Activated Jak2 with the V617F Point Mutation Promotes G1/S Phase Transition*

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The family of non-receptor Janus tyrosine kinases (Jaks) is comprised of four members, including Jak1, Jak2, Jak3, and Tyk2 (see for review Ref. 1). The Jaks contain seven regions with significant sequence homology between the kinases, termed Jak homology (JH) domains. The JH1 domain is located within the carboxyl terminus of the protein and contains the tyrosine kinase domain. The adjacent JH2 domain shows close homology to the JH1 domain. The JH2 domain lacks critical residues required for tyrosine kinase activity. This JH2 pseudokinase domain negatively regulates the kinase activity associated with the JH1 domain (2, 3). Jaks are thought to primarily tyrosine phosphorylate and thus activate STAT (signal transducer and activator of transcription) transcription factors, although it seems clear that there are other activities. The Jak/STAT pathway has been implicated in a variety of solid tumors, as well as hematologic disorders. In particular, oncogenic fusion proteins of Jak2 with ETV6, PCM1, or BCR have been described in different leukemias and myeloproliferative disorders (4–7). It has been suggested that elevated Jak2 tyrosine kinase activity contributes to transformation, likely in part through the STAT5 transcription factor. Genetic modeling suggests that STAT5 genes support immature hematopoiesis, as mice with a targeted disruption of the STAT5A and STAT5B genes have reduced myeloid progenitor counts (8). Furthermore, STAT5 may also be required for normal fetal erythropoiesis (9). A close link of Jak2 kinase activity to STAT5 activation is also consistent with the fact that Jak2 knock-out mice have severely disrupted hematopoiesis that resembles the phenotype in mice with erythropoietin or erthropoietin receptor gene disruption (10–14).

An additional activating mutation of Jak2 has been recently reported in patients with polycythemia vera, essential thrombocytocemia, idiopathic myelofibrosis as well as in several myeloproliferative disorders and infrequently in myelodysplastic syndromes (15–21). The single point mutation leading to a V617F substitution in the JH2 domain of Jak2 has been associated with the proliferative phenotype, likely by leading to deregulated kinase activity. The detailed molecular requirements for Jak2 activation are still being worked out and may require the autophosphorylation on multiple sites. We sought to further define signaling mechanisms activated by Jak2V617F and identify targets that would aid in the development and implementation of targeted therapies for the treatment of Jak2V617F-related disorders.

EXPERIMENTAL PROCEDURES

Cells—The human CML cell line K562 and the human erythroleukemia cell line HEL were grown in RPMI 1640 (Mediatech, Herndon, VA) containing 10% fetal calf serum (Tissue Culture Biologicals, Tulare, CA); HEL cells were supplemented with 1 mM sodium pyruvate (Invitrogen). The murine BaF3 pre-B cell line with doxycycline-inducible constitutive active STAT5 was maintained as described before (22). STAT5 expression was induced by treatment with 1 μg/ml doxycycline (Sigma) in the absence of murine interleukin-3. BaF3.EpoR cells (expressing the erythropoietin receptor, EpoR) and BaF3.EpoR cells expressing Jak2V617F without enhanced green fluorescence protein (EGFP) were kindly provided by Dr. A. D’Andrea (Dana-Farber Cancer Institute, Boston, MA) and Dr. G. Gilliland, respectively (Brigham and Women’s Hospital, Boston, MA). BaF3 and BaF3.EpoR cell lines were kept in interleukin-3-containing medium

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2 The abbreviations used are: JH, Jak homology; EpoR, erythropoietin receptor; GFP, green fluorescent protein; EGFP, enhanced GFP; PDTC, pyrrolidine dithiocarbamate; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; ROS, reactive oxygen species; DCF-DA, 2’,7’-dichlorofluorescin diacetate; NAC, N-acetylcysteine.

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JaK2V617F Promotes G1/S Phase Transition

unless otherwise indicated. Additional BaF3 cell lines were generated by infection with pMSCV/Jak2.JRES.EGFP or pMSCV/Jak2V617F.JRES.EGFP containing viruses, kindly provided by Dr. G. Gilliland. In some experiments cells were treated with Jak inhibitor 1 (Calbiochem, La Jolla, CA), N-acetylcy steine (Sigma), 4,5-dihydroxy-1,3-benzenedisulfonyl acid (Tiron; Sigma), pyrrolidine dithiocarbamate (PDTC; Sigma) or transfected with specific human Jak2 siRNA (siGenome™, Dharmacon, Lafayette, CO). Cell growth and viability was determined by trypan blue exclusion or by a colorimetric method (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI). In some experiments, comparisons between test and control samples were evaluated using Student’s t test.

Electroporation of siRNA—Human Jak2-specific and control siRNA were used to transfect HEL cells in 100 µl of Nucleofector solution R (Amaxa, Gaithersburg, MD) according to the manufacturer’s directions using the Nucleofector device (Amaxa). In some experiments, the pmaxGFP vector (Amaxa) was transfected to survey transfection efficiency. Cells were transferred to culture medium after electroporation and incubated at 37 °C in 5% CO2. Comparisons among siRNA-transfected samples were done by an analysis of variance, followed by the Dunnett’s t test for post-hoc analysis.

Immunoblotting—Proteins were extracted from whole cells in buffer containing Tris (50 mM, pH 8.0) (Invitrogen), NaCl (150 mM) (Fisher Scientific), Nonidet P-40 (1% v/v) (Calbiochem), deoxycholic acid (0.5% w/v) (Fisher Scientific), sodium dodecyl sulfate (0.1% w/v) (Bio-Rad), NaF (1 mM) (Sigma), Na3VO4 (1 mM) (Sigma), and glycerol (10% v/v) (Invitrogen) supplemented with protease inhibitor mixture tablets (Complete, Roche Diagnostics). Polyclonal rabbit antibodies against STAT5 (C-17), cyclin D2 (M-20), p27Kip (C-19), Jak2 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), Jak2 (Upstate Biotechnology, Lake Placid, NY) or phosphorylated Jak2 (C-20) (Upstate Biotechnology, Lake Placid, NY) were used to detect Jak2 phosphorylation (Phospho-Jak2 (Tyr-1007/1008), C-6, Cell Signaling, Beverly, MA), and mouse monoclonal antibodies against phosphotyrosine (kindly provided by Dr. T. Roberts, Dana-Farber Cancer Institute) or β-actin (Sigma) were used for immunoblotting or immunoprecipitation.

Apoptosis Assays—Annexin V-positive staining was determined by FACS analysis (Annexin-V-Fluor Staining Kit, Roche Diagnostics) according to the manufacturer’s directions in cells that were treated with Jak inhibitor 1, cells transfected with siRNA, or left untreated.

Cell Cycle Analysis—Cells were fixed with 70% (v/v) ethanol in phosphate-buffered saline and incubated on ice for 30 min. RNase-treated samples (10 µg of RNase/ml for 20 min at 37 °C) were stained with propidium iodide (5 µg/ml) (Sigma) at 4 °C for at least 10 min. Cell cycle parameters were determined by FACS analysis using murine bone marrow cells in vivo (16). In the search for an appropriate cell line model, we confirmed the presence of the Jak2V617F mutation in the erythroid leukemia cell line HEL, but not in the erythroid Ph+ cell line K562 (not shown and Ref. 15). Also, a specific pyridone-containing tetracycle compound that has previously been shown to be a specific Jak kinase inhibitor (23) was used in HEL cells. Treatment of HEL cells with this Jak inhibitor I led to a dose-dependent reduction of cell growth in a three-day culture, with an IC50 of ~300 nM (Fig. 1A). At a concentration of 1 µM, there was more than 90% inhibition of cell growth in HEL cells as compared with untreated cells, which is consistent with recently published data (15). There was no inhibition of cell growth in the BCR-ABL-transformed K562 cells at 1 µM Jak inhibitor I, and the IC50 was not reached under these conditions. Interestingly, we found that even though Jak inhibitor I reduces growth of HEL cells, it did not have an impact on overall cellular tyrosine phosphorylation (Fig. 1B). To determine the mechanism of reduced cell growth induced by the Jak kinase inhibitor, we looked at cellular targets known to be involved in cell growth. In control experiments, the Jak inhibitor I was found to reduce tyrosine phosphorylation of the Jak2 substrate STAT5 on its activation site Tyr-694 at concentrations greater than 300 nM (Fig. 2A, top panels), and tyrosine phosphorylation of Jak2 was reduced at comparable concentrations (not shown). Further experiments showed that Jak kinase inhibitor I also reduced cyclin D2 expression and increased p27Kip expression (Fig. 2A, bottom panels), suggesting a role for Jak2 in regulating G1 phase to S phase transition. Next, cell cycle distribution in response to the inhibitor by propidium iodide staining was determined. Cells were left untreated or treated with Jak inhibitor I, and the different phases of cell cycle distribution were determined (Fig. 2B). The percentage of cells in G1 phase increased from 18 to 52% in cells that were treated for 48 h with Jak inhibitor I (up to 1 µM) in a dose-dependent manner, whereas the percentage of cells in S phase decreased from 62 to 37%, and those in G2/M phase decreased from 20
to 11%. On average, we observed a 28 ± 3.2% increase in G1 phase, a 21 ± 2.1% decrease in S phase, and a 7 ± 1.2% decrease in G2/M phase (means ± S.E., n = 3) upon 1 μM Jak inhibitor I treatment. This suggests that Jak inhibitor I leads to a significant G1 cell cycle arrest in the transformed cells, which is consistent with our findings of cyclin D2 and p27Kip regulation by this drug. However, there was no significant increase of cells in sub-G1 phase. We also looked at the change in Annexin V-positive staining of cells, an indication for increased exposure of phosphatidylserine to the outer cell membrane during apoptosis.

Using HEL cells, we found that treatment with Jak inhibitor I (24 h, 1 μM) led to a marginal increase in Annexin V-positive cells compared with untreated cells (not shown). However, prolonged exposure of HEL cells to the drug (48 h, 1 μM) led to a small increase of 6.1 ± 3.6% (n = 3) in Annexin V-positive staining (Fig. 2C). In the control cells less than 10% of the total population showed signs of apoptosis. These data demonstrate that Jak inhibitor I-induced G1 cell cycle arrest primarily contributes to the reduced cell growth of treated HEL cells, in combination with a weak proapoptotic signal.

Expression of Jak2V617F Is Required for Transformation of HEL Cells—Lessons learned from tyrosine kinase inhibitors such as imatinib mesylate indicate that efficacy does not always correlate with the specificity of the drug. The previous experiments demonstrate that Jak inhibitor I efficiently suppresses cell growth of HEL cells at concentrations where K562 cell growth is not affected. The efficacy of the Jak inhibitor also strongly correlates with a reduction in tyrosine phosphorylation of the Jak2 substrate STAT5. Because it is possible that this small molecule drug also inhibits other kinases at these concentrations, we used a human Jak2-targeted siRNA approach. HEL cells were transfected with siRNAs targeting four different regions in Jak2 or with control siRNA. Three siRNAs (defined as 6, 7, and 10) were found to efficiently reduce Jak2 expression, whereas one siRNA (defined as 9) only slightly reduced Jak2 compared with control siRNA transfected cells (Fig. 3A). We next asked whether reduction in Jak2 expression would also regulate phosphorylation of STAT5. Consistent with the previous data, only siRNA6, siRNA7, and to a somewhat lesser extent siRNA10 significantly reduced STAT5 phosphorylation (Fig. 3B). Again, siRNA9 had little effect on STAT5 phosphorylation compared with control transfected cells. These data suggest that Jak2V617F expression is required for optimal phosphorylation of STAT5 at its activation site. The inhibitor studies suggested that Jak activation regulates cell cycle progression and that inhibition of Jak2 induces G1 cell cycle arrest. Consistent with this assumption, reduced cyclin D2 and increased p27Kip expression was found to correlate with reduced Jak2 expression and thus reduced phosphorylation of STAT5 (Fig. 3B, bottom panels).

Finally, the effect of Jak2 RNA interference on cell growth of HEL cells was determined. The transfection of siRNA6, siRNA7, and siRNA10 consistently led to a reduction in cell growth compared with control transfected cells (Fig. 4A). An analysis of variance indi-
cated that there were differences between the siRNAs (p < 0.0001). Using simultaneous 99.5% confidence limits for these differences, we found in the post-hoc analysis that siRNA6, siRNA7, and siRNA10 are all statistically significantly different from the control siRNA at the 0.005 level; only siRNA9 failed to differ from the control at any reasonable level of significance for this experiment, including 0.05. Even after a 72-h period, we only measured a small increase in Annexin V-positive staining of less than 4.5% of the total population compared with control transfected cells (not shown). This suggests that apoptosis was not the main underlying reason for reduced cell growth by Jak2 siRNA. In contrast, there was an increase in G1 cell cycle distribution of between 9 and 24% within 48 h after transfection with the indicated siRNAs. (means ± S.E., n = 3). *, indicates that significant differences (p < 0.005) were observed between treated and control cells using an analysis of variance and Dunnett’s t test.

![Graph](image)

**Figure 4.** Jak2 expression is required for transformation of HEL cells. HEL cells were transfected with either control siRNA or human Jak2-specific siRNA. A, relative growth of HEL cells 72 h after siRNA transfection was calculated as a percentage compared with control transfected cells (n = 3). Cell cycle distribution was determined in HEL cells 48 h after transfection with the indicated siRNAs. (means ± S.E., n = 3). *, indicates that significant differences (p < 0.005) were observed between treated and control cells using an analysis of variance and Dunnett’s t test.

![Graph](image)

**Figure 5.** Activation of the STAT5 pathway by Jak2V617F is sufficient for elevated levels of ROS and suppression of p27Kip1. A, HEL cells were treated for 24 or 48 h with Jak inhibitor I (1 μM), and intracellular ROS levels were measured by DCF-DA staining (top panels). HEL cells were treated for 48 h with NAC or Jak inhibitor I, and cellular proteins were detected by immunoblotting as indicated (bottom panels). B, untreated BaF3 cells with doxycycline (DOX)-inducible constitutive active STAT5 (Control) were used and compared with cells treated with 1 μg/ml doxycycline or cells maintained in interleukin-3 (IL-3)-containing medium. Intracellular ROS levels were measured by DCF-DA staining (top panels), or cellular proteins were detected by immunoblotting as indicated (bottom panels). C, relative growth of HEL cells in response to antioxidants was calculated as a percentage compared with cells left untreated. Cells were treated for 3 days with N-acetylcysteine, Tiron, or PDTC (n = 4). *, indicates that significant differences (p < 0.05) were observed between treated and control cells.

was found to have only a small effect on intracellular ROS levels after a 1-day treatment but reduced ROS by ~50% after a 2-day treatment compared with untreated cells (Fig. 5A, top panels). We next looked at the direct effects of the antioxidant N-acetylcysteine (NAC) on the expression of p27Kip1 and cyclin D2 in HEL cells. Both NAC (20 mM) and Jak inhibitor I were found to reduce p27Kip expression as well as reduce cyclin D2 levels (Fig. 5A, bottom left panel), consistent with an important role for redox-sensitive pathways in the regulation of G1 phase to S phase transition. Interestingly, NAC was also found to strongly inhibit activation of STAT5 using a phosphospecific antibody against its activation site (Fig. 5A, bottom right panel).

These results demonstrate that activation of the Jak2 pathway is required for optimal induction of ROS in HEL cells and regulation of p27Kip1 levels. To further determine whether activation of the Jak2 substrate STAT5 is sufficient to increase cellular levels of ROS, we used a BaF3 cell line with doxycycline-inducible constitutively active STAT5 (22). Cells maintained in the absence of growth factor were compared with doxycycline-treated cells or cells stimulated with interleukin-3-containing medium as a positive control for increased ROS (26). Doxycycline treatment by itself does not alter intracellular ROS levels (not shown). Induction of active STAT5 expression led to a significant increase in ROS, which was comparable to the increase observed after interleukin-3 treatment (Fig. 5B, top panel). In control experiments the induction of STAT5 in these cell lines was determined by immunoblotting (Fig. 5B, first bottom panel). Surprisingly, active STAT5 also led to a significant reduction in p27Kip levels compared with growth factor-deprived cells in the absence of doxycycline. We also observed an
increase in cyclin D2 levels in the presence of activated STAT5 through doxycycline induction (Fig. 5B, bottom panels). Overall, these results demonstrate that activation of the Jak2/STAT5 pathway is sufficient for the induction of elevated levels of ROS and cyclin D2 and the suppression of the cell cycle inhibitor p27Kip.

We also looked at the effect of the antioxidants NAC, Tiron, and PDTC on cell growth in HEL cells (Fig. 5C). HEL cells were treated with 0–30 mM NAC, 0–10 μM Tiron, or 0–3 μM PDTC, and relative cell growth was measured in a 3-day culture. All three drugs led to a dose-dependent reduction in cell growth and reduced the cell number by more than 90% at concentrations of 30 mM NAC, 3 μM Tiron, or 3 μM PDTC. Overall these results suggest that a high oxidative state is important to maintain cell growth in HEL cells. Also, the Jak2/STAT5 pathway is likely to significantly contribute to elevated ROS levels and the reduction in p27Kip levels.

Jak2V617F Regulates p27Kip, Cyclin D2, and ROS in Erythropoietin Receptor-expressing BaF3 Cells—In addition to the Jak2V617F-expressing HEL cells, we generated Jak2V617F- or wild-type Jak2-expressing BaF3 cells. BaF3 cells are interleukin-3-dependent and can be transformed to grow factor independence by a variety of activated tyrosine kinases, such as BCR-ABL (28), TPR-Met (29), or others. Jak2V617F expressed in BaF3 cells or growth factor receptor-expressing BaF3 cells can lead to cytokine hyper-responsiveness as well as to partial factor-independent growth (15, 16, 30). Virus-infected cells were sorted for EGFP-positive cells and monitored for equally elevated EGFP and Jak2 expression levels (not shown). Surprisingly, we found that jak2V617F readily induced partial growth factor independence in BaF3 cells only in the presence of either the TpoR or the EpoR. Wild-type Jak2 by itself did not increase cell growth in either cell line (not shown). Using the parental BaF3 and BaF3.EpoR (EpoR) cells or cells infected with retroviruses to express Jak2V617F, cell cycle distribution was determined in cells deprived for 24 h of interleukin-3. The percentage of interleukin-3-deprived BaF3 cells showed only small changes in cell cycle distribution compared with Jak2V617F-expressing cells (Fig. 6A, top left panels). Overall, we observed a 3.7 ± 5.7% increase of cells in G1 phase and a 4 ± 3.4% increase in S phase, as well as a 6.7 ± 7.3% decrease in G1/M phase (means ± S.E., n = 3). In contrast, co-expression of the EpoR strongly reduced the percentage of cells in G1 phase and increased the percentage of cells in S phase (Fig. 6A, top right panels). On average, we observed a 34.7 ± 7.0% decrease in G1 phase, as well as a 34 ± 8.7% increase in S phase and a 0.7 ± 1.8% increase in G1/M phase (means ± S.E., n = 3).

Wild-type Jak2 did not have an effect on cell cycle distribution (not shown). This suggests that Jak2V617F promotes G1/S phase cell cycle transition in EpoR-expressing BaF3 cells. The design of this assay allows one to appreciate the apparent qualitative differences of BaF3.EpoR cells compared with parental BaF3 cells. It will be interesting in the future to see whether the differences between these cells are because of intrinsic factors or reflect differences in the amount of functionally expressed receptors. To partially address this point, we looked at the effects of mutated Jak2 on the EpoR. Parental BaF3.EpoR cells and cells infected to express Jak2 or Jak2V617F were maintained in interleukin-3-free medium for 18 h, and the tyrosine phosphorylation of the EpoR was detected by immunoblotting (Fig. 6B). The EpoR was found to be tyrosine phosphorylated in Jak2V617F-expressing cells but not in control cells or cells expressing wild-type Jak2. Expression of Jak2 and actin proteins was determined in whole cell lysates (Fig. 6B, bottom panels). This suggests that Jak2V617F can signal through the EpoR, and differences compared with other models may be contributed in part to activation of signaling mechanisms downstream of the activated EpoR. In this context, it should be pointed out that James et al. (16) have shown that parental BaF3 cells can be partially transformed by Jak2V617F, and it is possible that clonal effects contributed to this phenomenon. Nevertheless, our data clearly suggest that overexpression of the EpoR strongly enhances Jak2V617F signaling in BaF3 cells.

Based on these findings, we used the BaF3.EpoR cell line model to confirm the role of activated Jak2 in the regulation of cyclin D2 and p27Kip. Consistent with the signaling mechanisms regulated by Jak2V617F in HEL cells, expression of this oncoprotein was sufficient to lead to low expression of p27Kip and high expression levels of cyclin D2 when compared with interleukin-3-treated cells that were used as a positive control (Fig. 6C, right panels). The up-regulation of p27Kip and the down-regulation of cyclin D2 was not prevented during growth factor deprivation in the parental cells line or in wild-type Jak2-expressing cells (Fig. 6C, left panels). The regulation of p27Kip and cyclin D2 expression correlated with phosphorylation of STAT5 on its activation site (Fig. 6C). These two cell lines, but not Jak2V617F-expressing cells, underwent G1 cell cycle arrest within 24 h upon interleukin-3 withdrawal (not shown). Further, similar to experiments using HEL cells, functional expression of Jak2V617F was required for elevated levels of intracellular ROS in BaF3.EpoR cells (Fig. 6D, right panel). The increase in ROS by Jak2V617F was comparable to the increase in response to interleukin-3 stimulation in BaF3.EpoR cells (Fig. 6D, left panel) and the response was, in contrast, not transient but rather chronically elevated. Finally, using the antioxidants N-acetylcysteine, the abnormal up-regulation of cyclin D2 by Jak2V617F during growth factor deprivation was reverted to the phenotype observed in the parental BaF3.EpoR cells (not shown). These data observed in BaF3.EpoR cells transformed by Jak2V617F are consistent with the results obtained from the human erythroleukemia cell line HEL, further implicating Jak2V617F in the regulation of cell cycle regulatory proteins and ROS.
Jak2V617F Promotes G1/S Phase Transition

DISCUSSION

Our goal was to further define the molecular mechanisms involved in transformation by Jak2V617F and identify targets that would aid in the development and implementation of targeted therapies for the treatment of Jak2V617F-related myeloproliferative disorders. A pyridone-containing tetracycle Jak kinase inhibitor (23) specifically reduced cell growth of HEL cells with an IC50 of 300 nm. The reduction in cell growth correlated with reduced tyrosine phosphorylation of STAT5 on its activation site. STAT5 is a known substrate of Jak2 (31), and we and others have previously implicated activation of STAT5 in transformation by the BCR-ABL oncogene (32). This is also of special interest because activation of STAT5 by itself is sufficient to transform hematopoietic cells (33). It is possible that activation of this transcription factor is a common feature in myeloproliferative disorders. Downstream targets of STAT5 will therefore deserve additional attention. Reduced cell growth may be a direct consequence of reduced STAT5 phosphorylation or may involve other Jak2 substrates, such as VAV (34). The main mechanism involved in growth inhibition by Jak inhibitor I was found to be G1 cell cycle arrest with decreased cyclin D2 expression and increased p27Kip expression. Cyclin D proteins and p27Kip are critical inhibitors I. Functional Jak2 siRNA also significantly reduced cell growth and led to a reduction in STAT5 tyrosine phosphorylation. These studies emphasize the causal relationship between the functional expression of Jak2 and STAT5 activation, determined by phosphorylation on its activation site and underline the relative specificity of the Jak kinase inhibitor I. Functional Jak2 siRNA also significantly reduced cell growth in HEL cells compared with control transfected cells. Similar to the inhibitor studies, the Jak2 siRNA-induced G1 cell cycle arrest with a decreased expression of cyclin D2. Again, reduced Jak2 expression only slightly induced apoptosis. Using a similar approach, James et al. (13) found that Jak2 siRNA reduced erythropoietin-independent erythroid colony formation in cells from polycythemia vera patients, which could be rescued by erythropoietin treatment. Nevertheless, the Jak2 siRNA had no specific effect on cell growth when Jak2V617F-transformed cells were compared with normal cells (16). However, the expression levels of Jak2 in these primary cells in response to Jak2 siRNA or control siRNA were not determined. Our studies demonstrate the requirement for active Jak2 expression in HEL cells containing the V617F mutation. It will now be interesting to see whether the combination of Jak2 siRNA and Jak inhibitor I can increase the efficacy of either approach like previously shown for the Flt3 inhibitor MLN518 and specific Flt3 siRNA (39). Another notable aspect of signaling by Jak2V617F is the apparent requirement for expression of specific cytokine receptors. Although Jak2V617F can directly transform BaF3 cells (16), we found that efficient induction of cell cycle progression requires erythropoietin receptor co-expression. An intriguing possibility could be that different in vivo disease phenotypes associated with Jak2V617F are due to lineage-specific genes such as certain growth factor receptors. The direct involvement of any of these receptors in a proliferative phenotype would require further evaluation.

Of additional interest is the role of ROS in signaling through Jak2V617F. Both the inhibition of Jak2 activity in HEL cells and the constitutive active STAT5 expressed in BaF3 cells were found to regulate ROS, likely in part through the JakV617F/STAT5 pathway. This is also supported by our findings that Jak2V617F-expressing BaF3.EpoR cells have elevated levels of ROS compared with the parental cell line. Several antioxidants were tested and found to dramatically reduce cell growth in a dose-dependent manner, consistent with an important role of a high oxidative state for cell growth in these cells. We had shown before that treatment of a growth factor-deprived hematopoietic cell line with the ROS hydrogen peroxide is sufficient to promote G1 to S phase cell cycle transition (26) and reducing agents are known to induce G1 cell cycle arrest in a variety of cell models (40–42). It is possible that regulation of the cell cycle inhibitor p27Kip downstream of STAT5 in combination with redox-dependent processes may significantly contribute to the regulation of G1/S phase transition. The molecular mechanisms involved in the suppression of p27Kip by constitutively active STAT5 are of future interest and would require further evaluation.

In summary, identification of the mutational status of Val-617 in Jak2 is of clear diagnostic and potential therapeutic value in patients with myeloproliferative disorders. Using a known Jak kinase inhibitor, we have characterized the dramatic effects on G1/S phase transition and oxidative stress. Because a major effect of inhibiting Jak2V617F is G1 cell cycle arrest, it is likely that the efficacy of targeting this pathway will be increased in vivo by combination with antioxidant therapy or drugs that regulate the expression of cyclin D2 or p27Kip.

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