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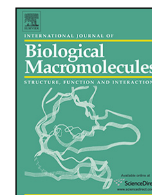

Effects of the I682F mutation on JAK2's activity, structure and stability

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ABSTRACT

Janus kinase 2 (JAK2) plays important roles in the regulation of varieties cellular processes including cell migration, proliferation and apoptosis. JAK2 I682F genetic mutation existed in the 4–8% of B-cell acute lymphoblastic leukemia (B-ALL). However, roles of this mutation in the development of B-ALL are still unknown. In order to investigation the mechanism of the JAK2 I682F mutation led to B-ALL, series of mutations were constructed. Mutations I682F, I682G, I682D and I682S significantly increased JAK2's activity and decreased its structural stability, while the I682L mutation almost had no effect on JAK2's activity and structural stability. Furthermore, the spectroscopic experiments implied that mutations I682F, I682G, I682D and I682S impaired the structure of JAK2 JH2 domain, and led JAK2 to the partially unfolded state. It may be this partially unfolded state that caused JAK2 I682F constitutive activation. This study provides clues in understanding the mechanism of the JAK2 I682F mutation caused B-ALL.
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1. Introduction

Janus kinase 2 (JAK2) is a non-receptor tyrosine kinase. It has been implicated in signaling by the members of type II cytokine receptor family (e.g., interferon receptors), the GM-CSF receptor family (IL-3R, IL-5R and GM-CSF-R), the gp130 receptor family (e.g., IL-6R), and the single chain receptor (e.g., Epo-R, Tpo-R, GH-R, PRL-R) [1]. On cytokine stimulating, activated JAK2 subsequently turns on downstream signaling pathway such as signal transducer and activator of transcription 5 (STAT5) and leads to the proliferation and differentiation of the hematopoietic cells [2]. There are many JAK2 mutations detected in the hematological malignancies, such as the JAK2 V617F mutation for MPN [3,4] and JAK2 R683S (G) mutations for B-cell acute lymphoblastic leukemia (B-ALL) [5–8]. Our previous studies indicated that JAK2 R683S (G) mutations disrupted the JH1/JH2 domain interactions, activated JAK2-STAT5 signal pathway and finally led to the development of B-ALL [9]. Thus, JAK2 might be a potential therapeutically target to design specific inhibitors for diseases caused by the deregulation of down stream signaling pathway [1,2,5–8].

JAK2 is a multidomain protein possessing seven conserved JAK homology (JH) domains 1–7 [10]. The JH1 domain is a highly conserved kinase domain. The JH2 domain which was presumed to be a pseudokinase is proven to be a kinase now [3,4]. The JH2 domain phosphorylates amino acid residues Ser523 and Tyr570 and plays vital roles in keeping JAK2 in low activity [3,4]. Previous studies indicated that domain–domain interactions were important for keeping the correct conformation of multidomain proteins [11–13]. Lindauer et al. [14] implies that the JH2 domain interacts with JH1 domain and holds the JH1 domain in a closed, inactive conformation. Our previous studies also suggested that the JAK2 C618R mutation led to JAK2 activation in the absence of ligand by disrupting the JH1/JH2 domain interactions [15]. Moreover, Bandaranayake et al. [4] also indicated that the JAK2 V617F mutation rigidified the α-helix C in the N lobe of JH2 domain, which facilitated the *trans*-phosphorylation of the JH1 domain. Further studies revealed that domain–domain interactions also involved in the JH2 domain dimerization both in pre-dimerized cytokine receptors and after receptor rearrangement. Thus, these studies suggest that the correct conformation of JH2 domain is essential for JAK2 autoinhibition.

Abbreviations: JAK, Janus kinase; JH, JAK homology; STAT5s, ignal transducer and activator of transcription 5; B-ALL, B-cell acute lymphoblastic leukemia; MPDs, myeloproliferative disorders; ALL, acute lymphoblastic leukemia; WT, Wild type; ANS, 1-anilino-8-naphthalenesulfonic acid; E_{max}, the emission maximum of the intrinsic fluorescence.

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To achieve their functions, domains of the mutidomain proteins are connected sequentially by the linker in the primary structure. In some cases, the sequences of linkers do not contribute to the structural stability and activity of these proteins [16], whereas in others, linkers may participate in the folding or function of these proteins [17,18]. However, little is known about roles of the amino acid residues located at the linker in JAK2's folding, activity and structural stability. Recent studies suggest that the JAK2 I682F mutation is identified in approximately 3–5% of the high risk B-ALL in children [5–8]. According to the crystal structure of JH2 domain, amino acid I682 located in the linker between the N and C lobes of the JH2 domain [3,4]. Due to the vital roles of the linker in protein functions [17,18], the amino acid residue I682 might be important for keeping the structural conformation of the JH2 domain. However, the mechanism of I682F mutation caused JAK2 hyperactivation is still unclear.

In this research, series of the amino acid residue I682 mutations were constructed to investigate roles of the linker between the N and C lobes of JH2 domain in JAK2's activity, stability and folding. Our study suggested that the amino acid residue I682 played an important role in JAK2's activity, structural stability and folding. Mutation I682F disrupted the compact structure of the JH2 domain, significantly increased JAK2's activity and decreased its structural stability. Furthermore, this mutation also led JAK2 to the partially unfolded state and JAK2 constitutive activation. Thus, these results herein might provide clues in understanding the mechanism of the JAK2 I682F mutation caused B-ALL.

2. Materials and methods

2.1. Cloning, site-directed mutagenesis and expression of the mutant JAK2

The pFastHTB plasmid with human cDNA (pFastHTB-human WT-JAK2) inserted was used as a template for mutagenesis [9,15].

All mutations (I682F, I682G, I682D, I682L and I682S) were obtained by the site-directed mutagenesis (Stratagene Technologies). Then mutant JAK2s were cloned into the pFastHTB plasmid, sequenced and transformed into the DH10Bac^{TM} *Escherichia coli* to obtain recombinant Bacmid DNA, respectively. The recombinant Bacmid DNAs were used to transfect Sf9 insect cells to generate viruses. And then the recombinant enzymes were purified from baculovirus-infected Sf9 cells using the NTA-Ni resin as described previously [19].

2.2. Enzyme assay and the determination of kinetic parameters

The JAK2's activity was assayed as described previously with little modifications [19]. The reaction mixture was incubated at 30 °C, and then reactions were stopped by adding an equal volume of 20 mM EDTA. An aliquot of the reaction mixture was spotted on to the P80 filter paper. After extensive washing with 0.1% phosphoric acid solution, bound radiolabeled phosphate was counted using a scintillation counter. The K_m^{STAT5}, K_m^{ATP} and protein concentrations were determined as described previously [9]. All the reactions were carried out at least three times.

2.3. Thermal stability of JAK2

The thermal stability of the WT and mutant JAK2s were determined by activity assay after being incubated at given temperatures. The enzyme solutions were incubated at given temperatures varying from 25 to 65 °C for 10 min, then cooled on ice and the activity was measured at 30 °C. The data were normalized to JAK2's activity measured at 25 °C. The aggregation of JAK2s at a given temperature was monitored by measuring the turbidity at 400 nm. The final protein concentrations of JAK2s were 2.3 μM.

2.4. Unfolding and refolding experiments

For the unfolding experiment, the WT and mutant JAK2s were added to the standard buffer (10 mM Tris–HCl, pH 8.0) with different concentrations of GdnHCl dissolved for 24 h at equilibrium state. The refolding experiment was initiated by diluting denatured JAK2s into the standard buffer with final GdnHCl concentrations ranging from 0 to 3 M. The intrinsic fluorescence of JAK2s folding was collected on an F-4500 spectrofluorometer using a 1-cm path-length cuvette. For the ANS-fluorescence measurements, 50-fold molar excess of ANS was added to samples. Then samples were equilibrated for 30 min in the dark, and extrinsic fluorescence was measured on an F-4500 spectrofluorometer. Far-UV circular dichroism (CD) spectra were recorded on a Jasco 715 spectrophotometer with a 1 mm path-length cell. All the experiments were carried out at 25 °C.

2.5. Parameter *A* and phase diagram analysis of the intrinsic fluorescence data

To more clearly characterize effects of mutations on JAK2s folding, Parameter *A* and phase diagram analysis were used to compare the folding pathway of the WT and mutant JAK2s. Parameter *A*, which reflects the spectral shape of intrinsic Trp fluorescence [20], was obtained by dividing the fluorescence intensity at 320 nm (I_{320}) by the intensity at 365 nm (I_{365}) during unfolding and refolding process. The "phase diagram" analysis which is a sensitive tool to detect folding intermediates was carried out as described previously [21]. In the phase diagram, each straight line in the phase diagram reflects an "all-or-none" process, and the joint position of two lines indicates that an intermediate appeared at the corresponding GdnHCl concentration.

2.6. Modeling the structure of the WT and mutant JAK2s

The location of the amino acid residue I682F in JAK2's JH2 domain was determined by using the crystal structure of JAK2 JH2 domain (PDB: 4FVP) [3,4]. For the full-length homology model of JAK2, multiple sequence alignment was firstly performed using the program ClustalW and the 3D structure of JAK2 was obtained by the software Modeller 9.11 [22] and AutoDock 4.2 [23]. Then simulations were performed using the NAMD analysis package developed by the Theoretical and Computational Biophysics Group at the University of Illinois at Urbana-Champaign [24]. High hydrophilic cavities inside the protein were filled with water molecules using the program DOWSER [24], and the protein was enclosed in a water box with 10 Å padding. Sodium and chloride ions were added to neutralize the system. All simulations were performed using the CHARMM force field [25] with particle mesh Ewald electrostatics and periodic boundary conditions. All the structure of the WT and mutant JAK2s were created as described previously [3,4,9].

3. Results and discussion

3.1. Kinetic parameters analysis

The recombinant WT JAK2 showed similarly enzymatic characteristics to that of the native JAK2, indicating that the N-terminal His_6-tag had no effect on its activity (Table 1).

In order to detect effects of mutations on JAK2's activity, kinetic parameters were determined. As shown in Table 1, the activity of I682F, I682G, I682D and I682S mutant JAK2s was 5–6 folds higher than that of the WT JAK2, while the K_m^{STAT5} values of these mutations

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