Kinetic Study of Human Full-Length Wild-Type JAK2 and V617F Mutant Proteins

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Abstract: The Janus kinase 2 (JAK2) is a drug target in particular because a missense mutation in this gene (V617F) has been identified in various human diseases. We report here the first kinetic study of the human full-length wild type and V617F JAK2 proteins and of their isolated kinase domain. The kinetic parameters of both full-length proteins are similar revealing that the mutation does not affect JAK2 catalytic activity suggesting that it has a more complex role in the regulation of JAK2 activity. Our study also shows that the domains located outside the kinase domain have little influence on JAK2 catalytical activity.

INTRODUCTION

The Janus kinase (JAK) family of non-receptor tyrosine kinases comprises 4 members: JAK1, JAK2, JAK3, and TYK2. These proteins play a crucial role in cytokine signalling and development [1]. The JAK proteins associate with the intracellular region of various cytokine and hormone receptors. The binding of a cytokine to its receptor leads to the activation of JAK proteins, which in turn phosphorylates STAT proteins on specific tyrosine residues. Upon phosphorylation, the STAT proteins dimerize and translocate into the nucleus, where they induce the transcription of target genes. The deregulation of JAK-STAT pathways is at the origin of various human disorders and medicinal chemistry programs are ongoing in order to identify JAK inhibitors at various pharmaceutical companies [2]. JAK2 is considered to be an attractive target for oncology [3], because STAT5, which is a downstream target of JAK2, is deregulated in various types of cancer [4] and translocations in leukemia cause constitutive activation of JAK2 fusion proteins [5]. Furthermore, an activating point mutation (valine-617 to phenylalanine, V617F), identified in the majority of polycythemia vera patients [6] and occurring at high frequency in patients with essential thrombocythemia and primary myelofibrosis [7], has been shown to be sufficient to recapitulate most aspects associated with these diseases in mouse models.

JAK2 is constituted from the N- to the C-terminus of a FERM, a SH2-like, a pseudokinase, and a kinase domain [8]. The pseudokinase domain plays an important role in JAK2 autoinhibition [9] and, interestingly, the V617F mutation is located within this domain. Immunoprecipitation experiments from HeLa cells transfected with JAK2 V617F have shown that this protein has an enhanced kinase activity as measured by Western blot [10] suggesting an active role of this mutant in some human diseases.

The purification of full-length JAK2 protein has been reported in the literature [11, 12], but the kinetic properties of this enzyme have not been measured. Furthermore, the comparison of the kinetic properties of human full-length wild-type JAK2 and V617F mutant has never been described. In this report we describe the kinetic study of the recombinant human full-length wild-type JAK2 and full-length JAK2 V617F proteins and of their isolated JAK2 kinase domain (called hereafter fl wt JAK2, fl V617F JAK2, and JAK2 KD, respectively).

MATERIAL AND METHODS

Materials

Pyridone 6 and CP-690,550 can be purchased from Calbiochem and Cardiff Chemicals, respectively. Plasmids pAcG2TtevJAK2 FL WT, pFast-HTB JAK2 FL V617F, and pAcG2TtevJAK2 KD (aa 840-1132) encoding for Glutathione S-transferase (GST)- and Histidine (His)-tagged human fl wt, fl V617F, and JAK2 KD, respectively, were kindly provided by Michael Eck (Dana-Farber Cancer Institute, Boston).

Cloning and Generation of Baculoviruses

The GST-tagged variant pFastBac::GST2-JAK2 FL V617F was generated by replacing the His-tag in pFast-HTB JAK2 FL V617F by the GST-tag isolated from pFastBacGST2 (Invitrogen). The constitutively active full-length L226C EpoR gene in pFastBac::EpoR L226C was generated with the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) with an EpoR cDNA template from a Novartis cDNA collection following the manufacturer's instructions. All DNA constructs were sequences verified. Generation of baculovirus DNAs, transfections, virus amplifications and plaque assays was performed according to the manual for the Bac-to-Bac Baculovirus Expression System (Invitrogen).

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Protein Expression and Purification

Full-length JAK2 proteins. The proteins were co-expressed with EpoR L226C in *Spodoptera frugiperda* Sf9 cells for 48 h at 27 °C. Suspension culture cells were infected at a density of $1x10^6$/ml and the multiplicity of infection for each virus pair was optimised. Prior storage at -80°C, the cell pellets were pre-incubated as described by Ma *et al.* [12]. All purifications steps were carried out at 4 °C. The cells were lysed in lysis buffer (50 mM Tris.HCl pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM DTT, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1xEDTA-free complete protease inhibitors (Roche Diagnostics), and 12.5 U/ml Benzonase (Novagen)) for 30 min and centrifuged. Glutathione Sepharose 4B beads (GE Healthcare) were incubated for 4 h with the soluble fraction of the lysate and washed with buffer A (50 mM Tris.HCl pH 8, 120 mM NaCl, 0.5 mM reduced L-Glutathione). The JAK2 proteins were eluted with buffer B (buffer A with 5 mM L-Glutathione). Glycerol (10% final) was added and the proteins concentrated (Amicon Ultra-15 column (Millipore), 100000 molecular weight cut-off). Brij-35 was added (0.1% final) before storage at –80 °C.

JAK2 KD protein. All purifications steps were carried out at 4 °C. The *Spodoptera frugiperda* Sf9 cells were lysed with 12 ml lysis buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1xEDTA-free complete protease inhibitors, and 12.5 U/ml Benzonase). The soluble fraction of the lysate was incubated with Glutathione Sepharose 4B beads for 2 h. The beads were washed with buffer C (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM DTT, and 10% glycerol) and JAK2 KD eluted with buffer D (buffer C with 10 mM reduced L-glutathione). The eluted protein was concentrated by centrifugation and Brij-35 (0.1% final) was added before storage at –80 °C.

Protein concentration and purity was determined by Bradford assay and SDS-PAGE analysis, respectively.

Peptide Synthesis

Peptides were synthesized starting with the first amino acid being attached to the CLEAR resin (Peptides International, Louisville, USA) and using fluorenylmethoxycarbonyl-strategy in combination with 2,4,5-trichlorophenyl ester activation. The purity of the peptides was determined by analytical HPLC.

Radiometric Filter-Binding Assay

Kinase assays were carried out at room temperature (23-25 °C) in 96-well plates (Brand). The reactions were initiated by the addition of the enzymes (JAK2 KD: 0.2 to 1 ng; full-length proteins: 5 ng) into solutions containing the indicated amounts of peptide and ATP diluted in reaction buffer (50 mM Tris.HCl pH 7.5, 1 mM DTT, 0.05 mg/ml BSA, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.01% Brij-35, and 0.9 μCi $^{33}\gamma$P-ATP (GE Healthcare)). The reactions were run for 10 min and stopped by the addition of stop solution (0.125 M EDTA pH 8). The solutions were transferred onto pre-activated filter plates (MultiScreen-IP plates, Millipore), the membranes were washed twice with 0.5% phosphoric acid and dried at room temperature. Microsint 40 (Perkin Elmer) was dispensed in each well and the bound radioactivity was counted in a TopCount NXT (Packard). When more than 15 μM peptide was present in the assay, the solutions were diluted in stop solution before loading to the membrane to prevent filter saturation.

For IC_{50} determinations, the compounds diluted in DMSO (1% final) were incubated for 10 min at room temperature with the enzymes, peptide (15 μM for JAK2 KD and 8 μM for full-length proteins), and $^{33}\gamma$P-ATP/ATP (0.45 μM). The reactions were stopped by the addition of stop solution and the bound radioactivity measured.

Data Analysis

The kinetic data from experiments carried out at saturating concentration of one substrate were analysed by non-linear regression analysis with the equation:

$$v = \frac{V_{\max}^{app}[S]}{K_m^{app} + [S]}$$

where v is the rate of the reaction, V_{max}^{app} is the apparent maximal rate, [S] is the concentration, and K_m^{app} the apparent Michaelis constant of the varied substrate.

When both substrate concentrations were varied the following equation was used:

$$v = \frac{V_{\max}[ATP][P]}{\alpha K_{ATP} K_P + \alpha K_P[ATP] + \alpha K_{ATP}[P] + [ATP][P]}$$

where v is the rate of the reaction, V_{max} is the maximal rate, [ATP] and [P] are the ATP and peptide concentrations, $K_{(ATP)}$ and $K_{(P)}$ are the dissociation constants for ATP and peptide. α defines the degree of regulation of the 2 substrates for each other [13].

Competition experiments with products or dead-end inhibitors were analysed with the equations for linear competitive, uncompetitive, and mixed inhibition [13]. Statistical analyses (F test and Akaike's information criterion) were realised to identify the best fitting model.

RESULTS AND DISCUSSION

JAK2 and its mutated form, V617F, are thought to play an important role in several haematological disorders and many efforts are being made to understand their cellular function and to identify inhibitors of these enzymes [2]. In sharp contrast to these efforts, little has been done to characterize these two proteins at the enzymatic level. However, the study of the enzymatic properties of fl wt and V617F JAK2 proteins is of interest for several reasons. First, the kinetic properties of the V617F mutant protein and wt JAK2 have never been compared and therefore the effect of this mutation on JAK2 catalytical activity is not known. Second, a deeper knowledge of JAK2 kinetic properties will allow a more detailed mechanistic understanding of JAK2 inhibitors. Finally, the availability of the purified fl JAK2 permits the initiation of drug discovery strategies aiming at the identification of inhibitors that bind to regions of the protein located outside its kinase domain, a strategy successfully used for other protein kinases [14]. To this end, we decided to study the kinetic properties of human fl wt JAK2, fl V617F JAK2, and JAK2 KD.

The ability of the three proteins to phosphorylate different peptidic substrates was tested (Fig. **1A**). In agreement with previous results, JAK2 proteins phosphorylate the STAT1 and STAT5 peptides [9, 11] and, as shown here, the STAT3 and IRS1 peptides (Fig. **1B**). The three proteins phosphorylate these short synthetic substrates in a similar manner indicating that, within our experimental conditions, they do not have a different substrate selectivity.

The V617F mutation could, in the context of the full-length protein (allosteric effects), affect the binding properties of JAK2 inhibitors. The potency (IC_{50}) of two known JAK specific inhibitors, CP-690,550 [15, 16] and pyridone 6 [17] was therefore determined. Similar IC_{50}s were measured indicating that neither the V617F mutation nor the regions located outside the kinase domain affect the potency of these two compounds.

Kinetic experiments were carried out at saturating concentration of one substrate varying the concentration of the other. Similar kinetic parameters were measured with both full-length proteins in the presence of two different peptidic substrates (IRS1 and STAT5) (Table **1**). This shows for the first time that the V617F mutation does not directly affect the kinetic properties of the human full-length JAK2 enzyme suggesting a more complex effect of this mutation on JAK2 properties (e.g., role in the activation mechanism of JAK2). The kinetic parameters of JAK2 KD are, except for V_{max}^{app}, similar to the ones of fl JAK2 indicating that the FERM, SH2-like, and pseudokinase domains have a minimal effect on JAK2 catalytic activity.

Since both fl wt and V617F proteins behave in a similar fashion, we focused our efforts on the fl wt JAK2 and JAK2 KD proteins. Initial rates were measured from experiments where both ATP and STAT5 peptide concentrations were varied. Double reciprocal plots of initial velocities versus either ATP or STAT5 peptide concentration are linear and display a pattern of intersecting lines at a single point (Fig. **2A**). This shows that these two enzymes follow a sequential mechanism where both substrates bind to the enzyme prior to the release of products. Competition experiments with dead-end inhibitors (STAT5 peptide with Tyr694 replaced by phenylalanine (F-STAT5) and adenosine 5'-(β-γ-imido) triphosphate (ADPNP)) and product inhibitors (adenosine diphosphate (ADP) and a STAT5 peptide with phosphorylated Tyr694 (P-STAT5)) were conducted. ADP and ADPNP are competitive inhibitors for ATP and mixed inhibitors for STAT5 peptide (Fig. **2B** and **C**). No reproducible inhibition profiles were obtained with F-STAT5 and P-STAT5 peptides because they are too weak inhibitors. Three possible mechanisms can be proposed: a random Bi Bi mechanism and an ordered Bi Bi or a Theorell-Chance mechanism with ATP binding first to the free enzyme and ADP released last. Fig. (**2A**) shows that the lines intersect on the abscissa suggesting that there is little interaction between both substrates therefore favouring a random Bi-Bi mechanism. Assuming such mechanism, the kinetic parameters for fl wt JAK2 and JAK2 KD were determined (Table **2**). α is close to 1 for both enzymes and $\alpha K_{(ATP)}$ (JAK2 KD: 0.56 μM; fl wt JAK2: 0.4 μM) and $\alpha K_{(STAT5)}$ (JAK2 KD: 93 μM; fl wt JAK2: 42 μM) values are in very good agreement with the $K_{m(ATP)}^{app}$ and $K_{m(STAT5)}^{app}$. This together with the data presented on Table **1** show that the regions located outside the kinase domain have little influence on JAK2 catalytical activity. The reaction mechanism of several protein kinases has been recently re-

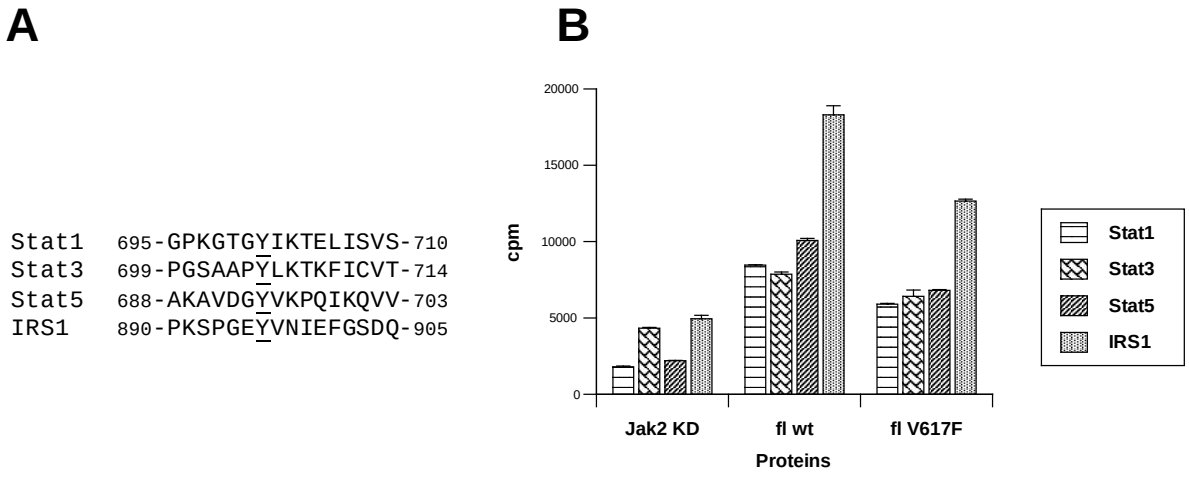

Fig. (1). Peptidic substrates of JAK2. **A.** Primary sequence of the peptide substrates. Numbers: position of first and last residue in the primary sequence of the corresponding human protein. Phosphorylation sites are underlined. **B.** Kinase activity of the JAK2 proteins measured with different peptide substrates. [Peptide] = 15 μM; [ATP] = 0.45 μM.

Table 1. Inhibition of JAK2 Proteins and Kinetic Parameters Determined at Saturating Concentration of one Substrate

Proteins	IC_{50} (nM) CP-690,550	IC_{50} (nM) Pyridone 6	V_{max}^{app} (pmol/min/ng)	$K_{m(ATP)}^{app}$ (μM)	$K_{m(IRS1)}^{app}$ (μM)	$K_{m(STAT5)}^{app}$ (μM)
JAK2 KD	1.5±0.1	1.5±0.3	0.23±0.07	0.415±0.007	15±1	82±13
fl wt JAK2	2±0.6	2.5±0.3	0.03±0.01	0.48±0.04	8.5±0.2	48±11
fl V617F JAK2	2.5±0.6	2.3±0.1	0.012±0.002	0.4±0.1	8±1	45±12

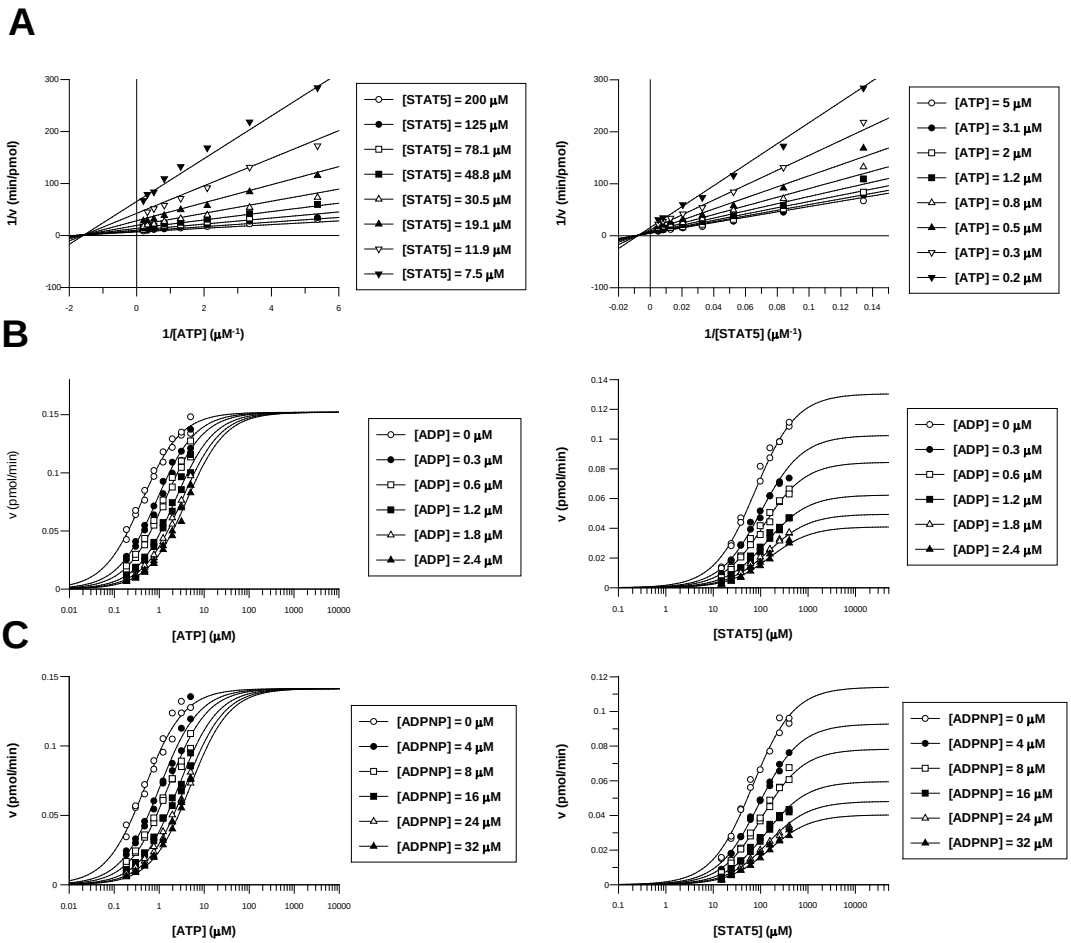

Fig. (2). Two-substrate steady-state kinetics of fl wt JAK2 and JAK2 KD and inhibition studies. **A.** Double-reciprocal plots from the initial velocity experiments presented in A. Left panel plot 1/v versus 1/[ATP]; right panel plot 1/v versus 1/[STAT5]. Inhibition by ADP (**B**) and ADPNP (**C**) with varied ATP (left panel) and peptide (right panel). A logarithmic scale is used to allow an easier visualization of the effect of the inhibitor on both V_{max} and K.

viewed [14]. This published survey shows that the vast majority of the protein kinases follows, as does JAK2, a random Bi Bi mechanism. Therefore the mechanism proposed here for the JAK2 protein is not unusual for protein kinases. However, it should be kept in mind that the substrates usually utilized in biochemical assays are short synthetic peptides and not large full-length proteins. It would therefore be very interesting to determine the kinetic mechanism of the JAK2 protein when a larger substrate, for example the full-length STAT5 protein, is used in the assay.

Table 2. Kinetic Parameters of fl wt JAK2 and JAK2 KD

Proteins	V_{max} (pmol/min/ng)	$K_{(ATP)}$ (μM)	$K_{(STAT5)}$ (μM)	α
JAK2 KD	0.40 ± 0.01	0.68 ± 0.06	113 ± 7	0.83 ± 0.01
fl wt JAK2	0.025 ± 0.04	1 ± 0.1	105 ± 5	0.4 ± 0.1

In summary, we have studied the kinetic properties of fl wt JAK2, fl V617F JAK2, and JAK2 KD proteins. The two full-length proteins have similar kinetic properties revealing that the V617F mutation does not affect the catalytic activity of the JAK2 protein. This suggests that the disease relevant gain-of-function mutation confers an advantage by a mechanism that it is not purely intramolecularly linked to an alteration of its enzymatic properties. The purification of human fl JAK2 opens also the opportunity to identify compounds that inhibit JAK2 by binding to regions located outside the kinase domain. Such compounds have already been identified for other protein kinases, as for example AKT [18].

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