Janus Kinase 2: A Critical Target in Chronic Myelogenous Leukemia

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Abstract
The Bcr-Abl tyrosine kinase is the causative factor in most chronic myelogenous leukemia (CML) patients. We have shown that Bcr-Abl is associated with a cluster of signaling proteins, including Janus kinase (Jak) 2, growth factor receptor binding protein 2–associated binder (Gab) 2, Akt, and glycogen synthase kinase (GSK)-3β. Treatment of CML cell lines and mouse Bcr-Abl+ 32D cells with either Jak2 short interfering RNA or Jak2 kinase inhibitor AG490 inhibited pTyr Gab2 and pSer Akt formation, inhibited the activation of nuclear factor-κB, and caused the activation of GSK-3β, leading to the reduction of c-Myc. Importantly, BaF3 cells expressing T315I and E255K imatinib-resistant mutants of Bcr-Abl underwent apoptosis on exposure to AG490 yet were resistant to imatinib. Similar to wild-type Bcr-Abl+ cells, inhibition of Jak2 by Ag490 treatment resulted in decrease of pSer Akt and c-Myc in imatinib-resistant cells. These results identify Jak2 as a potentially important therapeutic target for CML.

Introduction
Chronic myelogenous leukemia (CML) is caused by hematopoietic progenitor cells that possess the Philadelphia chromosome, which encodes the Bcr-Abl oncoprotein (1). The driving force for the leukemia is the activated tyrosine kinase of Bcr-Abl, which stimulates several signal transduction pathways (1), including the Janus kinase (Jak) 2 pathway (2) and, separately, the signal transducer and activator of transcription 5 (STAT5) pathway (3). Imatinib mesylate is a selective inhibitor of the Bcr-Abl tyrosine kinase, and this drug is the frontline treatment for CML patients (4).

Bcr-Abl activates the Jak2 tyrosine kinase (2, 5), a member of the Jak family. The activation step involves phosphorylation of critical Tyr1007 (2, 5). Activation of Jak2 is also very important for Bcr-Abl-mediated oncogenicity (2). AG490, a member of the tyrphostin family of protein kinase inhibitors, is a potent and specific inhibitor of Jak2 kinase activity, and this drug is the frontline treatment for CML patients (4).

In Bcr-Abl+ 32D cells, the activation of Jak2 does not lead to STAT5 activation (2, 5), which is activated by Bcr-Abl (3, 8). One major effect of the activation of Jak2 by the Bcr-Abl oncoprotein is the increase in c-Myc expression (5), which is required for leukemia induction (9).

Results
Jak2 kinase inhibitor AG490 inhibited Akt phosphorylation, activated GSK-3β, and decreased the expression of c-Myc. We examined the effects of AG490 on the growth and survival of 32Dp210 cells. To determine the effects on the signaling molecules downstream of Jak2, we examined 32Dp210 cells and the CML cell line K562 after treating the cells with AG490. We incubated 32Dp210 cells with 100 μmol/L AG490 for 1 to 5 hours and measured the effects on the levels of pSer173 Akt, pSer9 GSK-3β, and c-Myc. The pAkt and pGSK-3β (but not the unphosphorylated proteins) and c-Myc protein levels were sharply reduced after 2 to 5 hours of treatment of 32Dp210 cells with AG490 (Fig. 1A). Treatment of the CML cell line K562 with 100 μmol/L AG490 also decreased pGSK-3β and c-Myc (Fig. 1B). The amount of reduced expression was estimated by dividing the intensity of the bands normalized by β-actin loading controls by the normalized intensity
AG490 inhibited activation of nuclear factor-κB. It is well known that nuclear factor-κB (NF-κB) activation increases levels of c-Myc transcripts, and Akt kinase activity is known to regulate NF-κB. The cells were incubated with different concentrations of AG490 for 16 hours. Compared with the untreated control, the activation of NF-κB was strongly reduced in the presence of AG490 (IC50, 12 μmol/L; Fig. 1D; Supplementary Fig. S1). Therefore, the reduction of NF-κB activation by AG490 in 32Dp210 cells indicates that the Bcr-Abl/Jak2 pathway also regulates NF-κB activation.

Treatment of 32Dp210 and CML cell lines with Jak2 siRNA inhibited the phosphorylation of Akt but induced the activation of GSK-3β. To establish the critical role of Jak2 in this Bcr-Abl pathway, we transfected Jak2-specific siRNAs in 32Dp210 cells, K562, and other human CML cell lines. At 48 hours after transfection with Jak2 siRNAs, the transfected cells were collected, and Western blotting was carried out with the various specific antibodies. After Jak2 siRNA treatment of 32Dp210 cells, Jak2 expression was reduced >75% compared with the untreated control (Fig. 2A). Analysis of the same lysates for pAkt and pGSK-3β showed significantly lower levels of these phosphoproteins and a lower level of c-Myc protein in 32Dp210 (Fig. 2A) and K562 cells (Fig. 2B). Like AG490 treatment, Jak2 siRNA–transfected cells also showed strongly reduced activation of NF-κB compared with the control (Fig. 2C).

The effect of Jak2 siRNA on K562 cells was dose dependent (Supplementary Fig. S2A). We also tested several other CML cell lines besides K562, including BV-173 and KBM7, for the effects of Jak2 knockdown (Supplementary Fig. S2B-C). Transfection with Jak2 siRNAs reduced the level of Jak2 protein, the expression of the c-Myc protein, and the phosphoproteins pAkt and pGSK-3β. Because Gab2 is involved in the activation of phosphatidylinositol 3-kinase (PI3K; refs. 1, 13), we also measured levels of pTyr Gab2 using a sequence-specific antibody that detects one of the several phosphoproteins and a lower level of c-Myc protein in 32Dp210 (Fig. 2A) and K562 cells (Fig. 2B). Like AG490 treatment, Jak2 siRNA–transfected cells also showed strongly reduced activation of NF-κB compared with the control (Fig. 2C).

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Inhibition of the Jak2 tyrosine kinase in Bcr-Abl+ cells inhibited survival. Trypan blue dye exclusion showed that cell viability was significantly reduced in 32Dp210 cells and partially reduced in K562 cells [it has other oncogenic events besides Bcr-Abl (e.g., lack of p53)] in a dose-dependent manner at 72 hours of treatment with AG490 (Supplementary Fig. S4A-B). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays showed similar results (data not shown). AG490 also induced apoptosis in 32p210 cells in a dose-dependent manner using the Annexin V and DNA fragmentation assays (Supplementary Fig. S4C-D). However, the K562 cells were relatively resistant to AG490 treatment (data not shown). We note that cells treated with AG490 for 16 hours had no effect on viability (Supplementary Fig. S5A). Therefore, the effects of AG490 on signal transduction in Fig. 1 were not caused by nonspecific toxic effects of AG490.
AG490 treatment of imatinib-resistant Bcr-Abl+ BaF3 cells induced apoptosis. Several mutations in the ATP-binding domain of Bcr-Abl have been shown to render cells from CML patients resistant to imatinib (15). To determine whether the Jak2 pathway would still be required in imatinib-resistant Bcr-Abl+ cells, we treated imatinib-sensitive and imatinib-resistant Bcr-Abl+ BaF3 cells with 50 μmol/L AG490 and measured apoptosis induction after 72 hours (Fig. 3A). The results showed that T315I-mutant Bcr-Abl+ cells, although resistant to imatinib, were sensitive to AG490 treatment. In fact apoptosis induction by AG490 was higher in resistant than sensitive cells (Fig. 3A). Similarly, the E255K mutant was also more responsive to AG490 than the imatinib-sensitive cells. The imatinib-resistant mutant Bcr-Abl proteins used the Bcr-Abl/Jak2/Akt network for maintaining growth and survival of imatinib-resistant cells (Fig. 3B), suggesting that drugs that inhibit Jak2 may be useful for treatment of imatinib-resistant CML patients.

GSK-3β is associated with Bcr-Abl/Jak2/Akt signaling network. To identify the Bcr-Abl-associated proteins (termed the network), detergent lysates of the mouse 32D and 32Dp210 cell lines were analyzed by immunoprecipitation with either Jak2 antibody-protein A conjugate (for Jak2-associated proteins) or by the addition of other specific antibodies (e.g., GSK-3β). These same antibodies were used to identify Bcr-Abl network–associated proteins by Western blotting of the immunoprecipitates (Fig. 4). Immunoprecipitation and Western blotting of the detergent-soluble lysates from either 32D cells maintained in IL-3 or 32Dp210 cells with the anti-Jak2 antibody detected Akt and GSK-3β (Fig. 4A) and Bcr-Abl in the 32Dp210 lysate (data not shown). If the same lysates were used for immunoprecipitation with anti-Akt and probed with the respective antibodies, Bcr-Abl (only in the 32Dp210 lysate) and GSK-3β were detected (Fig. 4B). Immunoprecipitation with anti-GSK-3β of these lysates followed by Western blotting with the appropriate antibodies identified cyclin D1, a target of GSK-3β kinase, and Bcr-Abl but only in the 32Dp210 lysate (Fig. 4C). Controls for specificity are shown in Supplementary Fig. S6A-B. Thus, Bcr-Abl protein network constitutes a signaling complex, which contains Bcr-Abl, Jak2, Akt, and GSK-3β. Other proteins, such as the PI3K and Gab2, are likely to be in the Bcr-Abl/Jak2 network (12, 13).

Discussion

We have presented evidence that Jak2 plays a critical role in the signal transduction through the Bcr-Abl/Jak2/Gab2/PI3K/Akt/GSK-3β network, which leads to the activation of many...
downstream targets, including c-Myc (Fig. 4D; refs. 2, 5, 11–13). This pathway regulates c-Myc expression by (a) transcriptional activation of c-Myc transcription through NF-κB and (b) enhanced stability of c-Myc protein by inactivation of GSK-3β kinase by phosphorylation at Ser9 of GSK-3β by pAkt (Fig. 4D). We propose that Jak2 may be a target for treatment of CML patients, especially those who are resistant to imatinib because AG490 treatment of hematopoietic cells transformed by imatinib-resistant mutants of Bcr-Abl (15) was induced to undergo apoptosis (Fig. 3A).

The presence of a multimeric protein complex composed of Bcr-Abl, Jak2, Gab2, PI3K, Akt, and GSK-3β is supported by various immunoprecipitation experiments using anti-Akt, anti-Jak2, and anti-GSK-3 (Fig. 4A-C). In CML cells, Bcr-Abl activates Jak2 (2, 5). Our studies (Figs. 1 and 2) indicate that, in Bcr-Abl+ cells, a dedicated Jak2 pathway is involved in activating the Gab2/PI3K/Akt pathway but not STAT5 activation (2, 5). Like others, we term this multimeric complex the Bcr-Abl network (Fig. 4D; refs. 12, 13). This Bcr-Abl network includes (a) the Bcr-Abl/Jak2 complex (2, 5), which leads to phosphorylation of Gab2 at the Tyr within the YxxM motif that is significantly reduced in lysates of Bcr-Abl+ cells following Jak2 knockdown (Fig. 2); (b) the Bcr-Abl/Grb2/Gab2 complex, which is formed by autophosphorylation of Tyr177 of Bcr-Abl and is involved in activation of the PI3K (11); (c) the p85 regulatory subunit and the p110 catalytic subunit of the PI3K, in which the Src homology (SH) 3 domain of p85 interacts with the Bcr-Abl network (13) and the SH2 domain of p85 binds to the pYxxM in Gab2 (12, 13); (d) the active PI3K, which forms phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 3,4-biphosphate and then attracts components of the Bcr-Abl/Jak2 network to the inner face of the cell surface membrane through pleckstrin homology domains; (e) the Akt activation pathway (PIP3, PDK1, and Akt); and (f) GSK-3β. Imatinib effectively reduced the effects of Bcr-Abl on all members of the Bcr-Abl/Jak2 network that were examined (data not shown). Signal transduction by this Bcr-Abl/Jak2 protein network results in phosphorylation and activation of Akt, which down-regulates the kinase activity of GSK-3β and thus leads to the stabilization of c-Myc and activates NF-κB to cause elevation of c-Myc transcripts (Fig. 4D). Although the possible involvement of other signaling networks cannot be excluded, our findings suggest that this Bcr-Abl network plays a central role in the cell proliferation and survival of Bcr-Abl+ leukemia cells.

Our findings with knockdown of Jak2 in CML cell lines (K562, BV173, and KBM7) further strengthen the role of Jak2 in the Bcr-Abl/Jak2/Gab2/Akt/GSK-3β network (Fig. 2; Supplementary Fig. S2). These results were very similar to the effects of AG490 treatment of K562 and 32Dp210 cells and thus confirm that the Jak2 kinase is a major component of the Bcr-Abl network that leads to the enhancement of c-Myc expression and possibly other important targets that contribute to the oncogenic phenotype induced by Bcr-Abl.

The PI3K pathway is well known to be a transducer of the Bcr-Abl tyrosine kinase signal, which is essential for cell proliferation and survival (12–14). As expected, we observed that treatment of 32Dp210 cells with the PI3K inhibitors wortmannin

**Figure 4.** Bcr-Abl and Jak2 proteins are part of a protein network. A to C, 32Dp210 cells were immunoprecipitated with several antibodies, and the immunoprecipitates were detected by Western blotting. D, model for the role of Jak2 in the Bcr-Abl/Jak2/Gab2/PI3K/Akt/NF-κB/GSK-3β network that enhances c-Myc protein levels in Bcr-Abl+ hematopoietic cells.
and LY294002 diminished pAkt and c-Myc levels and, at the same time, activated the kinase activity of GSK-3β (data not shown). Thus, the Bcr-Abl effects on c-Myc are likely to be mediated through the Bcr-Abl/Jak2/Gab2/P13K/Akt/GSK-3β pathway. It has been further shown that Grb2 binding to pTyr177 of Bcr-Abl leads to Gab2 binding and activation of P13K (11). Our findings indicate that inhibition of Jak2, either by treatment of CML cells with AG490 or knockdown of Jak2 by use of Jak2 siRNAs, reduced the level of tyrosine phosphorylation of the YxxM sequence in Gab2. The Gab2 pYxxM sequence binds the regulatory subunit of the PI3K, which leads to the activation of the P13K (Fig. 4D; ref. 14).

Another important issue is whether Jak2 or another tyrosine kinase downstream of Jak2 is the actual kinase that phosphorylates Gab2 at the YxxM sequence (Fig. 4D; ref. 14). The kinase specificity of Jak2 is not well known, which raises the possibility that Jak2 may phosphorylate the YxxM site. Alternatively, another tyrosine kinase downstream of Jak2 and activated by Jak2 might catalyze the phosphorylation of Gab2 at the YxxM sequence.

Several fusions involving the jak2 gene have been reported to induce myeloid leukemias (16–18). In addition, polycythemia vera patients were shown to have a mutation in Jak2 that activates the kinase (19).

Adaptor proteins, such as Gab1, Gab2, insulin receptor substrate (IRS)-1, and IRS-2, have been shown to have a critical role in coupling tyrosine kinase activation to P13K recruitment in protein networks (20). Our studies have focused on Gab2, but they do not eliminate the involvement of other Gab isoforms.

In summary, we have presented evidence to support the concept that the Bcr-Abl network contains Jak2/Gab2/P13K/Akt/GSK-3β. Importantly, our findings have shown that mouse hematopoietic cells transformed by the T315I mutant of Bcr-Abl were strongly induced to undergo apoptosis by AG490 treatment (Fig. 3A). Thus, specific inhibitors of Jak2 may be useful for the treatment of imatinib-resistant forms of CML.

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References

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