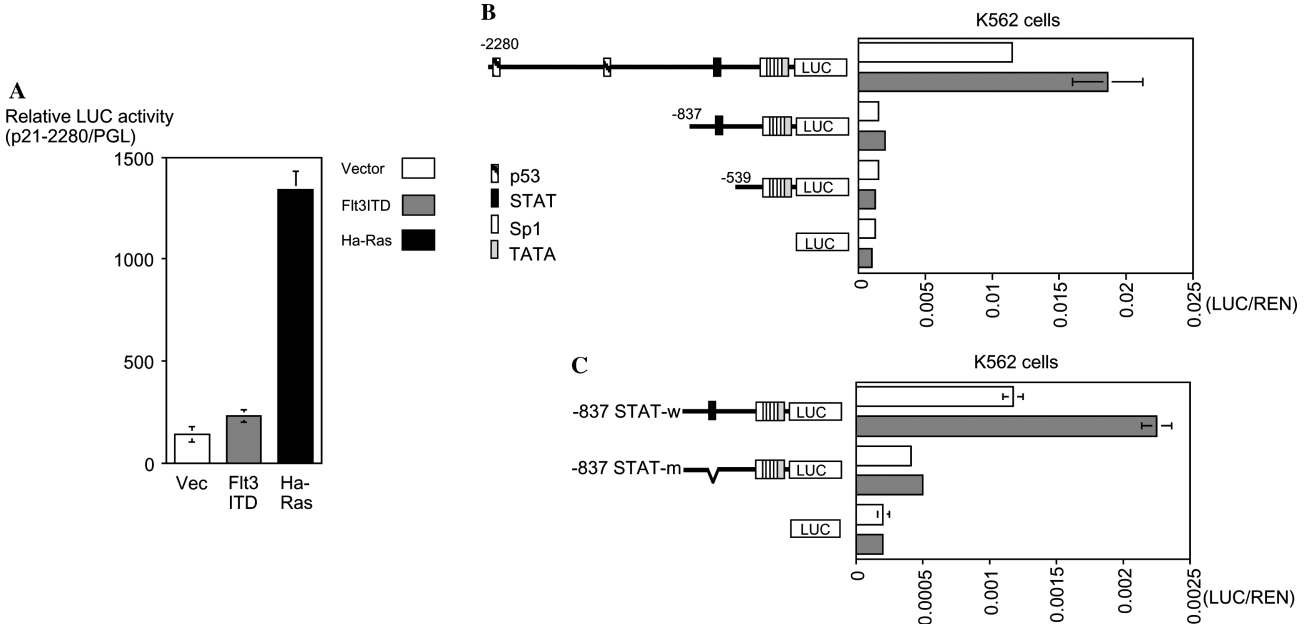

Fig. 1. Up-regulation of p21 promoter by −692 to −684 STAT site by Flt3-ITD in K562 cells. (A) Upregulation of p21 promoter by Flt3-ITD and Ha-Ras. The full length p21 promoter (p21-2280) was co-transfected with the indicated expression vectors. Relative luciferase activities were calculated by comparing normalized luciferase activities (luciferase divided by *Renilla* activity) to normalized activity driven by the pGL-basic plasmid in order to exclude non-specific activation of PGL-basic luciferase reporter by effectors. (B) Deletion analysis of human p21 promoter activity in K562 cells. The results are expressed as the ratio of luciferase to *Renilla* activity in transfected cells. (C) Effect of internal deletion of −692/−684 STAT site in p21 promoter in K562 cells. Wild type or an internal deletion construct of −692 to −684 STAT site (see Materials and methods) was transfected into K562 cells in the presence or absence of Flt3-ITD expression vector. The data are mean values (± standard deviation) from four independent experiments.

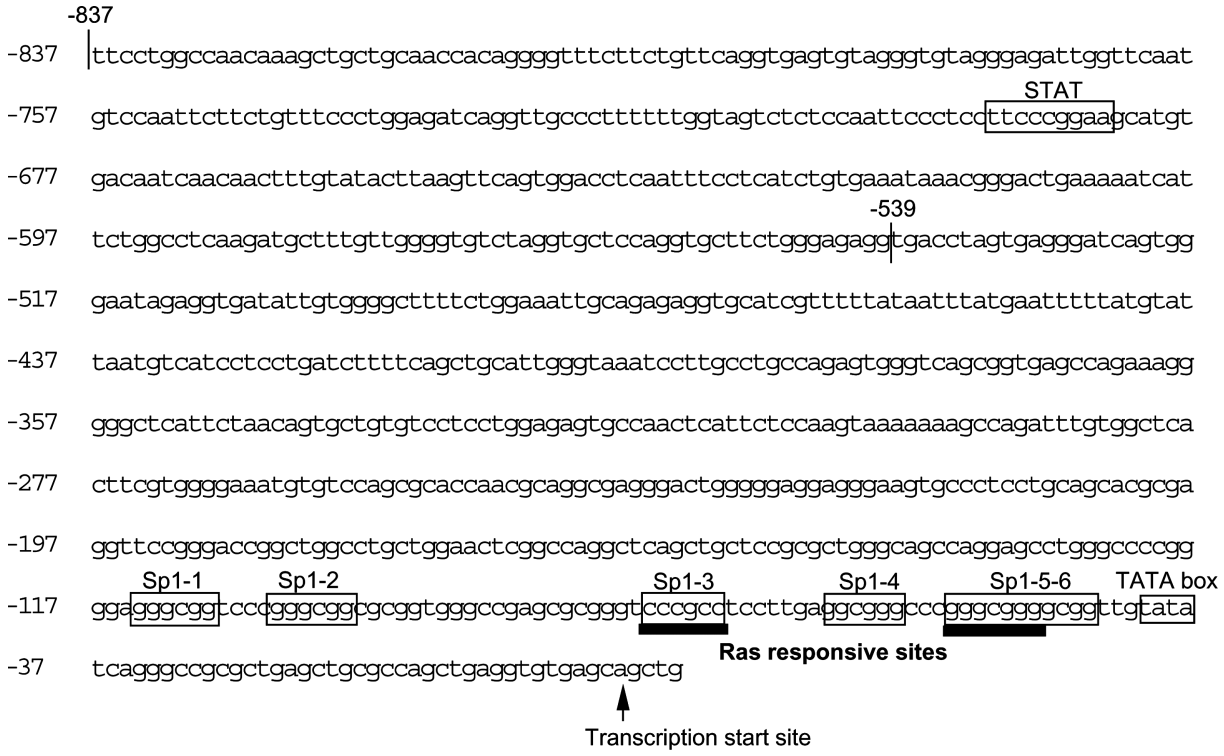

Fig. 2. The 5′-upstream region of the human p21 gene. The nucleotide sequence of the 5′-upstream region (up to −837 from transcription start site) is presented [16,18]. STAT, Sp1 binding sites,s and TATA box are shown as indicated. Ras responsive sites [16] are shown by the black lines.

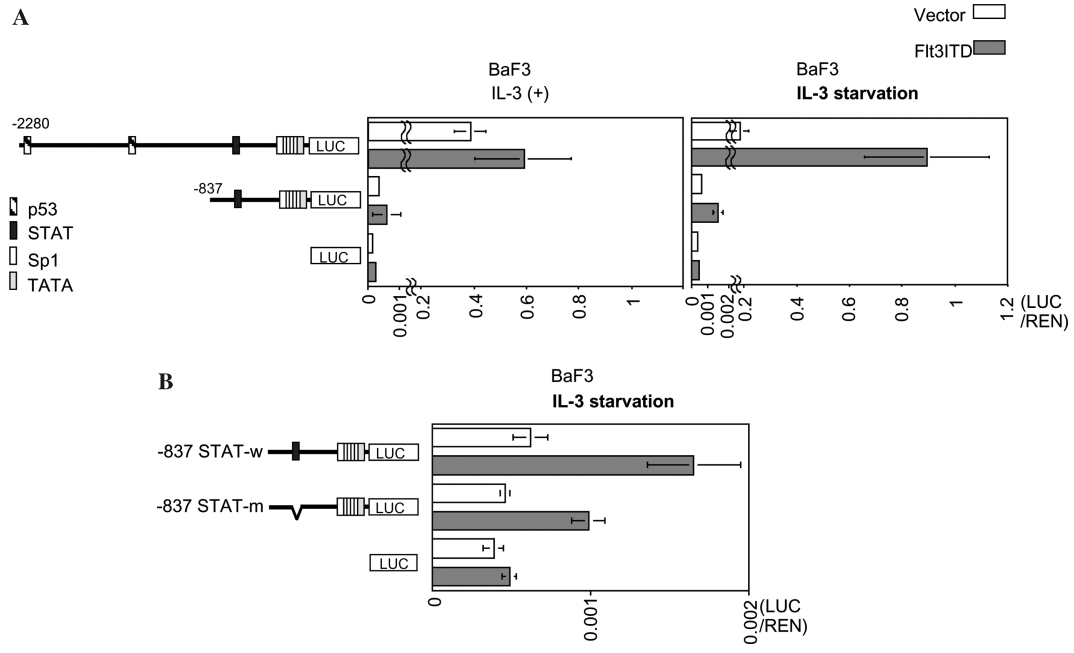

Fig. 3. Transactivation of p21 promoter by Flt3-ITD in IL-3 starved BaF3 cells. (A) Deletion analysis of human p21 promoter activity. Twenty-four hours after transfection, IL-3 starved BaF3 cells (right panel) were IL-3 deprived for 6 h and cells were harvested. The results are shown as the ratio of luciferase to *Renilla* activity in transfected cells. (B) The effect of internal deletion of −692/−684 STAT site in p21 promoter in IL-3 starved BaF3 cells. The average normalized luciferase activity (± standard deviation) from four independent experiments is plotted.

STAT5a specifically binds to −692 to −684 site in p21 promoter

After identifying a major response element for p21 induction by Flt3-ITD, next we tried to identify the protein binding to this element by electrophoretic mobility shift assays (EMSA). We transfected BaF3 cells with a bicistronic Flt3-ITD vector also encoding green fluorescent protein (GFP). Nuclear extracts were prepared from the sorted GFP positive cells and used for EMSA. Profiles of GFP positive cells are shown in Fig. 4A. The DNA–protein binding reactions were carried out using oligonucleotides representing the wild type −698 to −679 sequence in the p21 promoter (Fig. 4B). As shown in Fig. 4C, a specific DNA–protein complex found on this site (arrow), that increased in intensity with expression of Flt3-ITD in IL-3 starved BaF3 cells (Fig. 4C, lanes 2 and 3). The complex was effectively competed with wild-type (lane 4) but not with mutant unlabeled oligonucleotide (lane 5). STAT5a is preferentially activated among other STAT proteins by Flt3-dependent signaling [26,27]. In accordance with this fact, addition of anti-STAT5a antibody resulted in the supershift of the band (lane 7). This supershift is also observed in K562 cells (data not shown). We further checked another STAT protein STAT5b and STAT3, but the addition of anti-STAT3 (lane 10) or anti-STAT5b (lane 12) supershift antibody had no effect on this complex in our condition. Taken together, our data indicate that, STAT5a specifically binds to −692/−684 STAT site and Flt3-ITD enhances the binding of the protein to site, and this may mediate increased expression of p21.

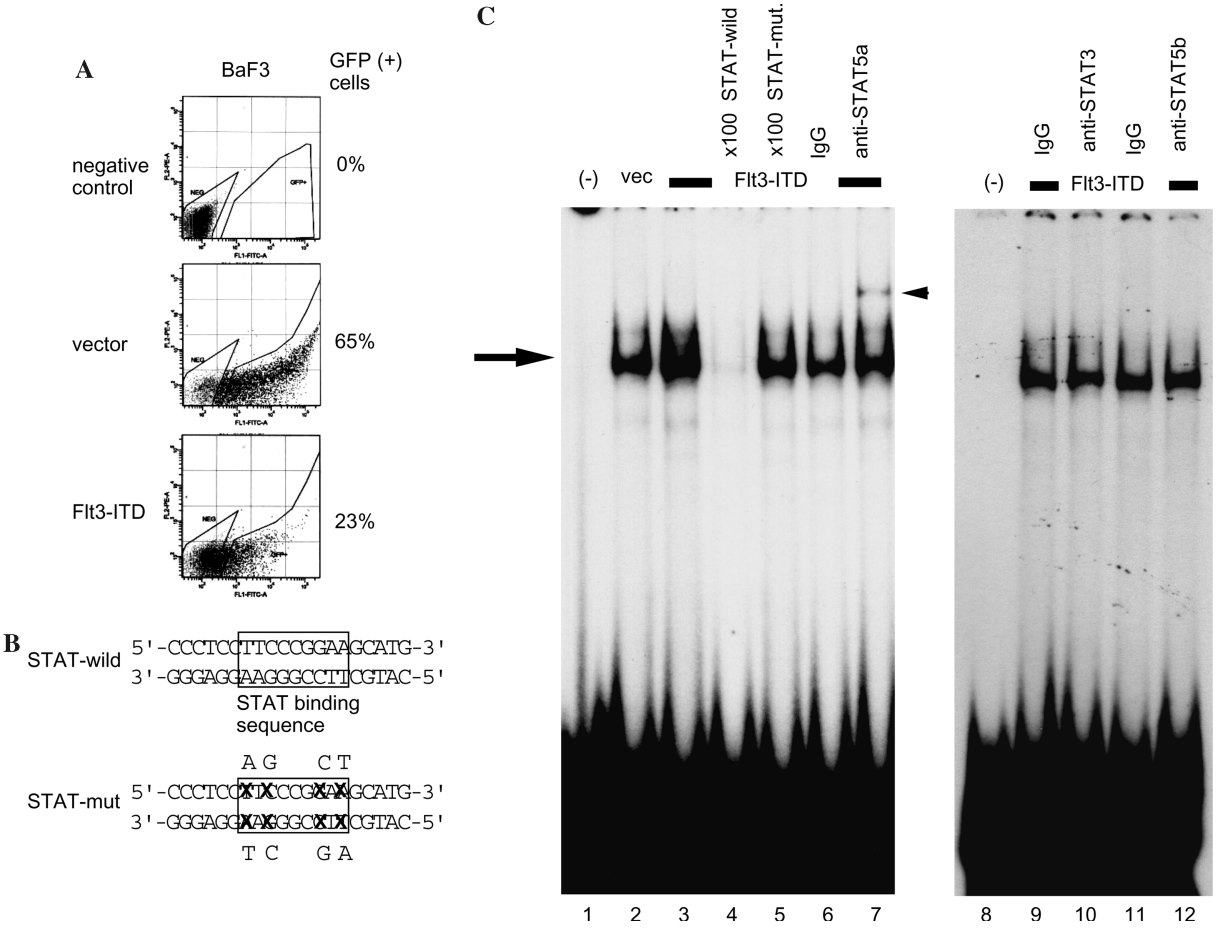

Fig. 4. STAT5a specifically binds to −692/−684 STAT site and Flt3-ITD enhances the binding of the protein binds to this site. (A) Flow cytometric profiles of MSCV-Flt3-ITD-GFP or MSCV-GFP transfected BaF3 cells. GFP positive cells used in this assay were collected from right squared area. The numbers on the right indicate the transfection efficiency of the experiment. (B) Oligonucleotides used for probe and/or competitor assays for electrophoretic mobility shift assays are shown. (C) Electrophoretic mobility shift assays. Five microgram aliquots of nuclear extracts from vector transfected (lane 2) or Flt3-ITD expression vector transfected (lanes 3–7, 9–12) BaF3 cells were incubated with end-labeled oligomers corresponding to the STAT site (−692/−684) within the p21 promoter in the absence (lanes 1–3) or presence (lanes 4, 5) of a 100-fold molar excess of the indicated oligonucleotides or anti-STAT5a (lane 7), STAT3 (lane 10), and STAT5b (lane 12) antibody. The arrow indicates the complex of the probe and STAT binding protein, and the arrowhead represents the ternary complex of the probe, STAT5a, and antibody.

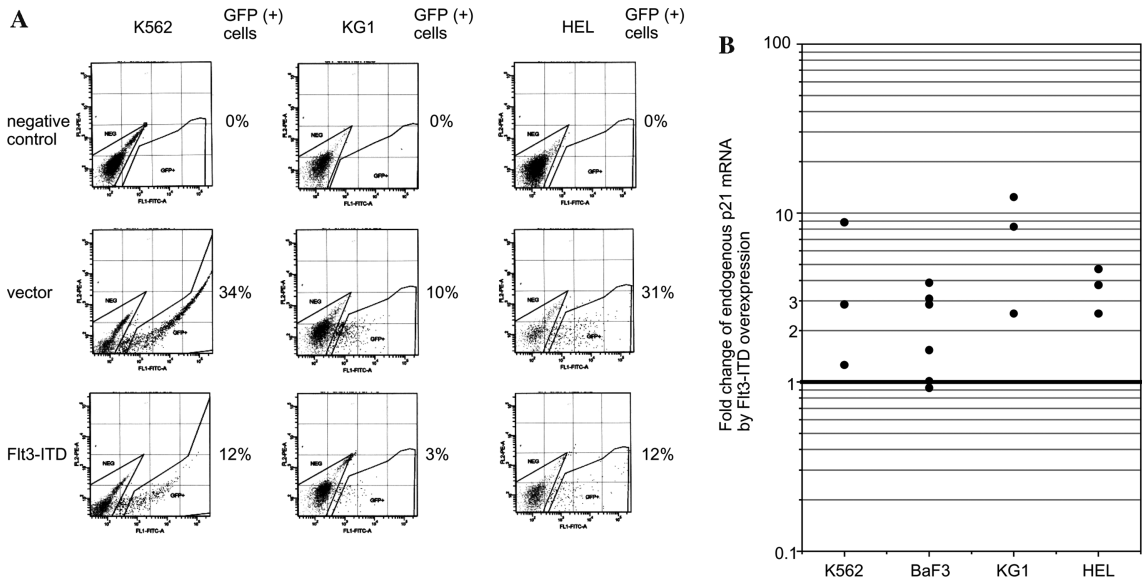

Fig. 5. Flt3-ITD induces endogenous p21 expression in various cell lines. (A) Flow cytometric profiles of MSCV-Flt3-ITD-GFP or MSCV-GFP transfected cells. Similar to Fig. 4A. K562, BaF3, KG1, and HEL cells were used for this assay. Profiles of BaF3 cells are shown in Fig. 4A and presented are profiles of K562, KG1, and HEL cells. Each panel is representative of three independent experiments. (B) Expression of endogenous p21 in Flt3-ITD overexpressed cells. The fold change of expression of p21 was obtained by the comparative ΔCt method as described in Materials and methods. Each dot is an average of triplicate samples from one experiment. The experiments were performed independently three to six times for each cell line and mRNA used in these experiments was isolated from independent replicate transfections.

Flt3-ITD induces the endogenous expression of p21 in various cell lines

After clarifying the induction mechanism of p21 promoter by Flt3-ITD, we next checked whether the endogenous expression of p21 is affected. We used four different kinds of Flt3 non-mutant [28] hematopoietic cell lines, K562, BaF3, KG1 (human myelogenous leukemia), and HEL (human erythroleukemia) cells. The cells were transfected with MSCV-Flt3-ITD-GFP or MSCV-GFP plasmid and sorted by flow cytometry and GFP positive cells (Fig. 5A) were collected. mRNAs were prepared from these cells for real time PCR. Quantification of mRNA was calculated by the comparative Ct (ΔΔCt) method [22] as described in Materials and methods. As shown in Fig. 5B in all four cell lines, Flt3-ITD expression led to an up to 10-fold increase in expression of endogenous p21 compared to vector transfected cells.

Discussion

To our knowledge, this is the first description about the upregulation of p21 by the Flt3 mutation. Interestingly, p21 is involved in the resistance of BCR-ABL expressing cells against cytotoxic agents [29]. High BCR-ABL levels concomitantly increased the expression of p21 [29]. In another study, high levels of constitutive p21 expression were associated with chemoresistance and represented an important prognostic factor for response to therapy and survival in AML patients [30]. It is possible that upregulation of p21 may be one reason why Flt3-ITD mutation is associated with a poor outcome [6,31]. Quite recently, it was reported that constitutive cytoplasmic expression of p21, induced by protein kinase C activation, is playing a role in the antiapoptotic functions [32]. They reported that AML blasts with high cytoplasmic p21 were less sensitive to VP-16 induced apoptosis as compared to AML cases with low or undetectable p21 expression. Another group currently published that the diversity of Flt3-ITD induced antiapoptotic pathways, compared to wild type Flt3 expressing cells, that might contribute to malignant progression by conferring a survival advantage through the suppression of apoptotic cell death [33]. Together with these facts, upregulation of p21 by Flt3-ITD may contribute to the antiapoptotic phenotype leading to malignant progression of Flt3 mutated leukemia cells.

We identified the functional importance of STAT5a to the p21 promoter by Flt3-ITD activation. Quite interestingly, Zhang et al. [26] reported that STAT5a, but not other STAT proteins, plays a preferential role in the proliferative response of primary hematopoietic progenitor cells by Flt3 ligand stimulation. They showed Flt3 ligand did not act on progenitors from marrow cells from STAT5a knockout mice but did in cells lacking STAT5b. As we identified the functional importance of STAT5a to the p21 promoter, this suggests that p21 expression may play a role in the Flt3-ITD induced cell proliferation.

The p21 gene product is also specifically upregulated by histone deacetylase inhibitors [34]. We recently find that transcriptional co-repressor SMRT (silencing mediator of retinoic and thyroid hormone receptors) function is inhibited by overexpression of Flt3-ITD and this inhibition is largely dependent on nuclear export of SMRT by Flt3-ITD (Takahashi et al., submitted). SMRT is widely expressed and recruits histone deacetylase to many promoters including p21. Hence, induction of p21 by Flt3-ITD is dependent on the inhibition of SMRT function and this may contribute to the upregulation of p21 by Flt3-ITD.

In conclusion, we demonstrated the induction of p21 by leukemia specific mutation, Flt3-ITD, through STAT5a dependent mechanism. This may indicate that the involvement of p21 in Flt3-ITD induced leukemogenesis.

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