Identification of an Acquired JAK2 Mutation in Polycythemia Vera*

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Polycythemia vera (PV) is a human clonal hematological disorder. The molecular etiology of the disease has not yet been identified. PV hematopoietic progenitor cells exhibit hypersensitivity to growth factors and cytokines, suggesting possible abnormalities in protein-tyrosine kinases and phosphatases. By sequencing the entire coding regions of cDNAs of candidate enzymes, we identified a G:C \( \rightarrow \) T:A point mutation of the JAK2 tyrosine kinase in 20 of 24 PV blood samples but none in 12 normal samples. The mutation has varying degrees of heterozygosity and is apparently acquired. It changes conserved Val1617 to Phe in the pseudokinase domain of JAK2 that is known to have an inhibitory role. The mutant JAK2 has enhanced kinase activity, and when overexpressed together with the erythropoietin receptor in cells, it caused hyperactivation of erythropoietin-induced cell signaling. This gain-of-function mutation of JAK may explain the hypersensitivity of PV progenitor cells to growth factors and cytokines. Our study thus defines a molecular defect of PV.

Polycythemia vera (PV)\(^{1}\) is a clonal myeloproliferative disorder characterized by increased production of red cells, granulocytes, and platelets (1–3). It mainly affects people between 40 and 60 years of age with an annual incidence of about 14 per million in the population. Thus far there is no effective cure for the disease. Phlebotomy is the mainstay of treatment for the disease, and hydroxyurea, interferon-\(\alpha\), and anagrelide drug therapies and \(^{32}\)P radiation therapy have commonly been used (3). The mortality rate is high if the disease is untreated or is associated with leukemia. Despite extensive studies in recent years, the molecular etiology of PV remains unknown.

A major feature of PV is that hematopoietic progenitors in patients display hypersensitive responses to many growth factors and cytokines (1–3). Despite these abnormal responses, the numbers of receptors for the growth factors and cytokines on the surface of these cells are normal, suggesting a primary defect in a shared signaling pathway in these cells. Tyrosine phosphorylation is a fundamental regulatory mechanism for cell growth and development, and this process is controlled by coordinate actions of protein-tyrosine kinases (PTKs) and phosphatases (PTPs) (4). Both families of enzymes have highly diverse structures and functions. Their activities are tightly regulated under normal conditions, and deregulation of the enzymes produces human diseases. According to the human genomic data base, there are about 90 PTKs and 38 phosphotyrosine-specific PTPs (5–7). Both mutation of PTKs and PTPs have been implicated in human cancers. Recent studies using a large scale sequencing-based approach revealed that PTK and PTP genes are mutated more often in human cancers than previously anticipated. A minimum of 30% of colorectal cancers contains at least one mutation in PTKs, and 28% of them has a mutation in PTPs (8, 9). It is highly expected that mutations of PTKs and PTPs may also be a major manifestation of other types of malignancies such as PV.

In earlier studies, we found that PV erythroid progenitor cells contain a hyperactive PTP that corresponds to PTP-MEG2 (10, 11). However, this abnormality is caused by altered distribution of the enzyme rather than a mutation in its primary structure, suggesting that PTP-MEG2 is not the primary cause of the disease. Considering the general importance PTKs and PTPs and the feature of PV hematopoietic progenitor cells, we have performed a large scale DNA sequencing analysis of a number of candidate PTPs and PTKs. Here we report the identification of a JAK2 mutation in a majority of the PV samples. We have further demonstrated the JAK2 mutant has a gain-of-function feature.

**EXPERIMENTAL PROCEDURES**

**Materials**—Polyclonal antibodies against JAK2 and the erythropoietin receptor (EPOR) were from Santa Cruz Biotechnology. Polyclonal antibodies against regular and phosphorylated forms of STAT5, ERK1/2, and Akt were from Cell Signaling Technology. Peripheral blood samples were obtained from healthy volunteers and PV patients. Approval was obtained from the Vanderbilt University institutional review board for these studies. All of the PV patients met the established criteria for PV (3). The blood samples (usually 10 ml for isolation of mononuclear cells and 400 ml for purification of erythroid colony-forming cells) were collected in heparin at a final concentration of 20 units/ml. Light density mononuclear cells were collected by centrifugation on Ficoll (1.077 g/ml) following the standard protocol. Erythroid colony-forming cells were purified as described previously (10).

**PCR and Sequencing Analysis**—Total RNAs were isolated from peripheral blood mononuclear cells and erythroid colony-forming cells by using the TRIzol reagent (Invitrogen), and genomic DNAs were extracted by phenol/chloroform after proteinase K digestion following standard techniques. First strand cDNAs were prepared by reverse transcription of total RNAs with random primers. For amplification of the JAK2 cDNA, the primers used were 5'-CTTCGCGCCGCGGCGGCAAAATGTTCT-3' and 5'-CTTTGGTCTCTGTTAATCTACCTTTTGCTCTCAG-3' for initial PCR and 5'-TGCTAGGAAGATGGCCTGCTAC-3' and 5'-CTTTGCCTCCAGCCTATTACCCCTTA-3' for nested PCR. For amplification of the JAK2 genomic DNA, the PCR primers were 5'-

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1 The abbreviations used are: PV, polycythemia vera; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; EPOR, erythropoietin receptor; GST, glutathione S-transferase; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; JH, JAK homology; SH, Src homology; IR, inhibitory region; IL, interleukin.

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JAK2 Mutation in Polycythemia Vera

Identification of a JAK2 Mutation in PV—Initially, we hypothesized that PV might be caused by mutations of PTKs and/or PTPs. To identify the mutations, our basic strategy was to sequence the entire coding regions of a number of PTPs and PTKs selected based on their known involvement in erythropoiesis. For PTPs, we analyzed SHP-1, SHP-2, TC-PTP, RPTPα, DEP, PTP-MEG1, PTP-MEG2, and CD45. These are the major PTPs identified in erythroid colony-forming cells (11). For PTKs, we chose members of the Src, Abl, JAK, and platelet-derived growth factor (PDGF) receptor families. We isolated total RNAs from peripheral blood mononuclear cells and purified erythroid colony-forming cells. Upon synthesis of first strand cDNAs by reverse transcription, PCR was performed with primers corresponding to the 5' and 3' ends of the coding regions of candidate PTPs and PTKs. For samples that failed to yield products in the first PCR, nested PCR was performed with a set of inner primers. To avoid mutations caused by PCR amplifications, high fidelity DNA polymerases (Pfu-ultra and Phusion) were used. Complete sequencing analyses of the PCR products revealed sporadic single nucleotide polymorphisms, point mutations, and abnormal splicing of several PTPs and PTKs (not shown) and, most importantly, defined a point mutation of JAK2 in the majority of PV samples (Fig. 1). The JAK2 mutation is a G:C→T:A transversion, which causes substitution of Val617 by phenylalanine. At the 5' end of JAK2, this portion of JAK2 contains the autophosphorylation site corresponding to amino acid residues 883–1132 at the C-terminal end of JAK2. This portion of JAK2 contains the autophosphorylation sites Tyr911 and Tyr1007. The fusion protein, designated GST-JAK2CT, was purified from Escherichia coli by using glutathione-Sepharose columns. Wild type JAK2 and the JAK2<sup>V617F</sup> mutant were cloned into the pcDNA3 vector (Invitrogen) for expression in HeLa cells. Upon transfection with the FuGENE 6 transfection reagent (Roche Applied Science), cells were lysed in a buffer containing 50 mM β-glycerophosphate (pH 7.3), 5 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1% Triton X-100, 0.1 mg/ml GST-JAK2CT, 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 μM ATP, and 2 mM dithiothreitol. The reactions were allowed to proceed at room temperature for 20 min and then stopped by addition of the SDS gel sample buffer. Tyrosine phosphorylation of GST-JAK2CT was determined by Western blotting analysis with an anti-phosphotyrosine antibody.

RESULTS

Identification of JAK2 mutation in cDNAs and genomic DNAs. The data show sequencing of JAK2 PCR products from cDNAs (from both 5' and 3' directions) and the corresponding genomic DNA (from 3' side only) of one normal and four PV mononuclear cell samples. The mutation point is underlined. Percentages of the mutant in the total PCR products were calculated based on the average signals of nucleotides A and C in the sequencing analysis. see PV1 in Fig. 1), whereas in three cases (e.g. PV4 in Fig. 1), nearly 100% of the PCR products corresponded to the mutant. The mutation is apparently not confined to erythroid progenitor cells, because no increase in the portion of mutant JAK2 was seen with RNAs isolated from purified erythroid colony-forming cells (data not shown), reflecting the multiple lineage feature of PV. Because the point mutation occurs somewhere in the middle of the JAK2 cDNA, it should not affect the efficiency of PCR amplification. Therefore, the ratio of wild type and mutant JAK2 PCR products should reflect the ratio of the wild type and mutant-JAK2 mRNA in the original samples. We also analyzed the sequence of JAK2 at the genomic level. The mutation point is located at exon 14 of the JAK2 gene (the translation initiation codon is in exon 3). We designed PCR primers...
to amplify a fragment (1678 bp) of the gene covering exons 13 and 14 with an intron in between. Samples showing homoygous mutation of JAK2 at the cDNA level also gave rise to homozygous mutation with genomic DNAs. Interestingly, in all cases, the genomic DNA gave rise to significantly lower amounts of the mutant PCR products than the correspondent cDNA samples. In fact, several samples that showed low percentages of the JAK2 mutant in the PCR products with cDNAs produced hardly any mutant JAK2 PCR product with genomic DNAs (e.g., PV1 in Fig. 1). Apparently, analysis of cDNA is more sensitive in detecting the mutation of JAK2 in PV than in genomic DNAs. This is because not all hematopoietic cells express JAK2, and those expressing high levels of JAK2 mRNA are also affected by the mutant enzyme. These cells, bearing the mutant JAK2, presumably have gained an advantage to grow and thus give rise to more mRNA products without contributing much to the total genomic DNA in the blood. Altogether, the different degrees of mutation detected with both cDNAs and genomic DNAs indicate that the mutations of JAK2 in PV are not derived from germ lines but rather represent acquired events occurred in certain hematopoietic progenitor cells.

**Structural Analysis of the JAK2 Mutant**—The structure of JAK family PTKs can be divided into seven JAK homology (JH) domains (12, 13). Our sequence search of the GenBank database by using the BLAST program revealed the presence of four major domains in JAK2 (Fig. 2). The C-terminal tyrosine kinase domain and pseudokinase domain correspond to the JH1 and JH2 domains, respectively. We also identified a putative SH2 domain that has an inhibitory role. The SH2 domains bind specific tyrosine-phosphorylated motifs, but what the SH2 domain of JAK2 binds is not yet defined. The JH4–JH7 region constitutes the band 4.1 domain (also known as the FERM domain), which are known to mediate protein–protein interactions (14). All of the catalytic domains of protein kinases consist of 12 subdomains with conserved key amino acid residues (15, 16). The V617F mutation point resides in subdomain IV of the pseudokinase domain in the ATP binding lobe. The pseudokinase domain has the basic structural features of PTKs but has no catalytic activity. Growing evidence indicates that it regulates activities of the kinase domain. The pseudokinase domain suppressed basal JAK2 activity by lowering the $V_{\text{max}}$ of the kinase domain but does not affect the $K_m$ value; three inhibitory regions, namely IR1 (residues 619–670), IR2 (725–757), and IR3 (758–807), have been defined in the pseudokinase domain (17). Val617 is just N-terminal to the IR1, and it is conserved in JAK2 of various animals from fish, frog, bird, to mice. It is also conserved in human JAK1 and Tyk2 and is replaced by Met in JAK3. One can predict that the replacement of Val by Phe in JAK2 should cause major conformation changes. This may disrupt the inhibitory function of the pseudokinase domain and thus cause deregulation of the kinase domain. Earlier studies have shown that deletion of the pseudokinase domain leads to hyperactivation of JAK2. In fact, the Tel-JAK2 fusion protein with constitutive activation of its kinase domain causes myeloid and lymphoid malignancies (18). Considering the phenotype of PV disease, it is conceivable that JAK2V617F may be a gain-of-function mutant and have increased kinase activity.

**JAK2 Mutant Possesses Enhanced Kinase Activity and Causes Hyperactivation of Signaling Components Downstream of EPOR**—To prove the potential gain-of-function feature of JAK2V617F, we compared the tyrosine kinase activity of wild type JAK2 and the mutant enzyme. Both the wild type and mutant enzymes were expressed in HeLa cells carried by the pcDNA3 vector. The enzymes were purified from cell extracts by using antibodies immobilized to agarose beads. For the substrate, we used a GST fusion protein containing the C-terminal region of JAK2 where the autophosphorylation sites reside. The data shown in Fig. 3 demonstrate that the GST fusion protein was phosphorylated by JAK2. More importantly, JAK2V617F produced a much stronger phosphorylation than wild type JAK2. Furthermore, JAK2V617F itself was autophosphorylated, and JAK2V617F showed a higher level of autophosphorylation. Together, these data indicate that the V617F mutation increases the kinase activity of JAK2.

To analyze whether JAK2V617F produces a gain-of-function phenotype in cells, we co-expressed EPOR with JAK2 or JAK2V617F in HeLa cells. The cells were treated or left untreated with erythropoietin, and activation of various signaling components was accessed by using phosphospecific antibodies. As expected, expression of JAK2V617F resulted in a much higher activation of STAT5, ERK1/2, and Akt than the wild type enzyme (Fig. 4). Significant activation of these signaling components was seen even in the absence of erythropoietin. These data demonstrate that JAK2V617F causes hyperactivation of signal transduction pathways induced by EPO, which presumably is responsible for the EPO-independent growth of PV erythroid progenitor cells and the hypersensitivity of these cells to growth factors and cytokines.

**DISCUSSION**

In the present study, we identified an acquired mutation of JAK2 in 83% of PV samples that we analyzed. This mutation causes replacement of a key valine residue by phenylalanine in the pseudokinase domain of JAK2 that has an inhibitory role.
structural analysis predicts gain-of-function phenotype. Our further study by overexpressing the mutant enzyme demonstrated that the mutant enzyme has enhanced kinase activity and causes hyperactivation of signal transduction pathways downstream of EPOR. We thus defined a molecular defect of the PV disease. This should have major implications in the diagnosis and treatment of PV disease. Increasing evidence has shown that tyrosine kinases are excellent targets for cancer drug therapies. JAK2 thus becomes a potential target for developing therapeutic drugs to treat PV.

JAK2 is a PTK involved in signaling pathways by members of the single chain receptors (e.g. EPOR, TPOR, GHR, PRLR), the IL-3 receptor family (IL-3R, IL-5R, and GM-CSF-R), the gp130 receptor family, and the class II receptor cytokine family (19, 20). JAK2 knock-out mice exhibited an embryonic lethal phenotype, dying on day 12.5 of gestation due to a failure in definitive erythropoiesis (21, 22). This is similar to what occurs in EPO−/− and EPOR−/− mice (23), indicating that JAK2 has pivotal functions for signal transduction initiated by growth factors and cytokines required in definitive erythropoiesis. A primary growth factor involved in erythropoiesis is EPO (24). Its receptor, EPOR, does not possess kinase activity, but it is associated with the latent JAK2. Binding of EPO to EPOR activates JAK2 and results in activation of PI3K/Akt, STAT5, and ERKs (25). Growth and expansion of erythroid progenitor cells absolutely requires the presence of EPO. However, some PV erythroid progenitor cells possess the ability to grow autonomously. This implies that gain-of-function mutation of JAK2 was not sufficient to transform primary cells. This suggests that other abnormalities such as loss of tumor suppressors may be associated with PV.2

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