Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors

Tekla Hornakova,[1,2] Lorraine Springuel,[1,2] Julien Devreux,[1,2] Alexandra Dusa,[1,2] Stefan N. Constantinescu,[1,2] Laurent Knoops,[1,2,3]* and Jean-Christophe Renauld[1,2]*

*Shared senior co-authorship

[1]Ludwig Institute for Cancer Research, Brussels Branch; [2]de Duve Institute, Université Catholique de Louvain, Brussels, and [3]Division of Hematology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

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Correspondence: Jean-Christophe Renauld, Ludwig Institute for Cancer Research, Avenue Hippocrate, 74, B-1200 Brussels, Belgium. E-mail: renauld@bru.licr.org

ABSTRACT

Background

Activating mutations in JAK1 and JAK2 have been described in patients with various hematologic malignancies including acute lymphoblastic leukemia and myeloproliferative neoplasms, leading to clinical trials with JAK inhibitors. While there has been a tremendous effort towards the development of specific JAK inhibitors, mutations conferring resistance to such drugs have not yet been observed.

Design and Methods

Taking advantage of a model of spontaneous cellular transformation, we sequenced JAK1 in selected tumorigenic BaF3 clones and identified 25 *de novo* JAK1 activating mutations, including 5 mutations already described in human leukemias. We further used this library of JAK1 mutation-positive cell lines to assess their sensitivity to ATP-competitive inhibitors.

Results

While most JAK1 mutants were sensitive to ATP-competitive JAK inhibitors, mutations targeting Phe958 and Pro960 in the hinge region of the kinase domain rendered JAK1 constitutively active but also resistant to all tested JAK inhibitors. Furthermore, mutation of the homologous Tyr931 in JAK2 wild-type or JAK2 V617F mutant found in patients with myeloproliferative neoplasms also conferred resistance to JAK inhibitors, such as INCB018424, which is currently in clinical use.

Conclusions

Our data indicate that some activating mutations not only promote autonomous cell proliferation but also confer resistance to ATP-competitive inhibitors. *In vivo*, such a mutation can potentially occur as primary JAK-activating mutations but also as secondary mutations combining oncogenicity with drug resistance.

Key words: JAK, tyrosine kinase, mutations, inhibitors, STAT.

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Introduction

Persistent activation of signal transducer and activator of transcription (STAT) factors is characteristic of many types of leukemia and solid tumors. In normal cells, cytokine-induced activation of STAT proteins provides the stimulus for proliferation, survival, self-renewal, differentiation and functional activation. In leukemic cells, like in other cancers, STAT proteins are frequently constitutively activated in the absence of cytokine stimulation, providing malignant cells with persistent signals and self-sufficiency for survival and proliferation.[1]

The most common mechanism for constitutive activation and phosphorylation of STAT factors is the dysregulation of tyrosine kinases. In 2005, the identification of an activating mutation in JAK2 (the V617F mutation) as a STAT5-activating and disease-causing genetic alteration in a significant proportion of patients with myeloproliferative neoplasms (MPNs) has emphasized the oncogenic role of the JAK tyrosine kinases in hematologic malignancies.[2-5] JAK2 is a member of the Janus tyrosine kinase family comprising three other mammalian non-receptor tyrosine kinases (JAK1, JAK3 and TYK2) that associate with cytokine receptors lacking intrinsic kinase activity to mediate cytokine-induced signal transduction and activation of STAT transcription factors.[6] All JAKs share a similar protein structure and contain a tyrosine kinase domain at the C-terminus flanked by a catalytically inactive pseudokinase domain with kinase-regulatory activity, by an atypical SH2 domain and by a FERM domain that mediates association to the membrane-proximal region of the cytokine receptors.[7,8] Soon after the discovery of JAK2 V617F, we and others described that activating JAK1 mutations are relatively common in adult patients with T-cell acute lymphoblastic leukemia (ALL) and participate in ALL development allowing for constitutive activation of STAT5.[9-11] Several STAT5-activating JAK1 mutations were also reported in AML and breast cancer patients.[10]

Because of its central role in the pathophysiology of MPNs, the JAK2 V617F is an attractive therapeutic target. Several JAK2 inhibitors are now under development or/and undergoing clinical trials for MPNs. Some of these JAK2 inhibitors were also shown to be remarkably active on JAK1 and have, therefore, a strong therapeutic potential for JAK1 mutation-positive patients. One of the most promising JAK1/JAK2 inhibitors is INCB018424,[12] currently in phase III clinical trials for MPNs. The major drawback of tyrosine kinase inhibitor therapy is the development of secondary resistance caused by the acquisition of new mutations. The best example of this scenario comes from BCR-ABL positive neoplasms. Here patients treated with imatinib become resistant because of the acquisition of mutations in the ABL kinase domain.[13]

In our laboratory, we have previously described an *in vitro* model of spontaneous transformation of the IL-3-dependent hematopoietic BaF3 cell line towards growth factor-independent tumorigenic clones with constitutive STAT5 activation.[14] This model was developed from the study of BaF3 cells transfected with an IL-9Rα mutant lacking the STAT-recruiting site (BaF3 phe116). This BaF3 phe116 almost completely fails to proliferate and activate STATs in response to IL-9.[15] However, upon prolonged culture with IL-9, a small number of cells manage to survive and even proliferate, allowing an IL-9-dependent cell line (BaF3 phe116/9) to be selected.[14] In contrast to parental BaF3 phe116 cells, those IL-9-selected cells could progress to autonomous cells (BaF3 Aut) after a second selection step in the absence of cytokine. These autonomous cells show a cytokine-independent activation of JAK1 and STAT5 and are highly tumorigenic when injected in mice, which is not the case for parental BaF3 phe116 and BaF3 phe116/9.[14,16] We previously showed that upregulation of the endogenous JAK1 gene was associated with the first step of transformation, namely increased sensitivity of BaF3 phe116 cells to IL-9, and promotion of the second step of transformation, namely progression towards cytokine-independent BaF3 autonomous cells.[16]

In this study, we show that 80% of the autonomous BaF3 clones, selected in our *in vitro* model, acquired activating point mutations in the kinase or pseudokinase domain of JAK1. These JAK1 mutations provide cells with tumorigenic potential by inducing constitutive activation of the JAK-STAT pathway, which supports their autonomous proliferation. We took advantage of this collection of JAK1 mutation-positive autonomous cell lines to study the sensitivity of different JAK1 mutations to JAK inhibitors. For the first time, we report that mutations of the Phe958 and Pro960 not only constitutively activate JAK1, but also render the mutated JAK1 protein resistant to ATP-competitive inhibitors. The homologous mutation in JAK2, namely Y931C, also renders JAK2 wild-type or V617F mutant resistant to all tested ATP-competitive inhibitors.

Design and Methods

Cell culture and cytokines

BaF3 mouse hematopoietic pro-B cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and IL-3 (150 U/mL), which was produced by transfected CHO cells. Recombinant human IL-9 was produced in the baculovirus system and purified by affinity chromatography in our laboratory. The generation of BaF3 phe116 as well as BaF3 phe116/9 cells and the selection of autonomous cells has been previously described.[14] The frequency of autonomous cells was assessed as previously described.[14] IL-9-selected BaF3 phe116/9 and non-selected BaF3 phe116 cells were grown in the presence of IL-3, while autonomous clones were selected and subsequently amplified in the absence of IL-3.

RNA extraction, cDNA synthesis, PCR and sequencing

Total RNA was isolated from 10^6 IL-3 dependent BaF3 phe116, BaF3 phe116/9 or autonomous BaF3 (BaF3 Aut) clones using TriPure reagent (Roche) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA with an oligo (dT) primer (Roche) and M-MLV RT (Invitrogen). PCR amplification was performed from cDNA corresponding to 20 ng of total RNA at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min with a total of 39 cycles. Depending on the region of JAK1 to be sequenced, different sets of primers were used to amplify and sequence JAK1 PCR product (available upon request). PCR product was purified using Chromaspin technology (Clontech): 50-100 ng of PCR product was used for sequencing with the DYEnamic ET Dye Terminator Kit (Amersham Biosciences) according to the manufacturer's instructions. Two independent PCR reactions were performed to rule out the possibility of Taq-induced mutations during amplification.

Interestingly, all the nucleotides mutated in the murine JAK1 sequence were conserved in the human JAK1 sequence (*data not shown*). Therefore, the numbering of mutated amino acids was based on the human JAK1 sequence, allowing currently used nomenclature for JAK1 protein mutations in literature to be adopted and to localize our JAK1 mutations in the previously described three-dimensional structure model of human JAK1 protein.[9]

Reverse transcription-quantitative PCR (RT-PCR)

Reverse transcription was performed as described above. Quantitative PCR reactions were performed using primer sets corresponding to murine JAK1 or murine Actin with qPCR™ Mastermix for SYBR Green I (Eurogentec). The sequences of primers (final concentration 300 nM) were: mJAK1 5'-GGAGTGCAGTATCTCTCCTCTCT-3' (forward) and 5'-CCATGCCCAGGCACTCATTTTCA-3' (reverse); mActin 5'-GCTGGAAGGTGGACAGTGAG-3' (forward), 5'-CTCTGGCTCCTAGCACCATGAAG-3' (reverse). To amplify corresponding cDNA, PCR conditions were used as previously described.[17,18] Results were analyzed using MyiQ software. Starting quantity of the transcripts was calculated using standard curves from a cDNA clone.

Plasmid construction, stable DNA transfections and analysis of transfected cells

All JAK1 and JAK2 mutants were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene) and subcloned into the pMX-GFP (for JAK1) or pMEGIX-GFP (for JAK2) biscistronic retroviral vector upstream of the IRES as previously described.[17-19] The mutated JAK1 and JAK2 constructs were verified by sequencing the full-length JAK1 and JAK2 using DYEnamic ET Dye Terminator Kit (Amersham Biosciences). For stable transduction, typically 0.5×10^6 BaF3 cells were infected by retroviruses produced by BOSC packaging cells using a previously described standardized protocol.[20] Cells were subsequently sorted by FACS according to equal levels of GFP.

Western blots

For Western blot analysis, 10^6 BaF3 cells were starved for 4 h and lysed in 250 μL of Laemmli buffer (BioRad). We separated proteins using 12% pre-cast Tris-Glycine gels (Invitrogen) and transferred them to nitrocellulose membranes (Hybond-C, Amersham). Phosphorylation of signaling proteins was investigated using the following phospho-specific antibodies from Cell Signaling Technology: anti-pY1034/1035 JAK1 (#3331S), anti-pY1007/1008 JAK2 (#3771S), anti-pY705 STAT3 (#9131S), anti-pY694 STAT5 (#9351S) and anti-p44/42 MAPK (#9101L). Blots were re-probed with anti-JAK1 (#3332), anti-JAK2 (#24B11) (Cell Signaling Technology, Beverly, MA, USA), anti-STAT5 (Santa Cruz) or anti-β-Actin (Sigma) antibodies, as control. All the antibodies were used at dilutions recommended by the manufacturer.

JAK inhibitors and proliferation assay

To test the effect of JAK inhibitors, 10,000 stably-transduced or JAK1 mutation-acquired autonomous BaF3 cells were seeded in 96-well plates in the presence of JAK inhibitors CMP6 or INCB018424 at different concentrations (10 - 0.001 nM) and DMSO as control. After 48 h, tritiated thymidine was added to the cells for 4 h. Cells were then collected on microfiltered plates, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT, USA).

JAK1 structures

The three-dimensional structural model of the kinase and pseudokinase domain of JAK1 has been previously described[9] and was obtained by employing Deep View software and the Swiss Model server after manual regulation of the best alignment. Structural data from recently solved crystal structure of JAK1 kinase domain in complex with CPM, an ATP-competitive inhibitor (PDB 3EYH),[21] were used to analyze more precisely the impact of the kinase domain-located JAK1 mutations. Molecular graphic images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, USA.[22]

Statistical analysis

Significant differences were determined using InStat software. The mean of 2 groups was compared with Student's t-test. Normality tests were used to test the assumption of a normal distribution. Mean values ± SEM are shown.

Results

De novo *mutations in kinase and pseudokinase domain of JAK1 spontaneously occur during selection of BaF3 autonomous clones*

The transformation of BaF3 phe116 into autonomous tumorigenic clones is a two-step model of tumorigenesis. The first step requires spontaneous upregulation of JAK1, while the mechanism involved in the second step of transformation is not known.[16] As constitutive phosphorylation of JAK1 was detectable in autonomous clones,[14] we systematically sequenced JAK1 from autonomous clones and found that the vast majority of them (139 of 164) were heterozygous for a single *de novo* mutation. Altogether, 25 different missense mutations were identified as listed in the *Online Supplementary Table S1* and *Online Supplementary Figure S1*. Among the 25 different mutations identified, 12 affect residues located in the pseudokinase domain and 13 in the kinase domain (Figure 1A and B). Most of the remaining JAK1 mutation-negative clones have acquired the IL-3 autocrine loop (*data not shown*). An unknown genetic or epigenic event remains to be identified in 3 last autonomous clones that are JAK1 mutation-negative and IL-3 autocrine loop-negative.

To better highlight the localization of mutated residues, we took advantage of the three-dimensional model structure of kinase (JH1) and pseudokinase (JH2) domain of JAK1 previously described.[9] As shown in Figure 1B, half of the affected residues (6 of 12 *de novo* mutations) of the pseudokinase domain (F635V, S646F, Y652H and V658I/L/F), including 3 mutations (S646F, Y652H and V658F) that were previously identified in T-ALL patients,[10,11,23] are located within the pocket formed between the JH2 αC helix and an adjacent loop comprising Val658 and Tyr652 residues. These two regions of the JH2 domain were predicted to interact with the activation loop of the kinase domain in the active conformation.[24] The enrichment of this region in activating mutations suggests that this area represents a hotspot for mutations that might share a common mechanism to activate JAK1. Three of the JH1-located mutations (K1026E, Y1035C and S1043I) are also targeting the activation loop of the JH1 domain that is part of the interface between the kinase (JH1) and the pseudokinase (JH2) domain, further sup-

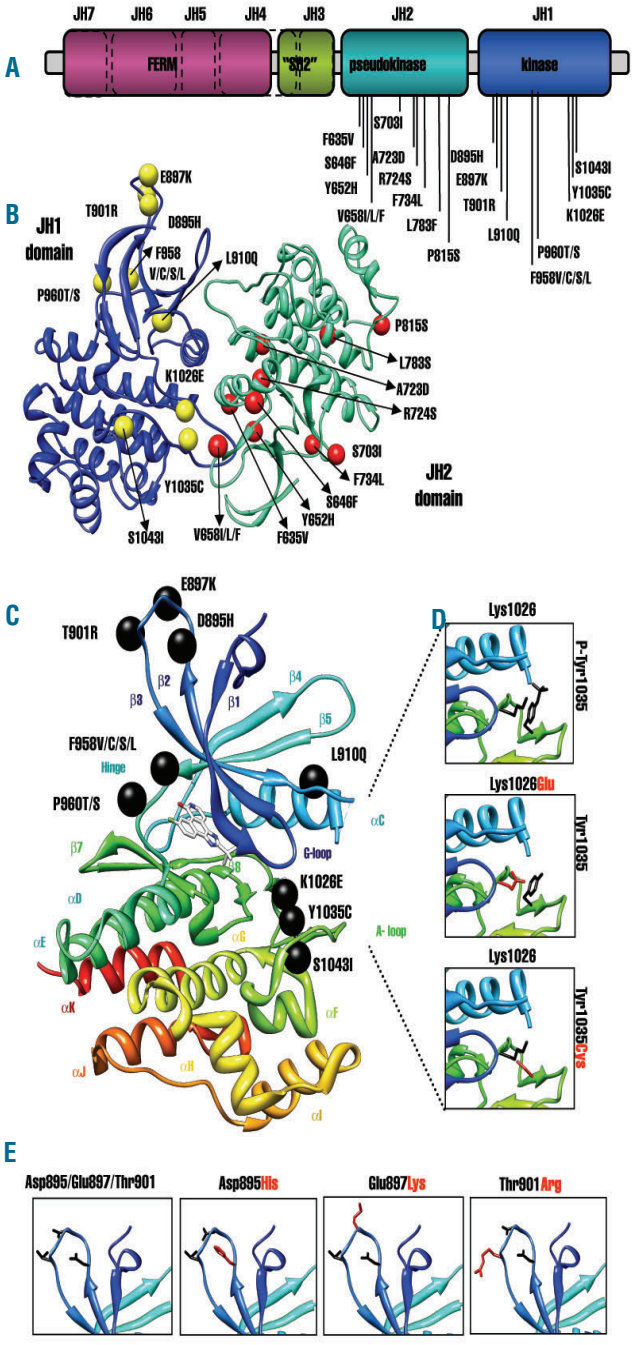

Figure 1. *De novo* mutations in kinase and pseudokinase domains of JAK1 spontaneously occur during selection of BaF3 autonomous clones. (A) Localization of 25 identified missense point mutations affecting 19 residues in the schematic structure of JAK1 with its functional domains and (B) in the three-dimensional modeled structure of kinase and pseudokinase domain published by Flex[9]. Ten affected residues (red balls) in pseudokinase (in green) and 9 (yellow balls) in kinase domain (in blue) are shown. (C) Localization of 9 affected residues (black balls) in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 (PDB 3EYH).[21] The JAK inhibitor is presented in white using a ball-and-stick representation. (D) Magnification of structure of the activation loop showing the side chains of the residues at positions 1026 and 1035 for the wild-type JAK1 (top panel), the K1026E mutant (middle panel), and the Y1035C mutant (lower panel). (E) Magnification of the β2/β3 linking loop highlighting the side chains of the residues at positions 895, 897 and 901 for the wild-type JAK1 (left panel), the Asp895His mutant (left middle panel), the Glu897Lys (right middle panel) and the Thr901Arg mutant (right panel).

porting this hypothesis.

For the mutations located in the JH1 kinase domain, we took advantage of the recently solved crystal structure of the JAK1 JH1 domain to detail their localization.[21] As shown in Figure 1C, D and E, from 13 JH1-located mutations, 3 (K1026E, Y1035C and S1043I) are located in the activation loop (A-loop), 4 mutations affect the same residue (F958V, F958C, F958S, F958L) in the hinge region of the kinase domain at the entry of the ATP-binding pocket, 3 others (D895H, E897K and T901R) are located at the top of the kinase domain in the loop formed between two antiparallel β-strands (β2 and β3) and one mutation affects the loop formed between the β-strand-3 (β3) and the αC helix of the JH1 domain (L910Q). Altogether, beside the mutations targeting the JH1/JH2 interface, the other activating mutations found in this study point to two other hotspots for the regulation of JAK enzymatic activity: the hinge region and in the loop formed between β2- and β3-strand in the kinase domain. Moreover, structural modeling of these hotspots also suggests that each of them might have a distinct molecular mechanism for kinase activation.[25]

JAK1 mutations spontaneously occurring in autonomous BaF3 cells are JAK-STAT pathway activating mutations

We previously showed that progression of BaF3 phe116/9 cells toward cytokine-independence was associated with constitutive activation of JAK1 and STAT5.[14] We, therefore, examined whether all JAK1-mutated autonomous clones (BaF3 Aut) showed a similar signaling profile. We chose one representative JAK1 mutation-positive autonomous BaF3 clone for each of the 25 JAK1 mutations and performed Western blot analysis to assess the activation status of JAK1, STAT5 and ERK1/2. As shown in *Online Supplementary Figure S2A*, none of these proteins were phosphorylated in BaF3 phe116 or IL-9-selected BaF3 phe116/9 in the absence of cytokine. In contrast, all autonomous BaF3 clones showed constitutive activation of STAT5 and, for most of them, constitutive phosphorylation of JAK1 and ERK1/2 was also detected, confirming that all JAK1-mutated autonomous clones harbored constitutive activation of the JAK-STAT and the MAP kinase pathways. As expected, the total level of JAK1 was 2-3 times higher in BaF3 phe116/9 and in autonomous clones as a consequence of the first selection step, which consists of spontaneous JAK1 upregulation.[16] To confirm that the acquisition of mutations in JAK1 is responsible for autonomous BaF3 cell proliferation, we produced mutated JAK1 constructs for the first 13 of the 25 JAK1 mutations that we had found. We transduced parental BaF3 cells with either mutated or wild-type (WT) JAK1. In contrast to cells stably transduced with WT JAK1, which quickly died after IL-3 starvation, all JAK1 mutated constructs were able to promote the autonomous growth of BaF3 cells (*data not shown*). Moreover, similarly to autonomous BaF3 harboring spontaneously acquired mutations, all JAK1 mutants induced the constitutive activation of STAT5 and MAP kinase pathways when ectopically expressed in BaF3 cells (*Online Supplementary Figure S2B*) for 8 representative mutants. As a control for this experiment, we used an autonomous BaF3 cell line obtained by transfection with an oncogenic mutant of M-RAS (Glu71Lys), which shows constitutive activation of the MAP kinase but not the

JAK/STAT pathway (*Online Supplementary Figure S2B*). Altogether, these results confirmed that the JAK1 mutations occurring spontaneously *in vitro* were JAK1/STAT5-activating mutations.

The F958V mutation confers resistance to ATP-competitive JAK inhibitors

JAK inhibitors represent a promising therapy for JAK mutation-positive neoplasms. We used our JAK1 mutation-positive autonomous cell lines to evaluate sensitivity of different JAK1 mutants to treatment with CMP6 inhibitor (JAK inhibitor I) by assessing tyrosine phosphorylation of JAK1 and STAT5, one of the main JAK1 phospho-substrates. As shown in Figure 2A, treatment of BaF3 Aut(V658I) with CMP6 completely blocked phosphorylation of JAK1 and STAT5, while the same treatment did not affect either JAK1 or STAT5 phosphorylation in the BaF3 Aut(F958V) cells. Similar results were obtained with BaF3 stably transduced with the corresponding JAK1 mutants (Figure 2B), confirming that the F958V mutation confers resistance to CMP6. To visualize the Phe958 to Val mutation, we used the crystal structure of the JAK1 kinase domain with CMP6 inhibitor (Figure 2C). The Phe958 is in direct interaction with CMP6 through its main-chain atoms as previously reported in this model (Figure 2C, left panel). The replacement of the Phe958 by Val abolishes this interaction (Figure 2C, right panel).

We further measured proliferation of BaF3 Aut(V658F) and Aut(F958V) in the presence of different concentrations of CMP6 or INCB018424. The BaF3 Aut(F958V) cells were more resistant to the effect of these inhibitors when compared to the Aut(V658F) cells, with a 10-15 times increase in the IC50 (Figure 2D and E). In contrast, no difference was observed in the sensitivity of these cell lines to cytosine arabinoside (ARA-C), a standard chemotherapeutic drug which targets different pathways (*data not shown*). Conversely, JAK inhibitors did not affect MAP kinase-dependent proliferation of control autonomous cells transfected with an activated mutant of M-RAS. In line with these results, treatment of Aut(V658) cells with INCB018424 blocked constitutive phosphorylation of JAK1 and STAT5, while their phosphorylation was preserved in Aut(F958V) cells even after treatment with 10 μM of inhibitor (Figure 2F). Altogether, these results show that the F958V mutation confers resistance to ATP-competitive JAK inhibitors.

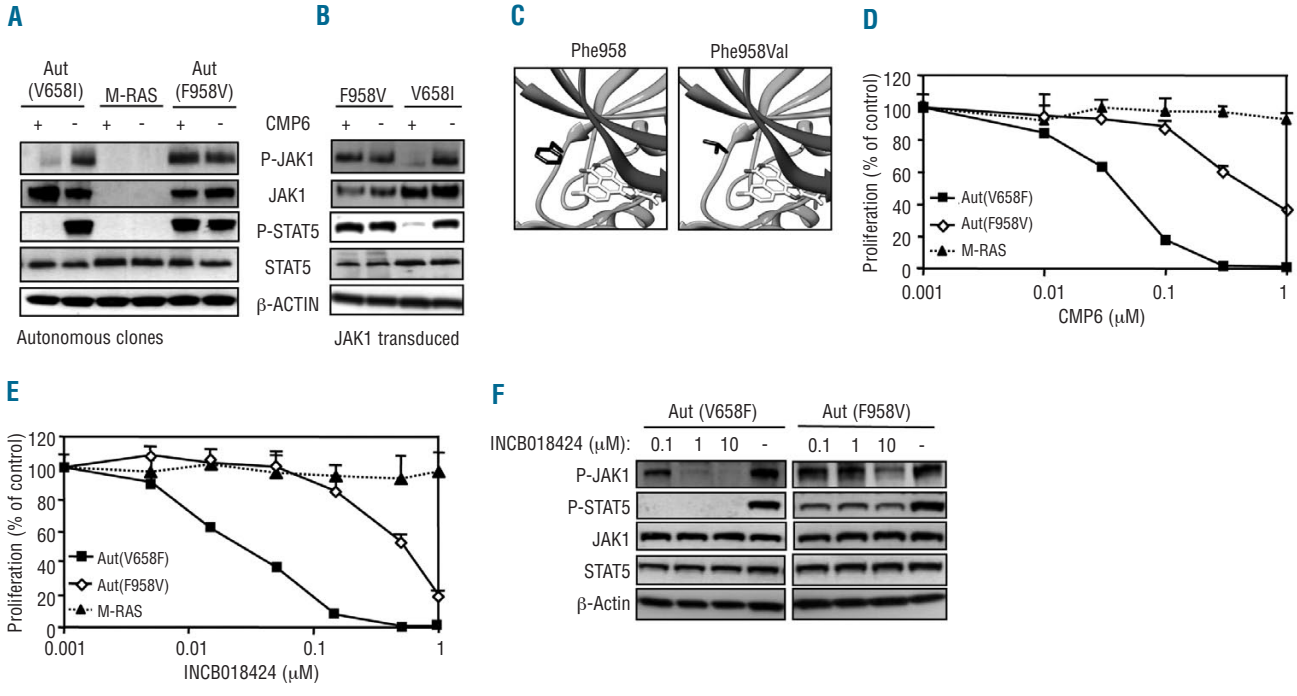

Figure 2. The F958V mutation confers resistance to ATP-competitive JAK inhibitors. (A) 10^6 BaF3 Aut(V658I), Aut(F958V) and BaF3 cells stably transduced with M-RAS or (B) JAK1 V658I and F958V mutants were treated for 30 min with 500 nM CMP6 inhibitor or with 0.1 % DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-p1034/35 JAK1 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments. (C) Magnification of the hinge region and ATP-binding pocket in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 (PDB 3EYH) in ribbon representation[21] showing the side chains of the Phe and Val residue at positions 958 for the wild-type JAK1 (left panel) and the F958V mutant (right panel), respectively. (D and E) 10,000 BaF3 Aut(V658F), Aut(F958V) cells or BaF3 cells stably transduced with activated M-RAS as a control, were seeded in 96-well plates and treated with increasing concentration of (D) CMP6 or (E) INCB018424 JAK inhibitor. After 48 h, tritiated thymidine was added to the cells for 4 h and thymidine incorporation was measured. Results are mean ± SD of triplicate cultures. Similar results were obtained in at least 3 independent experiments with 2 different autonomous clones for each JAK1 mutation. (F) 10^6 BaF3 Aut(V658I), Aut(F958V) were treated for 30 min with 100 nM, 1 μM or 10 μM INCB018424 inhibitor or with DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT5 was detected using specific anti-pY1034/35 JAK1 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK1, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.

Mutations targeting JAK1 Phe958 and Pro960 confer JAK inhibitor resistance

To assess the sensitivity of other JAK1 mutation-positive cell lines to JAK inhibition, we performed a proliferation assay with increasing doses of INCB018424. The concentration of INCB018424 necessary to inhibit 50% of cell proliferation is represented in the *Online Supplementary Figure S3A*. Out of the 25 mutants, 6 showed a significant reduction in their sensitivity to INCB018424 (*Online Supplementary Figure S3A*). The other 19 mutants had the same sensitivity as the V658F (*data not shown*). All the mutants affecting Phe958 conferred BaF3 resistance to the inhibitor, as already shown for the F958V, with a 5 to 15-fold increase in the IC_{50} compared to V658F mutation-positive cells. The mutations of Pro960 to Thr or Ser, affecting a residue close to the Phe958, also reduced sensitivity to INCB018424 with an approximately 2 to 4-fold increase in IC_{50}. Similar results were observed when we tested CMP6 and other ATP-competitive inhibitors (*data not shown*). In line with these observations, all F958- and P960-targeting JAK1 mutations showed reduced inhibition of STAT5 phosphorylation in response to CMP6, while all the other mutations had similar IC_{50} to that of the parental BaF3 cells growing in the presence of IL-3 (*data not shown*).

One of the main mechanisms of secondary resistance in patients treated with tyrosine kinase inhibitors is acquisition of new inhibitor-resistant mutations. The JAK1 V658F mutation is constitutively active[26] and has been described in ALL and AML patients.[10,11] This makes it a good candidate for JAK inhibitor therapy. We decided to investigate whether the acquisition of a secondary JAK inhibitor resistant mutation in the kinase domain would make JAK1 V658F insensitive to JAK inhibition. We, therefore, transduced parental BaF3 cells with the V658F (JAK inhibitor sensitive), F958C (JAK inhibitor resistant) and the V658F/F958C double JAK1 mutants and selected these cells for autonomous proliferation. We then measured the proliferation of these cells with increasing concentrations of INCB018424 and calculated the IC50 (*Online Supplementary Figure S3B*). As expected, BaF3 cells expressing the F958C mutant were less sensitive than BaF3 V658F, while the level of JAK1 mutant expression was identical (*Online Supplementary Figure S3C*). Importantly, cells expressing the double mutants V658F/F958C showed identical sensitivity to the inhibitor as cells expressing F958C mutant alone (*Online Supplementary Figure S3B*). These results show that acquisition of a JAK inhibitor resistant mutation such as the F958C in cells dependent on activated JAK1 for growth is a potential mechanism of secondary resistance to JAK inhibitor therapy.

Long-term culture of F958V-positive BaF3 cells with JAK inhibitor leads to increased expression of F958V JAK1 that correlates with increased resistance to JAK inhibitor

We demonstrated that the F958V JAK1 mutation makes BaF3 cells resistant to JAK inhibitors. Therefore, proliferation of the F958V-positive cells in the presence of the JAK inhibitor CMP6 is not completely abolished and allows further expansion and proliferation of these cells in the presence of the JAK inhibitor CMP6 for days or even weeks (*Online Supplementary Figure S4A*). We tested the sensitivity of the F958V-positive cells to CMP6 before and after long-term culture in the presence of this inhibitor. Cells cultured with CMP6 for two weeks were more resistant to CMP6 than F958V-positive cells which had never been exposed to this inhibitor before (*Online Supplementary Figure S4B*). The JAK1 cDNA from several clones from this long-term culture have been entirely sequenced to rule out secondary acquired mutations. No secondary mutations have been detected in these cell lines. However, this increase in intrinsic resistance correlated with increased expression of JAK1 protein and RNA levels (*Online Supplementary Figure S4C and D*). Similar results were observed using F958C-positive cells (*Online Supplementary Figure S4D*). Altogether these results show that treatment of cells expressing JAK inhibitor-resistant mutants with JAK inhibitor allows an increase in the intrinsic resistance of these cells by increasing the level of mutated JAK1.

JAK2 Y931C, the homologous mutation of JAK1 F958C, confers resistance to JAK inhibitors

Because of its central role in the pathophysiology of MPNs, the JAK2 V617F is an attractive therapeutic target.[8] Currently several JAK2 inhibitors are under development or/and undergoing clinical trials for MPNs.[27] Identification of JAK inhibitor-resistant mutations in JAK2 is, therefore, of great importance. We, therefore, introduced the Y931C, a homologous mutation of JAK1 F958C, into WT or V617F JAK2 protein and selected these cells for autonomous proliferation. All three JAK2 mutants, but not JAK2 WT or control BaF3 cells, were able to proliferate in the absence of IL-3 (Figure 3A). The ability to proliferate autonomously was associated with constitutive JAK2 and STAT5 activation in these cells (Figure 3B). Proliferation of the autonomous cells was measured in the presence of increasing concentrations of INCB018424 or CMP6 to calculate the IC_{50} (Figure 3C and D). The JAK2 Y931C mutation dramatically reduced sensitivity of BaF3 cells to INCB018424 compared to JAK2 V617F-positive BaF3 cells (Figure 3C). Moreover, cells expressing the V617F/Y931C double JAK2 mutant showed identical sensitivity to the inhibitors as cells expressing Y931C mutant alone, with a 20 to 50-fold increase in the IC50 (INCB018424 and CMP6, respectively) compared to V658F mutation-positive cells. Similar results were observed when we tested other ATP-competitive inhibitors (*data not shown*). The resistance of the JAK2 Y931C positive cells to inhibitor was associated with persistent JAK2-phosphorylation in the presence of JAK inhibitor (Figure 3E). These results show that acquisition of a JAK inhibitor-resistant mutation such as the Y931C in cells dependent on activated JAK2 V617F for growth is a potential mechanism of secondary resistance to JAK inhibitor therapy in MPN patients.

Discussion

The acquisition of growth signal self-sufficiency is one of the hallmarks of cancer.[28] In this paper, we show that a broad spectrum of spontaneous mutations in one of the endogenous JAK1 alleles is the main mechanism that confers to BaF3 cells cytokine-independent growth capability *in vitro* and full tumorigenicity *in vivo* (*Online Supplementary Figure S5*), secondary to the upregulation of the JAK1 transcript. We use this *in vitro* model of spontaneous tu-

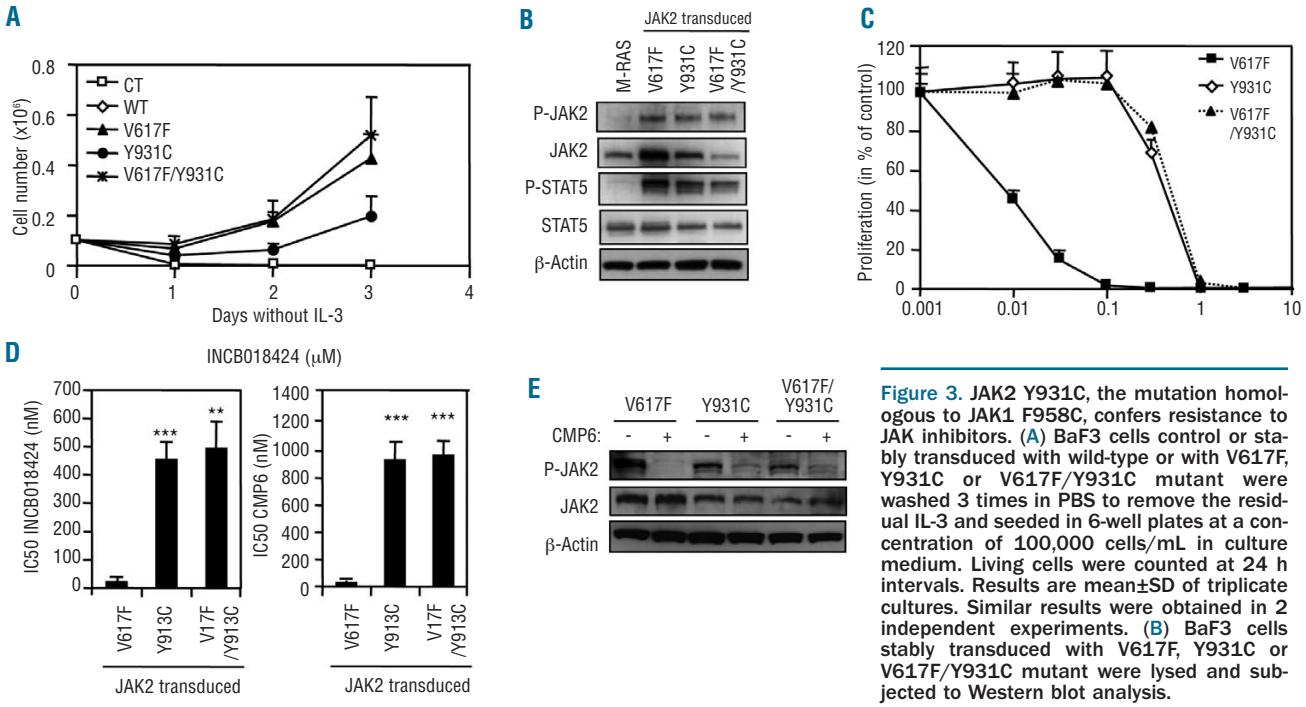

Figure 3. JAK2 Y931C, the mutation homologous to JAK1 F958C, confers resistance to JAK inhibitors. (A) BaF3 cells control or stably transduced with wild-type or with V617F, Y931C or V617F/Y931C mutant were washed 3 times in PBS to remove the residual IL-3 and seeded in 6-well plates at a concentration of 100,000 cells/mL in culture medium. Living cells were counted at 24 h intervals. Results are mean±SD of triplicate cultures. Similar results were obtained in 2 independent experiments. (B) BaF3 cells stably transduced with V617F, Y931C or V617F/Y931C mutant were lysed and subjected to Western blot analysis. Phosphorylation of JAK2 and STAT5 was detected using specific anti-pY1007/8 and anti-pY694 STAT5. Membranes were re-probed with anti-JAK2, anti-STAT5 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments. (C) 10,000 BaF3 cells stably transduced with V617F, Y931C or V617F/Y931C JAK2 mutants were seeded in 96-well plates and treated with increasing concentration of INCB018424 JAK inhibitor. After 48 h, tritiated thymidine was added to the cells for 4 h and thymidine incorporation was measured. Results are mean ± SD of triplicate cultures. Similar results were obtained in at least 3 independent experiments. (D) IC50 for INCB08424 and CMP6 inhibitor calculated based on dose-dependent proliferation curves of BaF3 cells stably transduced by V617F, Y931C and V617F/Y931C double JAK2 mutant. Results are mean ± SEM of at least 3 independent experiments (P<0.01; ***P<0.001). (E) 10^6 BaF3 cells stably transduced with JAK2 V617F, Y931C and V617F/Y931C double JAK2 mutant were treated for 30 min with 300 nM CMP6 inhibitor or with DMSO as a control (- condition), lysed and subjected to Western blot analysis. Phosphorylation of JAK2 was detected using specific anti-pY1007/8 JAK2. Membranes were re-probed with anti-JAK2 and anti-β-Actin antibodies as a control. Similar results were obtained in 3 independent experiments.**

morigenesis to generate a library of cell lines with 25 different *de novo* JAK1-activating mutations, 6 of which are targeting two residues, Phe958 and Pro960, located in the hinge region at the periphery of the ATP-binding pocket. The important property of these mutations targeting Phe958 and Pro960 is that they not only make the kinase constitutively active, but also give increased resistance to ATP-competitive inhibitors, including INCB018424, which is currently used in clinical trials for JAK2 V617F-positive MPNs.[12]

The major drawback of tyrosine kinase inhibitor therapy is the development of secondary resistance caused by the acquisition of new mutations, as best exemplified by imatinib-resistant mutations in BCR-ABL positive CML.[13] Multiple BCR-ABL kinase domain mutations conferring imatinib-resistance have been described including the T315I mutation, which targets the gate-keeper Thr315 in ABL kinase. This gatekeeper Thr residue is not conserved in the JAK kinase family, but the Phe958 residue of JAK1 is the homologous residue of Phe317 in ABL kinase, which is close to the gatekeeper residue and was also found to be mutated in imatinib-resistant BCR-ABL positive patients[13,29,30] (*Online Supplementary Figure S6A and S6B*). Along the same lines, in non-small cell lung cancer (NSCLC), acquired resistance to Gefitinib/Erlotinib kinase inhibitors has also been associated with a secondary mutation of the gatekeeper Thr790 residue of the epidermal growth factor receptor (EGFR). The T790M mutation has been shown to activate the wild-type EGFR, acting both as a drug-resistance and as an activating mutation, similar to JAK mutations reported here.[31]

The gatekeeper Thr residue in ABL and EGFR is located at the periphery of the nucleotide-binding site and directly interacts with imatinib, so that mutation into more bulky amino acids such as Met and Ile sterically hinders the binding of imatinib and other ATP-competitive inhibitors. However, at least for the EGFR, increased affinity for ATP appears to be the primary mechanism by which the T790M mutation confers drug resistance, and might also explain its activating property.[32] Based on the crystal structure of the JAK1 kinase domain (PDB 3EYH),[21] replacement of Phe at position 958 by the less voluminous Val, Cys, Ser or Leu residues is predicted to change the orientation of the side chain to the opposite direction (Figure 2B). This rules out the possibility that resistance to inhibitors results from a steric hindrance as proposed for the Thr315 mutations of ABL.[13,33] However, the crystal structures of JAK1 and JAK2 also show that Phe958 or Tyr931, respectively, are in direct contact with the CMP6 inhibitor.[21] The replacement of Phe958 by Val/Cys/Ser/Leu abolishes this

interaction and should decrease the affinity for this compound (Figure 2C). Further structural data are necessary to determine whether a critical interaction also takes place between Phe958 and INCB018424 or other inhibitors used in this study, but the observation that these JAK mutations confer resistance to different ATP-competitive inhibitors and that they are activating mutations is more in line with a mechanism of increased affinity for ATP, as shown for the T970M EGFR mutation.[32] The homologous residues to JAK1 Phe958 are either Phe or Tyr in other kinases such as ABL, SRC, PDGFR, KIT and JAK2, suggesting that an aromatic side chain at this position is required for proper regulation of the kinase activity. The effect of mutations affecting Phe958 in JAK1 and Tyr931 in JAK2, combined with the F317L/I mutations in drug-resistant BCR-ABL patients supports the notion that this residue plays a critical functional role for the ATP access into the ATP-binding pocket.

The clinical relevance of our *in vitro* model is highlighted by the fact that 5 of the 25 activating mutations (S646F, Y652H, V658F, R724H, L783F) have been previously reported in human patients. The 6 drug-resistant mutations have not yet been described in patients, but our observation that Tyr931 mutation confers resistance of JAK2 V617F to several JAK inhibitors, including INCB018424, suggest that these mutations might contribute to the development of drug-resistant clones during treatment of JAK2 V617F-positive MPNs with such inhibitors.

It has been shown in imatinib-resistant CML that drug resistance conferred by mutations does not necessarily correlate with proliferative advantage and increased kinase activity.[34] Other, non-activating mutations or drug-resistance mechanisms, might be acquired by tumor cells. However, as proposed for the T790M mutation in the EGFR,[31] the significant gain-of-function property conferred by the mutations that we describe here may favor their initial presence before drug selection, and rapid selection during tyrosine kinase inhibitor-based therapy. These JAK mutants should, therefore, be considered to be an invaluable tool to evaluate the activity of novel, potentially more potent, ATP-competitive JAK inhibitors for MPNs and other neoplastic processes associated with JAK kinase mutations.

Authorship and Disclosures

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