

## Prediction of the structure of human Janus kinase 2 (JAK2) comprising the two carboxy-terminal domains reveals a mechanism for autoregulation

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**The structure of human Janus kinase 2 (JAK2) comprising the two C-terminal domains (JH1 and JH2) was predicted by application of homology modelling techniques. JH1 and JH2 represent the tyrosine kinase and tyrosine kinase-like domains, respectively, and are crucial for function and regulation of the protein. A comparison between the structures of the two domains is made and structural differences are highlighted. Prediction of the relative orientation of JH1 and JH2 was aided by a newly developed method for the detection of correlated amino acid mutations. Analysis of the interactions between the two domains led to a model for the regulatory effect of JH2 on JH1. The predictions are consistent with available experimental data on JAK2 or related proteins and provide an explanation for inhibition of JH1 tyrosine kinase activity by the adjacent JH2 domain.**  
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### Introduction

Janus kinases (JAK proteins, JAKs) are receptor associated protein tyrosine kinases and are of critical importance for cytokine-mediated signal transduction. Cytokine binding to the extracellular parts of their receptor chains leads to the formation of homo- or heteromeric receptor complexes involving at least two receptor chains. Through the formation of such a complex the receptor-associated JAK proteins are brought together at the inside of the cell membrane, which in turn induces trans-phosphorylation of the JAK proteins (Leonard and O'Shea, 1998; Yeh and Pellegrini, 1999). Once the JAKs are activated, a cascade of further signalling events is triggered, involving phosphorylation of selected receptor chain tyrosines, binding of the so-called STAT proteins and phosphorylation of these STATs (Bazan, 1990; Cosman, 1993; Kishimoto *et al.*, 1994). The phosphorylated STATs then dimerize, translocate to the nucleus and initiate gene activation (Briscoe *et al.*, 1996a).

So far, four mammalian JAKs have been identified (JAK1, JAK2, JAK3 and TYK2) (Duhé and Farrar, 1998). The members of the JAK family display some remarkable features. They consist of seven regions of conserved homology, known as JAK homology (JH1–JH7) domains. The two carboxy-terminal domains are a tyrosine kinase (JH1) and a tyrosine kinase-like domain (JH2). The remaining five domains (JH3–JH7) do not show significant sequence similarity to other known proteins and are thought to interact with regulatory proteins or with the cytoplasmic domains of cytokine receptors

(Gauzzi *et al.*, 1997; Saltzman *et al.*, 1998; Yamamoto *et al.*, 1999). For the interferon- $\gamma$  receptor it has been shown that  $\beta$ -chain-associated JAK2 could be exchanged for another Janus kinase without any consequences for activation or activity of the so-called STAT proteins (Kotenko *et al.*, 1996). Similar results were obtained by Jiang *et al.* (1996); their studies showed that JAK2 could be substituted for JAK1 in a mitogenic response to interleukin-2. In contrast to these studies, which indicate little contribution from the JAKs to post-receptor specificity, a comparison between JAK1 and JAK3 indicated a better ability of JAK1 to phosphorylate STAT5 (Liu *et al.*, 1997). Schaper *et al.* (1998) studied the response of JAK1, JAK2 and TYK2 deficient human fibrosarcoma cell lines by stimulation with soluble interleukin-6. They found that only JAK1 and not JAK2 or TYK2 deficient deficient cell lines display reduced activity of phosphorylation of the associated STAT proteins. The question thus remains of how JAK kinases confer specificity to cytokine-induced signal transduction.

A number of structure–function studies have been performed on JAK proteins. These studies involved deletion of entire domains of the JAKs, introduction of single point mutations or generation of mutants with short deletions. JAK proteins lacking the amino terminal domains (JH3–JH7) have been shown to be unable to bind to their cognate cellular receptors. (Tanner *et al.*, 1995; Chen *et al.*, 1997; Yan *et al.*, 1998). Deletion of the tyrosine kinase domain resulted in inactive JAKs (see, for example, Duhé and Farrar, 1995; Velazquez *et al.*, 1995). Deletion of the kinase-like domain was shown to have different effects on the function of the respective JAK protein. A TYK2 mutant without the JH2 domain lacked *in vitro* catalytic activity and *in vivo* function (Velazquez *et al.*, 1995). By contrast, similar JAK2 mutants were able to function *in vivo* (Frank *et al.*, 1995) and showed increased catalytic activity (Sakai and Kraft, 1997). Another study found several indications for an inhibitory role of the JH2 domain in regulation of JAK2 activity, through interaction with the JH2 domain (Saharinen *et al.*, 2000). For JAK3 the JH2 domain seems to be required for full catalytic activity, but appears at the same time to inhibit kinase activity (Chen *et al.*, 2000). A number of point mutations have been found to affect catalytic activity or regulation of the JAK proteins. In the tyrosine kinase domain mutation of an invariant lysine (K882 in human JAK2) has been found to generate defective kinases for JAK1/JAK2 (Briscoe *et al.*, 1996b) and TYK2 (Gauzzi *et al.*, 1996). Substitution of Asp by Ser in the DFG triplet at the start of the activation loop in JH1 rendered human JAK1 inactive (Briscoe *et al.*, 1996b). A double mutation (W1020G/E1024A) in JAK2 inactivated this kinase (Zhuang *et al.*, 1994). In JAK2 Y1007 and Y1008 have been found to be sites of trans- or autophosphorylation *in vivo* and *in vitro* (Feng *et al.*, 1997). Mutation of Y1007 to phenylalanine (Y1007F) reduced the kinase activity significantly, whereas mutation of Y1008 (Y1008F) had no influence on kinase activity (Feng *et al.*,

1997). Y1007 of JAK2 has recently been identified to interact with a JAK binding protein (JAB), which negatively regulates kinase activity (Yasukawa *et al.*, 1999). Mutations within the JH2 domain can also have a significant effect on kinase activity and indicate a regulatory role of this domain. Point mutations of a glutamate residue in *Drosophila* HOP (E695K) and murine JAK2 (E665K) hyperactivated the corresponding JAK-STAT pathways (Luo *et al.*, 1997). By contrast, the corresponding mutation in JAK3 (E639K) had no effect (Chen *et al.*, 2000). A short deletion ( $\Delta$ 586–592) in the kinase-like domain of JAK3 has been found to inhibit kinase activity (Chen *et al.*, 2000).

From a mechanistic viewpoint, the importance of the JAKs stems from the fact that they are the first proteins involved in the intracellular part of signal transduction. There is also mounting evidence that JAKs would be suitable targets for therapeutic intervention in many diseases. It has been shown that selective inhibition of JAK2 blocks leukemic cell growth *in vitro* and *in vivo* (Meydan *et al.*, 1996). Blockade of JAK3 induced apoptosis in human leukemia cell lines (Sudbeck *et al.*, 1999). Malaviya and Uckun (1999) found that JAK3 plays a pivotal role in IgE receptor/Fc $\epsilon$ RI mediated mast cell responses and they suggest that targeting JAK3 could provide the basis for new and effective treatment for mast cell-mediated allergic reactions.

The explosive growth of sequence databases created by ongoing research in the human genome project continuously increases the gap between protein sequences and known three-dimensional protein structures. However, it has been observed that newly determined structures increasingly tend to fall into structural folds already known (Murzin, 1996, 1998). This indicates that the number of folds is finite (Chothia, 1992; Orengo *et al.*, 1994; Govindarajan *et al.*, 1999). The prediction of the three-dimensional structure of proteins has made significant progress over the last few years (Koehl and Levitt, 1999). Also, it has been shown that protein models should be good enough for high-level functional analysis (Wei *et al.*, 1999). Model structures with a more moderate resolution are at least sufficient for identifying enzymatic active sites with specific residue geometry (Fetrow and Skolnick, 1998; Fetrow *et al.*, 1998). Therefore, in the absence of experimentally determined structures, prediction of the three-dimensional structure of proteins by computer represents an important technique in order to explore structure and function of proteins.

Because there are no experimental structures of JAK proteins available to date, we decided to initiate prediction studies of these important proteins. JAK2 was chosen as a representative because of its involvement in leukemic cell growth (Meydan *et al.*, 1996) and the fact that extensive experimental data are available for comparison with the predictions. In this paper we present the prediction of the three-dimensional structures of the two carboxy-terminal domains (JH1 and JH2) of JAK2. At first the structures of these two domains were predicted separately. This allowed for comparison of the tyrosine kinase and kinase-like domains and structural and functional differences could be highlighted. Then a model for the relative orientation of the two domains was developed. Analysis of the combined structure led to prediction of interactions between the domains and yielded a model for regulation of kinase activity through JH1–JH2 interactions. The structures were validated as much as possible through comparison with experimental studies and were found to be consistent with these studies.

## Methods

Multiple sequence alignment was performed with the programs ClustalW (Thompson *et al.*, 1994) and Dialign (Morgenstern, 1999). For identification of folds likely to be adopted by the JH1 and JH2 domains the following fold recognition methods were used: Threader (Jones *et al.*, 1992), Predict Protein (Rost and Sander, 1993, 1994; Rost *et al.*, 1994), 123D (Alexandrov *et al.*, 1996), ToPLign (Thiele *et al.*, 1999) and UCLA-DOE (Fischer and Eisenberg, 1996). Generation of the 3D structures was accomplished by application of restraint-based homology modelling using the program MODELLER (Šali and Blundell, 1993).

For predicting interactions between amino acids, a novel technique was developed. This procedure is based on a method applied to RNA molecules, where it is referred to as ‘comparative sequence analysis’ (Chiu and Kolodziejczak, 1991; Gutell *et al.*, 1992). Here the interactions between nucleotides of RNA molecules are identified by statistical analysis of a set of aligned RNA sequences that are related. Given a set of aligned sequences, the task is to identify correlated mutations at two positions  $x$  and  $y$  along the alignment. From such a correlation one can infer that in 3D the residues at positions  $x$  and  $y$  are in contact. The underlying assumption is that this correlation actually stems from the evolutionary pressure to respond to a mutation at  $x$  by a compensatory mutation at  $y$  (if the residues at  $x$  and  $y$  are in contact in the 3D structure), in order to preserve structure and/or functionality. During the analysis, the 20 amino acids can be considered as each being a class of its own or they can be grouped into different physicochemical classes, e.g. according to hydrophobicity, size, charge, etc. Calculation of the so-called mutual information  $M(x,y)$  between all possible position pairs provides a quantitative measure of the interactive strength in those pairs. The mutual information is expressed as a sum of entropy-like terms that contain the frequencies (or probabilities) of the amino acids (or the classes to which they belong) and the frequencies of the possible pairings at  $x$  and  $y$ . More specifically, the mutual information in the positions  $x$  and  $y$  is defined as

$$M(x,y) = \sum_{a_x a_y} f_{a_x a_y} \ln \frac{f_{a_x a_y}}{f_{a_x} f_{a_y}} \quad (1)$$

where  $f_{a_x}$  refers to the frequency at which (the probability with which) an amino acid  $a$  (or an amino acid class) can be found at position  $x$  and  $f_{a_x a_y}$  is the frequency at which a specific residue pair can be found at positions  $x$  and  $y$ . Another, equivalent, way of calculating  $M(x,y)$  is

$$M(x,y) = H(x) + H(y) - H(x,y) \quad (2)$$

with

$$H(x) = - \sum_{a_x} f_{a_x} \ln f_{a_x} \quad (3a)$$

and

$$H(x,y) = \sum_{a_x a_y} f_{a_x a_y} \ln f_{a_x a_y} \quad (3b)$$

where  $H$  is the estimated composition entropy or Shannon entropy (Shannon and Weaver, 1963). Putative residue interactions will be identified according to the magnitude of the mutual information values. This approach is regarded as being more robust, as it does not rely on a normal distribution

of the underlying data required for conventional correlation analysis. By introducing the necessary modifications, the method was ported to proteins. Before it was applied to JAK2 proteins, we tested the method by studying a set of aligned protein tyrosine kinase sequences. Most of the correlated mutations were in good agreement with the known three-dimensional structures of the corresponding proteins and included residues either in the catalytic core of the kinases or between adjacent  $\alpha$ -helices.

A detailed description of the use of this method for proteins is in preparation and an outline of its application to JAK2 follows: 495 receptor associated or integrated tyrosine kinase sequences were retrieved from the protein sequence databases SWISSPROT and SWISSNEW (Bairoch and Apweiler, 1997). The sequences were aligned with ClustalW (Thompson *et al.*, 1994) and their phylogenetic relation was calculated (Clamp, 1998). The topology of the corresponding phylogenetic tree indicated that the sequences could be combined in three sets: Set A contained 10 mammalian JAK sequences that are closely related to the human JAK2 sequence. Set B included set A and 175 other sequences which are less related to the human JAK2 sequence than those in set A. Set A is therefore a subset of set B ( $A \subset B$ ). Set C contained all 495 sequences ( $A \subset B \subset C$ ). With these sets a comparative sequence analysis was performed. For the analysis different amino acid classification schemes were set up according to (1) hydrophathy, (2) protonation state, (3) hydrogen bond donor/acceptor capabilities of the amino acid side chains and (4) a class containing 20 different members corresponding to the individual amino acids. Correlated mutations were identified by high mutual information values.

The modelled structures were refined by 1000 steps of energy minimization using the Amber force field (Pearlman *et al.*, 1994). Analysis of the final structures was carried out with the program Procheck (Laskowski *et al.*, 1993). Checks performed included Ramachandran plots,  $\chi_1$  versus  $\chi_2$  plots, bump checks and evaluation of various stereochemical parameters. All parameters were found to be within the normal tolerances.

## Results

### Sequence alignment

Multiple sequence alignment of the two carboxy-terminal homology domains (JH1, JH2) for 10 JAK sequences from different species is shown in Figure 1. Comparison of the sequences indicates strong sequence homology among all members of the JAK family. More specifically, the JAK homology domains JH1, JH2 and JH4 share strong pairwise sequence identity (JH1 34%, JH2 26–28%, JH4 20–25%), whereas the other domains show lower sequence identity (<19%), but are similar to each other.

### Structure of human JAK2 homology domain JH1

First, possible folds of this domain were evaluated by fold recognition methods. Several proteins of known three-dimensional structure were identified as possible templates by high scoring values, as shown in Table I. Interestingly, the highest scoring structures not only share the function of a tyrosine kinase with the JAK proteins, they also play a similar role in signal transduction, as they are receptor-integrated tyrosine kinases. These structures were then used as template structures in the prediction process. The alignment of the template sequences with the target sequence is shown in Figure 2. The

sequences share a rather high sequence identity of 22%. Both the hydrophathy profile and the secondary structure elements (assigned and predicted) align very well. Using the alignment shown, restraint based modelling as implemented in MODELLER (Šali and Blundell, 1993) was applied for calculating the three-dimensional structure of the JAK2-JH1. Several structures satisfying the input restraints were obtained and were superimposed by their C $\alpha$  atoms (residues: R839 to T1049 and L1078 to S1115). The difference between these structures was marginal, as indicated by an averaged r.m.s. value of  $0.56 \pm 0.08$  Å. The alignment shown in Figure 2 reveals a loop insertion within the JAK2-JH1 domain. This loop is located between amino acids T1049 and L1078. Secondary structure prediction (Rost and Sander, 1994) indicated a short  $\alpha$ -helix between amino acids M1062 and M1064, but with a probability of <87%. Therefore, the region between residues T1049 and L1078 was modelled separately by employing loop database searches (Jones and Thirup, 1986; Moulton and James, 1986).

### Structure of human JAK2 homology domain JH2

The JAK homology domain JH2 is known as a tyrosine kinase like domain because it lacks the functionality of a tyrosine kinase (Duhé and Farrar, 1998). Application of fold recognition methods yielded high scoring values for several proteins as summarized in Table I. Notably, the four highest scoring structures were identical with the set of proteins identified for JAK2-JH1. For prediction of JAK2-JH2 the same procedure as for JAK2-JH1 was applied. Figure 3 shows the sequence alignment of the template and the target sequences. The hydrophathy profile and the secondary structure elements (assigned and predicted) aligned very well.

A motif common to tyrosine kinases is the DFG motif at the start of the activation loop (conserved domain VII according to the nomenclature for protein kinases devised by Hanks *et al.*, 1988). The JH1 domain of JAK2 contains this motif and it is easy to align with other tyrosine kinases. The JH2 domain lacks this motif and in previous studies (Wilks *et al.*, 1991; Ziemiecki *et al.*, 1994) the DPG sequence of JH2 was aligned with the common DFG motif (Figure 4A). As a consequence, however, two large gaps are introduced in the alignment, the activation loop of JH2 is shortened significantly and an additional set of eight amino acids would have to be accommodated in the  $\beta$ -sheet structure after the catalytic loop. Such an insertion would also destroy the conserved pattern of amino acids in this  $\beta$ -sheet structure. We therefore decided to align the activation loop of JH2 with other kinases as shown in Figures 3 and 4B. Compared with JH1, the lysine and glycine residues (K688 and G690) immediately before the DFG motif are preserved in this alignment and the aspartate residue of this motif is mutated into the corresponding amide (N691). Considering the low degree of amino acid conservation within the activation loop itself, the new alignment did not have a significant effect on the homology within this loop.

Different structures satisfying the input constraints were then obtained by distance constraint-based modelling. Superimposition of these structures by their C $\alpha$  atoms indicated that they are very similar (average r.m.s. value:  $0.60 \pm 0.04$  Å).

### Combined structure of JAK2-JH1 and JAK2-JH2

Evidence for assembling JAK2-JH1 and JAK2-JH2 was gathered from two different sources. The first was to use the crystal structure of a dimer of the FGF receptor tyrosine kinase domain (Mohammadi *et al.*, 1996) as a basis for the combined

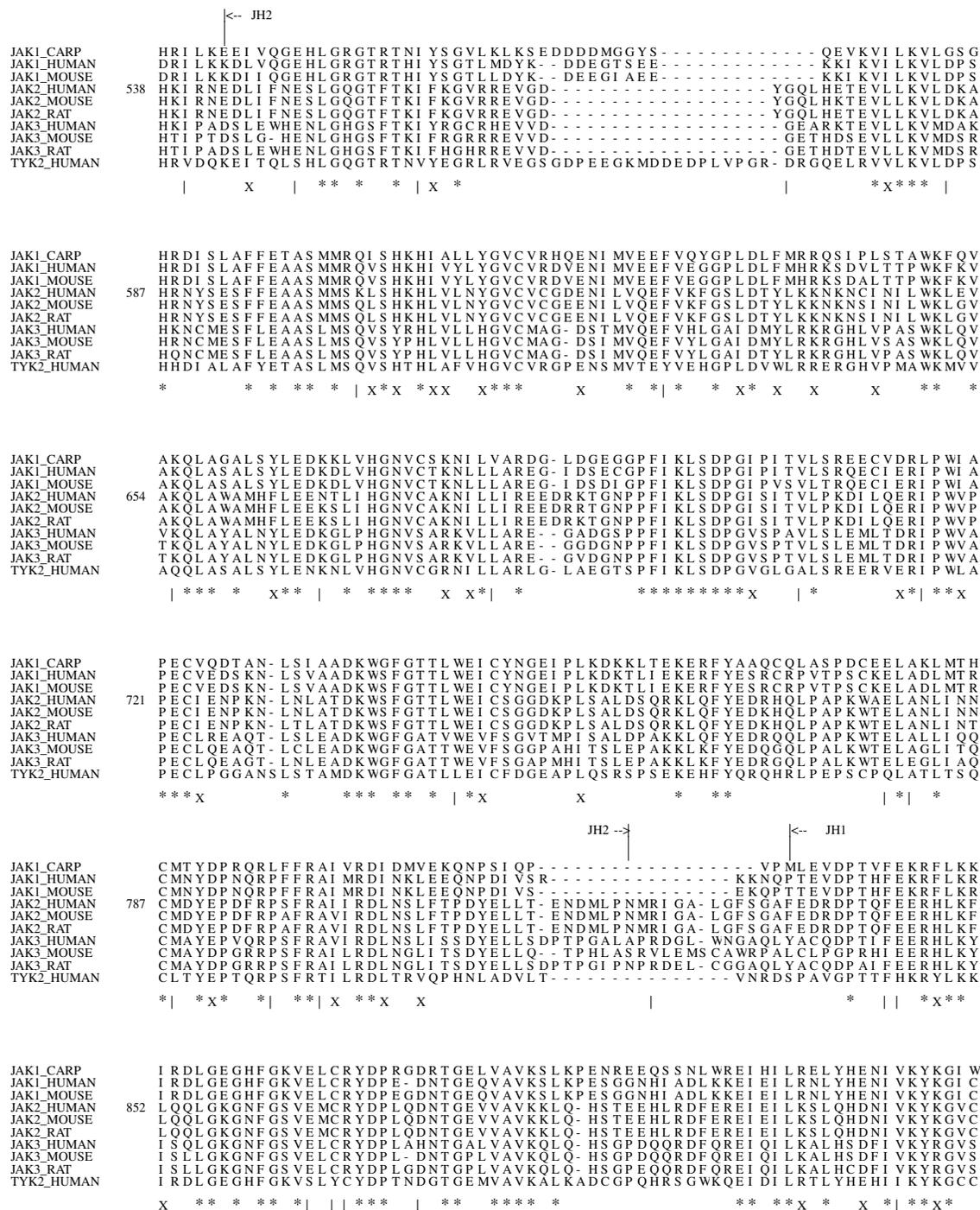
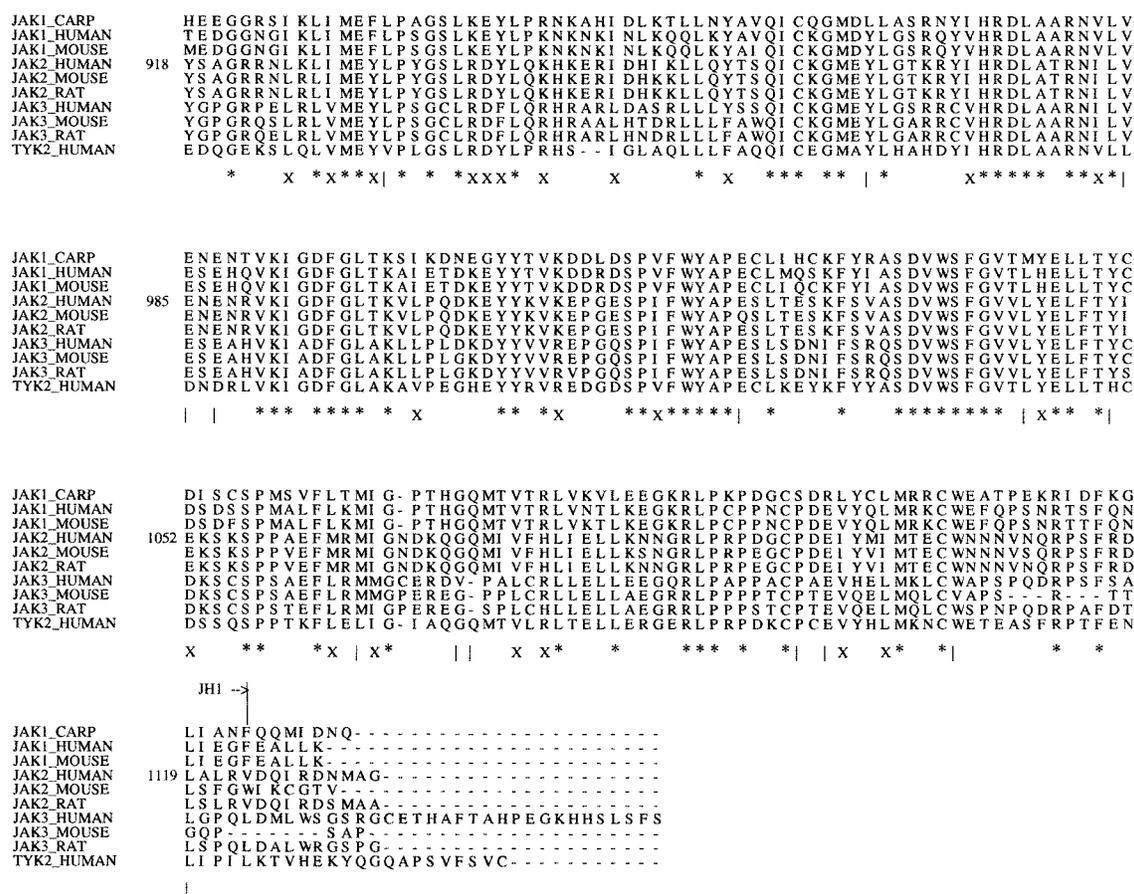


Fig. 1. (continued next page).

structure. In the paper by Mohammadi *et al.*, the possibility of dimerization of the FGF receptor tyrosine kinase domain was discussed and the potential biological significance of three different dimers was considered. In the crystal two of the dimers had a relatively small interface (950 and 670 Å<sup>2</sup>). These values are relatively low for protein–protein interactions of biological relevance (Conte *et al.*, 1999). The third dimer had a much larger interface area (1650 Å<sup>2</sup>). In the corresponding interface the first N-terminal α-helix of each tyrosine kinase domain is oriented almost parallel to the other one and the helices are located at the interface. The dimer can be described as axial symmetric. The symmetry axis is located between the

α-helices and parallel to them. This type of dimerization has been supported also by experimental evidence, as a mutation located in the interface of the dimer suppressed the functionality of the protein (Mohammadi *et al.*, 1996). Therefore, the two amino-terminal JAK2 domains were combined according to the dimer of the FGF receptor tyrosine kinase domain. This was accomplished by fitting the N-terminal α-helices of JAK2-JH1 (residues E889 to S904) and of JAK2-JH2 (residues R588 to K603) on to corresponding helices of the dimer of the FGF receptor tyrosine kinase domain. The second source of evidence was the application of comparative sequence analysis to a set of aligned JAK sequences, as described in the Methods section.



**Fig. 1.** Multiple sequence alignment of Janus kinase homology domains JH1 and JH2. The numbering scheme refers to the mature sequence of human JAK2. Boundaries of the homology domains are indicated by arrows. Sequence homology is indicated by symbols below the sequences as follows: \*, amino acid identity; l, amino acid identity with one exception; X, common physicochemical properties (such as hydrophobicity, charge or aromaticity).

**Table I.** Likely protein folds for JAK2-JH1 and JAK2-JH2

| Template structures |                                  | Fold recognition methods <sup>a</sup> |                |          |           |         |
|---------------------|----------------------------------|---------------------------------------|----------------|----------|-----------|---------|
| PDB code            | Reference                        | Predict protein                       | 123D           | UCLA-DOE | Threading | ToPLign |
| lirk                | Hubbard <i>et al.</i> (1994)     |                                       | X <sup>b</sup> | X        | X         | X       |
| lir3 A              | Hubbard (1997)                   | X                                     |                |          | X         |         |
| lfgk A              | Mohammadi <i>et al.</i> (1996)   |                                       |                | X        |           |         |
| 3lck                | Yamaguchi and Hendrickson (1996) |                                       |                | X        | X         |         |

<sup>a</sup>For references, see Methods section.

<sup>b</sup>Protein structures with significant scoring values are indicated by X.

This procedure indicated several correlated residue mutations in the two N-terminal helices of JH1 and JH2, thereby supporting the hypothesis that the two helices are in contact in the protein structure.

The loop between JAK2-JH1 and JAK2-JH2 domains was then modelled by employing loop database searches (Jones and Thirup, 1986; Moult and James, 1986). The final structure of the two carboxy-terminal domains of JAK2 is shown in Figure 5A. Its structural details (Figure 5B and 5C) will be discussed in the next section.

A structural comparison of the JH1/JH2 model with the FGF receptor kinase dimer is displayed in Figure 6. Overall the structures are very similar and the r.m.s. fit for the two structures is 1.91 Å using 436 C $\alpha$  atoms. The largest differences between the structures concern the loop regions at the ‘outside’

of the proteins, away from the interface. If the structures are fitted on to each other using only residues at or near the interfaces (50 residues in total), the r.m.s. deviation for this subsection is 1.36 Å.

## Discussion

### Human JAK2 homology domain JH1

In the model JAK2-JH1 adopts a fold which is typical for the architecture of tyrosine kinases (Hubbard *et al.*, 1994). The domain consists of two lobes. The N-terminal lobe contains a twisted  $\beta$ -sheet of five anti-parallel  $\beta$ -strands and one  $\alpha$ -helix. The C-terminal lobe is mainly  $\alpha$ -helical and three of the helices are oriented almost parallel to each other. Catalysis of phosphotransfer is thought to take place in the cleft between



**Fig. 2.** Alignment of the sequences of the template structures with the JAK2-JH1 sequence. Secondary structure assignments are shown underneath each sequence in grey. The secondary structure of JAK2-JH1, as predicted by the Predict Protein Server (Rost and Sander 1993, 1994; Rost *et al.*, 1994), is indicated as follows: C, coil; H, helix; E, extended/β-strand. The assigned secondary structure for the known structures follows the DSSP notation (Kabsch and Sander, 1983): B, residue in isolated β-bridge; E, extended strand; G, 3/10 helix; H, a-helix; I, p-helix; S, bend; T, hydrogen bonded turn. The functional loops (activation loop, A-Loop; catalytic loop, C-Loop; nucleotide-binding loop, NBL) are indicated by arrows. Amino acid homology is indicated as in Figure 1.

the two lobes. Particularly important in this respect are the nucleotide binding loop (residues G856 to G861), catalytic loop (residues K970 to N981) and the activation loop (residues D994 to E1024).

The activation loop (red in Figure 5) begins with the conserved DFG motif (residues D994 to G996) and ends with the conserved motif APE (residues A1022 to E1024) (Hanks *et al.*, 1988). The conformation of this loop can vary significantly

from one kinase to another, as shown by a comparison of the inactivated insulin receptor and FGF receptor kinases (Hubbard *et al.*, 1994; Mohammadi *et al.*, 1996b). In the present study this loop was modelled initially on the FGF receptor kinase, as this protein then served as a basis for the combined JH1/JH2 model. Of the residues in the DFG motif D994 points towards the active site and F995 away from it, similar to the corresponding residues (D641/F642) in the FGF

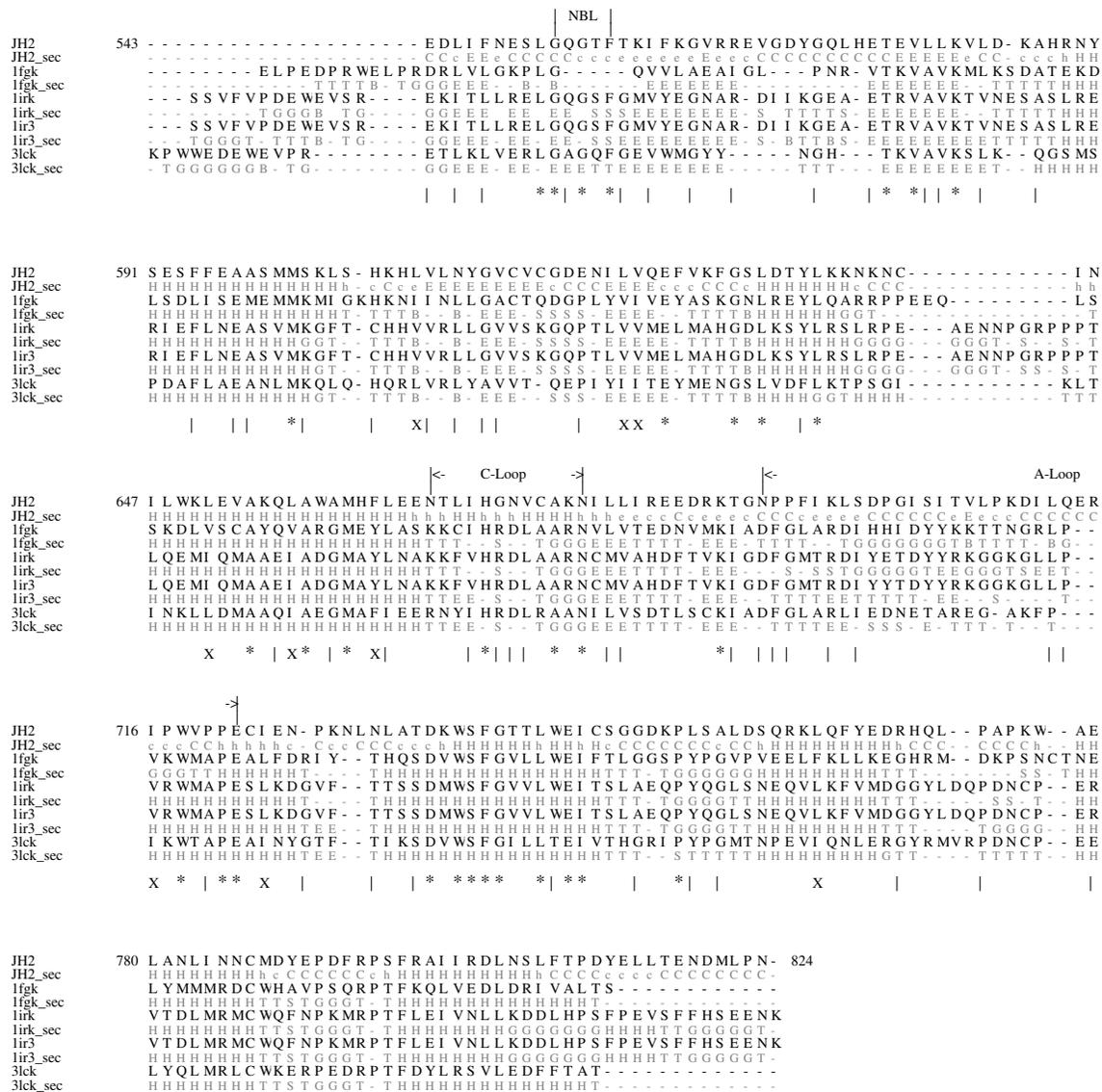


Fig. 3. Alignment of the sequences of the template structures with the JAK2-JH2 sequence. Details as in Figure 2.

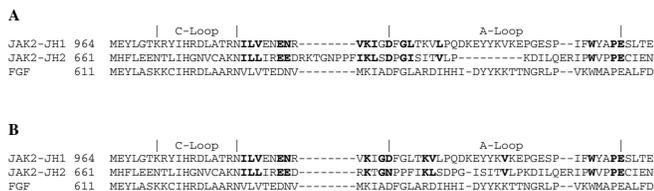


Fig. 4. Details of the alignment of the activation loop of JH2. (A) Alignment from previous studies; (B) alignment used in the present study. Bold residues indicate identity or homology between JH1 and JH2, after the catalytic and within the activation loop. The FGF receptor kinase sequence is shown as a reference.

receptor kinase. The activation loop contains two conserved tyrosine residues (residues Y1007 and Y1008), which are crucial for the tyrosine kinase activity of JAK2-JH1 (Songyang and Cantley, 1995). In the model both tyrosine residues point away from the active site. Y1007 is surrounded by several hydrophobic residues (I1018, L1026 and I1079), similar to Y653 in the FGF receptor kinase, which interacts with V664, L672 and F710. The OH group of Y1007 forms a hydrogen bond with D1004. This hydrogen bond is different from that

formed by Y653 in the FGF receptor kinase; this tyrosine is hydrogen bonded to the backbone oxygen of L672. The corresponding residue in JAK2 JH1 is L1026 and a slight change in structure would enable it to interact with Y1007. Y1008 is more solvent exposed and exhibits hydrophobic contacts with V1075. Based on sequence homology alone it is very difficult to decide which conformation the inactivated activation loop in JH1 would take up. The hydrophobic residues that interact with Y1007 and Y1008 are conserved in the sequence alignment (Figure 2), with the exception of V1075, which is replaced by a serine in the insulin receptor kinase. Also, it would be possible to model the activation loop analogous to the conformation taken up in the insulin receptor kinase and Y1007 would consequently hydrogen bond with D976, similar to the Y1162–D1132 pair in the insulin receptor kinase. In both loop conformations, however, Y1008 would be significantly more solvent exposed than Y1007, which could provide an explanation for the different role of these two tyrosines in trans- or autophosphorylation (Feng *et al.*, 1997).

In the catalytic loop (orange in Figure 5), the residues corresponding to D976 and N981 are invariant in the protein

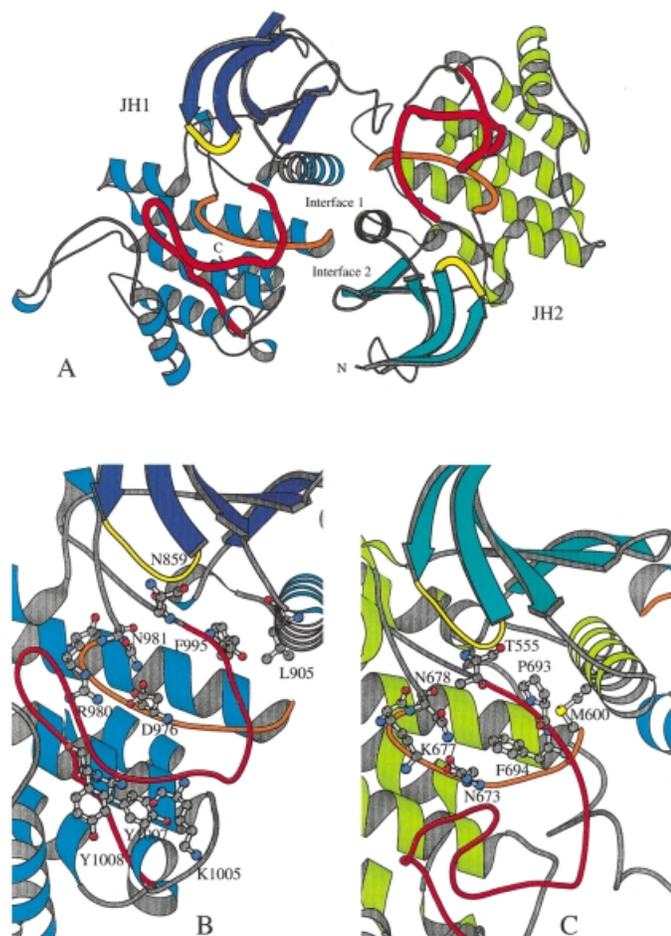


Fig. 5. Combined structure of JAK2-JH1 and JAK2-JH2. The general colouring scheme for JH1 is blue and JH2 is displayed in green. Colours for selected loops: activation loop, red; catalytic loop, orange; nucleotide-binding loop, yellow. (A) Overall structure. N-terminus, C-terminus and interfaces are annotated. (B) Catalytic core of JH1. (C) Catalytic core of JH2. Figure drawn with MOLSCRIPT (Kraulis, 1991).

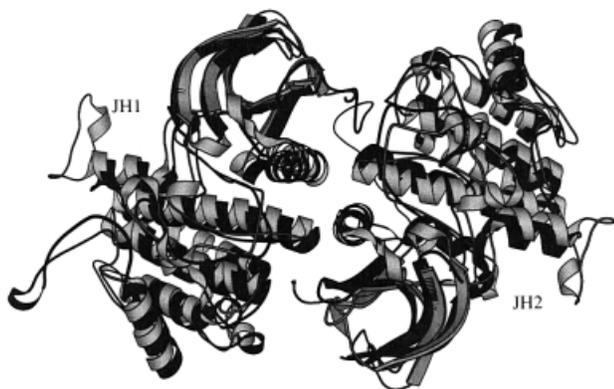


Fig. 6. Superposition of JAK2-JH1/JH2 (dark grey) with the FGF receptor kinase dimer (light grey). Orientation and domain arrangement of JAK2 as in Figure 5A. Figure drawn with MOLSCRIPT (Kraulis, 1991).

tyrosine kinase families and nearly invariant in the protein serine kinase families (Hubbard *et al.*, 1994). Within the known structures the residue corresponding to D976 of the model is the catalytic base of the phosphotransfer reaction and hydrogen bonded to the amino acid equivalent to N981. In the JH1 model the residues D976 and N981 are close in

space. Analysis of the structure with the program HBExplore (Lindauer *et al.*, 1996) indicated that the side chains of these two residues are connected by hydrogen bonds. An arginine (R980) within the catalytic loop, four residues along the sequence from the catalytic base, provides charge neutralization and forms a hydrogen bond with the catalytic base. It has been discussed that the residues corresponding to D976, N981 and R980 are involved in coordination of  $Mg^{2+}$  ions (Hubbard *et al.*, 1994). In the model these residues are close in space and stabilized by hydrogen bonding interactions. They are also orientated in such a way that they could interact with a putative  $Mg^{2+}$  ion.

The nucleotide binding loop (yellow in Figure 5), which is also referred to as the glycine-rich loop, is located between two  $\beta$ -strands in the N-terminal lobe of the tyrosine kinase. Compared with insulin tyrosine kinase (IRK), the nucleotide binding loop of JAK2-JH1 contains a mutation. The serine in IRK is replaced by an asparagine in JAK2-JH1 (Asn859). Members of the JAK1 and TYK2 family contain a histidine at the position of Asn859. The nucleotide binding loop of the lymphocyte kinase contains a glutamine at the corresponding position (Yamaguchi and Hendrickson, 1996). In IRK the backbone amide hydrogen of the serine is hydrogen bonded to the  $\beta$ -phosphate of ATP, indicating that the side chain is less important for ATP binding (Hubbard, 1997). This would also explain the low degree of conservation of this residue among the tyrosine kinases. Another feature known to be important for nucleotide binding is a hydrophobic pocket next to the nucleotide binding loop. The corresponding amino acids in JAK2-JH1 are F995 and L905.

#### Human JAK2 homology domain JH2

The model of JAK2-JH2 also consists of two lobes (Figure 5). Overall it is structurally similar to JAK2-JH1. The domain contains the equivalent of the activation loop (residues N691 to E722), the catalytic loop (residues N667 to N678) and the nucleotide binding loop (residues G552 to T557).

The loop between N691 and E722 corresponds to the activation loop in functional tyrosine kinases (red in Figure 5). Although it takes up a similar conformation in the model, it does not contain the main features of an activation loop. The conserved motif DFG, which indicates the start of the activation loop in tyrosine kinases, is mutated to an NPP motif. The conserved APE motif, which is thought to constitute the end of the activation loop, is replaced by a PPE motif. The most important change, however, is that the activation loop of JAK-JH2 contains no tyrosine. The corresponding residues are S703 and I704. This clearly renders the loop non-functional.

The catalytic loop (orange in Figure 5) is structurally and sequentially similar to the corresponding loops in the template structures and the JH1 domain. The residues corresponding to N981, R980 and D976 in JAK2-JH1 are N678, K677 and N673, respectively. They are located at equal positions in the catalytic loop compared with JAK2-JH1. The major difference is the change from a negatively charged residue (D976 in JH1) to a neutral one (N673 in JH2). On the whole, the catalytic loops of the JH1 and JH2 are rather similar.

The sequence of the nucleotide binding loop (yellow in Figure 5) of JAK2-JH2 is very similar to the corresponding loops in the tyrosine kinase structures used as templates and the JAK2-JH1 domain. The residue, which is frequently mutated throughout the tyrosine kinases, is T555. Another difference between JH1 and JH2 concerns the hydrophobic

pocket. The residue corresponding to F995 in JH1 is P693. The other key residue of the hydrophobic pocket, L905 of JH1, is replaced by M600 in JH2.

In principle, the model of the JAK-JH2 domain has a structure similar to those of other functional tyrosine kinases and contains all important loop regions. However, close inspection of these loops indicates that some of the main features required for tyrosine kinase activity are not present. This is in good agreement with the determined properties of the JH2 domain as a tyrosine kinase-like domain (Duhé and Farrar, 1998).

A mutation (E695K) in the JH2 domain of the Hop JAK kinase has been reported to cause an increase of the kinase activity (Luo *et al.*, 1997). The corresponding residue in JAK2 is E665. In the present model this residue displays several interactions with surrounding amino acids. In particular it forms a hydrogen bond with the  $\delta$ -hydrogen of H662 and the backbone amide hydrogen of F798. Other, positively charged, side chains in the vicinity include R799 and K736. Therefore, according to the model, mutation of E665 from a negatively charged residue to a positively charged amino acid could cause severe distortion of the local conformation. This in turn could lead to a local rearrangement of the whole structure. Such a change could have an effect not only on the structure of the JAK-JH2 domain but also on the relative orientation of the JAK-JH1 and JAK-JH2 domains. As it is known that the JH2 domain is responsible for negative regulation of the JAK-STAT pathway (Brechtold *et al.*, 1997; Barahmand-Pour *et al.*, 1998), a change in the relative orientation of these two domains would influence the kinase activity of JH1.

#### Combined structure of JAK2-JH1 and JAK2-JH2

Evidence for the spatial arrangement of the two domains was gathered from two different sources. The first was the observed dimer formation in the crystals of the tyrosine kinase domain of the fibroblast growth factor (Mohammadi *et al.*, 1996). Therefore, two  $\alpha$ -helices (one N-terminal helix from each domain) were oriented relative to each other as described for the dimer in the crystal. The resulting helix-helix interface (Interface 1 in Figure 5A) displayed a network of polar interactions, predominantly between complementary charged amino acids (Figure 7A). The second source of evidence was the detection of correlated residue mutations within the two  $\alpha$ -helices. Two of these mutations were found to be in contact in the model (S599-E900 and S599-S904, Figure 7A). This provided further support for assembly of the domains as described.

Another indication for the validity of the inter-domain arrangement could be derived from modelling the connecting loop between the domains. The sequence alignment in Figure 1 indicates different loop lengths for different JAK types. As all of these proteins exhibit very similar functions, one can assume that the JH1 and JH2 domains in these proteins show essentially identical inter-domain arrangements. Therefore, each of the connecting loop sequences in Figure 1 was modelled between JAK2-JH1 and JAK2-JH2. For each loop sequence a suitable fragment could be found by the application of loop database searches, including also the relatively short loop in JAK1. This indicates that the predicted JH1-JH2 arrangement could be valid for all JAK types.

The combined model of JAK2-JH1 and JAK2-JH2 revealed a second interaction site (Interface 2 in Figure 5A) between the two domains (Figure 7B). At this site the interactions take

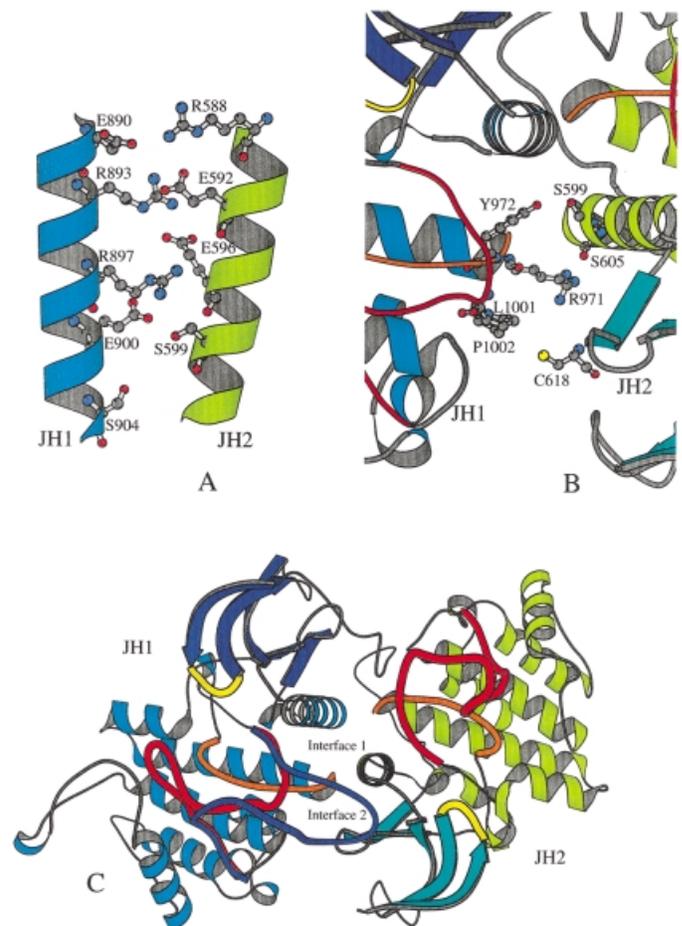


Fig. 7. Interactions between the JH1 and JH2 domains of JAK2. (A) Side view of Interface 1 in Figure 5A. The two helices belonging to JH1 and JH2 are shown. (B) Top view of Interface 2 in Figure 5A. The activation loop of JH1 is modelled in its inactivated conformation (red). (C) Comparison of the inactivated (activation loop in red) and activated (activation loop in blue) conformations of JAK2-JH1 in the combined model. Figure drawn with MOLSCRIPT (Kraulis, 1991).

place between the activation loop (residues D994 to E1024) of JAK2-JH1 and a loop connecting two  $\beta$ -strands of the N-terminal lobe of the JAK2-JH2 (residues V617 to E621). In particular, the hydrophobic residues V1000 and P1002 of the activation loop (JAK2-JH1) appear to interact with C618. A comparison of these loop regions within the JAK family showed that the hydropathy of these residues is conserved within the JAK2 and JAK3 family. In JAK3 an alanine corresponds to C618 and a leucine to V1000. In the human TYK2 protein the corresponding residues in JH2 would be G619 and P620. Within the JAK1 family the hydropathy pattern in these two loop regions is totally different and polar residues are present instead. Therefore, in the latter family the interactions between the two loops would be predominantly polar. Adjacent to this interface (Figure 7B) some additional interactions can be found between residues of the catalytic loop of JH1 (R971 and Y972) and residues of the N-terminal  $\alpha$ -helix of JH2 (S599 and S605). These contacts could also contribute to the regulatory effect of JH2 on JH1.

Deletion of the JAK2-JH2 domain led to intrinsic kinase activity of JAK2 (Brechtold *et al.*, 1997; Barahmand-Pour *et al.*, 1998). The interactions described above would be able

**Table II.** Unfavourable interactions between the activated A-loop in JH1 and JH2 residues

| JH1 residue | JH2 residue                           |                                     |
|-------------|---------------------------------------|-------------------------------------|
|             | Within 2.0 Å<br>(including hydrogens) | Within 1.5 Å<br>(without hydrogens) |
| V1000       | S599                                  | S599                                |
| L1001       | S602                                  | S602                                |
|             | P1002                                 |                                     |
| Q1003       | M601                                  | N612                                |
|             | S602                                  |                                     |
|             | N612                                  |                                     |
|             | L611                                  |                                     |
| D1004       | N612                                  | Y613                                |
|             | Y613                                  |                                     |
| K1005       | N612                                  | Y613                                |
|             | Y613                                  |                                     |
|             | G614                                  |                                     |
|             | V615                                  |                                     |
|             | L614                                  |                                     |
| V1010       | C618                                  | C618                                |
| K1011       | F595                                  | V617                                |
|             | V617                                  |                                     |
|             | C618                                  |                                     |

to explain the observed influence of the JH2 domain on the tyrosine kinase activity of JH1. More evidence of how the JH2 domain could influence the activity of the tyrosine kinase domain is provided by a comparison of JH1 with two crystal structures of the insulin receptor kinase. Initially the activation loop of JH1 was modelled in its inactivated conformation and this structure of JH1 was then used to generate the combined JH1/JH2 model shown in Figure 5A. In this, inactivated, conformation stabilizing hydrophobic interactions between L1001 and P1002 of the activation loop and residues around C618 of JH2 can be found (Figure 7B). It has been shown that protein kinases undergo major conformational changes in the activation loop upon ligand binding. We therefore generated a second structure of JH1, in which the activation loop was modelled using the activated form of the insulin receptor kinase (Hubbard, 1997) as a basis. In this conformation there is unrestricted access to the binding sites for ATP and substrate. When this loop was inserted into the combined JH1/JH2 structure (Figure 7C), it exhibited several very close contacts or unfavourable interactions with residues in the JH2 domain (Table II). The JH2 residues participating in these contacts are located either at the end of the N-terminal  $\alpha$ -helix or in the  $\beta$ -strand ending with C618. L1001 and P1002, which were interacting with C618 in the inactivated structure, now overlap with S602 and N612, respectively. Residues Q1003 to K1005 display a range of short contacts with amino acids L611 to V615 of JH2. V1010 and K1011 exhibit very close contacts with V617 and C618. If the proposed JH1/JH2 domain arrangement is correct, the range of unfavourable interactions with the activated form of JH1 would imply that loop motion from an inactivated to an activated conformation in JH1 is inhibited by the JH2 domain.

That the  $\beta$ -strand of JH2 ending with C618 is involved in regulation of JH1 activity is also supported by a recent study on JAK3 mutants (Chen *et al.*, 2000). A deletion mutant of JAK3 lacking residues L586 to M592 (the corresponding residues in JAK2 would be L611 to V617) was shown to

inhibit JH1 activity. In the view of the present JAK2 model this finding could be interpreted in such a way that the activated conformation of the activation loop of JH1 in JAK3 is stabilized by the non-mutated  $\beta$ -strand of JAK3 JH2. Another possible interpretation would be that during transition to the activated conformation in the deletion mutant of JAK3 the JH1 activation loop is stabilized in a conformation which is detrimental to kinase activity.

A more general explanation for the influence of JH2 on JH1 activity is provided by the fact that phosphorylation of the tyrosine residues within the activation loop causes a rearrangement in tyrosine kinases (Hubbard, 1997). This rearrangement can be described in terms of a rotation of the N-terminal lobe with respect to the C-terminal lobe. For JAK2-JH1 this rotation could be influenced by the interaction of the JH2 domain with the N- and C-terminal lobes of the JH1 domain. Internal rotation within the JH1 domain would be hindered by the interactions with JH2. This in turn would reduce the activity of the JH1 domain.

In order to test the predictions, one could introduce point mutations in the loop of JH2 which is predicted to interact with the activation loop of JH1. One possibility would be to substitute a cysteine for L1001 or P1002. According to the model, this new residue would then be able to form a covalent bond with C616. Subsequent alteration of the activity of JAK2 would give an indication of the validity of the model described above. Conversely, within the JH2 domain, it would be interesting to evaluate the effect of mutations in the  $\beta$ -strand before C618. According to the present model, one could prepare either deletion mutants between L611 and C618 or introduce point mutations in this  $\beta$ -strand.

Summarizing, the structures of the two carboxy-terminal domains (JH1 and JH2) of human JAK2 were predicted. Comparison of the structures of the two domains revealed some important differences. Overall, the two isolated domains are similar in shape, but crucial differences are apparent in the activation loops. The activation loop of JH1 contains all the features necessary for function, whereas the corresponding loop of JH2 lacks some important amino acids. In JH2 the catalytic base of the catalytic loop is replaced by a neutral residue. The hydrogen pattern, however, appears to be preserved. The nucleotide binding loops of JH1 and JH2 do not show any major differences.

Two different sources of evidence were used for establishing the relative orientation of the two domains. The resulting combination of JAK2-JH1 and JAK2-JH2 yielded some new insights, which could not be derived from the isolated domains. According to the prediction there are two main interactions between JH1 and JH2. The first is between two  $\alpha$ -helices of the two domains. The second concerns a loop between two  $\beta$ -strands of JH2 and the activation loop of JH1. These interactions are predicted to have a significant effect on the kinase activity of JH1, which is in line with experimental evidence that JH2 has a detrimental effect on JH1 activity. Support for the model also comes from the prediction that the activation loop of JH1 in its activated conformation undergoes unfavourable interactions with a  $\beta$ -strand in JH2; this is consistent with the finding that the corresponding residues in JAK3-JH2 have a profound effect on kinase activity.

It may be difficult to verify every structural detail of the model, but on the whole the predictions are consistent with available experimental data on JAK2 or related proteins and these data do not contradict the predictions. We therefore hope

that the model will be used as a working hypothesis for the design of further experiments, which will help to verify or reject it. The predicted structure is intended to serve several purposes. First, it will provide the starting point for further prediction studies of JAK proteins, with the ultimate goal of generating structures for the whole proteins. Second, it should stimulate the design of experiments in order to find out more about the structure and function of these proteins. Also, it could serve as a basis for the design of molecules interacting selectively with JAK2 for therapeutic benefit.

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