Brief report

Activation mechanisms of STAT5 by oncogenic Flt3-ITD

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Mutations in the receptor tyrosine kinase Flt3 represent a very common genetic lesion in acute myeloid leukemia (AML). Internal tandem duplication (ITD) mutations clustered in the juxtamembrane domain are the most frequent and best characterized mutations found in Flt3. Oncogenic activation of Flt3 by ITD mutations is known to activate aberrant signaling including activation of STAT5 and repression of myeloid transcription factors Pu.1 and c/EBP-alpha. However, the mechanisms of STAT5 activation by Flt3-ITD remain unclear. Using small molecule inhibitors and cell lines deficient for Src family kinases or Jak2 or Tyk2, here we show that Flt3-ITD–induced STAT5 activation is independent of Src or Jak kinases. Also, overexpression of SOCS1, an inhibitor of Jak kinases, inhibited IL-3– but not Flt3-ITD–mediated STAT5 activation. Furthermore, in vitro kinase assays revealed that STAT5 is a direct target of Flt3. Taken together, our data provide the mechanistic basis of STAT5 activation by Flt3-ITD.

Introduction

Previously, we and others have reported aberrant activation of STAT5 by Flt3-ITD mutations.1-7 Activation of STAT5 is increasingly recognized as important for self-renewal of hematopoietic stem cells.8,9 Constitutive activation of STAT5 has also been observed in human leukemias.9 In animal models, activation of STAT5 has been shown to be essential for induction of leukemias by Tel-Jak2 and recently by Bcr-Abl and Flt3-ITD.10,11 Therefore, elucidation of mechanisms of STAT5 activation by Flt3-ITD may help to selectively target oncogenic signals of Flt3-ITD.

Materials and methods

Cell lines

SYF (Src, Yes, and Fyn) cells are derived from Src, Yes, and Fyn knockout mice and express no Src family kinases.12 γ2A and U1A cell lines are deficient for Jak2 and Tyk2 kinases, respectively.13 All cell lines, except 32D, were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. The 32D cells were cultured in DMEM medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. The 32D cells were cultured and

Results and discussion

Flt3-ITD–induced STAT5 activation is independent of Src or Jak kinases

STAT5 is generally activated by one of the 3 mechanisms: (1) by Jak kinases, (2) by Src family kinases (SFK), or (3) directly by RTKs such as the EGFR, PDGFRB, or insulin receptors.14,15 Although phosphorylation of Jak2 or Tyk2 by Flt3-ITD has been shown in previous studies, the role of Jak kinases in Flt3-ITD signaling remains controversial and inconclusive.16,17 To systematically explore the mechanisms of STAT5 activation by Flt3-ITD, we first tested chemical inhibitors to inhibit SFKs or JAKs.18,19 Treatment of 32D-Flt3-ITD with AG490 resulted in a decrease of STAT5 phosphorylation, but also in proportional decrease of STAT5 phosphorylation, making the interpretation of this result difficult (Figure 1A). Therefore, we analyzed the effects of Jak2 knockdown using siRNA, which did not have any effect on Flt3-ITD–mediated STAT5 activation (Figure 1B). Failure of Flt3-ITD to induce phosphorylation of any of the 4 Jak kinases (Figure 1C and data not shown) also indicated minor, if any, involvement of JAKs in Flt3-ITD–induced STAT5 activation. The SFK inhibitors PP-2 and PP-1 had marginal effect on STAT5 phosphorylation by Flt3-ITD (Figure 1D and data not shown). Also, they inhibited Flt3 autophosphorylation, indicating off-target effects of the compounds (Figure 1D and data not shown). It has been published earlier that 32D cells
respectively, was confirmed by probing total cell lysates with Jak2 or Tyk2 antibodies. 

Expressing Flt3-ITD were serum starved and lysates were analyzed for activation of STAT5 as described in panel A. Deficiency for Jak2 or Tyk2 in /H9253 described in panel E. (H-I) Jak2 or Tyk2 is not involved in Flt3-ITD–mediated STAT5 activation. Jak2 (U1A)–deficient and control cells (2C4 and 2ftgh) stably expressing Flt3-ITD were serum starved and lysates were analyzed for activation of STAT5 as described in panel A. Deficiency for Jak2 or Tyk2 in γ2A or U1A cells, respectively, was confirmed by probing total cell lysates with Jak2 or Tyk2 antibodies. 

Figure 1. Flt3-ITD–induced STAT5 activation is independent of Jak or Src kinases. (A) Flt3-ITD phosphorylates STAT5 in AG490-resistant manner. The 32D Flt3-ITD cells were starved for 6 hours in the presence or absence of PKC412 (100 nM) or AG490 (50 μM). Cell lysates were immunoblotted against activation-specific phospho-Flt3 (Y591) as indicated by Western blot analyses with an antibody that recognizes the activation state–specific consensus phosphorylation site in all these proteins (Figure 1E). 

Effect of SOCS1 on Flt3-ITD–mediated growth of 32D cells

Since redundant functions of different Jak family members may account for our results in cells deficient for single Jak, we analyzed the effects of SOCS1, a potent molecular inhibitor of Jak family members, on STAT5 activation by Flt3-ITD.22,23 SOCS1 is a transcripational target and negative regulator of STAT5 activation.22,23 Several SOCS family members including SOCS1 and SOCS3 were found to be up-regulated in Flt3-ITD–expressing cell lines (C.C., H.S. unpublished observation, respectively.)
February, 2006; and Mizuki et al). To define the role of Jaks in Flt3-ITD signaling, 32D, 32D-Flt3-ITD, or 32D-Flt3-WT cells were transduced with a retroviral construct encoding SOCS1 and EGFP. Surprisingly, overexpression of SOCS1 had no effect on growth of Flt3-ITD cells, as the proportion of SOCS1-transduced EGFP-positive cells remained constant over time (Figure 2A). Also, SOCS1 was found to have marginal effect on FL-stimulated growth of Flt3-WT cells (Figure 2A). In contrast, overexpression of SOCS1 severely inhibited IL-3–mediated growth and resulted in drastic reduction of SOCS1-positive cells, suggesting selective inhibitory effects of SOCS1 on IL-3– but not on Flt3–mediated growth (Figure 2A). Similar to earlier reports using BAF/3 cells, SOCS1-transduced 32D cells underwent rapid apoptosis in the presence of IL-3 (data not shown). Furthermore, addition of IL-3 to Flt3-ITD-SOCS1 cells also resulted in a slower decrease of the proportion of SOCS1-positive cells, indicating that expression of Flt3-ITD could indeed rescue 32D cells from SOCS1-induced inhibition of IL-3–mediated growth.

To further characterize the effect of SOCS1 on Flt3-ITD–mediated proliferation, we generated stable SOCS1-overexpressing Flt3-ITD bulk cultures. These cells were able to grow factor independently, similar to Flt3-ITD cells without SOCS1 (data not shown). Furthermore, in thymidine incorporation assays, both Flt3-ITD and Flt3-ITD-SOCS1 cell lines proliferated factor independently but required Flt3 kinase activity, as the Flt3-specific inhibitor PKC412 inhibited the proliferation of both cell lines (Figure 2C). Interestingly, addition of IL-3 could...
rescue the effects of PKC412 on Flt3-ITD but not on Flt3-ITD-SOCS1 cells, again confirming Jak-independent proliferation of 32D-Flt3-ITD. Similar to SOCS1, the overexpression of another Jak family inhibitor, SOCS3, inhibited IL-3– but not Flt3-ITD–mediated growth (Figure 2B).

**Effect of SOCS1 and SOCS3 on Flt3-ITD–mediated STAT5 activation**

To further characterize the effects of SOCS1 and SOCS3 on Flt3-ITD–induced STAT5 activation, a comprehensive signaling analysis was performed on Flt3-ITD versus Flt3-ITD-SOCS1 or Flt3-ITD-SOCS3 cell lines. Flt3-ITD, Flt3-ITD-SOCS1, and Flt3-ITD-SOCS3 activated STAT5 comparatively, which could be inhibited by PKC412 (Figure 2D–E). Addition of IL-3 resulted in a slight increase of STAT5 phosphorylation and induction of the STAT5 target genes Pim-1 and Pim-2 in Flt3-ITD but not in Flt3-ITD-SOCS1 cells. Addition of IL-3 also rescued the inhibitory effects of PKC412 on STAT5 activation in Flt3-ITD but not in Flt3-ITD-SOCS1 cells. These results suggest different mechanisms of STAT5 activation by Flt3-ITD and IL-3, and demonstrate Jak-independent activation of STAT5 by Flt3-ITD. Interestingly, simultaneous inhibition of SFK and Jak kinases also had no effect on Flt3-ITD–mediated STAT5 phosphorylation, suggesting that SFK and Jak are dispensable for Flt3-mediated STAT5 activation (Figure 2F).

Our finding that Flt3-ITD strongly up-regulates endogenous negative regulators of STAT5 such as SOCS proteins and activates STAT5 in a SOCS-resistant manner is reminiscent to earlier findings on SOCS1-resistant transformation by v-Abl. Thus, similar to v-Abl, up-regulation of SOCS proteins by Flt3-ITD may skew cytokine signaling in acute myeloid leukemia (AML) blasts.

**STAT5 is a direct target of Flt3 in vitro**

After confirming SFK- and Jak-independent activation of STAT5 by Flt3-ITD, we asked whether STAT5 is a direct target of Flt3. Therefore, we analyzed whether recombinant Flt3 could phosphorylate recombinant STAT5 in vitro, using a phospho-specific STAT5 (Y694) antibody to detect STAT5 phosphorylation at a functionally relevant site. As shown in Figure 2G, Flt3 kinase directly phosphorylated STAT5 at similar levels as the PDGFRβ, which we used as a positive control, since it is known to directly phosphorylate STAT5.

Taken together, our data provide the mechanistic basis of STAT5 activation by oncogenic Flt3-ITD mutations and show that Flt3-ITD directly activates STAT5 in a SFK- and Jak-independent manner.

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**Authorship**

**Contribution:** H.S. and C.C. conceptualized the original idea and designed experiments; A.U. and F.-D.B. purified the recombinant proteins and performed in vitro kinase assays; C.C. carried out all the other experiments; C.C., H.S., C.B., J.S., L.T., B.S., W.E.B., and C.-M.T. were involved in discussions, data analysis, and writing the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**References**

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