Published in final edited form as:

Nat Struct Mol Biol. 2014 July ; 21(7): 579–584. doi:10.1038/nsmb.2849.

Molecular basis for pseudokinase-dependent autoinhibition of JAK2 tyrosine kinase

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Abstract

Janus kinase-2 (JAK2) mediates signaling by various cytokines, including erythropoietin and growth hormone. JAK2 possesses tandem pseudokinase and tyrosine kinase domains. Mutations in the pseudokinase domain are causally linked to myeloproliferative neoplasms (MPNs) in humans. The structure of the JAK2 tandem kinase domains is unknown, and therefore the molecular bases for pseudokinase-mediated autoinhibition and pathogenic activation remain obscure. Using unbiased molecular dynamics simulations of protein-protein docking, we produced a structural model for the autoinhibitory interaction between the JAK2 pseudokinase and kinase domains. A striking feature of our model, which is supported by mutagenesis experiments, is that nearly all of the disease mutations map to the domain interface. The simulations indicate that the kinase domain is stabilized in an inactive state by the pseudokinase domain, and they offer a molecular rationale for the hyperactivity of V617F, the predominant JAK2 MPN mutation.

Janus kinases (JAK1–3, TYK2) are protein tyrosine kinases that mediate cytokine signaling¹. JAKs possess an N-terminal FERM (band 4.1, ezrin, radixin, moesin) domain and a Src homology-2 (SH2)-like domain, which are responsible for cytokine-receptor association², and tandem protein kinase domains: a pseudokinase domain and a tyrosine kinase domain. JAKs are activated through cytokine-induced *trans*-phosphorylation, either as heterodimeric receptor-JAK complexes (all JAKs) or as homodimeric receptor-JAK2

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AUTHOR CONTRIBUTIONS

Y.S., Conceiver of project, supervisor of MD simulations, and manuscript author; K.G., biochemical experiments and manuscript author; D.U. and H.H., biochemical experiments and protein production; E.T.K., MD simulations; K.Y., protein docking analysis; O.S., supervisor of biochemical studies and manuscript author; D.E.S., supervisor of MD simulations and manuscript author; S.R.H., Conceiver of project, supervisor of biochemical experiments, and manuscript author.

complexes. Signaling through JAK-STAT (signal transducer and activator of transcription) pathways are essential for cell growth, differentiation, proliferation and survival, particularly in hematopoiesis, as well as for the initial events in innate and adaptive immunity¹.

Mutations in *JAKs* are causally linked to human myeloproliferative neoplasms (MPNs), which are clonal proliferative disorders affecting different myeloid lineages³. The more common MPNs—polycythemia vera, essential thrombocythemia, and primary myelofibrosis—are caused in most cases by mutations in the pseudokinase domain of *JAK2* (refs. 3,4). Mutations in the pseudokinase domain of *JAKs* have also been linked to acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)⁴. All of these pseudokinase domain mutations result in constitutive activity of the tyrosine kinase domain. V617F in the *JAK2* pseudokinase domain is the most commonly identified mutation in MPNs^{5–7}, responsible for ~95% of cases of polycythemia vera, and this mutation also been implicated in non-small-cell lung cancer⁸. These clinical data, as well as biochemical data^{9,10}, implicate the pseudokinase domain as a negative regulatory domain necessary to maintain low basal *JAK2* activity, yet the molecular basis for pseudokinase-mediated autoinhibition remains elusive.

We demonstrated previously that the pseudokinase domain of *JAK2* in fact possesses low catalytic activity, phosphorylating two negative regulatory sites in *JAK2* (ref. 11), and we subsequently determined its crystal structure¹². Numerous crystal structures of the tyrosine kinase domain of *JAK2* have been determined, but attempts to crystallize the tandem kinase domains of *JAK2* have been unsuccessful to date. Here, we used long time-scale molecular dynamics (MD) simulations, guided by biochemical knowledge of the system, to generate a structural model for the autoinhibitory interaction between the pseudokinase and kinase domains of human *JAK2*. Our model, which is supported by extensive mutagenesis data, can rationalize nearly all of the gain-of-function disease mutations in *JAK2*.

RESULTS

Generation of the *JAK2* JH2–JH1 model

To generate a structural model for the autoinhibitory interaction between the *JAK2* pseudokinase domain (JAK homology-2, JH2) and tyrosine kinase domain (JH1), which could then be tested experimentally, we simulated the JH2–JH1 interaction without any presumption of the binding pose, in a manner similar to small molecule-protein binding¹³. We placed atomic structures of JH2 and JH1 in an arbitrary, untethered, and non-contacting pose within a box of explicit solvent molecules (Fig. 1, state 1). From this starting pose, we ran 14 independent MD simulations of 3 μ s each, which, in each case, resulted in a JH2–JH1 configuration in which the two domains were in contact (Fig. 1, state 2). We did not include the 29-residue JH2–JH1 linker at this early stage of the simulations, because initial simulations with the linker resulted in entanglement of JH1 or JH2 with the linker, which could not be easily resolved in the relatively short (3 μ s) simulation time. We knew from mutagenesis data in the literature, and the lack of sequence conservation, that the JH2–JH1 linker was not a critical component of autoinhibition. Therefore, for computational efficiency, we chose to omit the linker initially and focus on direct JH2 and JH1 interactions.

Visual inspection revealed that there was a large variation of JH2–JH1 poses from these 14 simulations (Fig. 1, state 2). One of the 14 poses (pose 2) possessed two key features: the JH2–JH1 interface included α -helix C (α C) in JH2, which we and others had identified previously as a structural element in the regulation of JH1 by JH2 (refs. 12,14), and the C-terminus of JH2 and the N-terminus of JH1 could readily be connected by the JH2–JH1 linker. For these reasons, we chose to pursue pose 2. We also subjected these 14 simulations to two empirical protein-docking scoring functions (EMPIRE¹⁵ and OSCAR¹⁶), and pose 2 scored better than the others (Supplementary Fig. 1a). However, because our final model differs substantially from this initial pose (Supplementary Fig. 1b and as described below), and the interaction energy of the linker is likely to be non-negligible, the docking scores were not of fundamental consequence.

In the next phase of modeling, we added the JH2–JH1 linker to JH2–JH1 pose 2 (Fig. 1, state 3) and performed four simulations of the resulting system (residues 536–1131). In these simulations, the addition of the linker caused major movements of JH1 relative to JH2. One simulation of 1.7 μ s (Fig. 1, states 3 and 4) resulted in a root-mean-square deviation (RMSD) of ~ 9 Å for JH2 and JH1 (C α atoms) relative to the starting position. The resultant JH2–JH1 pose from this simulation was appealing because additional interdomain contacts were established, which were between the “backside” (β 7– β 8 loop) of JH2 and the N lobe of JH1 (β 2– β 3 loop) (described in detail below). In addition, a negative regulatory phosphorylation site, Tyr570 (refs. 11,17,18), in the β 2– β 3 loop of JH2, which was phosphorylated from the outset of the simulations, settled into a positively charged pocket in the N lobe of JH1 (described in detail below).

Because of the negative regulatory role of the SH2–JH2 linker¹⁹—in particular Ser523 (refs. 20,21), a JH2 phosphorylation site¹¹—we then added residues 520–535 to the model with Ser523 phosphorylated (Fig. 1, state 5). We simulated JAK2 residues 520–1131, encompassing the SH2–JH2 linker (C-terminal half), JH2, and JH1, for 40 μ s. After several microseconds, a defined interaction between JH2, JH1, and the SH2–JH2 linker was established, which we term the JH2–JH1 autoinhibitory pose (Fig. 1, state 6). (A potential mechanism by which JH2 autoinhibits JH1 in this pose is presented below.) This configuration was highly stable: in the 40 μ s simulation, the RMS fluctuation of C α atoms (in JH2 and JH1) was only 2.6 Å with respect to the average structure. (Structural coordinates for the JAK2 JH2–JH1 model (a representative snapshot from the simulation) and an animation of model generation (Supplementary Video 1) are included in Supplementary Information.)

Description of the model

The most striking feature of our model for the autoinhibitory interaction between JH2 and JH1 of JAK2 is the positioning of nearly all of the mapped disease mutations⁴, and other gain-of-function mutations¹⁹, in or proximal to the interdomain interface (Fig. 2a and Supplementary Fig. 1c). The JH2–JH1 interface can be subdivided into four regions: region 1, the β 2– β 3 loop of JH2 and the β sheet in the N lobe of JH1 (Fig. 2b); region 2, β 7– β 8 of JH2 and the β 2– β 3 loop of JH1 (Fig. 2c); region 3, the end of α C in JH2 and the kinase hinge region of JH1 (Fig. 2d); and region 4, the SH2–JH2 linker, α -helix C (α C) of JH2 and

α D of JH1 (Fig. 2e). Although residues in the JH2–JH1 linker also interacted with JH2 and JH1 during the simulations, these interactions were generally less stable and will not be enumerated.

In region 1 (Fig. 2b), pTyr570 in the β 2– β 3 loop of JH2 is inserted into the pocket formed by the curved β sheet in the N lobe of JH1, salt-bridged to Lys883 (β 3), Lys926 (β 5), and Arg922 (β 4– β 5 loop). In region 2 (Fig. 2c), the simulations showed a stable salt bridge between two residues, Arg683 (β 7) in JH2 and Asp873 (β 2– β 3 loop) in JH1; mutation of each residue (R683S, D873N) has been linked to acute lymphoblastic leukemia (ALL)⁴. Thr875, also in the β 2– β 3 loop, is the site of another disease mutation (T875N; acute megakaryoblastic leukemia⁴). In addition to Arg683, Lys607 (K607N; acute myeloid leukemia⁴) (α C– β 4 loop) was also observed to salt bridge with Asp873 during the simulation. In region 3 (Fig. 2e), Pro933 in the JH1 hinge region, which links the N and C lobes, formed a small hydrophobic cluster with Met600 and Leu604 (α C and just after) in JH2. Val878 (β 3) and Tyr931 (hinge) in JH1 also contribute to this hydrophobic cluster. P933R was mapped as an activating mutation in ALL²². Finally, in region 4 (Fig. 2e), the SH2–JH2 linker made contacts with α C of JH2 and α D of JH1. In addition to SH2–JH2 linker-mediated contacts between the domains, stable salt bridges were formed between Glu592 (α C, JH2) and Arg947 (α D– α E loop, JH1) and between Arg588 (α C, JH2) and pSer523 (SH2–JH2 linker). Arg947 also interacted with pSer523 during the simulation. Notably, the mutation R588A was shown previously to be partially activating²³.

Experimental validation of the model

To provide experimental validation for the autoinhibitory model of JAK2 JH2–JH1 derived from the MD simulations, we explored charge-reversal mutations in each of the four regions of the JH2–JH1 interface. In region 1 (Fig. 2b), we generated the individual point mutations Y570R (charge reversal of pTyr570; JH2) and K883E (JH1) and the double mutation Y570R K883E in full-length JAK2 and transfected them into COS7 cells. We measured JH1 activation-loop phosphorylation (pTyr1007–1008)—the standard read-out of JAK2 activation—and downstream STAT1 phosphorylation and STAT3-mediated gene transcription. The expectation was that the single point mutants would be partially activated, because of destabilization of the JH2–JH1 interaction, but that the activation state of the double mutant would be suppressed, due to formation of the “reverse” salt bridge and restoration of the autoinhibited state. Indeed, both Y570R and K883E were activated by a factor of $\sim$ 4 relative to wild-type JAK2, and, strikingly, the activation state of the double mutant was similar to wild type, i.e., suppressed (Fig. 3a and Supplementary Fig. 2a), consistent with reverse salt-bridge formation.

In region 2 (Fig. 2c), we probed the interaction between Arg683 (JH2) and Asp873 (JH1) (both ALL mutations). We first generated the charge-reversal mutants R683E, D873R, and R683E D873R. Although R683E was activated by a factor of $\sim$ 20 (Fig. 3b and Supplementary Fig. 2a), D873R was not activated, and testing of D873R in the context of JH1 alone revealed that this mutation (and also D873K) compromised JH1 activation-loop phosphorylation (Supplementary Fig. 2b), even though Asp873 is at a considerable distance from the JH1 active site (Fig. 2a). We then tested the actual disease mutant, D873N, and the

double mutant R683E D873N. D873N was activated by a factor of ~17, whereas activation of the double mutant was substantially reduced compared to the two single mutants (Fig. 3b and Supplementary Fig. 2a). These data argue for a direct interaction between these two residues. It is conceivable that Asn873 interacts more favorably with Glu683 (in R683E D873N) than with Arg683 (in D873N), given that asparagine–glutamate interactions were found empirically to be energetically more favorable than asparagine–arginine interactions²⁴. Consistent with this interpretation, in simulations, the single mutations (R683E, D873N) partially destabilized the JH2–JH1 interaction, while the double mutation (R683E D873N) restored wild-type stability (Supplementary Fig. 3a). Further support of a direct interaction of Arg683 with JH1 (Asp873) comes from a crystal structure of JAK2 JH2 R683S (data not shown), which shows that this disease mutation does not affect the structure (global or local) of JH2. Thus, it is unlikely that substitution of Arg683 destabilizes the JH2–JH1 interaction indirectly through structural perturbation of JH2.

In region 3 (Fig. 2d), we took advantage of a disease (ALL) mutation, P933R, which substitutes a positively charged residue in the hinge region between the JH1 kinase lobes. Residue 603 (Lys603 in human, Gln603 in mouse) in JH2 is opposite Pro933 in our JH2–JH1 model, and we tested whether substitution of a negatively charged residue at 603 could suppress the hyperactivation of P933R, by creating a favorable charge interaction across the interface. For this purpose, we generated mutants (in mouse JAK2) Q603E, P933R, and Q603E P933R. Q603E had activity comparable to wild-type JAK2, which was expected given that this residue is not conserved in mammalian species, P933R was activated by a factor of ~13, and, as predicted from the model, Q603E suppressed the activation of P933R (Q603E P933R) (Fig. 3c and Supplementary Fig. 2a). Because in this case only one of the two single mutants in the putative JH2–JH1 interface was activated, we confirmed that Q603E (JH2) does not cause impairment of JAK2 (e.g., loss of receptor engagement) by verifying that Q603E and Q603E P933R could be activated by erythropoietin (Epo) in γ 2A cells co-transfected with Epo receptor (Supplementary Fig. 2c).

Finally, in region 4 (Fig. 2e), we created charge-reversal mutants E592R (JH2), R947E (JH1), and E592R R947E. R947E was activated by a factor of ~4 (Fig. 3d and Supplementary Fig. 2a). E592R, however, was not activated, which was unexpected because mutation to alanine (E592A) was shown previously to be partially activating²³. MD simulations of E592R could rationalize the experimental result: Arg592 can form a salt bridge with pSer523, along with Arg947 (Supplementary Fig. 3b), to stabilize the autoinhibitory state. Importantly, the double mutant (E592R R947E) was not activated (Fig. 3d and Supplementary Fig. 2a), i.e., E592R suppressed the hyperactivation of R947E, consistent with formation of the reverse salt bridge (Arg592–Glu947), which indeed formed and was stable in the simulation of E592R R947E (Supplementary Fig. 3b). As for Q603E above, because E592R (JH2) was not activated on its own (yet suppressed R947E), we confirmed that this mutation does not impair JAK2 by co-expressing E592R and E592R R947E with Epo receptor and stimulating with Epo (Supplementary Fig. 2c).

Thus, based on the JH2–JH1 model, we predicted and confirmed experimentally three novel activating mutations in JAK2—two in JH1 (K883E and R947E) and one in JH2 (Y570R)—each of which could be suppressed with a mutation across the JH2–JH1 interface. We also

predicted and confirmed that a mutation in JH2 (Q603E) could suppress a disease mutation in JH1 (P933R).

Autoinhibitory mechanism

In our model for the interaction between JAK2 JH2 and JH1, the activation loop of JH1 is unencumbered, and the active site is accessible to substrates (Fig. 2a). However, our simulations suggest that the interaction with JH2 leads to a more extended configuration of the JH1 lobes (Supplementary Fig. 4a), which is reminiscent of the effect of the SH2 and SH3 domains on the kinase domain of Abl in the autoinhibited state²⁵. Because substantial lobe movements occur in protein kinases during the phosphoryl-transfer process²⁶, and the JH2 interaction with JH1 in the model involves both lobes of JH1, this interaction should suppress JH1 catalytic activity. In addition, the simulations indicate that binding of JH2 to JH1 destabilizes the catalytically important $\beta 3$ - αC (Lys882–Glu898) salt bridge in JH1 (Fig. 4a) and might facilitate the so-called “DFG flip” in the activation loop^{25,27}. Indeed, the DFG-out, catalytically inactive state was reached in the simulation of JH2–JH1, starting from the DFG-in (active) state (Fig. 4b). Taken together, the simulations suggest that the interaction of JH2 with JH1 stabilizes an inactive state of JH1 (Fig. 5).

While phosphorylation of Ser523 and Tyr570 are posited to fortify the JH2–JH1 autoinhibitory interaction (Fig. 2b,e), phosphorylation of the JH1 activation loop (Tyr1007–1008), which stabilizes the active state, conversely might destabilize the JH2–JH1 interaction. This concept is supported by MD simulations of JH2–JH1, in which phosphorylation of the JH1 activation loop leads to a higher JH1 RMSD than when the activation loop is unphosphorylated (Supplementary Fig. 4b). Presumably, a high degree of conformational freedom for JH1 is necessary for phosphorylation of other sites in JAKs (e.g., Tyr813 in JAK2), the cytokine receptor, and recruited STAT proteins.

Mechanism of V617F activation

Val617, the site of the predominant MPN-causing mutation, V617F^{5–7}, is not situated directly in the JH2–JH1 interface, but rather is proximal to the SH2–JH2 linker (Fig. 2e), which was shown previously to be important for maintenance of the JAK2 basal state¹⁹. To gain insights as to how this mutation results in constitutive activation of JAK2, we simulated V617F JH2–JH1. Analysis of the simulation trajectories suggest that the bulky phenylalanine at residue 617 destabilizes the position of the SH2–JH2 linker between JH2 and JH1 (Supplementary Fig. 4c), which results in increased conformational heterogeneity of JH1 relative to JH2 (Supplementary Fig. 4d). Accordingly, the catalytically active conformation of αC in JH1 ($\beta 3$ - αC salt bridge; see Fig. 4b) is more stable in V617F than in wild-type JAK2 (Supplementary Fig. 4e). A mutation in αC of JH2, F595A, was shown previously to suppress V617F^{14,28}, and in a simulation of the double mutant V617F F595A, the SH2–JH2 linker position is again stable between JH2 and JH1 (Supplementary Fig. 4c).

Applicability to other JAKs

The proposed autoinhibitory interaction between JH2 and JH1 of JAK2 should be applicable to the other JAKs as well, in particular JAK1, which shares several disease mutations with JAK2, including V658F (V617F in JAK2) and R724S (R683S in JAK2). Indeed, a 12- μ s

simulation of JH2 and JH1 of JAK1 (whose interdomain linker is 14 residues shorter than in JAK2) showed that the key interface interactions are conserved, with most of the known activating mutations in JAK1 clustered in the interface (Supplementary Fig. 5a). During the simulation, salt bridges were established between Arg724 in JH2 (Arg683 in JAK2) and Asp899 and Glu897 (Asp873 and Leu871 in JAK2) in the $\beta 2$ – $\beta 3$ loop of JH1, despite Arg724 being located >9 Å from these acidic residues at the start of the simulation. Although the $\beta 2$ – $\beta 3$ loop in JAK1 JH2 does not contain a known phosphorylation site, Glu609 in the loop is observed in the simulation to interact with Lys888 ($\beta 2$) and Lys911 ($\beta 3$ – αC loop) in the N lobe of JH1 (Supplementary Fig. 5a), similar to the interaction in JAK2 between pTyr570 and Lys883 ($\beta 3$) and Lys926 ($\beta 5$) (Fig. 2b). (Structural coordinates for the JAK1 JH2–JH1 model (a representative snapshot from the simulation) are included in Supplementary Information.)

DISCUSSION

In this study, we used long time-scale MD simulations to generate a molecular model for the autoinhibitory interaction between the pseudokinase domain (JH2) and tyrosine kinase domain (JH1) of JAK2. Our goal in performing the MD simulations was to see whether we could generate a plausible model for the JH2–JH1 interaction, which we could then test experimentally. While the particular MD simulation approach we took, which entailed decisions based on biochemical and structural knowledge of JAK2, is far from a “turnkey” method for *ab initio* modeling of protein-protein interactions, the current work highlights the potential of MD simulations as a powerful tool for structural elucidation of such interactions.

In our model, nearly all of the activating disease mutations are present in the JH2–JH1 interface, thus providing a molecular rationale for oncogenic activation through mutation: destabilization of the JH2–JH1 interaction results in more facile JH1 *trans*-phosphorylation (Fig. 5). Although the MD simulations of JH2–JH1 can provide insights into specific oncogenic mutations, such as D873N or V617F (Supplementary Figs. 3a and 4c–e), they are not able to predict, for example, the relative degree to which a mutation in JAK2 will be activating in cells. Moreover, whether destabilization of the SH2–JH2 linker is the sole mechanism by which V617F is activated will require additional structural and mechanistic studies.

Our JAK2 JH2–JH1 model is fundamentally different from models proposed previously^{23,29,30}, in which only V617F among the many MPN mutations is present in the respective JH2–JH1 interfaces (Supplementary Fig. 5b). In the prevailing model in the field²⁹, JH2 sterically prevents the JH1 activation loop from adopting an active conformation, and the SH2–JH2 linker plays no role in the JH2–JH1 interaction. In our model, JH2 binds to the “backside” of JH1, stabilizing an inactive conformation of JH1, and the SH2–JH2 linker serves as a bridging element between JH2 and JH1. The conformation of the SH2–JH2 linker in our model differs from that in the crystal structure of JAK1 JH2 (ref. 31), but this may be due to the absence of JH1 in the crystallized protein.

After our study was completed, a crystal structure of TYK2 JH2–JH1 was reported³². Our simulations-based models for JAK2 and JAK1 JH2–JH1 are in striking accord with the TYK2 structure. All of the key JH2–JH1 interactions in the JAK2 and JAK1 models are present in the TYK2 structure, in particular, those between the β 7– β 8 loop in JH2 and the β 2– β 3 loop in JH1 (Fig. 2c) and between the end of α C in JH2 and the hinge region in JH1 (Fig. 2d). On average (over the simulation), the JAK2 model is 3.7 Å (RMSD for C α atoms in JH2–JH1) away from the TYK2 crystal structure (PDB code 4OLI), and the JAK1 model is 3.3 Å away.

The JH2-mediated autoinhibitory mechanism described above would serve to limit *trans*-phosphorylation of JAK molecules associated either with heterodimeric receptors juxtaposed through ligand binding or with preformed homodimeric receptors (e.g., Epo receptor) reconfigured by ligand binding. For JAK2, which is the only JAK to associate with preformed homodimeric receptors, phosphorylation of Ser523 (refs. 11,20,21) and Tyr570 (refs. 11,17,18), which is unique to JAK2, provides an additional mechanism of JH2–JH1 stabilization (Figs. 2b,e and 5).

Finally, there is considerable interest in developing V617F-specific inhibitors of JAK2 for treatment of MPNs, which would minimize the toxicities associated with concomitant inhibition of wild-type JAK2 (ref. 33). By providing an understanding of how JH2 and JH1 interact in the basal state, our model should be valuable for the screening and design of small molecules that could fortify this interaction, which could potentially serve as novel therapeutic inhibitors of V617F or other oncogenic JAK2 mutants.

ONLINE METHODS

Molecular dynamics simulations

Simulation systems were set up by placing JH2–JH1 in a cubic simulation box (with periodic boundary conditions) of at least 100 Å per side and approximately 100,000 atoms in total. The system for the simulation of the unbiased association of JH2 and JH1 was 120 Å per side and approximately 165,000 atoms in total. Explicitly represented water molecules were added to fill the system, and Na⁺ and Cl[−] ions were added to maintain physiological salinity (150 mM) and to obtain a neutral total charge for the system. The systems were parameterized using the CHARMM36 force field with TIP3P water^{35–37} and then equilibrated in the NPT ensemble at 1 bar and 310 K for 10 ns. Equilibrium MD simulations were performed on the special-purpose molecular dynamics machine Anton³⁸ in the NVT ensemble at 310 K using the Nose-Hoover thermostat³⁹ with a relaxation time of 1.0 ps and a time step of 2.5 fs. All bond lengths to hydrogen atoms were constrained using a recently developed implementation⁴⁰ of M-SHAKE⁴¹. The Lennard-Jones and the Coulomb interactions in the simulations were calculated using a force-shifted cutoff of 12 Å (ref. 42). The DFG flip in the JH1 activation loop (Fig. 4b) was completed in two separate JH2–JH1 simulations. In the first one, JH1 started in the catalytically active conformation, characterized by an intact Lys882–Glu898 salt bridge, and Asp994 was deprotonated. In the course of this simulation, the Lys882–Glu898 salt bridge was disrupted due to the interaction of JH1 with JH2 (Fig. 4a). From this conformation (disrupted salt bridge), a second simulation was launched with Asp994 protonated, and the DFG flip was completed

in this simulation after approximately 12 μ s. As shown previously⁴³, disruption of the salt bridge and protonation of this aspartic acid promote the DFG flip in a protein kinase.

Transfection and western blot analysis

Mouse JAK2 cDNA was engineered to include a C-terminal HA tag and was inserted into plasmid pcDNA6. Mutations were introduced using the QuikChange site-directed mutagenesis kit (Agilent). The mutants were verified by DNA sequencing. COS7 cells were transiently transfected with 10 μ g of the respective JAK2 cDNA using X-tremeGENE 9 (Roche) according to the manufacturer's instructions. 48 h after transfection, cells were lysed using RIPA buffer in the presence of protease inhibitors. JAK2 was immunoprecipitated from the cleared lysate using 2 μ g of anti-JAK2 antibodies (HR-758, cat. no. sc-278, Santa Cruz) and Protein A/G beads (Santa Cruz) and western-blotted with anti-JAK2 pTyr1007–1008 antibodies (cat. no. 44-426G, Invitrogen) at 1:500 dilution or anti-HA antibodies (HA-7, cat. no. H9658, Sigma) at 1:3,000 dilution. Whole-cell lysates from transfected COS7 cells (~2% input) were western-blotted with anti-pTyr701 STAT1 antibodies (D4A7, cat. no. 7649P, Cell Signaling) at 1:1,000 dilution or anti-STAT1 antibodies (cat. no. 610185, BD Biosciences) at 1:1,000 dilution. Validation for the various antibodies used are available on the respective manufacturer's website. The western-blot signals were detected using the fluorescence-based Odyssey imaging system (LI-COR Biosciences). Original images of blots used in this study can be found in Supplementary Figure 6.

JAK2-deficient γ 2A cells (fibrosarcoma cells) were transfected with the respective JAK2 plasmids, Epo receptor, and STAT5, using Fugene 6 (Promega) according to the manufacturer's instructions. After 12 h, cells were starved in serum-free media followed by stimulation with Epo (200 U/ml, NeoRecormon, Roche) for 30 min. Cells were lysed in buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 mM NaF, 10% (v/v) glycerol, 1% (v/v) Triton-X, and protease inhibitor cocktail), and cleared whole-cell lysates were western-blotted using anti-JAK2 pTyr1007–1008 antibodies (cat. no. 3771, Cell Signaling) at 1:1000 dilution, anti-pSTAT5 antibodies (C11C5, cat. no. 9359, Cell Signaling) at 1:2000, or anti-HA antibodies (cat. no. MMS-101P, Covance) at 1:3000 dilution.

Mouse JAK2 JH1 (residues 825–1132) was cloned in pcDNA3.1 (+) with a C-terminal HA tag. HEK 293T cells were transfected with the respective JAK2 JH1 plasmids using X-tremeGENE 9 (Roche) according to the manufacturer's instructions. 48 h after transfection, cells were lysed using RIPA buffer in the presence of protease inhibitors. JAK2 JH1 was immunoprecipitated from the cleared lysate using 20 μ l of EZview™ Red Anti-HA affinity gel (Sigma) and western-blotted with anti-JAK2 pTyr1007–1008 (Invitrogen) at 1:500 dilution or anti-HA antibodies (Sigma) at 1:3000 dilution.

Luciferase assay

COS7 cells were plated at 2×10^4 cells/well in a 96-well plate 36 h before transfection. Each well was transfected with 50 ng of JAK2 cDNA (wild type or mutant) or empty vector, 50 ng of APRE-luc (Acute phase response element-firefly luciferase reporter for STAT3), and 50 ng of pRG-TK (Renilla luciferase reporter) using X-tremeGENE 9 (Roche), according to

the manufacturer's instructions. 48 h after transfection, the cells were assayed for luciferase activity using the Dual-Glo Luciferase assay kit (Promega), and the luminescence was measured using a Tecan SpectraFluor Plus instrument.

Supplementary Material

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ACKNOWLEDGMENTS

This work was supported in part by the US National Institutes of Health grant R21 AI095808 (S.R.H.), Medical Research Council of Academy of Finland, Sigrid Juselius Foundation, Medical Research Fund of Tampere University Hospital, Finnish Cancer Foundation, and Tampere Tuberculosis Foundation (O.S.). The APRE-RLuc and pRG-TK plasmids were gifts from D. Levy and J. Belasco, respectively. We thank R. Bandaranayake for crystallographic support, A. Philippsen for animation support, and W.T. Miller, M. Mohammadi, and M.P. Eastwood for critical reading of the manuscript.

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Figure 1.

Steps of JAK2 JH2-JH1 model generation. (1) Starting positions of JAK2 JH2 (PDB code 4FVQ¹²), residues 536–810, and JAK2 JH1 (PDB code 3KRR³⁴), residues 840–1131. The center-of-mass distance is 67 Å, with a minimum separation of 26 Å. JH2 is colored orange and JAK2 JH1 is colored cyan, with the activation loop (residues 994–1016) colored red. Structural elements that will converge in the final model of JH2-JH1 (αC (JH2) with αD (JH1) and $\beta 7-\beta 8$ (JH2) with $\beta 2-\beta 3$ (JH1)) are labeled at key steps. (2) JH2-JH1 interaction poses after 14 3- μs MD simulations (different initial random velocities for each simulation),

superimposed on JH2. Pose 2, shown in solid coloring, was used in the subsequent modeling steps. (3) After adding the JH2–JH1 linker in an extended conformation. (4) After simulating JH2–JH1, residues 536–1131, for 1.7 μ s. (5) After adding the SH2–JH2 linker in an extended conformation. (6) After simulating JH2–JH1, residues 520–1131, for 40 μ s. RMSD values of JH1 and JH2 (Ca atoms) relative to the final model (state 6) are given in parenthesis for states 3 and 4.

Figure 2.

Model of JAK2 JH2-JH1 derived from MD simulations. (a) Autoinhibitory pose of JAK2 JH2-JH1. The coloring scheme is the same as in Fig. 1. Residues that cause JAK2 activation upon mutation (to the indicated residues) are shown in sphere representation (side chains) and colored pink (carbon atoms). Phosphorylated Ser523 and Tyr570 are shown in stick representation and colored according to their location. Oxygen atoms are colored red, nitrogen atoms blue, sulfur atoms yellow, and phosphorus atoms black. A red superscript in a residue label indicates the figure part showing a zoom-in of that region. The N-terminus

(residue 520) is labeled 'N', and the C-terminus (residue 1131) is labeled 'C'. The JH2–JH1 interface (the SH2–JH2 and JH2–JH1 linkers excluded) buries 1670 Å² of total surface area. **(b–e)** Regions of the JH2–JH1 interface near pTyr570 **(b)**, near Arg683–Asp873 **(c)**, near the hinge region of JH1 **(d)**, and near the SH2–JH2 linker **(e)**. Select residues are shown in stick representation, some with van der Waals surfaces. Black dashed lines represent salt bridges.

Figure 3.

Experimental validation of the JAK2 JH2–JH1 model. (**a–d**) Left: representative western blots of immunoprecipitated JAK2 from COS7 cells, wild type (WT) or the indicated JAK2 mutant, probed with anti-pTyr1007–1008 (pJAK2) (top) or anti-HA antibodies (bottom). The position of the 150-kDa molecular-weight marker is indicated. Middle: quantification of the pJAK2 signals normalized by JAK2 protein levels and plotted as fold-change relative to wild-type JAK2 (set to 1.0). Average values and standard deviations were derived from three independent experiments (N=3). Right: representative western blots of COS7 whole-cell lysates probed with anti-pTyr701 STAT1 antibodies (pSTAT1) (top) or anti-STAT1

antibodies (STAT1) (bottom) to detect endogenous STAT1 levels. The position of the 100-kDa molecular-weight marker is indicated. Original images of blots used in this study can be found in Supplementary Figure 6.

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Figure 4.

JH2-mediated autoinhibition of JH1 in JAK2. **(a)** Distance in JH1 between Lys882 ($\beta 3$) and Glu898 (αC). The distance is plotted as a function of simulation time for simulations of JAK2 JH2-JH1 or JH1 alone (JH1 activation loop was unphosphorylated for both). To simplify the salt-bridge presentation (to account for both O ϵ 1 and O ϵ 2 of Glu898), the actual distance displayed is between N ζ of Lys882 and C δ of Glu898, and the gray rectangle indicates the salt-bridging distance range. **(b)** DFG-in and -out states of the JH1 activation loop. Left: in the active state of JH1 (PDB code 3KRR³⁴), the Lys882-Glu898 salt bridge is formed, and Asp994 and Phe995 of the DFG motif in the activation loop adopt the DFG-in (active) conformation. Right: during the simulation of JH2-JH1, the Lys882-Glu898 salt bridge is disrupted and the DFG motif more readily adopts a DFG-out (inactive) conformation (shown is a snapshot taken after 12 μ s of the simulation). Coloring is the same as in Fig. 1.

Figure 5.

Model for JAK2 JH2-mediated autoinhibition of JH1. (Not shown are the FERM and SH2 domains of JAK2 and cytokine receptor.) A conformational equilibrium exists between JH1 in the JH2–JH1 autoinhibitory interaction (state I), in which JH1 is held in an inactive state (JH1, red), and configurations in which JH1 is disassociated from JH2 (orange) and is transiently active (state II; JH1, mixed red and green). The N and C lobes of JH2 and JH1 are labeled. Phosphorylated Ser523 and Tyr570 (magenta and mixed white and magenta spheres, respectively) stabilize the autoinhibited state by binding to positively charged residues in JH1 and JH2 (blue patches; see Fig. 2b,e). Ser523 is constitutively phosphorylated²⁰, whereas Tyr570 is sub-stoichiometrically phosphorylated in the basal state, and its phosphorylation level increases upon JAK2 activation¹⁸, which probably serves as a negative feedback mechanism (to stabilize the autoinhibited state). In the basal state (no cytokine), the two JAK2 molecules (only one JH2–JH1 shown) associated with a cytokine-receptor dimer are maintained in positions that limit *trans*-phosphorylation of the JH1 activation loop (Tyr1007–1008). Cytokine binding and receptor rearrangement juxtapose the two JAK2 molecules to facilitate JH1 *trans*-phosphorylation of the activation loop, which activates JAK2 (state III; JH1, green). Activating mutations such as D873N, R683S, or V617F destabilize the autoinhibited state, permitting *trans*-phosphorylation of the JH1 activation loop in the basal state.