Activating Janus kinase pseudokinase domain mutations in myeloproliferative and other blood cancers

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Abstract
The discovery of the highly prevalent activating JAK (Janus kinase) 2 V617F mutation in myeloproliferative neoplasms, and of other pseudokinase domain-activating mutations in JAK2, JAK1 and JAK3 in blood cancers, prompted great interest in understanding how pseudokinase domains regulate kinase domains in JAKs. Recent functional and mutagenesis studies identified residues required for the V617F mutation to induce activation. Several X-ray crystal structures of either kinase or pseudokinase domains including the V617F mutant of JAK2 pseudokinase domains are now available, and a picture has emerged whereby the V617F mutation induces a defined conformational change around helix C of JH (JAK homology) 2. Effects of mutations on JAK2 can be extrapolated to JAK1 and TYK2 (tyrosine kinase 2), whereas JAK3 appears to be different. More structural information of the full-length JAK coupled to cytokine receptors might be required in order to define the structural basis of JH1 activation by JH2 mutants and eventually obtain mutant-specific inhibitors.

Introduction: the JAK family and their functions
The human genome codes for four JAKs (Janus kinases, although JAK initially stood for 'just another kinase') [1]. They contain homologous domains, denoted JH (JAK homology) domains, which are, from the C-terminus to the N-terminus: JH1 (kinase domain), JH2 (pseudokinase domain), JH3–(part of) JH4 [SH2 (Src homology 2) domain] and (part of) JH4–JH7 [N-terminal FERM (band 4.1/ezrin/radixin/moesin) domain], that is required for attachment of JAKs to the cytosolic domains of cytokine receptors (Figure 1a) [2–4].

The N-terminal FERM–SH2 fragments of JAKs were shown to chaperone and stabilize receptors at the cell surface [4–6] (Figure 1b). In the absence of ligand, receptor–JAK complexes are inactive. Binding of cytokines to their receptors triggers a conformational change of usually pre-formed dimers [7,8] that are transmitted to the cytosolic domains, which leads to cross-phosphorylation and activation of JAKs that phosphorylate tyrosine residues on the cytosolic domains of receptors. These then recruit signalling proteins that become substrates of JAKs [8–11].

Key words: blood cancer, Janus kinase (JAK), myeloproliferative disorder, pseudokinase domain.
Abbreviations used: APL, acute promyelocytic leukaemia; DHFR, dihydrofolate reductase; Epo, erythropoietin; EpoRe, Epo receptor; FERM, band 4.1/ezrin/radixin/moesin; G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; JAK, Janus kinase; JH, JAK homology; PML, promyelocytic leukaemia; RARα, retinoic acid receptor α; SH2, Src homology 2; STAT, signal transducer and activator of transcription; Tpo, thrombopoietin; TpoRe, Tpo receptor; TYK2, tyrosine kinase 2.

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so that the activation loop residue Tyr$^{1007}$ becomes phosphorylated [23]. Protein fragment complementation using JAK2 fused at the C-terminus to complementing fragments of DHFR (dihydrofolate reductase), showed that in cells transfected with EpoR, Epo treatment for 30 min led to DHFR reconstitution [24]. Thus, upon cytokine addition, the C-terminal ends of JAK2 come into close proximity.

**JAK2 V617F in myeloid cancers**

A major interest was elicited in pseudokinase JAK domains when an activating mutation was discovered in a group of human blood cancers: MPNs (myeloproliferative neoplasms) [25]. The unique acquired somatic JAK2 V617F mutation is associated with $>$98% of polycythaemia vera and $>$50% of essential thrombocythaemia and myelofibrosis patients [25–28].

The V617F mutation leads to constitutive JAK2 signalling, via STAT5, STAT3, ERK (extracellular-signal-regulated kinase) 1/2 and Akt [25]. For activation, a cytokine receptor must be co-expressed [29], and the FERM domain of JAK2 V617F must be intact [30]. The three dimeric myeloid receptors, i.e. EpoR, TpoR and G-CSFR, form complexes with JAK2 V617F, and they signal constitutively.

Saturation mutagenesis at Val$^{617}$ showed that tryptophan, leucine, isoleucine and methionine were also activating mutations. V617Y does not induce activation [31], suggesting that the hydroxy group of tyrosine prevents activation.

V617W showed the strongest activity, followed by V617F and then V617I, V617L or V617M were weaker [31]. However, for obtaining V617W or V617M, three or two nucleotides should be mutated respectively, whereas JAK2 V617F is obtained by one nucleotide change. V617I can be obtained by one base pair change, and it can be detected in rare myelofibrosis patients [32]. The same mutation has been discovered in a family with hereditary thrombocytosis [33]. Weaker than JAK2 V617F, JAK2 V617I signals in association only with TpoR, and not with other receptors, thus explaining the thrombocytosis phenotype [34].
Mechanisms of activation by V617F at the structural level

Several studies have converged on the important role of one phenylalanine residue, Phe595 of helix C, in supporting the activation by V617F. By aligning JH2 with other kinases, by assuming that JH2 also is folded like a kinase domain and by comparing the X-ray crystal structures of those kinase domains, the closest residue in space [<4 Å (1 Å = 0.1 nm)] to Phe617 was shown to be Phe595 [35]. Mutations of Phe595 to several aliphatic residues (F595A, F595I, F595L, F595K) inhibited the constitutive activity of JAK2 V617F, but did not affect its ability to be activated when cells were treated with Epo (for EpoR cells) [35]. Mutations of Phe595 to aromatic residues (F595W or F595Y) supported constitutive V617F activity. Thus an aromatic stacking interaction is necessary for constitutive V617F activity. Another study, utilizing cytokine receptors other than EpoR or TpoR, also detected an important role for Phe595 in JAK2 V617F activity [36].

A major advance was represented by the X-ray crystal structure of the JAK2 JH2 domain with or without V617F that was solved by the Hubbard laboratory in a collaborative effort with the Silvennoinen laboratory [20]. The X-ray crystal structures showed that JH2 bound ATP in a non-conventional manner, that it adopted an active kinase fold and that the V617F mutation rigidified, and extended by one turn, the helix C of JH2, via an aromatic stacking between Phe617, Phe595 and Phe594 (Figure 2), which comes into this aromatic network only in the V617F mutant [20]. Computational approaches that succeeded in microsecond simulations by David Shaw’s group showed that the stability of helix C of JH2 is actually increased by the V617F mutation, and decreased by the F595A mutation [20].

Models for activation

It is not known at the moment how the kinase domains are regulated by JH2 or by JH2 V617F. The F739R mutation in helix F of the C-terminal lobe, which is predicted to disorganize the fold of the C-terminal lobe of JH2 only led to levels of activation that were much lower than those induced by the V617F mutation [20]. These data indicate that the V617F mutation activates JH1, and does not only remove the JH2 inhibition on JH1. JH2 domains might form heteromeric dimers with JH1 domains (in cis or in trans), or with themselves, and this could change between inactive and active states.

Two major theoretical models have been used to explore effects of V617F. One is the Lindauer–Kroemer model [37,38]. It was based on a dimer seen in the X-ray crystal structure of the FGFR1 (fibroblast growth factor receptor 1) kinase [39]. This is an antiparallel symmetric (antisymmetrical) JH1–JH2 interaction in the inactive state (also having JH2 as an inactive kinase) and defined two interfaces, one between helices C of JH2 and JH1 and the other between the Val617-containing loop and residues of JH1. In the active state of JH1, its activation loop was predicted to clash with residues around position 617. Amazingly, this 2001 model proposed that residues 617 and 618 might be mutated in active JAK2 mutants [37]. Molecular dynamics simulations based on this model suggested that Phe595 prevents activation normally, and forms an aromatic stacking interaction with Phe617 in V617F, thus preventing JH1 inhibition by JH2 [40]. However, single Phe595 mutations are not activating [35].

The second is an asymmetric model [41,42], whereby the N-terminal lobe of JH2 binds to the C-terminal lobe of JH1, possibly inhibiting JH1. This model is based on the mechanism of activation described for EGFR (epidermal growth factor receptor) kinase in a dimer where one kinase is the ‘activator’ and the other kinase binding with its N-terminal lobe being the ‘receiver’ [43,44]. The V617F mutation is presumed to alter this arrangement and subsequently to favour a dimer of JH1 kinases that leads to activation. Several interface residues were described between JH2 and JH1 interacting in the initial antiparallel symmetric

Figure 2 | Structural features of the JAK2 V617F α-helix C
In the mutant JAK2 V617F (PDB code 4FVR), the three aromatic residues Phe594, Phe595 and Phe617 are involved in a double T-shaped π–π stacking interaction where Phe595 plays a pivotal role in maintaining the non-covalent interaction leading to a change of conformation of JH2 α-helix C, which is presumed to be responsible for the activation of the kinase domain. The X-ray crystal structure of JH2 V617F displays a more rigid and longer JH2 α-helix C due to the aromatic interplay. In the mutant protein, substitution of Phe595 by a non-aromatic residue produces a kinase, which responds to stimuli in a similar manner to wild-type JAK2.
Figure 3 | The cytokine receptor–JAK2 complex

JAK2 is 1132 amino acids long, whereas the length of type I cytokine cytosolic domains vary between 122 (TpoR) or 236 (EpoR) amino acids. The prediction is that cytokine receptor cytosolic domains form functional units with the appended JAKs.

Kroemer model configuration or between JH2 and JH1 interacting in an intermediate conformation between the symmetric and asymmetric models (JH2 helix C binding to the activation loop of JH1) [42]. On the basis of mutagenesis data, it is thought that V617F-induced activation of JAK2 requires intramolecular effects along the full-length JAK2 [30, 45, 46].

Furthermore, cytosolic tails of cytokine receptors might be crucial for JAK2 V617F activation, both as scaffolds, and by promoting dimerization of JAK2 V617F (or other mutants) in preformed oligomeric receptor complexes [7, 8, 29, 47, 48]. Receptors have been notoriously difficult to crystallize, possibly because cytosolic distal regions might be largely non-structured. It was proposed that juxtamembrane regions are helical up until Box 1 and that they are rigid and their conformation is controlled by the transmembrane helix [8, 10]. Single-particle electron microscopy on an IL-6 (interleukin 6) receptor by the Garcia group in Stanford showed that these regions are rigid and closely packed in the full-length receptor that binds JAKs [49]. Given that JAKs are 4–6-fold larger than the average cytosolic domains of receptors (Figure 3), it is likely that receptors and JAKs form functional units. Thus a structure of the entire receptor–JAK complex would be necessary. Interestingly, a negative regulator, SOCS3 (suppressor of cytokine signalling 3), has been shown to target specific receptor–JAK2 complexes by simultaneously binding the JAK2 kinase domain and the receptor cytosolic domain [50].

A provocative possibility is that the V617F mutation somehow promotes the formation of the regulatory hydrophobic spine, aligning in an active conformation four non-polar residues located on β4 and αC in the N-terminal lobe and the phenylalanine and tyrosine residues of the DFG (Asp-Phe-Gly) and Y/HRD (Tyr/His-Arg-Asp) motifs [51]. During activation, the phenylalanine residue from the activation loop DFG moves into the spine (DFG-in conformation), pushing out the activation loop and allowing its activation. V617F might alter the spine of JH2, and this might promote formation of the hydrophobic spine of JH1, eventually achieving its activation. Further studies are required to verify this hypothesis.

V617F homologous JAK1 and TYK2 mutations

The V617F homologous mutations activate JAK1 (V658F) and TYK2 (V678F), but not JAK3 [52]. A methionine residue is present at the homologous 617 position in JAK3, and methionine is already activating for JAK2 [31], indicating that activating conformations for JAK3 differ from the other JAKs. JAK1 V658F was found in a fraction of acute adult T-ALL (T-lymphoblastoid leukaemia) and in several other leukaemic conditions [53–55].

In a mouse model of APL (acute promyelocytic leukaemia)-M3, expression of the PML (promyelocytic leukaemia)–RARα (retinoic acid receptor α) fusion protein induced leukemia in mice after 8 months. In those leukaemia blasts, one recurrent acquired mutation was JAK1 V658F [56]. Co-expression of PML–RARα and JAK1 V658F led to rapid APL-M3 in mice [56]. Thus acquisition of JAK mutations precisely at the V617F homologous position seems to be a common event in experimental leukaemia.
Other JAK pseudokinase domain mutations

In JAK2, exon 12 mutations around Lys539 in the linker between the SH2 and JH2 domains are strongly activating and associate with 2% of polycythaemia vera cases [57]. A salt bridge between Asp620 and Lys539 is predicted to prevent and associate with 2% of polycythaemia vera cases [57]. A JH2 linker stabilizes the conformation induced by V617F [20]. Interrupting this salt bridge might change the conformation of the Val617 loop in such a way [57,58] that it might somehow induce effects similar to those of bulky residues at Val617 [31]. Recent molecular dynamics simulations indicate that the SH2–JH2 linker stabilizes the conformation induced by V617F [42].

A deletion of I462-REED686 in JAK2 was first shown to be associated with Down’s syndrome B-cell precursor lymphoblastoid leukemia [59]. The arginine residue (Arg685) in the IREED sequence (located in the hinge between the JH2 N- and C-terminal lobes) was then found to be mutated to serine and glycine in paediatric lymphoblastic leukemia [54,60].

Several studies selecting in culture for active JAK2 and JAK1 mutations showed that most of such mutations were in the JH2 and SH2–JH2 linker. Certain active mutations were in JH1, and some were JAK2-inhibitor resistant [45,48,61]. A simple model where mutations in JH2 would only alleviate the inhibitory effect of JH2 on JH1 would imply that also mutations on the JH1 site of the interface should be frequently detected, which was not the case.

Finally, a number of activating mutations have been identified in JAK3 with some being in the helix C of the pseudokinase domain. One mutation (A572V) was associated with megakaryoblastic leukemia [62]. A similar mutation in JAK2 is not activating (A. Dusa, E. Leroy, C. Pecquet, C. Mouton and S.N. Constantinescu, unpublished work), again pointing to differences between JAK2 and other JAKs.

Conclusion

An emerging principle is that, in several pathologies, mutations in the pseudokinase domain of JAKs activate their kinase domain. Whereas the conformational change locally in JH2 induced by the prevalent V617F mutation has been visualized by X-ray crystal structure studies, the mechanism by which mutated JH2 domains activate JH1 domains remains unknown. Structural and biophysical studies are required for understanding how transmembrane cytokine receptors interact with JAKs, and how cytokine binding or mutations in JAKs lead to JH1 kinase domain activation.

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