Published in final edited form as:

Exp Hematol. 2007 November ; 35(11): 1624–1632.

Characterization of a Highly Effective Protein Substrate for Analysis of JAK2^{V617F} Activity

Zhe Li^{1,2}, Shu Xing^{1,2}, Shaofeng Wang^{1,2}, Wanting Tina Ho¹, and Zhizhuang Joe Zhao^{1,*}

¹ Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

² Edmond H. Fischer Signal Transduction Laboratory, College of Life Sciences, Jilin University, Changchun, China

Abstract

Objective— Identification of JAK2^{V617F} in myeloproliferative disorders makes JAK2 an important marker for disease diagnosis and a highly attractive target for therapeutic drug development. This study is intended to identify a sensitive and specific substrate for assays of the JAK2 enzymatic activity.

Methods— We expressed a glutathione S-transferase (GST) fusion protein designated GST-JAKS which carries a peptide sequence derived from the autophosphorylation sites of human JAK2. The protein was purified from *E. coli* cells and was used to analyze tyrosine kinase activities of purified enzymes and crude cell extracts from cells including mononuclear cells of JAK2^{V617F}-positive polycythemia vera blood. It was also used to perform JAK2 kinase assays to screen inhibitors of JAK2.

Results— GST-JAKS is strongly phosphorylated by activated forms of JAK2 including JAK2^{V617F} and recombinant protein containing its catalytic domain alone. It showed minimal responses to wild type JAK2 and was not phosphorylated by the epidermal growth receptor and the insulin receptor tyrosine kinases. Kinase assays with GST-JAKS provide a sharp contrast between wild type and mutant JAK2^{V617F} and are sensitive enough to detect minute amounts of JAK2^{V617F} found in crude cell extracts. The assays can be scaled up to screen for inhibitors of JAK2 in a dot blot format.

Conclusion— GST-JAKS is sensitive and specific protein substrate for JAK2 assays. It may have clinical applications in diagnosis of diseases related to abnormal JAK2 activity. It is also an excellent substrate for development of large scale assays to screen JAK2 inhibitors.

Keywords

Myeloproliferative disorders; tyrosine kinases; inhibitor; enzyme assays

Introduction

JAK2 is a member of the Janus kinase subfamily tyrosine kinases. It is involved in signaling transduction by members of the single chain receptors (e.g. receptors for erythropoietin,

* Address correspondence to: Prof. Zhizhuang Joe Zhao, Ph.D., Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104. Tel 405-271-9344. E-mail: joe-zhao@ouhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

thrombopoietin, growth hormone, and prolactin), the interleukin-3 receptor family (interleukin-3, interleukin-5, granulocyte macrophage colony stimulating factor receptors), the gp130 receptor family, and the class II receptor cytokine family (1,2). Knockout of JAK2 in mice caused an embryonic lethal phenotype due to a failure in definitive erythropoiesis (3,4), suggesting its essential role in hematopoiesis. JAK2 has major pathological implications. Earlier studies demonstrated that fusions of the JAK2 gene with the TEL(ETV6) gene gives rise to a constitutive Tel-JAK2 fusion protein and causes myeloid and lymphoid malignancies (5,6). Recently, a somatic activating point mutation in JAK2 resulting from a valine to phenylalanine substitution within the regulatory pseudokinase domain (JAK2^{V617F}) was found in Ph-negative myeloproliferative disorders (MPDs) including in most patients with polycythemia vera (PV) and in about 50% patients with essential thrombocythemia and idiopathic myelofibrosis (7–11). Infrequent occurrence of this unique mutation has also been reported in chronic myelomonocytic leukemia, atypical or unclassified myeloproliferative disorders, myelodysplastic syndrome, systemic mastocytosis, chronic neutrophilic leukemia, acute myeloid leukemia (12–16), and in blood samples from patients with non-hematological diseases (17), suggesting a general pathological importance of the JAK2^{V617F} mutation. JAK2^{V617F} has been proven to be pathogenic since it generates PV-like phenotypes in mouse bone marrow transplant models (18–21). Interestingly, more recent studies demonstrated a mutation of cMPL in idiopathic myelofibrosis (22,23) and mutations of JAK2 exon 12 in PV (24). Evidently, abnormal JAK2 activity represents an important marker for disease diagnosis and a potential target for therapeutic drug development.

The JAK family tyrosine kinases have a unique domain structure with a kinase domain (JH1) adjacent to a pseudokinase domain (JH2). The pseudokinase domain lacks catalytic activity but plays a key regulatory role by inhibiting the kinase activity of JH1 (25). The V⁶¹⁷-toF mutation occurs in the subdomain III of the pseudokinase domain and is predicted to cause major conformational changes thereby disrupting the inhibitory effects. Structural analysis thus suggests that JAK2^{V617F} may possess enhanced tyrosine kinase activity. Indeed, previous studies demonstrated that JAK2^{V617F} displayed enhanced tyrosine phosphorylation (7–10). Tyrosine phosphorylation of JAK2 is thought to be correlated with its catalytic activity (26) but not a direct measure of its tyrosine kinase activity. An ideal assay should detect phosphorylation of an added protein substrate in the presence of JAK2. For this purpose, we have made a GST fusion protein carrying a peptide sequence derived from the autophosphorylation sites of human JAK2. This protein, designated GST-JAKS can be effectively phosphorylated by JAK2^{V617F} (27). In this study, we have fully characterized the sensitivity and specificity of the substrate toward JAK2. Our data suggest that GST-JAKS is highly specific for activated forms of JAK2 and can be used for large scale screening of JAK2 inhibitors. In addition, it can be used to identify elevated JAK2 activity in PV blood samples.

Materials and Methods

Materials

Polyclonal anti-JAK2 and anti-pJAK2(1007/1008) antibodies were from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine 4G10 antibody was obtained from Upstate Biotechnology, Inc. Recombinant proteins containing the kinase domain of EGF receptor (sEGFR) and the kinase domain of the insulin receptor (BIRK) was isolated from baculovirus expression systems as previously described (28,29).

Collection of peripheral blood and separation of low-density mononuclear cells

De-identified peripheral blood samples from normal donors and PV patients were collected from local clinical laboratories followed a protocol approved by the Institutional Review Board at the University of Oklahoma Health Sciences Center. The blood was layered onto Ficoll-

Hypaque (1.077 g/mL) (Amersham Biosciences, Piscataway, NJ), and low-density mononuclear cells were separated by centrifugation. After washing with cold phosphate-buffered saline, the cells were extracted with a whole-cell extraction buffer containing 25 mM β -glycerophosphate (pH 7.3), 5 mM EDTA, 2 mM EGTa, 5 mM β -mercaptoethanol, 1% Triton X-100, 0.1 M NaCl, 1mM sodium vanadate, and a protease inhibitor cocktail (Roche Applied Science). The cell lysates were cleared by centrifugation in a microfuge at 19,000 $\times$ g, and clear extracts were used directly for JAK2 activity assays.

Expression and purification of GST-JAKS

To make the GST-JAKS cDNA construct, DNA oligos encoding a peptide with sequence of PQDKKEYYKVKE were synthesized and ligated into the BamHI and EcoRI site of pGex-2T vector. The peptide sequence was derived from the autophosphorylation sites of human JAK2. The recombinant fusion protein was expressed in *E. coli* cells and then purified by using a glutathione-Sepharose column. Over 50 mg GST-JAKS protein can be obtained from one liter of *E. coli* cell cultures.

Expression and purification of His-tagged proteins containing JAK2, JAK2^{V617F}, and the catalytic domain (JSH1 domain) of JAK2

We employed the recombinant baculovirus expression system from Invitrogen to express various forms of JAK2. Briefly, full length JAK2 or JAK2^{V617F} and the catalytic domain (JAK2-Cat) corresponding amino acid residues 830-1132 of the JAK2 molecule were cloned into the pBluebacHis2 vector. The resultant plasmid DNAs together with Bac-N-blue DNA were used to transfect Sf9 insect cells to generate recombinant viruses according to the manufacturer's protocol (Invitrogen). The recombinant enzymes were purified from baculovirus-infected Sf9 cells by using the NTA-Ni resin (Qiagen).

Tyrosine kinase activity assays

Phosphorylation of GST-JAKS by various tyrosine kinases in isolated forms or in cell extracts were carried out in a buffer system containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.2 mM ATP, and 2 mM dithiothreitol. For typical assays with a total volume of 60 μ l, 5–10 μ l cell extracts containing 10 μ g total proteins were used. The reactions usually ran for 20 min at room temperature before being stopped by an addition of the SDS gel sample buffer. Tyrosine phosphorylation of GST-JAKS was determined by immunoblotting analyses with an anti-phosphotyrosine or anti-pJAK2 antibody followed by horseradish peroxidase-conjugated secondary antibodies. Detection by the ECL method, capture of immunoblot images, and quantification of band signals were carried out by using FluorChem SP imaging system from Alpha Innotech. For calculation of absolute kinase activity, given amounts of phosphorylated GST-JAKS were used as a standard and were analyzed in parallel with unknown samples in immunoblotting assays. The absolute amounts of phosphorylated GST-JAKS were determined by Coomassie blue staining plus densitometry following separations of phosphorylated GST-JAKS from non-phosphorylated GST-JAKS on SDS gels (referred to Fig. 1 and 2). Protein concentrations of purified kinases, GST-JAKS, and cell extracts were determined by using the Bradford method (30).

Results

GST-JAKS is a highly sensitive and specific substrate for detection of activated forms of JAK2

We have constructed a pGex-GST-JAKS plasmid which encodes a GST fusion protein carrying the peptide sequence derived from the autophosphorylation sites (Y1007 and Y1008) of JAK2. When induced by 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), *E. coli* cells

transformed by the plasmid gave rise to a robust expression of the GST-JAKS in the exclusion body. From one liter of cell culture, over 50 mg of nearly homogeneous recombinant protein was usually obtained by using a single glutathione-Sepharose column. The substrate is sensitive enough to detect activity of JAK2^{V617F} in cell extraction obtained from COS7 cell transfected with JAK2^{V617F} carried by the pCDNA3 vector. As shown in Fig. 1, the mutant JAK2^{V617F} caused marked phosphorylation of the added protein substrate as detected by anti-phosphotyrosine antibody. In contrast, cell extract from control cells transfected with the plain vector did not give any phosphorylation. Furthermore, when GST was used in the assays in place of GST-JAKS, no such a phosphorylation was observed. This suggests that the phosphorylation band corresponded to GST-JAKS and that the phosphorylation occurred on the fused peptide rather than the GST moiety of the fusion protein. Note the phosphorylation apparently caused a mobility shift of GST-JAKS (see also later in Fig. 2). Interestingly, the substrate was not able to detect the activity of wild type JAK2 in the cell extracts although the latter was expressed at a level similar to that of JAK2^{V617F} and was autophosphorylated at a lower level. Our data further demonstrate that JAK2^{V617F} is a hyperactive tyrosine kinase and GST-JAKS is a sensitive substrate to distinct the activity of wild from mutant JAK2.

To determine further the substrate specificity, we compared phosphorylation of GST-JAKS by purified tyrosine kinases. First, we used sEGFR and BIRK, which represented the soluble kinase domains of the epidermal growth factor receptor and the insulin receptor, respectively. These two enzymes were purified from the baculovirus expression system. They are highly active and have been used repeatedly in our previous studies to phosphorylate various protein and peptide substrates for tyrosine phosphatase assays (28,29). The data shown in Fig. 2 indicated that while sEGFR and BIRK underwent strong autophosphorylation, an indication of their kinase activities, they failed to phosphorylate GST-JAKS at all. The data suggest that GST-JAKS is not a substrate of sEGFR and BIRK. We have also expressed JAK2, JAK2^{V617F}, and the catalytic JH1 domain of JAK2 (JAK2-Cat) in baculoviruses. These enzymes were purified to over 50% pure by using NTA-Ni columns (data not shown). Purified wild type JAK2 showed slight phosphorylation of GST-JAKS, essentially at a minimal level in comparison to the robust phosphorylation caused by JAK2^{V617F}. Note that autophosphorylation of JAK2 is quite significant, moderately below that of JAK2^{V617F}. This again suggests GST-JAKS is a better choice for kinase assays to discriminate wild JAK2 from mutant JAK2^{V617F}. We further compared the activity of JAK2-Cat. Lacking the inhibitory JH1 domain, the isolated JAK2-Cat appeared to be fully active. It produced a phosphorylation level equivalent to that obtained with JAK2^{V617F} with less than 1/10 of the enzyme concentration. Coomassie blue staining of the phosphorylated GST-JAKS product showed clear a mobility shift of GST-JAKS (Fig. 2, bottom panel). Nearly 40% of substrate had been turned into phosphorylated products with 20 min of reaction. Based on the mobility shift and total amounts of GST-JAKS used in assays, we were able to determine the absolute amounts of phosphorylated GST-JAKS and thus to calculate the absolute activity of the kinases. They are 5, 110, 1200 nmole/min/mg for JAK2, JAK2^{V617F}, and JAK2-Cat, respectively. The data demonstrate the JAK2^{V617F} is over 20-fold more active than JAK2 but only exhibit ~1/10 activity of JAK-Cat. This also suggests that JAK2^{V617F} is not fully active.

We further employed GST-JAKS to characterize the kinetics of JAK2-Cat. In this case, tyrosine phosphorylation of GST-JAKS was detected by using anti-pJAK2 which was raised against phospho-peptide derived tyrosine phosphorylation at sites 1007 and 1008 of JAK2. The antibody turned out to be more sensitive and more specific than anti-phosphotyrosine for detection of tyrosine phosphorylated GST-JAKS. The kinetic data are shown in Fig. 3. With substrate concentration fixed at 0.2 mg/ml, GST-JAKS phosphorylation displayed near linear increase within the first 20 min of reaction (Fig. 3A). The reaction rate slowed down thereafter, probably due to a reduction of substrate concentrations and/or inhibition caused by the phosphorylated product. With a fixed reaction time of 20 min, the phosphorylation rate

increased with increases in substrate concentrations, and the reaction apparently followed the Michaelis-Menten kinetics (Fig. 3B). The calculated K_m value is 0.15 mg/ml or 5.5 μM , and the maximum reaction rate $V_{\text{max}} = 1,500$ nmole/min/mg, corresponding to a turnover number (kcat value) of 0.7 per second. These values exhibited by JAK2-Cat toward GST-JAKS are comparable to those reported for many other activated forms of tyrosine kinase toward their specific substrates (31).

GST-JAKS can be used for screening of JAK2 inhibitors in a large scale

The experiments described above relied on SDS gels to separate GST-JAKS before immunoblotting analyses. This is rather time-consuming and is not suitable for large scale assays. We have noted that with JAK2-Cat as a kinase, GST-JAKS is essentially the only protein that is phosphorylated in the reaction mixture. Therefore, separation of reaction products on SDS gels becomes unnecessary. We thus spotted reaction mixtures onto nitrocellulose membranes and analyzed the phosphorylation by anti-pJAK2 antibody directly in a dot-matrix format. This has been proven to be high effective, and we have used the assay to screen inhibitor JAK2-Cat. Fig. 4 shows a typical assay. Included in the assays were tyrosine kinase inhibitors and anti-cancer drugs erlotinib, imatinib mesylate, and gefitinib together with AG490, a known inhibitor of JAK2. As observed with JAK2^{V617F} in our earlier studies (27), erlotinib acted as a potent inhibitor of JAK2-Cat. Significant inhibition was seen at 1 μM concentration, and at 5 μM , it inhibited the activity of JAK2-Cat by over 90%. Additional assays demonstrated that erlotinib inhibited the activity of JAK2-Cat toward GST-JAKS with an IC_{50} value of 1.5 μM , where IC_{50} represents the concentration of compounds required to achieve a 50% reduction in the kinase activity. In contrast, imatinib mesylate, gefitinib, and AG490 were essentially ineffective at a concentration of 10 μM . We also included 42 unknown compounds from a chemical library in our assays. Interestingly, two of them, namely, No. 19 and 33, also showed strong inhibition at a 10 μM concentration. Further screening of the chemical library is under way. This study provides a proof-of-concept for the use of GST-JAKS in screening of JAK2 inhibitors in large scales.

GST-JAKS can detect elevated tyrosine kinase activity in PV samples

The fact that GST-JAKS can specifically detect JAK2^{V617F} in crude extracts of COS7 cells encouraged us to check the JAK2 activity in blood samples from PV patients. For this purpose, we employed mononuclear cells isolated from peripheral blood. As shown in Fig. 5, the cells from normal blood gave rise to minimum phosphorylation of GST-JAKS, which was not significantly above the basal signal detected by the anti-pJAK2 antibody (not shown), but all four PV samples produced clear phosphorylation. The data suggest the GST-JAKS can be used to detect the abnormal JAK2 kinase activity in isolated mononuclear cells from JAK2^{V617F}-positive PV blood samples. This assay appeared to be highly sensitive. It took a cell extract with only 10 μg of total proteins for each analysis. It should be pointed out that all four PV samples are JAK2^{V617F}-positive, and contents of the mutant allele were approximately 40%, 20%, 50%, and 90%. Apparently, the data do not suggest a direct correlation between the level of GST-JAKS phosphorylation and the degree of JAK2^{V617F} mutation. This may be partly due to the heterogeneities of the mononuclear cells analyzed. Of course, we can not rule out involvement of other kinases and elevation of JAK2 activity caused by factors in addition to JAK2^{V617F}. Although we only used JAK2^{V617F}-positive PV cells in our analyses due to a lack of JAK2^{V617F}-negative patients in our hands, we speculate that GST-JAKS is also able to detect abnormal JAK2 activity resulting from other activation mechanisms. We believe GST-JAKS may be useful for diagnoses of PV and other diseases involving elevated JAK2 activity. To prove this, much more patient samples (JAK2^{V617F}-positive and -negative) and further purification and fractionation of cells and cell extracts are needed.

Discussion

We have developed a highly sensitive and specific substrate designated GST-JAKS for assays of the JAK2 kinase activity. The substrate displays strong activity toward activated forms of JAK2 but show minimal response to wild type JAK2 and thus provides a sharp contrast between wild type and mutant JAK2^{V617F}. It can detect minute amounts of JAK2^{V617F} found in the cell extracts and can be scaled up for inhibitor screening. It is more accurate and sensitive than the autophosphorylation of JAK2 employed in early studies. It should find wide applications in future studies related to JAK2.

As a protein substrate, GST-JAKS should serve as an important tool to study changes of JAK2 activity not only in normal cells upon stimulation but also in abnormal cells as in the case of MPDs. The fact that GST-JAKS can differentially detect JAK2 activity in crude cell extracts from mononuclear cells of JAK2^{V617F}-positive PV blood suggests its potential clinical applications. JAK2^{V617F} has been found in most patients with PV and in about 50% patients with essential thrombocythemia and idiopathic myelofibrosis (32,33). Currently, various gene-based analyses have been developed to detect such a mutant, and this has provided valuable information for diagnosis of the diseases and has helped to design proper treatments (34,35). However, the gene-based assays only tell the presence of the mutant JAK2^{V617F} gene rather than its activity. Since GST-JAKS directly detects enzymatic activity, it should identify abnormal elevations of the JAK2 activity due to mutations of the enzyme at other sites or mutations of other signaling components that regulate JAK2. In fact, recent studies have demonstrated a mutation of cMPL in idiopathic myelofibrosis which affects JAK2 activation (22,23) and mutations of JAK2 exon 12, which also produce constitutively active JAK2 enzyme, in several PV patients (24).

We believe that GST-JAKS may find wide applications in screening for JAK2 inhibitors. Finding potent inhibitors of tyrosine kinases represents a new trend in cancer drug discovery. It has been well accepted that tyrosine kinases are central regulators of signaling pathways and play a crucial role in controlling proliferation, differentiation, transformation, motility, and invasion. Perturbation of PTK signaling by mutations and other genetic alterations such as chromosomal translocation, interstitial deletion, and internal tandem duplication results in deregulated kinase activity and malignant transformation (36). Consequently, these mutant kinases are attractive therapeutic targets, as exemplified by imatinib mesylate (STI571, Gleevec) for treatment of *BCR-ABL*-positive chronic myelogenous leukemia (37), gefitinib (Iressa, ZD1839), and erlotinib (Tarceva) used for non-small cell lung cancer with mutation of the epidermal growth factor receptor (38,39). Because of its pathogenicity, JAK2^{V617F} obviously represents an attractive target for drug development to treat myeloproliferative disorders. Our study thus provided a needed substrate for assay development. It offers advantages over commonly used synthetic peptide substrates because 1) it is inexpensive since *E. coli* cells give an unlimited source of the protein and 2) it can be directly attached to a polystyrene plate surface and nitrocellulose membrane without covalent modifications as required for peptide substrates. Therefore, GST-JAKS can be readily applied for large scale inhibitor screenings. Finally, our study also demonstrates that GST is an effective carrier for expression of short peptide sequences used for assays of protein kinases.

Acknowledgements

This study is supported by grants HL076309 and HL079441 from the National Institutes of Health, USA. The authors have no conflicts of interest to declare.

References

1. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 2002;285:1–24. [PubMed: 12039028]
2. Verma A, Kambhampati S, Parmar S, Platanias LC. Jak family of kinases in cancer. *Cancer Metastasis Rev* 2003;22:423–434. [PubMed: 12884916]
3. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* 1998;93:397–409. [PubMed: 9590174]
4. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, Vanin EF, Bodner S, Colamonici OR, van Deursen JM, Grosveld G, Ihle JN. Jak2 is essential for signaling through a variety of cytokine receptors. *Cell* 1998;93:385–395. [PubMed: 9590173]
5. Lacronique V, Boureux A, Valle VD, Poirel H, Tran Quang C, Mauchauffé M, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* 1997;278:1309–1312. [PubMed: 9360930]
6. Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia. *Blood* 1997;90:2535–2540. [PubMed: 9326218]
7. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 2005;365:1054–1061. [PubMed: 15781101]
8. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005;7:387–397. [PubMed: 15837627]
9. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005;434:1144–1148. [PubMed: 15793561]
10. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* 2005;352:1779–1790. [PubMed: 15858187]
11. Zhao R, Xing S, Li Z, et al. Identification of an acquired JAK2 mutation in polycythemia vera. *J Biol Chem* 2005;280:22788–22792. [PubMed: 15863514]
12. Steensma DP, Dewald GW, Lasho TL, et al. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both “atypical” myeloproliferative disorders and the myelodysplastic syndrome. *Blood* 2005;106:1207–1209. [PubMed: 15860661]
13. Jones AV, Kreil S, Zoi K, et al. Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood* 2005;106:2162–2168. [PubMed: 15920007]
14. Scott LM, Campbell PJ, Baxter EJ, et al. The V617F JAK2 mutation is uncommon in cancers and in myeloid malignancies other than the classic myeloproliferative disorders. *Blood* 2005;106:2920–2921. [PubMed: 16204151]
15. Jelinek J, Oki Y, Gharibyan V, Bueso-Ramos C, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. *Blood* 2005;106:3370–3373. [PubMed: 16037387]
16. Levine RL, Loriaux M, Huntly BJ, et al. The JAK2^{V617F} activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood* 2005;106:3377–3379. [PubMed: 16081687]
17. Xu X, Zhang Q, Luo J, Xing S, Li Q, Krantz SB, Fu X, Zhao ZJ. JAK2(V617F): Prevalence in a large Chinese hospital population. *Blood* 2007 Jan 1;109(1):339–342. [PubMed: 16946305]
18. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood* 2006;107:4274–4281. [PubMed: 16478879]
19. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood* 2006;108:1652–1660. [PubMed: 16670266]
20. Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S, Van Etten RA. Molecular Pathogenesis and Therapy of Polycythemia Induced in Mice by JAK2 V617F. *PLoS ONE* 2006;1:e18. [PubMed: 17183644]

21. Bumm TG, Elsea C, Corbin AS, Loriaux M, Sherbenou D, Wood L, Deininger J, Silver RT, Druker BJ, Deininger MW. Characterization of murine JAK2V617F-positive myeloproliferative disease. *Cancer Res* 2006;66:11156–11165. [PubMed: 17145859]
22. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood* 2006;108:3472–3476. [PubMed: 16868251]
23. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Medicine* 2006;3:e270. [PubMed: 16834459]
24. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, Warren AJ, Gilliland DG, Lodish HF, Green AR. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med* 2007;356:459–468. [PubMed: 17267906]
25. Saharinen P, Vihinen M, Silvennoinen O. Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain. *Mol Biol Cell* 2003;14:1448–1459. [PubMed: 12686600]
26. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM, Ihle JN. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Mol Cell Biol* 1997;17:2497–2501. [PubMed: 9111318]
27. Li Z, Xu M, Xing S, Ho WT, Ishii T, Li Q, Fu X, Zhao ZJ. Erlotinib Effectively Inhibits JAK2V617F Activity and Polycythemia Vera Cell Growth. *J Biol Chem* 2007;282:3428–32. [PubMed: 17178722]
28. Zhao Z, Bouchard P, Diltz CD, Shen SH, Fischer EH. Purification and characterization of a protein tyrosine phosphatase containing SH2 domains. *J Biol Chem* 1993;268:2816–2820. [PubMed: 8428956]
29. Zhao Z, Shen SH, Fischer EH. Stimulation by Phospholipids of a Protein-Tyrosine Phosphatase Containing Two src Homology 2 Domains. *Proc Natl Acad Sci USA* 1993;90:4251–4255. [PubMed: 7683430]
30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
31. Fischer EH, Charbonneau H, Tonks NK. Protein tyrosine phosphatases. A diverse family of intracellular, transmembrane enzymes. *Science* 1991;253:401–406. [PubMed: 1650499]
32. Zhao ZJ, Vainchenker W, Krantz SB, Casadevall N, Constantinescu SN. Role of Tyrosine Kinases and Phosphatases in Polycythemia Vera. *Seminars in Hemotol* 2005;42:221–229.
33. Campbell PJ, Green AR. The myeloproliferative disorders. *N Engl J Med* 2006;355:2452–66. [PubMed: 17151367]
34. Tefferi A. JAK2 Mutations in Polycythemia Vera - Molecular Mechanisms and Clinical Applications. *N Engl J Med* 2007;356:444–445. [PubMed: 17267904]
35. Hoffman R, Prchal JT, Samuelson S, Ciurea SO, Rondelli D. Philadelphia chromosome-negative myeloproliferative disorders: biology treatment. *Biol Blood Marrow Transplant* 2007;13:64–72. [PubMed: 17222772]
36. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature* 2001;411:355–365. [PubMed: 11357143]
37. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031–1037. [PubMed: 11287972]
38. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500. [PubMed: 15118125]
39. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139. [PubMed: 15118073]

Fig. 1. Assay of JAK2 kinase activity in cell extracts with GST-JAKS as a substrate
 COS7 cells were transfected with the pCDNA3 vector control, JAK2, and JAK2^{V617F}. Crude cell extracts were used for kinase activity with GST or GST-JAKS as indicated. Tyrosine phosphorylation was detected by using anti-phosphotyrosine antibody, and the protein levels of JAK2/JAK2^{V617F} and GST-JAKS were revealed by anti-JAK2 and anti-GST antibodies, respectively.

Fig. 2. Specificity of GST-JAKS as a substrate for assays of tyrosine kinases

Kinase assays were performed in a reaction mixture containing 0.2 mg/ml GST-JAKS and indicated tyrosine kinases purified from the baculovirus expression system. Concentrations of the kinases were 0.08 μ g/ml for JAK2-Cat and 1 μ g/ml for sEGFR, BIRK, JAK2, and JAK2^{V617F}. The reactions were allowed to proceed at room temperature for 20 min and were then stopped by an addition of the SDS gel sample buffer. Samples were separated on 10% polyacrylamide gels, transferred to PVDF membranes, and finally subjected to immunoblotting analysis with antiphosphotyrosine antibody, or to staining with Coomassie blue.

Fig. 3. Time course and dose response of GST-JAKS phosphorylation catalyzed by JAK2-Cat Kinases assays were performed with 0.1 μ g/ml JAK2-Cat in the presence of 0.2 mg/ml GST-JAKS for indicate time periods (A) or of varying amounts of GST-JAKS for 20 min (B). Insets show typical immunoblotting data obtained with anti-pJAK2 antibody. Error bars denote standard deviation (n = 3).

Fig. 4. Screening of JAK2 tyrosine kinase inhibitors by using GST-JAKS in a dot blot format
Tyrosine kinase activity of JAK2-Cat was performed with GST-JAKS as a substrate in the presence of various chemical compounds. The reaction was stopped by an addition of the SDS gel sample buffer, and 1 μ l of samples were spotted onto nitrocellulose membranes. The level of tyrosine phosphorylation was detected by using anti-pJAK2. 1T and 5T, 1 and 5 μ M Tarceva (erlotinib), respectively; 10A, 10 μ M AG490; 10I, 10 μ M Iressa (gefitinib); 10G, 10 μ M Gleevec (imatinib mesylate), C, control (no inhibitor); B, blank (no GST-JAKS substrate added); sample 1–42, 42 individual compounds from a chemical library at a concentration of 10 μ M.

Fig. 5. Assays of JAK2 kinase activity in cell extracts from mononuclear cells of normal and PV blood samples

JAK2 kinases assays were performed as described in Fig. 1 except that crude extracts containing equal amounts of proteins from mononuclear cells of two different normal and 4 different PV blood samples were used as the JAK2 kinase. Phosphorylation of GST-JAKS was detected by using anti-phospho-JAK2 (top panel). Coomassie blue staining detected actin from the mononuclear cell extracts and the added GST-JAKS substrate, thus demonstrating equal protein loading (bottom panel).