The majority of polycythemia vera (PV) patients harbor a unique somatic mutation (V617F) in the pseudokinase domain of JAK2, which leads to constitutive signaling. Here we show that the homologous mutations in JAK1 (V658F) and in Tyk2 (V678F) lead to constitutive activation of these kinases. Their expression induces autonomous growth of cytokine-dependent cells and constitutive activation of STAT5, STAT3, mitogen-activated protein kinase, and Akt signaling in Ba/F3 cells. The mutant JAKs exhibit constitutive signaling also when expressed in fibrosarcoma cells deficient in JAK proteins. Expression of the JAK2 V617F mutant renders Ba/F3 cells hypersensitive to insulin-like growth factor 1 (IGF1), which is a hallmark of PV erythroid progenitors. Upon selection of Ba/F3 cells for autonomous growth induced by the JAK2 V617F mutant, cells respond to IGF1 by activating STAT5, STAT3, Erk1/2, and Akt on top of the constitutive activation characteristic of autonomous cells. The synergic effect on proliferation and STAT activation appears specific to the JAK2 V617F mutant. Our results show that the homologous V617F mutation induces activation of JAK1 and Tyk2, suggesting a common mechanism of activation for the JAK1, JAK2, and Tyk2 mutants. JAK3 is not activated by the homologous mutation M592F, despite the presence of the conserved GVC preceding sequence. We suggest that mutations in the JAK1 and Tyk2 genes may be identified as initial molecular defects in human cancers and autoimmune diseases.
Homologous Polycythemia Vera Mutation in JAK1 and Tyk2

EXPERIMENTAL PROCEDURES

Generation of the Different JAK Mutants—The cDNA for the human Tyk2 tagged with the vesicular stomatitis virus epitope YTDIEMR-LGK at the carboxyl terminus (21) was kindly provided by Dr. Sandra Pellegri, Institut Pasteur, Paris. Mutagenesis reactions for the human Tyk2, human JAK3, and murine JAK1 were performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were cloned in the bicistronic retroviral vector pMX-IRESGFP (JAK1) or pMX-IRESCD4 (Tyk2, JAK3) (22, 23) and verified by sequencing. The human JAK2 V617F mutant was described previously (1).

Cell Lines, Retroviral Transductions, and Cytokines—The JAK1-deficient U4C human fibrosarcoma cells (24) were a kind gift of Dr. Ian Kerr, Imperial Cancer Research Fund, London and Dr. George Stark, The Cleveland Clinic, Cleveland, OH. The Tyk2-deficient 11,1 human fibrosarcoma cells (25, 26) were a kind gift of Dr. Sandra Pellegri, Pasteur Institute, Paris. Ba/F3 cells are murine IL3-dependent cells. Wild type (wt) or mutant JAK1, JAK3, or Tyk2 cDNAs were transfected into the BOSC packaging cells to produce retroviruses, as described (23). Ba/F3 cells were then infected and GFP- or CD4-positive cells were sorted 72 h after infection. Ba/F3 cells were normally cultured in RPMI medium supplemented with 10% fetal calf serum and cytokines (IL3). After retroviral infection and sorting, cells were washed in RPMI medium and cultured in the absence of cytokines for assaying cytokine-independent growth or cultured in the presence of IGFl. Cell numbers were recorded using a Coulter cell counter. IL9 was a kind gift of Drs. Jean-Christophe Renaud and Jacques Van Snick, Ludwig Institute, Brussels, while murine Tpo, IL3, and IGFl were purchased from R&D Systems.

Dual Luciferase Assays—Transcriptional activity of signal transducer and activator of transcription (STAT) proteins was assessed by measuring luciferase expression in cells transfected with the following luciferase constructs: pGRR5-Luc, a reporter that responds to STAT5, STAT3, and STAT1 (27); pGL3bPpr2-Luc construct, a luciferase reporter that responds to STAT3 (28) or the pLHRE-Luc reporter, which preferentially responds to STAT5 (29), as described (23). Transcriptional activity induced by STAT3 upon JAK1 activation was measured in JAK1-deficient U4C cells that were transfected with the pGL3bPpr2-Luc construct, along with cDNAs coding for JAK proteins to be tested and components of the IL9 receptor complex. Transcriptional activity induced by STAT3 upon Tyk2 activation was measured in Tyk2-deficient 11,1 cells that were transfected with the cDNA coding for pGL3bPpr2-Luc construct, along with the STAT3 cDNA, the cDNAs coding for the JAK proteins to be tested and for the TpoR. Experiments were performed as described previously (1). Transcriptional activity of STAT5 and STAT3 in Ba/F3 cells expressing various mutant JAK proteins was assessed by using the general STAT reporter pGRR5-Luc.

Western Blotting—Ba/F3 cells were washed three times in RPMI to remove residual IL3 and fetal bovine serum and then starved for 6 h in 0.1% bovine serum albumin in RPMI. The cells were counted and diluted to 2.5 million cells per ml of medium. Four cell lines were analyzed for each JAK protein, namely the Ba/F3 parental cells, Ba/F3 cells sorted for wild type JAK (wt), or mutant JAK expression and cells selected for cytokine-independent growth due to the expression of the mutant JAK. Cells were divided in aliquots of 1 ml and stimulated with either IGFl or IL3 for 5, 15, or 30 min or left untreated. After stimulation, the cells were centrifuged and the pellets lysed in 250 μl of Laemmli buffer using a 20-gauge syringe. Proteins were separated by SDS-PAGE on 8% gels by loading the equivalent of ~250,000 cells per well and were then transferred to polyvinylidene difluoride membranes. After blocking the membranes in 5% milk/TBS-Tween, immunoblotting was performed by overnight incubations in the cold with primary antibodies in 0.1% TBS-Tween, using the dilution recommended by the manufacturer. After 3 × 5 min of washing in 0.1% TBS-Tween, the membranes were incubated with secondary anti-rabbit-horseradish peroxidase antibodies from Cell Signaling, diluted 1:5000 in 0.1% phosphate-buffered saline-Tween. After 3 × 5 min of washing, ECL was performed using Santa Cruz Western blotting Luminol Reagent and exposed on Kodak Biomax Light Film.

Phosphorylation of signaling proteins was investigated by using Western blots and probing with the following phospho-specific antibodies from Cell Signaling Technology: anti-pY1007/1008 JAK2, anti-pY1022/1023 JAK1, anti-pY1054/1055 Tyk2, anti-pY694 STAT5, anti-pY727 STAT3, anti-pS473 Akt, anti-pYpT Erk1/2, and anti-pY1131 IGFlR/pY1146. Antibodies to detect protein levels of STAT5, STAT3, Akt, and IGFlR were also from Cell Signaling Technology. For the detection of Erk1/2 levels an anti Erk1/2 rabbit anti-EETARFQPGYRS antiserum was used. To detect protein levels of JAK2 and Tyk2, we used the anti-JAK2 (C-20) and the anti-Tyk2 (C-20) antibodies from Santa Cruz Biotechnology, for JAK1 we used the anti-JAK1 from Upstate Biotechnology, and for JAK3 we used anti-JAK3 (C-21) antibodies from Santa Cruz Biotechnology.

RESULTS

Conservation of JAK2 Val617 and Surrounding Sequence—In mammals there are four known Jak5, JAK1, JAK2, Tyk2, and JAK3 (8). We aligned the protein sequences coding for the Janus kinases and noted that the JAK2 Val617 is conserved in JAK1 (Val568) and Tyk2 (Val578) and is replaced by methionine in JAK3 (Met582) (Fig. 1A). The preceding sequence, GVC, is stringently conserved in all four Janus kinases. Based on the x-ray crystal structure of the fibroblast growth factor receptor kinase domain (30), these residues are predicted to be part of a short
β-strand, while Val617 is predicted to be part of a loop (30, 31). As we suggested previously (1, 6), the V617F mutation may disrupt the inhibitory activity of the pseudokinase JH2 domain on the kinase JH1 domain, possibly by stabilizing an activated conformation of the activation loop.

**Constitutive Activation of JAK1 V658F and Tyk2 V678F Mutants in Ba/F3 Cells**—To test whether the introduction of a phenylalanine residue at the homologous position of JAK2 Val617 affects the activity of other JAKs, we constructed mutants JAK1 V658F, Tyk2 V678F, and JAK3 M592F and cloned them in bicistronic retroviruses, as described (23). Fig. 1B shows that the mutant JAK1 V658F is constitutively active as shown by its ability to render Ba/F3 cells autonomous for growth. On the same graph, the acquisition of cytokine-independent growth by Ba/F3 cells expressing the JAK2 V617F or the Tyk2 V678F mutants is shown. It is clear that all three mutant JAKs are constitutively active. It is difficult to compare the activities, since each is detected in Western blots by different antibodies, but nevertheless, for each type of JAK protein, levels of sorted wild type and mutant proteins were similar, as shown by Western blotting with antibodies directed to each JAK protein (Figs. 3B, 4B, and 5B, compare sorted Ba/F3 cells for expression of wild type and mutant). Thus, JAK1 V658F and Tyk2 V678F are constitutively active and trigger ligand-independent cell proliferation in Ba/F3 cells. Fig. 1B also shows that the JAK3 M592F mutant is not constitutively active, although this mutant is well expressed and can mediate IL9 signaling like the wild type JAK3 (data not shown). Thus, both JAK1 and JAK3 are active, although this mutant is well expressed and can mediate IL9 signaling like the wild type JAK3 (data not shown).

**Constitutive and Ligand-induced Activation of STAT Proteins by the Mutant JAK1 and Tyk2**—Furthermore, we explored the transcriptional activity induced by the mutant JAKs via STATs. For the JAK1 V658F mutant we used JAK1-deficient U4C cells (24, 32), where the STAT3 reporter construct pGL3bPpr2-Luc (28) was co-transfected with STAT3 cDNA. Cells were transfected either with the cDNA coding for wild type or mutant JAK1 alone or together with the cDNAs coding for the components of the IL9 receptor (IL9R), namely the IL9 receptor α (IL9Rα), the common γ chain, and JAK3, which are all required to reconstitute IL9 signaling in these cells. Fig. 2A shows that expression of JAK1 V658F leads to constitutive STAT3 activation. When the components of the IL9R complex were co-transfected, addition of IL9 increased the activity of STAT3 in cells transfected with the wild type JAK1, while no significant increase was seen for the mutant JAK1, presumably because the system was saturated by the other receptor components, i.e. γ chain and/or JAK3.

We also examined the activation of STAT5 and STAT3 transcriptional activity by the Tyk2 V678F mutant. We used Ba/F3 cells either sorted for expression of the mutant Tyk2 or cells that were subsequently selected for autonomous growth. As shown in Fig. 2B, strong activation of STAT5 signaling could be demonstrated in both the sorted and the selected cells when the pGRR5-luc, a reporter that responds to STAT5, STAT3, and STAT1 (27) was used. Similar results were obtained when the pHLRE-luciferase construct was employed, which preferentially responds to STAT1 (27) was used. Similar results were obtained when the pHLRE-luciferase construct was employed, which preferentially responds to STAT5 (29) (data not shown). We also explored signaling by the mutant Tyk2 in cells deficient in Tyk2, the 11.1 fibrosarcoma cells (25, 26). For signaling the activated TpoR requires mainly JAK2, but Tyk2 can also transduce the signal in the absence of JAK2. In 11.1 cells there is not enough JAK2 activity to support TpoR signaling (data not shown). When activation of STAT3 was examined using the pGL3bPpr2 luciferase reporter, which preferentially responds to STAT3 (28), we could note again that the Tyk2 V678F mutant is constitutively active in 11.1 cells and that addition of Tpo further augments this activity (Fig. 2C). This result shows that the mutant Tyk2 can replace the wild type Tyk2 and be appended to the TpoR cytosolic domain, as the 11.1 cells are deficient for Tyk2.

**Cross-talk with IGF1**—Previous reports have described a hypersensitivity of PV progenitors to IGF1 (18–20). Such hypersensitivity to growth factors became one of the hallmarks of PV, along with the Epo-independent or hypersensitive erythroid colony formation and down-modulation of the cell-surface TpoR (c-Mpl) in megakaryocytes and platelets (33, 34). Our hypothesis is that the JAK2 V617F mutant is responsible for both hallmarks of PV (6, 7). We compared the response of parental Ba/F3 cells and of Ba/F3 cells sorted for expression of wt JAK2 and the mutant JAK2 V617F. As shown in Fig. 3B, these sorted Ba/F3 cells expressed levels of JAK2 that were ~2-fold higher than the endogenous levels (Fig. 3B). These cells were maintained in IL3 and
were not subjected to selection for autonomous, cytokine-independent growth, which normally takes 5–6 days of culture in the absence of IL3. In Fig. 3A we show that when cells were stimulated with 100 ng/ml IGF1, only the JAK2 V617F cells responded with a major increase in cell proliferation. Cells overexpressing wild type JAK2 showed a very weak response at concentrations of and above 300 ng/ml; parental Ba/F3 cells showed a marginal response at 300 ng/ml. The proliferative response of Ba/F3 JAK2 V617F cells to IGF1 was immediate and did not require a lag period or selection for autonomous growth. Similar results were obtained when cells were grown in the absence of serum and in the presence of 3% human serum albumin (data not shown). These data show that Ba/F3 cells that express the mutant JAK2 V617F acquire the ability to proliferate in the presence of IGF1. Experiments where dose-dependent growth was examined showed that the JAK2 V617F cells respond to 10–30 ng/ml IGF1, while cells expressing the wild type JAK2 needed >10–20-fold higher concentrations for a minimal response.

Next we examined signaling of IGF1 and IL3 on parental Ba/F3 cells, cells sorted for expressing JAK2 V617F mutant or the wild type JAK2, and on cells rendered autonomous (selected) by the JAK2 V617F mutant. The IGF1Rβ chain was tyrosine-phosphorylated at the activation loop Y1146 to similar levels in all cells. In selected cells, as expected, we detected basal tyrosine phosphorylation of the JAK2 mutant. This high level of activation was further increased by addition of IGF1 and, as previously shown, by IL3 (1). Interestingly, selection clearly lead to a further 2–2.5-fold increase of the protein levels of the JAK2 V617F mutant over the levels in sorted cells, most likely by selection for high mRNA expression levels, as the GFP levels in these cells also were higher than in sorted cells. Two key changes in IGF1 signaling were induced by the expression of the JAK2 V617F and selection for autonomous growth. First, IGF1 acquired the ability to induce tyrosine phosphorylation of JAK2, STAT5, and STAT3 on top of the constitutive phosphorylation levels induced by the JAK2 mutant (1). Second, IGF1 induced significantly higher levels of activation of Erk1/2 and Akt, as shown by detection with anti-phospho-Erk and anti-phospho-Akt antibodies. The difference in activation of Erk1/2 between selected and sorted cells is not due to increased protein levels of Erk1 or Erk2, as similar levels were detected by Western blot with anti-Erk antibodies. We could not detect these effects of IGF1 on JAK2, STAT5, and STAT3 on cells that were only sorted for expression of JAK2 V617F mutant, presumably due to lack of sensitivity. However, our experiment shown in Fig. 3A is equivalent to cells undergoing selection because treatment with IGF1 was performed in the absence of IL3, and thus cells were submitted to the selection pressure. Thus, IGF1 induces proliferation in cells expressing the JAK2 V617F mutant and this correlates with IGF1-dependent activation of STAT signaling.

We then asked whether JAK1 and Tyk2 activated mutants synergize with IGF1R to induce IGF1-dependent proliferation and signaling. In
Figs. 4 and 5A we show that these mutants do not appear to synergize with IGF1. Both the JAK1 and Tyk2 mutants appear to be stronger in inducing cytokine-independent proliferation when compared with the JAK2 mutant. Also, for the case of Tyk2, both the wt and mutant Tyk2 appear to synergize with IGF1 at 30 ng/ml, albeit very weakly. Thus, it is possible that the high constitutive level of activation of these mutants may mask a synergic effect of IGF1 with the Tyk2 or JAK1 mutants. Nevertheless, these results, as well as those presented in Fig. 3B, raise the question of whether synergy can be demonstrated at the signaling level between the JAK mutants and IGF1. Figs. 4B and 5B show that in selected cells expressing the mutant JAK1 or Tyk2, IGF1 does not acquire the ability to activate STATs (STAT5 or STAT3) or JAKs themselves. This profile of STAT activation is clearly different from that of cells rendered autonomous by the JAK2 V617F mutant (Fig. 3B). Surprisingly, we detected an effect of IL3 on JAK1 tyrosine phosphorylation in sorted cells expressing either the wt or mutant JAK1 (Fig. 4B), but it has been previously reported that IL3 indeed can activate JAK1 (35).

In Fig. 5B we show that both in cells sorted for expression of the Tyk2 mutant as well as in cells rendered autonomous for growth by the Tyk2 mutant, phosphorylation of the Tyk2 V678F is constitutive. The transduced Tyk2 (“exog” in Fig. 5B) can be recognized as migrating slower than the endogenous Tyk2 (“endog” in Fig. 5B), since it contains a VSV tag at the carboxyl terminus. All cells transformed by constitutively active JAKs acquired a stronger response to IGF1 with respect to the phosphorylation of Erk1/2 and Akt (Figs. 3B, 4B, and 5B). While levels of Erk1/2 were similar between sorted and selected cells in the case of JAK2 V678F and JAK1 V658F cells, selection for autonomous growth of Tyk2 V678F cells lead to a 2-fold increase in Erk1/2 protein levels (data not shown). We suggest that cross-talk between IGF1R and JAKs leading to IGF-dependent activation of Erk1/2 and Akt may not be sufficient to promote proliferation, which appears to correlate with IGF1-dependent activation of STAT signaling.

DISCUSSION
Our main observation is that JAK1 and Tyk2 are activated by the homologous mutation of the JAK2 V617F mutant, which we described in polycythemia vera (1). The GCV sequence preceding Val617 in JAK2 is conserved in all four JAKs. In JAK3, there is a methionine at the homologous Val617 position, and the M592F mutation does not activate the enzyme.

From a structural viewpoint our hypothesis is that the V617F mutation relieves the putative inhibition of the JH1 kinase domain by the JH2 pseudokinase domain and may stabilize a transient activating conformation of the JH1 activation loop. Janus kinases appear to require an
intact JH2 domain for physiologic activation, as shown by random mutagenesis of the Tyk2 JH2 domain and isolation of several mutants that impaired activity (17). Deletion of the pseudokinase domain inactivates Drosophila JAK (9) and human Tyk2 (16), while deletion of JH2 in JAK2 partially activates the kinase activity (36). Thus, it appears that JAKs require a correctly folded JH2 domain and interactions with JH1 for function. An interaction between the JH1 and JH2 domains has been reported for JAK1 (37), for JAK3 (10) and JAK2 (11). A few years ago Lindauer et al. (31) modeled JAK2 JH2 and JH1, based on the x-ray crystal structure of the FGF receptor kinase domains, which were found as dimers that might block each other (30). The suggestion was that JH2 may inhibit JH1 via two interfaces and that the second interface, which contains Val617, may stabilize the inactive conformation of the activation loop of JH1 (31). In any event, these predictions fit quite well with the experimental results on JAK2 V617F activation and with the data reported in this manuscript on JAK1 and Tyk2. Whether phenylalanine plays a special structural role in interacting with the activating loop of JH1, or other amino acid residues could also activate the enzyme, is not known. Following the prediction by Lindauer et al. (31), no mutagenesis study was published on residues Val617 or Cys618, most likely because the usual mutations (such as V → A) may not lead to a change in activation. Current studies in our laboratories aim at testing the effect of all possible mutations at the Val617 position of JAK2 and at the highly conserved GVC sequence that precedes this Val617 residue. However, only the x-ray crystal structures of the JAK2 V617F mutant and of the activated wild type JAK2 will clarify whether the conformation of the V617F mutant resembles the activated state of the wild type JAKs or a different intermediate conformation. Interestingly, these structural features appear to be different for JAK3, since the JAK3 M592F mutant is not constitutively active.

Our results raise the possibility that mutations in the JAK1 or Tyk2 pseudokinase domains may be found in human diseases. Particularly for JAK1, the V658F mutation could be obtained by one base pair GTC → TTC change. In vivo, this mutation may lead to leukemia, autoimmune diseases, or cancers of different lineages, depending on the cell type where this mutant may appear. Reconstitution of lethally irradiated mice with primary hematopoietic stem cells retrovirally transduced with the mutant JAK1 or Tyk2 will assess the effects these mutants in the hematopoietic system. Knocking-in the mutated JAK1 or Tyk2 genes in the place of wild type JAK1 and Tyk2 would be necessary (like for the JAK2 V617F) to explore the in vivo effects of these mutants on lineages other than hematopoietic. Nevertheless, our data suggest that systematic sequencing of the JAK JH2 regions may be useful in human diseases, especially since there are only four Janus kinases that transduce signals from 25 or more cytokine receptors. In addition, small molecule inhibitors may be found for the V617F mutants, and their mechanisms of action may be common and specific for the mutant and not the wild type JAKs.

It appears that the mutant JAKs can bind to receptor cytosolic domains. For JAK2 V617F in γ2A cells, that lack JAK2, or for Tyk2 V678F in 11.1 cells, that lack Tyk2, stimulation of EpoR with Epo (1) or TpoR with Tpo (Fig. 2C) leads to enhanced signaling via the mutant JAKs only. Thus, the activated JAK mutants can transduce ligand-dependent signals. However, it is likely that signaling for the mutated JAKs differs from that induced by the activated JAKs, as the mutant JAKs are permanently active and presumably blocked in one conformation. It is tempting to speculate that negative regulators of JAK signaling may not be effective in down-modulating the mutated JAKs.

One hallmark of polycythemia vera is represented by hypersensitivity of erythroid progenitors to growth factors, such as the IGF1 (18, 19). Our results show that, indeed, JAK2 V617F leads to strong synergizes with IGF1R, leading to IGF1-dependent cell proliferation and further activation of JAK2, STAT5, and STAT3. This may be mediated by expression of adaptors that connect the IGF1R to the mutant JAKs or by a direct interaction between IGF1R and the mutated JAKs. Interestingly, in transfected NIH3T3 fibroblasts and in 293 cells it has been reported that IGF1 can activate JAK1, JAK2, and STAT3 (38) and that the IGF1R might directly interact with JAK1 (39). However, in Ba/F3 cells, only the JAK2 V617F mutant could be shown to be tyrosine-phosphorylated in an IGF1-dependent manner. Since in preliminary experiments we were not able to demonstrate co-immunoprecipitation between mutant JAKs and IGF1R, we suggest that continuous JAK2 V617F signaling induces the expression of adaptors that may transiently link IGF1R and JAK2 V617F. Synergy with IGF1R with respect to proliferation and activation of STAT signaling appears to be specific to the JAK2 mutant and not to JAK1 V658F or Tyk2 V678F. As shown in Figs. 3B, 4B, and 5B, all mutated JAKs promote enhanced IGF1-dependent activation of Erk1/2 and Akt, which in itself may not be enough to lead to cell proliferation. Furthermore, it was discovered that the Epo-hypersensitivity of a truncated EpoR that causes autosomal dominant erythrocytosis in humans (40) requires signals from the IGF1 in serum (41). Proteomics studies are needed for the identification of the adaptors mediating the STAT and the mitogen-activated protein kinase activation. One important aspect is that the JAK2 V617F mutant appears to induce three related myelo-proliferative diseases, PV, ET, and IMF. It is possible that different degrees of cross-talk with the JAK2 V617F mutant and IGF1R, along with down-modulation of TpoR cell surface levels (33), contribute to these different phenotypes.

Taken together our results show that mutation of the conserved valine residue corresponding to JH2 positions 617, 658, and 678 in JAK2, JAK1, and Tyk2, respectively, results in constitutive kinase activation, possibly reflecting a common mechanism of activation for these three mutant JAKs. The fourth mammalian JAK, JAK3, does not share the same mechanism of activation, as the homologous M592F mutation does not lead to constitutive activation. The mutant JAK2 synergizes with the IGF1R leading to IGF1-dependent proliferation and activation of STAT5 and STAT3. We suggest that mutations at the homologous JAK2 Val617 position in JAK1 and Tyk2 may be found in human diseases and that the hypersensitivity of polycythemia vera erythroid progenitors to IGF1 is due to cross-talk with the JAK2 V617F mutant.

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