Regulation of JAKs: Insights Gleaned from the Functional Protein Domains

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Abstract

Since their identification in the early 1990s, many studies have investigated the function of Janus kinases as well as their regulation. It took about 15 years until a first crystal structure of a Janus kinase domain was described and by today the structures of all four kinase domains have been explored. In this chapter we discuss the effects of the different JAK domains on the activity, trafficking and localisation of JAKs that were reported in mutagenesis studies in the last 20 years of JAK research. We take into consideration the recently solved crystal structures of the kinase domains as well as other structural information. In addition, we reflect on the lessons that the recently identified activating mutations in patients teach us.

Introduction

The family of Janus kinases (JAK) consists of four mammalian members: JAK1, JAK2, JAK3 and TYK2. JAK1, JAK2 and TYK2 are ubiquitously expressed, but expression of JAK3 is confined mainly to cells of the haematopoietic system (Yeh and Pellegrini 1999; Heinrich et al. 2003; Ihle and Kerr 1995). JAK kinases are involved in a variety of biological processes including haematopoiesis and regulation of the immune system. Cytokine receptors bind different JAKs (Heinrich et al. 2003; O'Sullivan et al. 2007; Pestka et al. 2004; Kovanen and Leonard 2004; Hintzen et al. 2008) and the specificity

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of various signalling proteins for phosphotyrosine motifs within this receptor determines the signalling characteristics of the different cytokines.

JAKs are constitutively associated via their FERM domain with the membrane proximal region of the type I and type II hematopoietic cytokine receptors and JAKs are absolutely required for downstream signal transduction. Currently there is no structural information of the cytoplasmic domains of the cytokine receptors and the exact mechanism of binding and activation of the JAKs in the receptor complex are largely theoretical. Ligand binding induces conformational changes in the receptor and allows juxtapositioning and transphosphorylation of the activation loop tyrosines in JAKs resulting in enhancement of catalytic activity. Subsequently, tyrosine residues in the receptors become phosphorylated allowing recruitment of SH2 domain containing signalling proteins such as members of the Signal Transducer and Activator of Transcription (STAT) family transcription factors.

Phosphorylation plays an important role in regulation of JAK activity. As noted, activation of JAKs in response to cytokine stimulation depends on phosphorylation of the activation loop which in all JAKs consists of tandem tyrosine residues. However, JAKs are phosphorylated at multiple sites. JAK2 has been the subject to most thorough phosphor amino acid analysis and approximately 20 tyrosine residues have been identified to be phosphorylated upon cytokine stimulation. Several of these sites have been functionally characterized and in addition to activation loop Y1007/1008, phosphorylation of Y637, Y813, Y868, Y966 and Y972 have been shown to potentiate JAK2 activity, while phosphorylation of Y119, Y221, Y317, Y570 and Y913 regulate JAK2 activity negatively (Argetsinger et al. 2004; Feener et al. 2004; Robertson et al. 2009). Interestingly, in the absence of cytokine stimulation, JAK2 is constitutively phosphorylated on a single residue, S523 which mediates negative regulation of JAK2 activation (Mazurkiewicz-Munoz et al. 2006; Ishida-Takahashi et al. 2006). The precise mechanisms how these phosphorylation events regulate JAK activity is known only for a few residues. Phosphorylation of Y119 in the FERM domain induces dissociation of JAK2 from the Epo receptor, and Y813 binds regulator protein SH2-B and increases JAK2 activity (Funakoshi-Tago et al. 2006; Kurzer et al. 2004).

Due to their critical role in central biological processes such as proliferation, the activity of JAKs needs to be tightly regulated by several mechanisms. Protein tyrosine phosphatases SHP-1 and CD45 are shown to regulate JAK phosphorylation. The family of Suppressor of cytokine signaling (SOCS) proteins plays an important role in negative regulation of JAKs and cytokine signaling. SOCS1-7 and CIS are SH2 domain containing proteins that are transcriptionally induced by cytokine stimulation. SOCS can regulate and control cytokine signaling by different mechanisms (Yoshimura et al. 2007). The hallmark of the family is the C-terminal SOCS-box that possesses Ubiquitin E3 ligase activity, and hyperphosphorylated forms of onco-genic or normal JAKs have been shown to become ubquitinated by SOCS and directed for proteasomal degradation (Kamizono et al. 2001; Ungureanu et al. 2002). SOCS1 and SOCS3 contain also a kinase inhibitory region (KIR) that can inhibit JAK function by serving as a pseudosubstrate (Yasukawa et al. 1999). SOCS proteins can also bind cytokine receptors and compete for SH2 domain binding sites.

Phenotypic analysis of knockout mice of all four JAKs has yielded valuable information for the understanding of their physiological role. These mice show phenotypes that are linked to cytokine signalling deficiencies. JAK1 and JAK2 deficiency is not compatible with life. JAK2 knock-out mice die at day 11 of embryogenesis because of the lack of erythropoiesis (Parganas et al. 1998; Neubauer et al. 1998). JAK1 knock-out mice die perinatally due to motoneuronal defects (Rodig et al. 1998). JAK3 knock-out mice exhibit a SCID (Severe Combined Immuno-Deficiency) phenotype (Nosaka et al. 1995; Park et al. 1995; Thomis et al. 1995). Finally TYK2-deficiency leads to hypersensitivity towards infections due to the absence of pro-inflammatory immune responses (Karaghiosoff et al. 2000; Shimoda et al. 2000).

JAKs are involved in inflammatory and immune disorders in which cytokines play crucial roles (Ghoreschi et al. 2009; Pesu et al. 2008) as well as in cytokine-dependent cancers such as multiple myeloma. JAK3 mutations and deletions lead to severe combined immunodeficiency (SCID) characterised by the absence of circulating T- and NK-cells, normal or increased numbers of nonfunctional B-cells and hypoplasia of lymphoid tissues (Pesu et al. 2008; Macchi et al. 1995). Activating JAK2 fusion proteins (TEL-JAK2, PCM1-JAK2, ETV6-JAK2 and SSBP2-JAK2) evoke lymphoid and myeloid leukemia and MPN-U (Peeters et al. 1997; Lacronique et al. 1997; Reiter et al. 2005; Murati et al. 2005; Bousquet et al. 2005; Adelaide et al. 2006; Griesinger et al. 2005; Poitras et al. 2008; Cirmena et al. 2008). Mutations in the Janus kinase 2 gene were found with high incidence in patients with myeloproliferative neoplasms (MPNs) (JAK2-V617F and a number of point mutations and deletions in exon 12) (James et al. 2005; Kralovics et al. 2005; Levine et al. 2005; Baxter et al. 2005; Zhao et al. 2005), in myeloid leukemia (JAK2-T875N) (Mercher et al. 2006), in acute lymphoblastic leukemia (ALL) (JAK2-L611S) (Kratz et al. 2006), and in acute megakaryoblastic leukemia (AMKL) (JAK2-V617F and JAK2-M535I) (Nishii et al. 2007). These constitutively active JAK2 mutants have been described to activate STAT5 and STAT3, MAP kinases and PI3K/AKT. Activating mutations in JAK1 have also been reported for ALL (Flex et al. 2008; Jeong et al. 2008) and gain of function mutations of JAK3 (A572V, A573V) were found in ALL and AMKL patients (Malinge et al. 2008; Walters et al. 2006). Figure 1 shows a selection of JAK mutations associated with disease (for a more detailed description of more JAK mutations see Pesu et al. 2005; Haan et al. 2010). JAK3 mutations in humans SCID are amino acid changes, a premature stop or frame shift mutations causing altered protein sequence (see also Fig. 1). A point mutation in the pseudokinase domain of TYK2 was reported to impair IL-12 and IFN-mediated signalling and was associated with resistance to collagen-induced arthritis in a murine model (Shaw et al. 2003). Moreover, it has recently been shown that polymorphisms at the TYK2 locus are associated with Systemic Lupus Erythematosus (Sigurdsson et al. 2005).

Sequence similarities between JAK family members initially led to the description of seven JAK homology (JH) domains (Wilks et al. 1991), which only partially match the functional domain structure of JAKs. The JH1 and JH2 domains correspond to the kinase and pseudokinase domain. The JH3 to JH7 regions form a FERM and an SH2 domain (Wilks et al. 1991; Girault et al. 1998).The JAK FERM
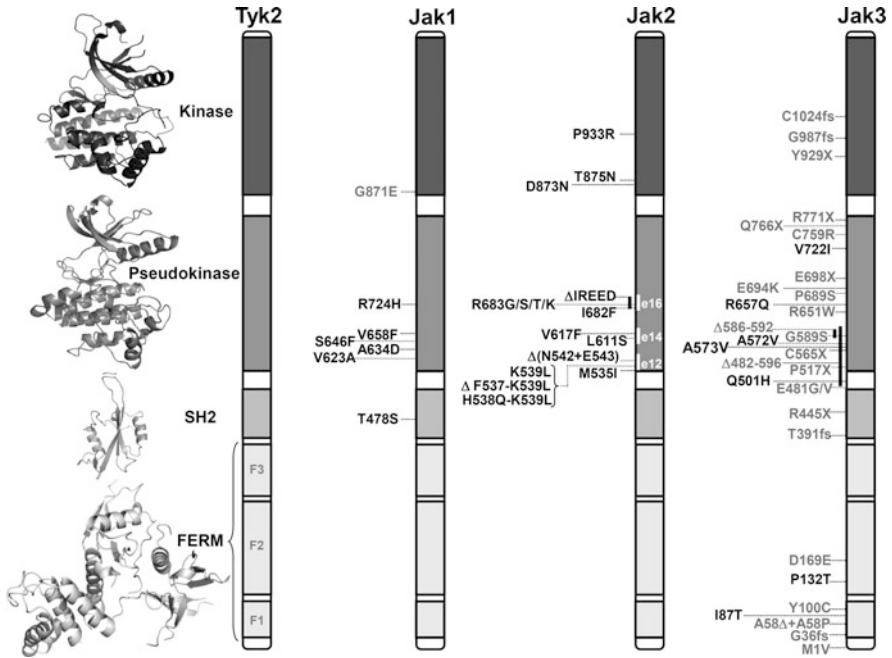
Fig. 1 Domain structure of Janus kinases and of a selection of mutations observed in patients. Model structures of the JAK1-FERM, -SH2, and pseudokinase domain (Haan et al. 2010), the solved crystal structure of the JAK2 kinase domain (PDB entry code: 2B7A) as well as the schematic domain structure for all JAKs are represented. Mutations indicated in black lead to constitutively active JAK proteins (only mutations with validated functions are shown). Mutations in grey represent mutations which lead to a loss of function (in JAK1) or are found in severe combined immunodeficiency (JAK3). Abbreviations used: X = stop codon; fs = frame shift; Δ = deletion; e12/14/16 = exon12/14/16; F1-3 = subdomains of the FERM domain

domain is quite divergent from other FERM domains so that structure prediction is not trivial. The SH2 domain too presents some special features discussed below.

Currently the only structural data available for any of the JAKs are the X-ray structures of the tyrosine kinase domains. While further structural information will be required to obtain complete understanding of the regulation of JAK kinases in physiological and pathogenic signalling, an overall picture of JAK regulation is emerging from various experimental settings. In this review we present biological, biochemical and clinical information about the different functional domains that reveal important information about regulation of JAK kinases.

JAK/Cytokine Receptor Interactions

Crystallographic data on the JAK N-terminal part and of cytokine receptors does not exist, thus the structure/function-relationship between cytokine receptors and Janus kinases still remains elusive as does the exact sequence of events involved in

Janus kinase activation. JAK binding to cytokine receptors is crucial for their function even in the context of constitutively active mutants. The JAK2-V617F mutant is rendered inactive if cytokine receptor binding is abrogated, and concomitantly looses its transforming potential (Lu et al. 2008; Wernig et al. 2008). Activating JAK2 fusion proteins (TEL-JAK2, PCM1-JAK2, ETV6-JAK2 and SSBP2-JAK2) however are constitutively active without cytokine receptor binding, and are activated by oligomerisation of the non-JAK part of the fusion protein, but this is of limited interest for the elucidation of the activation mechanism occurring in full length JAKs.

The FERM Domain

FERM domains are clover-shaped domains comprising three subdomains. The N-terminal subdomain F1 has a ubiquitin-like β-grasp fold. Subdomain F2 has an acyl-CoA-binding-protein-like fold, and subdomain F3 has a PH-domain (pleckstrin homology) fold (Pearson et al. 2000). Structural models of JAK FERM domains (based on structural data of a number of solved FERM domains (Haan et al. 2001, 2008, 2010)) have been used to explore the function of the postulated JAK FERM domain (Girault et al. 1998; Haan et al. 2001, 2008; Hilkens et al. 2001) (reviewed in Haan et al. 2006, 2010). The N-terminal FERM domain in JAKs binds to the membrane-proximal box1/2 region of cytokine receptors (Richter et al. 1998; Zhao et al. 1995; Chen et al. 1997; Cacalano et al. 1999; Kohlhuber et al. 1997). The involvement of rather long sequence stretches within the receptor and JAKs suggests that the interaction is mediated by multiple contacts. A defined JAK orientation on a cytokine receptor ultimately is critical for activation. The receptor-JAK interaction probably induces a restructuring of certain receptor residues into defined interaction interfaces. Such an "induced fit-like" scenario seems necessary to explain the binding of the largely non-structured (according to secondary structure predictions) region of cytokine receptors encompassing the box1 and box2 regions. In receptors such as gp130 or the EpoR this region counts 52 or 61 amino acids and could span a distance of about 19 or 23 nm, respectively. In contrast, the FERM domain of JAKs would at most measure about 6–7 nm across (Fig. 2). Alternatively, a non-structured cytoplasmic tail of a cytokine receptor could adopt a loop structure winding repeatedly through the clefts or along the surface of the FERM domain. Whichever scenario is correct, the involvement of several subdomains (FERM subdomains and SH2 domain) of the JAK and long stretches within the receptor harbours the potential for a very tight and long-lasting interaction. It seems to be a general phenomenon that the mere proximity of JAKs in receptor complexes is not sufficient for their activation and that further conformational changes are required (Constantinescu et al. 2001; Greiser et al. 2002; Watowich et al. 1999; Haan et al. 2002). There is evidence that rigidity of the α-helical transmembrane regions can extend into the intracellular (Constantinescu et al. 2001; Greiser et al. 2002; Zhu and Sizeland 1999) as well as to the extracellular region (Kubatzky et al. 2005). Secondary structure predictions suggest that the
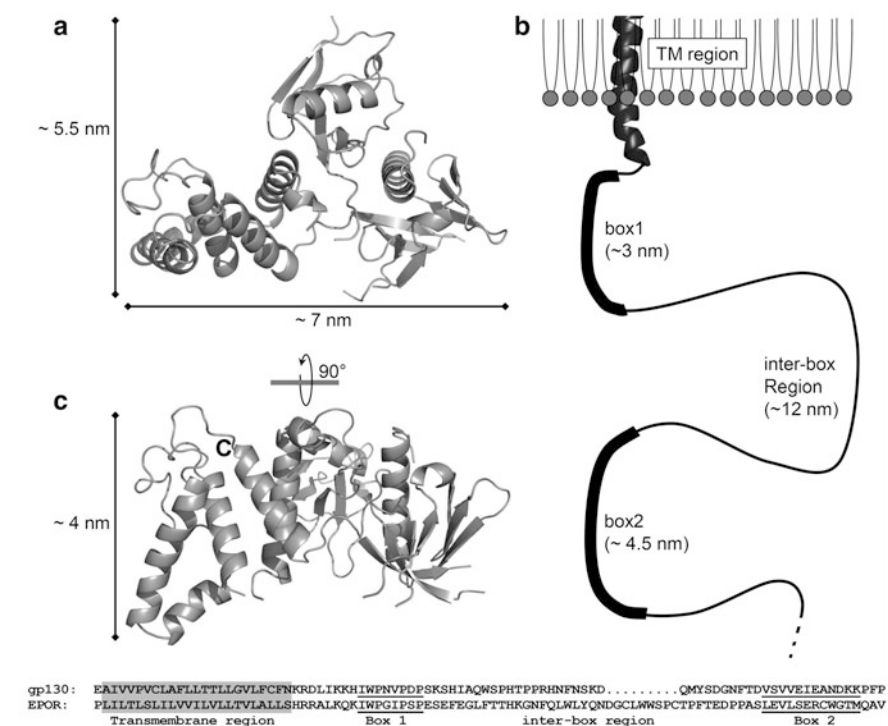
Fig. 2 Model structure of JAK-FERM domain compared to the non structured box region of a cytokine receptor. (**a**) Model structure of the JAK1-FERM domain with indicated dimensions. (**b**) Schematic representation of a non-structured box1–box2 region of the cytokine receptor gp130. The approximate dimensions for a non-structured polypeptide chain are given. (**c**) Alignment of the box1–box2 regions of gp130 and the EpoR

transmembrane α-helix may extend to the beginning of the box1 region (Fig. 2). The proline-rich box1 region might adopt other secondary structures (i.e. polyproline type II helical structure) or the receptor might have a less ordered conformation from there on. Interestingly, mutations or insertions of residues within this putative α-helical region of the cytokine receptor gp130, which did not have an influence on JAK1 binding, were nevertheless crucial for JAK1 phosphorylation and activation (Greiser et al. 2002; Haan et al. 2002). Thus, the role of the membrane proximal region in cytokine receptor signalling is not restricted to mere JAK binding. The W652 mutation in gp130 even behaved dominantly negative, since no signalling occurred when only a single cytoplasmic chain of a gp130 dimer contained the mutation. The corresponding mutation (W258) in the erythropoietin receptor (EpoR) also led to impaired JAK activation and is thought to be part of an α-helically organised region, whose precise orientation is necessary to promote signalling (Constantinescu et al. 2001). Thus, the continuation of the transmembrane helix into the cytoplasm seems to be important for JAK orientation on the cytokine receptor. This of course means that it might be

necessary to include receptor sequences into the research effort aiming at structurally solving the JAK N-terminal domains, which again adds a level of complexity to this unsolved problem. In the same line of evidence, not every peptide mimetic mediating EpoR dimerization led to signal transduction (Livnah et al. 1999). Also, signalling through the gp130 homodimer can be elicited using antibodies against the extracellular part of the cytokine receptor. Interestingly, efficient gp130 activation could only be achieved by two distinct agonistic monoclonal antibodies (Müller-Newen et al. 2000; Autissier et al. 1998) again supporting the notion that the sterical information is transduced through the transmembrane region into the cell to ultimately leads to JAK activation (Remy et al. 1999).

ERM proteins (ezrin, radixin, moesin) bind membranes by binding phospholipids with their FERM domains. Interestingly however, the residues which mediate phospholipid binding in the FERM domain of radixin (Hamada et al. 2000) are not conserved in JAKs. Furthermore mutations in Janus kinases which impair receptor binding lead to a cytoplasmic localisation of JAKs. This indicates that the JAKs are recruited to membranes solely by interaction with cytokine receptors.

The SH2 Domain

The FERM domain is followed by a predicted SH2 domain for which secondary structure prediction analysis of the JAK family members reveals the typical pattern of SH2 domains. The conservation of structural (conserved in all) and functional residues (conserved in only some JAKs) within the JAK SH2 domains shows a discrepancy to all other SH2 domains. The essential functional arginine residue at position βB5, conserved to 99.8% in SH2 sequences, is only conserved to 80% in all JAK SH2 sequences. Interestingly no classical SH2 domain function could be shown to date. Neither the IL6 nor the IFN-γ induced signalling capacity of JAK1 was affected by an SH2 domain inactivating point mutation (Radtke et al. 2005). A similar mutation in human JAK2 also did not interfere with IFN-γ signalling (Kohlhuber et al. 1997). JAK SH2 domain sequences show some additional unconventional features. The absence of a well conserved tryptophan which anchors the *N*-terminal tail at the back of the SH2 domain and directs it away from the phosphotyrosine recognition site, indicates that the domain preceding the SH2 domain, namely the FERM domain, could be positioned aside and not behind the SH2 domain. It was postulated that the SH2 domain may act as a spacer and structurally support and stabilise the FERM domain (Radtke et al. 2005). Recently a role for the SH2 domain has been proposed in the context of JAK2-V617F mutant (Gorantla et al. 2010).

Independently of any SH2 specific phosphotyrosine peptide binding function, truncation mutants and SH2 domain swapping mutants showed that the SH2 domain of JAK1 was structurally important for binding to the OSMR and consequently for efficient OSMR surface expression (Radtke et al. 2005). In contrast, for gp130, EpoR and the interferon-α receptor 1 (IFNαR1), the SH2 domain of JAK1,

JAK2 or TYK2, respectively, were not necessary for receptor binding, although the SH2 domain was required for the upregulation of receptor surface expression of EpoR and IFNαR1 (Hilkens et al. 2001; Ragimbeau et al. 2003; Huang et al. 2001).

Trafficking and Localisation of JAK/Cytokine Receptor Complexes

As mentioned above, the structural integrity of the FERM domain (and in some cases the SH2 domain) is crucial for receptor binding and constitutively active oncogenic JAK mutants require receptor interaction to transform cells. Thus, the trafficking and localisation of the JAKs is dependent on their structural features and is intimately linked to the regulation of JAK activity.

The JAK/Receptor Complex Is Comparable to a Receptor Tyrosine Kinase

The data from JAK/cytokine receptor interaction studies, from trafficking studies (Ragimbeau et al. 2003; Huang et al. 2001; Radtke et al. 2002, 2006; He et al. 2005; Royer et al. 2005;) and localisation studies suggest that JAK1 is recruited to membranes by tight association with cytokine receptors. The fact that JAK binding deficient cytokine receptor mutants or JAK mutants impairing receptor binding lead to a cytoplasmic distribution of JAKs shows that JAKs have no significant intrinsic membrane binding potential. A membrane-bound protein, like JAK1, without a transmembrane domain could conceivably also directly bind to the membrane by lipid modifications (e.g. myristoylation, palmitoylation, farnesylation), by lipid binding domains (e.g. FERM-, PH-, FYFE-domains), through membrane penetrating structures, by electrostatic forces, by binding to other membrane-associated proteins, or by a combination of some of these mechanisms. However, this does not seem to be the case for JAKs. As already mentioned above the residues which mediate phospholipid binding in the FERM domain of radixin (Hamada et al. 2000) are not conserved in JAKs. Also, after cytokine stimulation, JAK1 remained localised at the plasma membrane and did not change its localisation (Behrmann et al. 2004). Interestingly, the half-lives of cytokine receptors and JAKs e.g. gp130 and JAK1 are also identical (Siewert et al. 1999) and this again argues in favour of a "common fate" of the two proteins. FRAP experiments showed that the mobilities for overexpressed gp130-YFP and JAK1-YFP were equal. JAK1-YFP diffuses on the plasma membrane with the velocity of a transmembrane protein indicating that there is no rapid exchange of bleached JAKs from a transient cytoplasmic pool. It was also possible to show that immobilisation of gp130-CFP by a pair of cross-linking monoclonal antibodies also led to the immobilisation of JAK1-YFP (Giese et al. 2003). Thus, JAK molecules do not exchange between different receptors at the plasma membrane and the gp130/JAK1 complex at least can be considered as an un-dissociable entity resembling a receptor tyrosine kinase.

The Kinase/Pseudokinase Connection

One surprising finding of the analysis of the kinome consisting of 518 protein kinases was that appr. 10% of them, namely 48 proteins, contained pseudokinase domains (Manning and Cantley 2002). A protein is designated as a pseudokinase if it lacks one or several of the canonical motifs considered to be required for catalysis. In only five of these proteins a pseudokinase domain and an additional functional kinase domain are present in the same protein polypeptide. These are the four Janus kinases and the serine/threonine kinase GCN2. Recent structural data indicate that pseudokinases with significant sequence degeneration adapt a kinase fold that resembles that of their nearest functional relative (Scheeff et al. 2009). The general fold of the pseudokinase domain of JAKs is expected to follow closely a kinase structure.

The Kinase Domain

The kinase activity is mediated by the C-terminal kinase domain. All protein kinases possess a catalytic domain that comprises approximately 300 amino acids. They share the bilobal kinase fold: The N-terminal lobe is composed of five β-strands and a single α-helix. The C-terminal lobe is predominantly α-helical and contains the regulatory activation loop (A-loop). The sequential similarity of the JAK kinase domains is quite high and the solved structures of the JAKs also show little difference in and around the ATP binding pocket. The published crystal structures of all the JAK1, JAK2, JAK3 and TYK2 kinase domains have proven the existence of an additional helix within the C-lobe of the JAK kinase domain which was termed αH-helix for JAK2 and FG-helix in the case of JAK3 (Lucet et al. 2006; Boggon et al. 2005; Williams et al. 2009) and that has been shown to be crucial for kinase activity (Haan et al. 2009). This special feature in JAKs is lining the substrate binding site of the kinase and lies in close proximity to the catalytic cleft of the enzyme. One family member, JAK3 has some special features compared to the other JAKs. It is the only JAK family member in which an alanine residue directly precedes the DFG-motif (in contrast to a glycine residue in the other JAKs). This subtle difference could directly affect the conformation of the A-loop in the way that was already discussed for the inactive insulin receptor (GDFG-motif) and fibroblast growth factor receptor (ADFG-motif) kinase domains (Hubbard et al. 1998). JAK3 is also the only Janus kinase having a cysteine residue at position C909 in close proximity to the ATP binding pocket. Thus JAK3 would be a potential target for ATP-competitive inhibitors with an electrophilic group (so called irreversible inhibitors) (Haan et al. 2010) which would covalently attach to the mentioned JAK3 cysteine. The toxic potential is hard to evaluate but the amount of possible off-kinase-targets potentially reacting with the electrophile is a risk (Rishton 2003).

The Pseudokinase Domain

The sequence of the JH2 pseudokinase domain is conserved to the same extent as the JH1 domain among different JAKs (appr. 30% identity) and during evolution. However, the genomic organization of JH2 differs from JH1 thus suggesting that the domains have evolved individually. The structure of the JH2 domain for any of the JAKs has not yet been published, but the sequence homology to functional kinases suggests that it follows a similar fold. However, differences in some of the conserved sequence motifs considered to be required for catalytic activity are missing or altered in JH2. Specifically, JAKs lack the third Gly in the Glycine-rich (GxGXXG) ATP binding loop, and in the ATP orienting VAIK motif the Alanine is changed to Val, Leu or Ile. In the in DFG cation binding motif the Phenylalanine is changed to Proline. Most dramatic difference, however, is the lack of the catalytic base Aspartic acid in the subdomain VIb. Collectively these alterations have led to the conclusion that JH2 is catalytically inactive and been assigned as pseudokinase domain (Boudeau et al. 2006; Zeqiraj and van Aalten 2010). Recently, however, the pseudokinase status of several proteins, including CASK, haspin, WNK1, VRK3, HER3/ErbB3, and STRADα, has been changed and the studies have shown that ATP-binding and/or catalytic activity can be achieved through non-canonical mechanisms (Mukherjee et al. 2008; Eswaran et al. 2009; Shi et al. 2010; Zeqiraj et al. 2009; Scheeff et al. 2009). Each of these proteins utilizes a distinct mechanism for nucleotide binding and/or catalysis. Interestingly, HER3, which resembles JAKs in lacking the catalytic base aspartate was found to retain low level kinase activity and be able to phosphorylate its intracellular region in vitro (Shi et al. 2010). The crystal structure of HER3 showed that it assumes an atypical conformation for active kinases, particularly in αC helix and activation segment (Shi et al. 2010; Jura et al. 2009). It will be important to evaluate whether the JAK JH2 also possess catalytic activity.

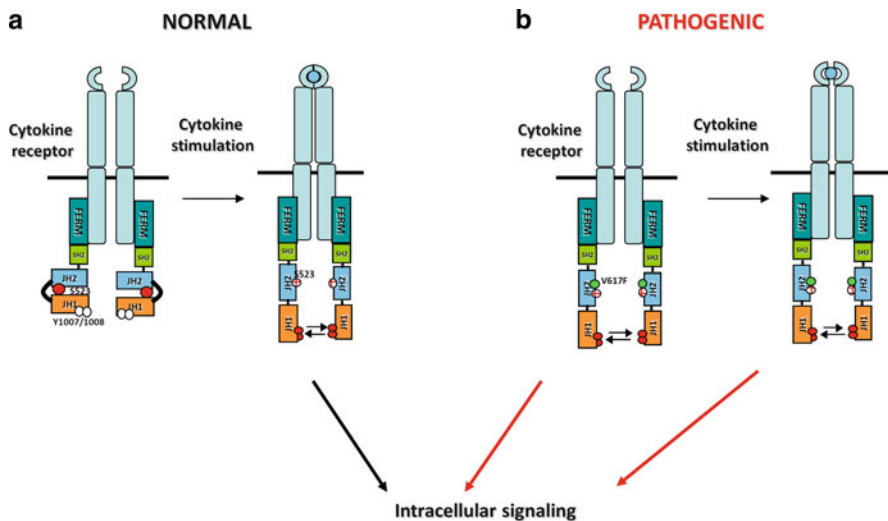## Regulation of Kinase Domain by the Pseudokinase Domain

The domain structure of JAK kinases is conserved from *Drosophila* to mammalians suggesting that the dual kinase domain structure is functionally important. The first insight into the functional role of JH2 domain was obtained from SCID patient, where mutations in JAK3, including those in the JH2 domain, were found to cause abrogation of JAK3 activation and IL-2 mediated signal transduction (Russell et al. 1995; Candotti et al. 1997). The next piece of information related to the function of JH2 domain came from the *Drosophila* system, where a point mutation in the JH2 domain was found to cause hyperactivation of the JAK kinase and hyperproliferation of hemolymph (Luo et al. 1997). Analogous mutation in mammalian JAK2 (E665K) also resulted in hyperactivity though the effect was mild (Luo et al. 1997). Thus, these genetic models provided seemingly controversial conclusion, in JAK3 JH2 domain was required for activity and signalling, while in Drosophila the domain was mediating a negative regulator function. However, biochemical and

functional studies have provided additional information about the role of JH2 domain in regulation of JAKs and cytokine signalling (Chen et al. 2000; Yeh et al. 2000). The studies on JAK2 demonstrated that deletion of JH2 domain increased basal activity but abolished the cytokine induced activation of JAK and downstream signalling (Saharinen et al. 2000). The function of JH2 appears to be conserved among JAKs, or at least between JAK2 and JAK3, since chimeric constructs encompassing the JH2 of JAK3 in JAK2 background was able to reconstitute cytokine induced signalling in JAK2 deficient cell line (Saharinen and Silvennoinen 2002). Biochemical and kinetic analysis of the JH2 domain in JAK2 in vitro showed that the JH2 domain did not affect Km but reduced the Vmax of JAK2 catalytic activity thus suggesting a non-competitive mechanism of inhibition (Saharinen et al. 2003). This finding, combined with the cellular interaction between JH1 and JH2 suggested that a physical interaction between JH1 and JH2 is mediating the inhibitory function (Saharinen and Silvennoinen 2002). Furthermore, three inhibitory regions have been identified in JH2 of which the first starts at the loop between β4 and β5 in the N-lobe of JH2. The *Drosophila* Hop mutation as well as the MPN causing V617F mutation reside both in this same region.

The evidence for the requirement of the JH2 domain for JAK activation and functional cytokine signalling is derived from clinical and artificial mutations as well as from functional studies. The underlying mechanism is still unknown but the data from receptor-JAK complex organizations provides insights into this paradigm. The binding of JAK1 and JAK2 to the juxtamembrane regions in gp130 and EpoR, respectively, is necessary but not sufficient for the induction of JAK activation (Constantinescu et al. 2001; Haan et al. 2002). In the case of JAK2 and EpoR, the induction of catalytic activity was suggested to involve an interaction between the active conformation of the α-helical juxtamembrane region and the JH1-JH2 domain. Collectively these data can be summed in a model of JAK regulation in cytokine receptors, where JAKs are maintained inactive through a JH1-JH2 interaction in the absence of cytokine stimulation. Ligand binding to the receptor induces a conformation change in the α-helical hydrophobic juxtamembrane region which relieves the inhibitory JH1-JH2 interaction and allows transphosphorylation of the JAKs and their activation and progression of signal transduction (Fig. 3).

The mechanisms by which JH2 mediates the regulatory functions is currently unknown but a recent study has evaluated the inter-domain interactions in kinase activity and substrate specificity using recombinant JAK2 kinase domains (O. Silvennoinen and I. Touw, personal communication). Using a peptide micro-array platform, the JH2 was found to drastically decrease the activity of the JH1 domain by increasing the Km for ATP. JH2 was also found to modulate the peptide preference of JAK2. Interestingly, the V617F mutation partially releases this inhibitory mechanism but did not significantly affect substrate preference or Km for ATP. These results provide the biochemical basis for the interaction between the kinase and the pseudokinase domain of JAK2. In addition, molecular modelling has provided insights into possible mechanism of JH2 function. The model of Lindauer et al based on the crystal structure of the FGF receptor dimer, suggest two interaction interfaces between JH1 and JH2 (Lindauer et al. 2001). It should be noted, that

Fig. 3 Schematic representation of the normal and pathological activation of the Janus kinases. (**a**) In the absence of ligand binding, the kinase activity is prevented via the interaction with the pseudokinase domain and involving pS523 (*red dot*) in the JH2 domain. Upon cytokine binding, receptor dimerization leads to kinase activation via transphosphorylation, by releasing the inhibitory JH1-JH2 interaction. (**b**) Cytokine-independent activation of Janus kinases mediated by mutations in the JH2 domain. MPD-causing mutations in the JH2 domain result in displacement of JH1-JH2 inhibitory interaction and altered pSer523 phosphorylation levels. JAKs can transphosphorylate even in the absence of cytokine binding, leading to cytokine-independent signalling and a hyperactive JAK2

there is currently no firm evidence that this model is relevant for JAKs, but nonetheless the model has proven to provide good predictions and explanations to structure/function analysis, particularly related to the V617F mutations. The main interface between JH1 and JH2 is composed of the N-terminal α-helices in both domains. The second interaction site is between the activation loop in JH1 and the loop between β4 and β5 in JH2, starting from V617. This interaction is expected to stabilize the inactive conformation in the activation loop. Recent molecular dynamics simulations largely agree with the original homology model, but provide evidence for additional interfaces consisting of hydrophobic interaction between F595 in JH2 with the activation loop and the interaction between β4 and β5 loop with a loop in JH1 (E1028-S1032) interacting with the activation loop (Lee et al. 2009). The V617F mutation is predicted to inhibit the inhibitory JH1-JH2 interaction by blocking the interaction of F595 and S591 with the activation loop and forcing the activation loop to its active fold. Dusa et al have addressed the function of F595 experimentally and their results also indicate a stacking interaction between F595 and the V617F mutant as a mechanism to activate the kinase (Dusa et al. 2010).

Lessons from Patient Mutations

Mutations Within the Pseudokinase Domain

The majority of the pseudokinase domain mutations affect the N-terminal lobe of the domain and modify residues involved in either the postulated interface with the kinase domain (Levine et al. 2005) or structurally important residues whose mutation can destabilize the N-lobe and thus also affect a possible interface between the pseudokinase and kinase domains. Mutations in the C-lobe of the pseudokinase domain are rare, which could suggest that the structural integrity of this region is essential for JAK function and/or that its surface does not participate in the kinase domain activity regulation. In the case of JAK2 the mutations can be attributed to be part of two structural hotspots (I and II) which are associated with different disease phenotypes. Mutations in hotspot I are associated with MPN while mutations located in hotspot II lead to a different clinical phenotype, namely lymphoblastic leukaemia (discussed in Haan et al. 2010). To date this genotype-phenotype specificity incorporates all activating exon 12, 14 and 16 mutations (Haan et al. 2010; Bercovich et al. 2008). The mutations in the different structural hotspots I and II might influence the recruitment to different signalling complexes including different cytokine receptors and lead to different signalling events. Such genotype-phenotype specificity is not yet obvious for the corresponding mutations in JAK1 and JAK3, where the same structural hotspots are affected by mutations.

A proposed theory, based on a molecular model of the full length JAK2, concerning the effects of the V617F postulates that the residue V617 is part of the binding interface by which the pseudokinase domain contacts the kinase domain and negatively regulates its activity (Levine et al. 2005). Accordingly, mutation of this residue to a larger hydrophobic residue should prevent optimal contact and reduce the affinity of the inhibitory interaction. However, it was shown that a V617Y exchange does not lead to constitutive activity, indicating that the situation may be more complex (Dusa et al. 2008). Although the hypothesis concerning the interface between the pseudokinase and kinase domain makes a lot of sense and explains much of the biological data, it must be noted that the true molecular mechanism could be different and that only a solved structure encompassing at least the pseudokinase and the kinase domains would provide reliable evidence for the mechanism.

Mutations Within the Kinase Domain

Most mutations are confined to a loop-region between the β2 and β3 strands of JAK2 (R867Q, D873N, T875N). Similarly, the other reported mutations (P933R in JAK2 and R879C/H/S in JAK1) affect residues which are exposed on the surface and do not affect the structure of the domain. Considering the kinase-pseudokinase interaction model by Lindauer and colleagues (Lindauer et al. 2001), none of the activating JAK2 mutations can be attributed to the proposed interface between the two domains.

Perspectives

Although there is an overall understanding of the basic functions of the different JAK subdomains it is yet unclear how these domains interact with each other and with the cytokine receptor and which structural changes are imposed on JAKs during activation of the cytokine receptor complex. It still remains mechanistically unclear how the disease-associated mutations in JAKs translate into a gain-of-function phenotype and thus the molecular basis of the mutational hotspots associated with either MPN or leukaemia remains elusive.

Here, we have reviewed data which demonstrate that the FERM domain of JAKs is crucial for receptor association and the SH2-like domain may also be involved in this interaction. Nevertheless, the real situation might still be more complex. The FERM domain has also been described to influence kinase activity. The structural integrity of the pseudokinase domain of TYK2 is essential for high-affinity-binding of cytokines to the IFNAR (Yeh et al. 2000; Gauzzi et al. 1997), pointing to an important role of TYK2 in "organising" the receptor complex. Also, data on JAK3 suggest that the kinase domain may affect receptor binding (Zhou et al. 2001). All this is indicative of a complex interplay of the different JAK subdomains with the cytokine receptor, which very likely reflects different activation states. Interestingly all of these yet unknown intramolecular interactions might be susceptible to interference with allosteric inhibitors.

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