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Mechanistic insights into activation and SOCS3-mediated inhibition of myeloproliferative neoplasm-associated JAK2 mutants from biochemical and structural analyses

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Abstract

JAK2 (Janus kinase 2) initiates the intracellular signalling cascade downstream of cell surface receptor activation by cognate haematopoietic cytokines, including erythropoietin and thrombopoietin. The pseudokinase (JH2) domain of JAK2 negatively regulates the catalytic activity of the adjacent tyrosine kinase (JH1) domain and mutations within the pseudokinase domain underlie human myeloproliferative neoplasms, including polycythaemia vera and essential thrombocythosis. To date, the mechanism of JH2-mediated inhibition of JH1 kinase activation as well as the susceptibility of pathological mutant JAK2 to inhibition by the physiological negative regulator, SOCS3, have remained unclear. Here, using recombinant, purified JAK2_{JH1-JH2} proteins, we demonstrate that, when activated, wild-type and myeloproliferative neoplasm-associated mutants of JAK2 exhibit comparable enzymatic activity and inhibition by SOCS3 in *in vitro* kinase assays. Small angle X-ray scattering (SAXS) showed that JAK2_{JH1-JH2} exists in an elongated configuration in solution with no evidence for interaction between JH1 and JH2 domains *in cis*. Collectively, these data are consistent with a model in which JAK2's pseudokinase domain binds and inhibits the activation of the tyrosine kinase domain of a neighbouring JAK2 molecule within a cytokine receptor complex, but does not influence the activity of JAK2 once it has been activated. Our data indicate that, in the absence of the N-terminal FERM domain and thus cytokine receptor association, the wild-type and pathological mutants of JAK2 are enzymatically equivalent and equally susceptible to inhibition by SOCS3.

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AUTHOR CONTRIBUTIONS

Leila Varghese, Nicos Nicola, Olli Silvennoinen, Jeffrey Babon and James Murphy designed the project; Leila Varghese, Nicholas Liao, Samuel Young, Artem Laktyushin, Jeffrey Babon and James Murphy performed experiments; Leila Varghese, Nicholas Liao, Isabelle Lucet, Nicos Nicola, Jeffrey Babon and James Murphy analysed data; Daniela Ungureanu, Henrik Hammaren and Olli Silvennoinen contributed vital reagents and protocols; Leila Varghese, Jeffrey Babon and James Murphy wrote the manuscript with input from all other authors.

INTRODUCTION

Nearly all patients diagnosed with polycythaemia vera, and the majority of those with the related myeloproliferative neoplasms (MPNs), essential thrombocythaemia and primary myelofibrosis, harbour a mutation in the pseudokinase domain of the protein kinase, JAK2 (Janus Kinase 2) [1–6]. The predominant acquired somatic missense mutation translates into the amino acid substitution of valine to phenylalanine at residue 617 and results in constitutive kinase activity of the protein. In contrast to wild-type JAK2, V617F mutant JAK2 initiates signalling in the absence of cytokine and this leads to aberrant proliferation of distinct haematopoietic cell subsets and gives rise to the disease phenotype.

JAK2 is a receptor-associated multi-domain kinase that initiates the intracellular signalling cascade induced by exposure to cytokines such as erythropoietin (EPO), thrombopoietin (TPO) and interleukin-3 (IL-3)-family cytokines. JAK2, like the other JAK family members (JAK1, JAK3, TYK2) consists of four distinct domains [7]. The N-terminal FERM domain binds to the intracellular Box 1 motif on cytokine receptors, such as the EPO and TPO receptors, whilst the C-terminal kinase domain is the catalytic domain responsible for phosphorylating downstream targets, including the STAT transcription factors. Between these two terminal domains is a non-standard SH2-like domain and a pseudokinase domain, which is responsible for ensuring that the catalytic domain is switched “off” in the absence of cytokine exposure. Since the discovery of the V617F JAK2 mutation, over 30 mutations in JAK2 have been identified in patients with MPNs and other proliferative blood disorders. The overwhelming majority of these occur in the pseudokinase domain of JAK2 and the linker region connecting the pseudokinase and SH2-like domains. It is presumed that these mutations disrupt the inhibitory effects of the pseudokinase domain but our understanding of how this, and the process of JAK2 activation itself, occurs mechanistically is largely speculative and key questions remain unanswered. Biochemical and structural studies of JAK2 have been hampered by the difficulty in producing full-length recombinant protein. Many structural snap-shots of the isolated kinase domains from JAK1, JAK2, JAK3 and TYK2 have been produced since the first X-ray crystal structures were determined [8–10], but only more recently have techniques been developed to produce the pseudokinase domain [11–13] and the pseudokinase-kinase (JH1-JH2) tandem domains [11]. This advancement allows us to visualize how the V617F mutation alters the conformation of the pseudokinase domain in isolation [14, 15]. In the absence of structural information on a multi-domain construct, however, it remains unclear how the JAK2 pseudokinase domain interacts with and negatively regulates the activity of the adjacent tyrosine kinase domain and, consequently, how mutations within the pseudokinase domain lead to constitutive activation of the tyrosine kinase domain.

Differential susceptibility or interaction of JAK2 mutants with cellular regulatory proteins is another possible mechanism for the disease pathogenesis. The contribution of SOCS family proteins, physiological regulators of JAK2, has been investigated but with somewhat conflicting results. SOCS proteins regulate JAK activity in a negative feedback loop. Their expression is induced by JAK activation [16] and they then down-regulate the JAK/STAT signalling cascade, either through direct inhibition of JAKs kinase activity [17, 18] or through targeting signalling components for proteasomal degradation [19]. Disruption of this

regulatory system could contribute significantly to the myeloproliferative phenotype and affect the onset and/or the severity of the disease. Epigenetic silencing of SOCS1 and SOCS3 has been detected in patients with myeloproliferative disorders [20–24]. SOCS1 and SOCS3 mRNAs are also reported to be upregulated in patients with V617F-associated myeloproliferative disorders [25, 26], and protein levels similarly increase with the induction of JAK2 V617F over-expression in cell lines [27]. This latter study found that SOCS1 and SOCS3 can inhibit V617F JAK2 and reduce the expression levels of mutant JAK2. In contrast, some reports arising from over-expression studies indicate that SOCS3 is unable to inhibit V617F JAK2 [28, 29] due to tyrosine phosphorylation of SOCS3. Whether or not mutant JAK2s can be directly inhibited by SOCS1 or SOCS3 in myeloproliferative disorders is therefore a point of contention.

In this manuscript we use newly developed methods to produce recombinant purified human JAK2 tandem kinase-pseudokinase domain constructs, including a panel of 14 constructs harbouring mutations that had been identified in patients with haematological disorders. We investigate these constructs biochemically and show that, when fully activated, none of the MPN-derived mutant constructs have an increased intrinsic kinase activity compared to wild-type. This suggests that inappropriate activation is the sole mechanism that leads to aberrant downstream signaling and that, once activated, their catalytic activity is indistinguishable to that of the wild-type enzyme. In addition, we show that all of the MPN-derived mutants are inhibited by SOCS3 *in vitro* with similar IC₅₀ values compared to wild-type JAK2_{JH1-JH2}. Finally, we use small angle X-ray scattering to gain insight into the relative orientation of JAK2's kinase and pseudokinase domains, and their interaction with SOCS3 and show that the pseudokinase and kinase domains do not exist in any fixed orientation relative to another, *in vitro*, but are instead connected by a flexible linker. Collectively our data are consistent with a model in which the pseudokinase domain regulates the activity of the kinase domain in *trans* and that the pathological mutations that affect this regulation do so by promoting inappropriate activation of the enzyme rather than altering its intrinsic catalytic activity. Our data indicate that the mechanism that promotes this aberrant activation is not encoded by the pseudokinase and kinase domains alone, as it is only evident in the context of the full-length protein and not in the tandem domain (pseudokinase-kinase) construct studied in the present work.

EXPERIMENTAL PROCEDURES

Recombinant JAK2 cloning and expression

A fragment of human JAK2 (hJAK2) that incorporates both the kinase (JH1) and pseudokinase (JH2) domains (JH1-JH2, residues 513–1132) was cloned as a His₆-tagged protein in pFastBac HTb (Life Technologies). Wild-type hJAK2_{JH1-JH2} and a panel of 14 different mutants were produced by oligonucleotide-directed PCR mutagenesis and all insert sequences were verified by Sanger sequencing (Biomolecular Research Facility, Australian National University or Micromon, Monash University). A composite construct encoding His₆ hJAK2_{JH1-JH2} and N-FLAG tagged hPTP1B was assembled using the MultiBac system, as described previously [30].

Viral stocks encoding His₆-tagged JAK2_{JH1-JH2} constructs were raised in *Spodoptera frugiperda* (Sf21) cells using the Bac-to-Bac baculovirus expression system (Life Technologies) following bacmid preparation, as described [31]. Expressions were performed by infecting 0.5 L Sf21 shaking cultures (density 3–4 × 10⁶ cells/mL) with 10% (v/v) viral supernatant and culturing for 48 hours. Purification of proteins from cell pellets was accomplished using a standardised two-step process: nickel-nitrilotriacetic acid (Ni-NTA) resin then Superdex 200 size-exclusion chromatography (GE Healthcare), essentially as described previously [11, 12, 32]. Proteins were eluted from the size-exclusion column in a buffer comprising 0.5 M NaCl, 20 mM Tris pH 8, 15% (v/v) glycerol and 1 mM TCEP. Fractions containing hJAK2_{JH1-JH2} were concentrated using centrifugal ultrafiltration, analysed by SDS-PAGE and Western blot, aliquoted, snap frozen in liquid N₂ and stored at –80°C until required.

A shorter form of hJAK2_{JH1-JH2} (residues 536–1132) was used in Small Angle X-ray Scattering (SAXS) experiments. In these constructs the linker region between the SH2-like domain and JH2 was excluded. These hJAK2 protein constructs were expressed and purified as described above, with the following modifications: 0.4 μM of the JAK inhibitor CMP-6 (EMD Biosciences) was added to Sf21 protein expression cultures for enhanced protein yield and stability; and following purification with Ni-NTA the His₆ tag was removed by incubation with 1 mg of Tobacco Etch Virus protease overnight at 4°C. Recombinant purified SOCS3 SH2 domain was prepared as described previously, with refolding in complex with the gp130 pY757 peptide [33]. hJAK2_{JH1-JH2}:SOCS3 SH2:gp130 phosphopeptide complex, was prepared by mixing hJAK2_{JH1-JH2} with an excess of SOCS3 SH2:gp130, prior to purification by Superdex-200 size-exclusion chromatography. As a final purification step, both *apo* JAK2_{JH1-JH2} and hJAK2_{JH1-JH2}:SOCS3 complex were subjected to ion-exchange chromatography (MonoQ; GE Healthcare) in a buffer system containing 20 mM Tris pH 8, 15% (v/v) glycerol and 1 mM TCEP over a gradient of 0 to 500 mM NaCl. Protein fractions were concentrated to 5–9 mg/mL by centrifugal ultrafiltration, aliquoted, snap frozen and stored at –80°C until required.

***In vitro* kinase inhibition assay**

hJAK2_{JH1-JH2} constructs were tested for their ability to be inhibited by SOCS3 by *in vitro* kinase assay, essentially as described [34]. For these assays a modified murine SOCS3, lacking the first 21 amino acids and with its PEST motif (residues 129–163) replaced by a 4xGly-Ser linker, and co-expressed and purified with elongins B and C for stability and solubility from *E. coli* (hereafter SOCS3/elonginBC; as described in [35]). Briefly, 4 nM of hJAK2_{JH1-JH2} was incubated in kinase buffer (20 mM Tris pH 8.0, 100 mM NaCl, 100 μM 2-Mercaptoethanol (Sigma), and 4 mM MgCl₂) with 1 mM ATP and 1 μCi γ[³²P]-ATP and used to phosphorylate 0.67mM of Stat5b peptide (residues 693–708;[36]). SOCS3/elonginBC inhibitor was included at 0–1.35 μM. Reactions were carried out at 22°C and terminated at two time points (7.5 and 15 min) to ensure linearity of product formation. The reactions were quenched by spotting onto P81 phosphocellulose paper (Whatman) and washed four times for 15 min in 5% (v/v) phosphoric acid. Following a final acetone wash, the paper was air-dried before phosphorimaging.

Small angle X-ray Scattering (SAXS) data collection and analyses

SAXS data were collected on the SAXS/WAXS beamline at the Australian Synchrotron [37] using the inline size-exclusion chromatography setup, as described previously [30, 38, 39]. Protein samples were injected onto a Superdex 200 5/150 (GE Healthcare) column, pre-equilibrated in 0.5 M NaCl, 20 mM Tris pH 8, 15% (v/v) glycerol and 1 mM TCEP to eliminate any aggregates prior to X-ray scattering data collection, and to ensure proper buffer matching between blanks and protein samples. Protein samples were eluted *via* a 1.5 mm glass capillary at 16°C through which the 1.033 Å X-ray beam was positioned. The beamline was equipped with a 1 M, 170 mm×170 mm Pilatus detector (Dektris) at a distance of 1.6 m from the scattering source, providing a q range of 0.0114 – 0.4 Å⁻¹. Data were collected at 2 s intervals over the course of the elution. 2D scattering data from the chromatography elution peak were radially averaged, normalized to sample transmission and an average of scattering profiles from prior to protein elution from the column was subtracted as background using the ScatterBrain software (Stephen Mudie, Australian Synchrotron). The ATSAS suite of software was used for all subsequent SAXS data analyses [40]. For an initial assessment of data quality, Guinier analysis was performed using PRIMUS [41]. The program GNOM [42] applied an indirect Fourier transform to the scattering profile to calculate the real-space, pairwise intra-atomic distance distribution function $P(r)$ and the maximum dimension of the scattering particle, D_{max} . Rigid body modelling was performed using *BUNCH* [43].

RESULTS

Enzymatic analyses of wild-type and V617F JAK2

JAK2^{V617F} is known to be aberrantly active *in vivo*. Whilst it is clear that this is due to inappropriate activation of the mutant enzyme when bound to homodimeric receptors, even in the absence of cytokines [44], it is also possible that, when fully activated, JAK2^{V617F} may also have a higher catalytic activity than wild-type JAK2. The Michaelis-Menten analyses that could answer such questions have been hampered, to date, by an inability to produce recombinant fragments of JAK2 that include the pseudokinase domain. We recently developed an expression and purification protocol for JAK2 constructs that contain both the pseudokinase (JH2) and kinase (JH1) domains [11] and used this to produce wild-type and mutant (V617F) JAK2_{JH2-JH1} as well as the JAK2_{JH1} domain alone. These constructs were purified to near homogeneity and subjected to Michaelis-Menten analysis using *in vitro* kinase assays. All constructs were incubated with ATP prior to the final step of their purification to ensure they were fully phosphorylated at pY1007/1008 in their activation loop in JH1. Both ATP and substrate concentrations were varied in order for both K_{MATP} and $K_{MSubstrate}$ to be measured. The substrate used was a tyrosine-containing peptide from STAT5b described previously [36]. We repeated the experiments 4–6 times, each time using a different preparation of enzyme, in order to ensure that any potential differences were not due to batch-specific differences in enzymatic activity. As shown in Table 1 (and Supplementary Figure 1) there was no reproducible, marked difference between the K_M values of all three constructs for either ATP or peptide substrate. The other kinetic constant accessible via Michaelis-Menten analyses, k_{cat} , could not be reliably ascertained as the specific activity of individual JAK2_{JH1-JH2} preparations (both wild-type and mutant) varied

significantly. Nevertheless, measurements of k_{cat} for JAK2_{JH1}, JAK2_{JH1-JH2} and V617F JAK2_{JH1-JH2}, were always within three-fold of each other (approximately 2 s^{-1}), suggesting that there is no dramatic difference in the kinetic parameters (data not shown). We conclude that there are no significant differences in the enzymatic activities of activated wild-type and mutant (V617F) JAK2_{JH1-JH2} and that the aberrant activity seen *in vivo* is due purely to inappropriate activation of JAKs.

Biochemical analyses of MPN-derived JAK2 mutants

Given that V617F JAK2_{JH1-JH2} showed no obvious difference in terms of catalytic activity compared to wild-type, we wished to investigate whether our JAK2_{JH1-JH2} constructs were more easily autoactivated. The primary readout for activation of JAK is phosphorylation of the JH1 activation loop tyrosines 1007/1008. Although it is known that full-length V617F JAK2 is aberrantly activated *in vivo*, it was unclear whether this mutation would induce increased autoactivation of our JAK2_{JH1-JH2} constructs, given the absence of upstream FERM and SH2-like domains in the absence of associated receptors. In addition to V617F, there are now over a dozen JAK2 mutants associated with myeloproliferative disease. We therefore extended our studies to include a number of these different mutations. Fourteen different MPN-derived JAK2 mutants were expressed and purified; these mutants incorporated many of the missense, insertion and deletion mutations reported in patients with MPNs and other blood disorders (Figure 1A). These included a selection from the three pseudokinase domain mutational “hotspots”, in exons 12, 14 and 16, as well as three kinase domain mutants (see Table 2). All purified JAK2_{JH1-JH2} constructs were active, indicating they were correctly folded, and displayed similar K_{MATP} and $K_{\text{MSubstrate}}$ values to those described above when tested in *in vitro* assays (data not shown).

In order to determine whether these mutations were sufficient to induce aberrant auto-activation, we examined the phosphorylation of their activation loop tyrosines using a pY1007/1008 specific antibody. As shown in (Figure 1B, upper panel) the 14 JAK2_{JH1-JH2} mutants were autophosphorylated at pY1007/1008 at similar levels to that seen in wild-type JAK2_{JH1-JH2}. To verify that any phosphorylation of the activation loop was due to auto-activation and not a contaminating insect cell kinase, K882R JAK2_{JH1-JH2}, which is known to be catalytically inactive [45] was included as a control. No autophosphorylation of Y1007/1008 in K882R JAK2_{JH1-JH2} could be detected, confirming that the phosphorylation of the activation loop of the other JAK2 constructs occurred via auto-activation (see Figure 1B, lane 5). These results are consistent with Sanz and colleagues’ finding for recombinant purified V617F JAK2_{JH1-JH2} [11] and suggests that the higher level of activation loop phosphorylation in *full-length* mutant JAK2 is due to the requirement that it be scaffolded to a receptor for constitutive *trans* activation [46] or that the high concentrations of recombinant JAKs in our expression system allow phosphorylation *in trans* even in the absence of receptor association.

Another key event in the control of JAK2 activation is the phosphorylation of tyrosine 570, an event thought to be an inhibitory signal [47, 48]. Phosphorylation of Y570 was recently suggested to be an auto-activation event catalysed by the pseudokinase (JH2) domain, which is down-regulated in MPN-derived JAK2 mutants [12, 14]. Using an antibody specific to

pY570, we did not observe any reproducible difference in the level of Y570 phosphorylation for any JAK mutants with the exception of Y570F JAK2_{JH1-JH2} (negative control) and K882R JAK2_{JH1-JH2} (Figure 1B, lower panel). The latter result was unexpected as it suggests that kinase domain activity or an active conformation of the kinase domain is absolutely required for Y570 phosphorylation.

SOCS3 is a potent inhibitor of MPN-derived JAK2_{JH1-JH2} mutants *in vitro*

One outstanding question in the field relates to the potentially ameliorating effect that the action of the physiological inhibitor of JAK2 activity, SOCS3, may have in regulating the onset or severity of the myeloproliferative phenotype induced by the V617F mutation. Some studies have suggested that V617F JAK2 is immune to regulation by SOCS3 [28, 29] whilst others have indicated the contrary [27]. We therefore tested the ability of SOCS3 to inhibit V617F JAK2_{JH1-JH2} *in vitro*, using our previously reported assay system that measures JAK2 phosphorylation of the STAT5b peptide [31, 34]. A complex of SOCS3 with elongins B and C was used as these are the physiological ligands for SOCS3 and improve its solubility and stability *in vitro* [35]. As shown in Figure 2, SOCS3 inhibited all JAK2_{JH1-JH2} mutant proteins with a similar inhibitory concentration for 50% maximal activity (IC₅₀) to wild-type JAK2_{JH1-JH2}. This shows that the MPN-derived mutations do not disrupt the SOCS3 binding site, an idea supported by the fact that our wild-type JAK2_{JH1-JH2} and V617F JAK2_{JH1-JH2} constructs both bound SOCS3 with similar affinity as judged by surface plasmon resonance analysis (Supplementary Figure 1B).

Structural studies of JAK2_{JH1-JH2} uncomplexed (*apo*) and in complex with SOCS3 by small angle X-ray scattering

A number of outstanding questions surround the molecular mechanism by which JAK2's tyrosine kinase (JH1) domain is negatively regulated by the pseudokinase (JH2) domain. To date, two recurrent models have been proposed to describe the binding and inhibition of JH1 by JH2, in which JH2 either binds *in cis* to the adjacent JH1 domain within the same JAK2 molecule (Figure 3A) or to the JH1 domain of another JAK2 molecule *in trans* (Figure 3B), although neither model has been adequately tested experimentally. Similarly, we sought to examine whether the SOCS3 SH2 domain binds JAK2_{JH1-JH2} in a mode comparable to that observed for the isolated JAK2_{JH1} domain [30, 34, 49]. Having prepared recombinant JAK2 comprising the JH2 and JH1 domains and the related SOCS3 SH2 domain complex (Figure 3), we proceeded to characterize the arrangement of domains in solution using small angle X-ray scattering (SAXS) experiments. SAXS data were collected at the Australian Synchrotron using the inline size-exclusion chromatography setup [37], with protein eluted in a buffer comprising 0.5 M NaCl, 20 mM Tris pH 8, 15% glycerol, 0.5 mM TCEP (summary statistics recorded in Table 3; Figure 4A, B). Comparable scatter patterns were obtained from experiments in which ion-exchange chromatography fractions (in 20 mM Tris pH 8, 10% glycerol, 0.5 mM TCEP containing ~0.2 M NaCl) were loaded directly into the capillary (data not shown). As shown in the insets accompanying the scatter profiles in Figures 4A and B, the Guinier plots for each of the *apo* JAK2_{JH1-JH2} and JAK2_{JH1-JH2}:SOCS3 complex SAXS profiles are linear, consistent with protein monodispersity and an absence of aggregation that would confound data analyses. Indirect Fourier transform analyses of these data using GNOM enabled us to generate plots of real-

space interatomic distance distributions ($P(r)$ plots, Figure 4C). As shown in Figure 4C, both the *apo* JAK2_{JH1-JH2} and JAK2_{JH1-JH2}:SOCS3 SH2 domain complex exhibit $P(r)$ distributions representative of elongated, non-spherical particles in solution. Furthermore, these analyses indicated that the maximum dimension of the scattering particle, D_{max} , is longer for the JAK2_{JH1-JH2}:SOCS3 complex (150 Å) than the *apo* JAK2_{JH1-JH2} (135 Å). Based on our previous characterisation of JAK2_{JH1} inhibition by the SOCS3 SH2 domain [30, 34, 49, 50] and the JAK2_{JH1-JH2} inhibition studies described above, we predicted that the SOCS3 SH2 domain would engage the JH1 domain within the JAK2_{JH1-JH2} construct via an analogous, phospho-independent binding mode. To test this hypothesis, we used size exclusion chromatography to examine whether SOCS3 SH2 domain could form a stable complex with dephosphorylated JAK2_{JH1-JH2} as we observed for phosphorylated JAK2_{JH1-JH2}. Indeed dephosphorylation of the JAK2_{JH1} activation loop residues, Y1007 and Y1008, by co-expression of JAK2_{JH1-JH2} with the cognate phosphatase, PTP1B, in insect cells did not impact the capacity of JAK2_{JH1-JH2} to form a stable complex with SOCS3 SH2 domain (Figure 4D and Supplementary Figure 2A). These data are consistent with our earlier interrogation of JAK2_{JH1} engagement by the SOCS3 SH2 domain [30] and suggest that an analogous mode of interaction occurs within the context of the JAK2_{JH1-JH2} tandem domains. Additionally, relative to molecular weight standards, these data illustrate that *apo* JAK2_{JH1-JH2} elutes at a volume consistent with the protein behaving as a monomer, with a small, but measurable, increase in molecular mass evident upon SOCS3 SH2 binding (Figure 4D).

We further analysed our SAXS data to define the orientations of the JH1 and JH2 domains relative to one another in solution. Using the crystal structures of JAK2_{JH1} and JAK2_{JH2} as rigid bodies, we used *BUNCH* [43] to fit the JH1 and JH2 models connected by a flexible bead linker to our experimental SAXS profile (Figure 4E; χ for fit to data=0.38; Supplementary Figure 2B). As anticipated from the $P(r)$ distribution (Figure 4C), the JAK2_{JH1-JH2} model derived from *BUNCH* is elongated, rather than globular. Similarly, we used *BUNCH* to perform rigid body modeling with the JAK2_{JH2} and JAK2_{JH1}:SOCS3 crystal structures as starting models to generate the models shown in Figure 4F (χ for fit to data=0.36; Supplementary Figure 2C). We elected to perform fitting using the JAK2_{JH1}:SOCS3 SH2 domain complex as a single rigid body rather than the component domains, since our biochemical (Figure 2) and size exclusion chromatography (Figure 4D) studies are consistent with SOCS3 inhibition of JH1 in the context of the JAK2_{JH1-JH2} tandem domains occurring *via* the same mode as observed for JAK2_{JH1} alone in earlier work [30, 34]. The resulting model reveals an elongated arrangement of domains (Figure 4F), similar to that observed for *apo* JAK2_{JH1-JH2}, although the longer maximum dimension, D_{max} , observed for the JAK2_{JH1-JH2}:SOCS3 complex relative to *apo* JAK2_{JH1-JH2} can be accounted for by the addition of SOCS3 SH2 domain to the complex. Overall, these data are most consistent with JAK2_{JH1-JH2} existing in an elongated conformation in solution, in which the JAK2_{JH2} and JAK2_{JH1} domains do not directly engage one another.

DISCUSSION

The JAK2 pseudokinase (JH2) domain was initially revealed as an essential negative regulator of the tyrosine kinase activity of the adjacent JH1 domain in 2000 [51], and

coupled with subsequent identification of mutations within the JH2 domain as the molecular basis for haematological malignancies, this has ignited interest in the physiological roles of this domain. Whilst the mechanism by which it exerts its activity upon the tyrosine kinase domain has been the subject of intense speculation over the past decade, biochemical examination of the interaction between the JAK2_{JH1} and JAK2_{JH2} domains has been thwarted by a dearth of methods to produce purified, recombinant constructs that incorporate both of these domains. Methods to produce such material have only recently been developed [11, 12].

We purified a recombinant JAK2_{JH1-JH2} construct and examined its structure using small angle X-ray scattering (SAXS). Strikingly, these experiments showed that there was no detectable interaction between the two adjacent domains when they were part of the same polypeptide chain. This is contrary to the prevailing model in which JH2 binds and inhibits its adjacent JH1 domain *in cis* (Figure 3A). Our SAXS data show that the two domains adopt a “beads-on-a-string”, elongated conformation with no stable contacts with each other. This raises several possibilities, either: (A) the N-terminal FERM and SH2 domains are required to scaffold a direct JAK2_{JH1}:JAK2_{JH2} interaction *in cis*; (B) the interaction between the two domains occurs *in trans*, whereby the JH2 domain of one JAK molecule inhibits the activation of the JH1 domain of a second JAK molecule; or (C) any JAK2_{JH1}:JAK2_{JH2} interaction is lost upon activation of the kinase (JAK2_{JH1}) domain. The latter hypothesis is possible given that the JAK2_{JH1-JH2} constructs we used in our SAXS experiments were fully phosphorylated on Y1007, while analogous analyses of the dephosphorylated protein were precluded by lower solubility. It is even possible that a combination of all three of these possibilities represents the physiological situation. We favour a *trans*-inhibition model in light of our data and that contained within a recent study which indicated that, within the physiological context of signalling by full length JAK proteins associated with the dimeric thrombopoietin or erythropoietin receptors, mutations within the JAK2_{JH2} domain preclude binding and inhibition of the JH1 domains of not only JAK2, but also the other JAK family members, JAK1 and TYK2, bound to a neighbouring receptor chain within a preformed dimeric receptor complex [52].

The fact that we see no evidence for JAK2_{JH1-JH2} tandem domains forming an intermolecular dimer *in vitro* (by size exclusion chromatography or small angle X-ray scattering) does not detract from the above hypothesis as, *in vivo*, individual JAK monomers are brought into close proximity by associating with dimeric (or oligomeric) receptors. This results in a much higher local concentration of JAK pairs than can be obtained by simply concentrating recombinant JAK_{JH1-JH2} constructs *in vitro*. It is notable that if the JAK2_{JH1}:JAK2_{JH2} interaction occurred *in cis*, then it would not be concentration- or receptor-dependent, as the two domains are part of the same polypeptide chain. Indeed, the observation that recombinant JAK2_{JH1-JH2} proteins were purified from insect cells in an activated form, as evidenced by Y1007/1008 phosphorylation (Figure 1B), is consistent with the JAK2_{JH2} domain not binding and inhibiting the adjoining JAK2_{JH1} domain *in cis*, in keeping with the elongated JAK2_{JH1-JH2} structure observed in SAXS experiments.

The V617F mutation in the JAK2 pseudokinase domain is associated with a number of human myeloproliferative disorders, especially polycythemia vera. This mutation causes

JAK2 to become constitutively active, whereas the wild-type enzyme is only activated after cytokine binds to the extracellular domain of the receptor to which JAK is bound. We conceived of two possibilities to best describe the molecular events that lead to this constitutive activation. Either (A) mutant JAK2 is hyper-activated and therefore phosphorylates itself and downstream substrates at an unusually high rate or (B) mutant JAK2 *trans*-phosphorylation is constitutive, while its activity in phosphorylating downstream targets is the same as that of activated wild-type JAK2. These two possibilities can also be described as (A) mutant JAK is aberrantly *active* or (B) mutant JAK is aberrantly *activated*. We investigated the first possibility by investigating the enzymatic activity of wild-type and V617F JAK2_{JH1-JH2}. We ensured that both enzymes were fully activated before commencing these experiments by pre-incubating them with ATP and monitoring the phosphorylation of their activation loop tyrosines (Y1007/1008) by Western blot. We found the K_M and V_{max} of the fully activated mutant (V617F) enzyme to be the same as fully-activated wild-type JAK2_{JH1-JH2}. This analysis was extended to a panel of fourteen different MPN-derived JAK2_{JH1-JH2} mutants and none of them displayed any significant difference in activity compared to wild-type JAK2_{JH1-JH2}. Moreover, wild-type and mutant JAK2_{JH1-JH2} constructs exhibited comparable auto-phosphorylation of the JH1 activation loop residues, Y1007/1008, and the negative regulatory phosphorylation site in JH2, Y570, in Western blot analyses, indicating no intrinsic defects or elevations in the catalytic activities of these JAK2_{JH1-JH2} constructs. These observations are consistent with the fact that the N-terminal FERM and SH2-like domains and receptor association are known to be a necessary prerequisite for the enhanced auto-activation seen for mutant JAK2 [44, 53–55]. The absence of Y570 phosphorylation in the K882R protein was unexpected, since in cells Y570 phosphorylation has been shown to occur in catalytically defective JAK2 mutants expressed in JAK2-deficient cell lines [12, 48] and in recombinant purified JAK2_{JH2} domain [12]. It is thus possible that the ATP bound conformations of both JAK2_{JH1} and JAK2_{JH2} are required for optimal phosphorylation of Y570. We conclude that, although MPN-derived JAK2_{JH1-JH2} mutants are “switched on” (activated) inappropriately, their catalytic activity once they are switched on is normal.

Apart from the pseudokinase domain, the other major regulator of JAK2 activity, *in vivo*, is SOCS3. SOCS3 inhibits the catalytic domain of JAK2 (in addition to JAK1 and TYK2) by binding near the active site and preventing substrate access. This is believed to be independent of the pseudokinase domain although that had never been formally tested. If that is the case then SOCS3 should inhibit mutant JAK2 and act, *in vivo*, to ameliorate the myeloproliferative phenotype. However, there have been conflicting reports as to whether MPN-derived JAK2 mutants are susceptible to inhibition by SOCS3. We sought to investigate this *in vitro* by performing kinase inhibition assays using SOCS3 and our panel of MPN-derived JAK2_{JH1-JH2} mutants. All JAK2_{JH1-JH2} mutants were inhibited by SOCS3 with similar affinity. This indicates that the JAK2_{JH2} domain (and mutations within) do not interfere with SOCS3 binding. This observation is consistent with our SAXS analysis of a SOCS3:JAK2_{JH1-JH2} complex. As observed for the JAK2_{JH1} domain in previous studies [30, 34], SOCS3 formed a stable complex with the JAK2_{JH1-JH2} tandem domains (Figure 4D). This complex is elongated, like the *apo* JAK2_{JH1-JH2}, but exhibits a longer maximum particle dimension (Figure 4C), owing to further extension arising from the addition of the

SOCS3 SH2 domain (Figure 4F). Together these data support a model in which JAK2 binds SOCS3 *via* the JAK2_{JH1} domain exclusively, explaining why the introduction of MPN mutations have no bearing on the capacity of SOCS3 to inhibit JAK2_{JH1} activity within the context of the JAK2_{JH1-JH2} tandem domains (Figure 3C). Our data show that MPN-derived JAK2_{JH1-JH2} mutants have the same *intrinsic* susceptibility to SOCS3 inhibition as wild-type JAK2_{JH1-JH2} however, *in vivo* there may be factors that confound this inhibition. This may include hyperphosphorylation of SOCS3 by mutant JAK2 as suggested in earlier studies [28, 29].

Our recent work suggests that few pseudokinases exhibit kinase activity [32, 56], however JAK2_{JH2} is one of those few that does [12]. It still remains unclear whether the weak enzymatic activity of JAK2's pseudokinase domain is intrinsic to the negative regulatory role it serves. By comparison, the pseudokinase domain of JAK1 does not undergo autophosphorylation ([13]; and DU and OS, unpublished work), suggesting that the negative regulation conferred by the JAK1 pseudokinase domain may arise principally as a consequence of its interaction with the tyrosine kinase (JH1) domain. These questions await the structural characterization of full-length JAK2, and our efforts lie in this direction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY STATEMENT

Unexpectedly, wild-type and myeloproliferative neoplasm-associated mutants of JAK2 pseudokinase (JH2)-kinase (JH1) exhibited comparable catalytic activities and inhibition by SOCS3 *in vitro*. These data were consistent with small angle scattering, in which the JH2 and JH1 domains behaved independently in the absence of receptor association.

Figure 1. Characterization of purified recombinant JAK2_{JH1-JH2} preparations

His-tagged JAK2_{JH1-JH2} domains were expressed in *Sf21* cells and purified by Ni-NTA affinity and size-exclusion chromatography. 0.5 µg of purified protein was analysed by SDS-PAGE and Coomassie stained (A) or transferred to PVDF membranes and treated with antibodies against phosphorylated activation loop Y1007/1008 (B: top panel) or phosphorylated Y570 (B: bottom panel). All JAK2_{JH1-JH2} proteins were N-terminally His₆ tagged, except for the C-terminally tagged wild-type control, WT-His. Western blots are representative of two independent experiments.

Figure 2. JAK2^{JH1-JH2} mutants are inhibited by SOCS3

A. SOCS3 had a comparable inhibitory effect on phosphotransfer to a STAT5b peptide substrate catalysed by purified recombinant wild-type and MPN mutant JAK2^{JH1-JH2} constructs in *in vitro* kinase assays. B. An overlay of the data from panel A illustrates that SOCS3-mediated inhibition of mutant JAK2 proteins is comparable to that toward wild-type JAK2^{JH1-JH2}, with all IC₅₀ values ~100 nM. Data are normalized to no inhibitor controls and averages of two independent experiments are shown. Error bars represent the range.

Figure 3. Models depicting candidate mechanisms of JAK2 pseudokinase (JH2) inhibition of the tyrosine kinase (JH1) domain

Schematic representations of two candidate mechanisms by which the JAK2 JH2 domain inhibits the catalytic activity of JH1 *in cis* (A) within the same JAK2 molecule or *in trans* (B) by binding the JH1 domain of another JAK2 protomer within an unactivated, dimeric class I cytokine receptor complex (such as that of gp130). The four domains of JAK2 are shown schematically: the N-terminal receptor-bound FERM domain, the SH2-like domain of unknown function, the regulatory pseudokinase domain (JH2) and the C-terminal tyrosine kinase domain (JH1).

C. SOCS3 (yellow) binds to JAK2_{JH1} via its SH2 domain to inhibit its kinase activity, and is simultaneously bound to the cytokine receptor, gp130.

Schematic representations of constructs used to test the models shown in panels A–C in SAXS and size exclusion chromatography experiments (Figure 4) are shown on the right of the respective model.

Figure 4. Small angle X-ray scattering analyses of *apo* JAK2_{JH1-JH2} and the JAK2_{JH1-JH2}:SOCS3 complex

Scattering intensity profiles for (A) *apo* JAK2_{JH1-JH2} and (B) JAK2_{JH1-JH2}:SOCS3 complex with inset Guinier plots for data in the range, $q \cdot R_g \leq 1.3$. Linearity of the Guinier plots indicates neither high molecular weight aggregates nor inter-particle interference contribute measurably to scattering. C. Fourier transformation of the scattering intensity yields the pair-wise inter-atomic distance distribution function, $P(r)$, calculated using GNOM. The maximum particle dimension, D_{max} , for *apo* JAK2_{JH1-JH2} is 135 $\text{\AA}$ (grey curve) and 150 $\text{\AA}$ for JAK2_{JH1-JH2}:SOCS3 SH2 domain complex.

D. Superdex-200 size exclusion chromatography analyses of *apo* JAK2_{JH1-JH2}, JAK2_{JH1-JH2}:SOCS3 complex, dephospho-JAK2_{JH1-JH2}:SOCS3 complex and SOCS3 SH2 domain. The elution volumes of molecular weight standards are marked above the chromatograms. *Apo* JAK2_{JH1-JH2} and *apo* dephosphorylated JAK2_{JH1-JH2} elution profiles superimpose (not shown) and as such we have omitted the latter profile for clarity. Peak fractions of JAK2_{JH1-JH2}:SOCS3 and dephospho-JAK2_{JH1-JH2}:SOCS3 were resolved by reducing SDS-PAGE before Coomassie Blue staining or anti-pY1007/1008 Western blot analysis (Supplementary Figure 2A).

E. Rigid-body modelling of *apo* JAK2_{JH1-JH2} domains using BUNCH modelling to fit the experimental scattering data (A). The lower structural cartoon depicts a 90° rotation of the

upper cartoon about the x-axis. The model comprises the crystal structures of JH2 (PDB: 4FVP; *dark blue*) and JH1 (PDB:2B7A; *light blue*) connected by a flexible linker of dummy atoms (*grey*), with relative positions of JH2 and JH1 optimised to fit experimental scattering data. The fit of *BUNCH* model to experimental data is shown in Supplementary Figure 2B.

F. Rigid body modelling of the JAK2_{JH1-JH2}:SOCS3 SH2 domain complex, as described for *apo* JAK2_{JH1-JH2} (E), but using JH2 (PDB: 4FVP; *dark blue*) and JH1:SOCS3 complex (PDB:4GL9; JAK2 JH1, *light blue*; SOCS3 SH2, *yellow*). The fit of *BUNCH* model to experimental data is shown in Supplementary Figure 2C.

Table 1

Kinetic parameters of JAK2 constructs

	K_M^{ATP} (μM) *	$K_M^{Substrate}$ (μM) *
JAK2_{JH1-JH2} (wild-type)	216 $\pm$ 40	620 $\pm$ 230
JAK2_{JH1-JH2} (V617F)	144 $\pm$ 45	402 $\pm$ 92
JAK2_{JH1}	105 $\pm$ 36	390 $\pm$ 111

* error indicates 95% CI. n=4

Table 2

Human JAK2 mutations associated with proliferative blood disorders cloned in this study.

Mutation	cDNA	Exon	Domain	Disease	Reference
FHK537-9>L	c.1611_1616del TCACAA	12	SH2-like:JH2 linker	IE, PV	[57]
K539L	c.1615_1616AA >CT	12	SH2-like:JH2 linker	IE, PV	[57, 58]
K607N	c.1821G>C	14	JH2	AML	[59]
L611S	c.1832T>C	14	JH2	ALL, PV	[60, 61]
V617F	c.1849G>T	14	JH2	PV, ET, MF, Atypical CML	[1, 3-6, 59]
D620E	c.1860C>A	14	JH2	PV	[62, 63]
I682del insMPAP	c.2046_2046C>GCCCCGCCCT	16	JH2	ALL	[64]
I682F	c.2044A>T	16	JH2	Paediatric B-ALL	[65, 66]
R683S	c.2049A>T	16	JH2	Paediatric B-ALL, ALL	[64-67]
R683G	c.2047A>G	16	JH2	Paediatric B-ALL, ALL	[64-67]
R683K	c.2048G>A	16	JH2	ALL	[64]
R867Q	c.2600G>A	20	JH1	Paediatric B-ALL	[65, 66]
D873N	c.2617G>A	20	JH1	Paediatric B-ALL	[65, 66]
P933R	c.2798C>G	21	JH1	Paediatric B-ALL	[65, 66]

IE, idiopathic erythrocytosis; ALL, acute lymphoblastic leukaemia; PV, polycythaemia vera; ET, essential thrombocytosis; MF, myelofibrosis; CML, chronic myelogenous leukaemia; AML, acute myelogenous leukaemia; B-ALL, B-cell acute lymphoblastic leukaemia.

Table 3

Data collection and scattering parameters for small angle X-ray scattering

Data collection parameters		
Instrument	Australian Synchrotron SAXS/WAXS beamline	
Beam geometry	120 μm point source	
Beam wavelength (Å)	1.033	
<i>q</i> range (Å ⁻¹)	0.0114 – 0.4	
Exposure time (s)	2	
Protein concentration (mg/mL) [§]	5–9	
Temperature (°C)	16	

Structural parameters		
	<i>apo</i> JAK2 _{JH1-JH2}	JAK2 _{JH1-JH2} : SOCS3 SH2: gp130 phosphopeptide
<i>I</i> (0) (cm ⁻¹) [from <i>P</i> (<i>r</i>)]	0.02948±0.00017	0.02027±0.00036
<i>R</i> _g (Å) [from <i>P</i> (<i>r</i>)]	38.11±0.35	41.26±0.96
<i>I</i> (0) (cm ⁻¹) [from Guinier]	0.0291±0.00002	0.02010±0.00034
<i>R</i> _g (Å) [from Guinier]	36.4±0.39	39.7±1.03
<i>D</i> _{max} (Å)	135	150

Software	
Primary data reduction	<i>ScatterBrain</i> (Australian Synchrotron)
Data Processing	<i>PRIMUS</i> , <i>GNOM</i>
Rigid body modelling	<i>BUNCH</i>
Computation of model intensities	<i>CRY SOL</i>
3-D graphics representations	<i>MacPyMOL</i>

[§] *via* in-line size exclusion chromatography