



**GENERATION OF K581A
MUTATION AND PRODUCTION OF
RECOMBINANT JAK2 PROTEIN**

Bachelor's Thesis
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TAMPEREEN AMMATTIKORKEAKOULU
Tampere University of Applied Sciences

ABSTRACT

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HOVINEN, EMILIA:

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Protein kinases are a large group of enzymes that play a major role in regulating signalling processes, DNA replication and transcription, cell differentiation and carbohydrate and lipid metabolism. JAKs are members of the Janus kinase family belonging to the group of non-receptor tyrosine kinases. The research group, led by Olli Silvennoinen, investigates the JAK-STAT signaling pathway, which regulates gene expression by mediating extracellular signals to nucleus *via* cell-surface receptors. Several cytokines and hormones activate the JAK-STAT pathway and its dysfunction affect the cell's ability to differentiate, proliferate, develop and survive. The objective of this study was to investigate the solubility, structure and function of kinase domain (JH1) and pseudokinase domain (JH2) of JAK2 protein. The purpose was to generate a kinase inactivating mutation K581A in the pseudokinase domain and produce recombinant JAK2 protein in Baculosystem.

JAK2 is an essential protein in erythropoiesis and myeloid differentiation. Mutations in JAK2 affect its structure and function in the JAK-STAT pathway, leading to myeloproliferative diseases. The production of inactive and soluble proteins has shown to be a difficult task. In this study, bac-to-bac expression system was used to produce desired recombinant proteins. Two different sizes of mutated DNA -constructs were transformed into competent DH10Bac cells for generating recombinant bacmid DNA, which was isolated and transfected into Sf9 cells. Fresh insect cells were infected with the baculovirus particles harvested from media. Western blot results indicate that the insect cells were able to express the mutant JAK2 -protein but not in soluble form.

The aim of the thesis was to produce stable and soluble protein which can be used in structural studies of two-domain JAK2. The mutations, K581A and D976N together could have interfered with the stability of the JAK2 and induce the protein insoluble. The catalytic activity of JAK2 is regulated by tyrosine residue phosphorylation in the kinase domain and only the crystal structures of pseudokinase (JH2) and kinase (JH1) domain have been resolved. In the future, more recombinant protein production is required for X-ray crystallography studies which will give more information on the three-dimensional structure and catalytic activity of JAK2 and allow the development of new therapeutic drugs for a trial. The constructs can be used again to produce high amounts of recombinant proteins, which can be purified for structural studies.

Key words: Recombinant protein, JAK2, K581A, phosphorylation, molecular cloning, Baculovirus system

TIIVISTELMÄ

Tampereen ammattikorkeakoulu
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HOVINEN, EMILIA:

K581A -mutaation luominen ja rekombinantin JAK2-proteiinin tuotto

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JAK-kinaasit ovat entsyymejä, joilla on tärkeä rooli solusignaloinnin säätelyssä, DNA:n kahdentumisessa ja transkriptiossa sekä solujen kyvyssä lisääntyä ja erilaistua. Janus kinaasit ovat sytokiinireseptoreihin sitoutuvia tyrosiinikinaaseja, jotka välittävät sytokiinin signaalin solun sisään autofosforylaation sekä solunsisäisten signalointireittien aktivoitumisen kautta. Professori Olli Silvennoisen tutkimusryhmä selvittää JAK-kinaasien säätelymekanismeja immuunivastetta heikentävissä sairauksissa. Sytokiinireseptorien avulla JAK-STAT signaaliketju säätelee geenien ilmentymistä välittämällä solun ulkopuolisia signaaleja tumaan. Useat sytokiinit ja hormonit aktivoivat JAK-STAT signaaliketjua. Häiriöt signaaliketjun toiminnassa vaikuttavat solun kykyyn lisääntyä, erilaistua, kehittyä ja selviytyä.

JAK2-proteiinilla on tärkeä tehtävä erythropoiesissa ja myeloidisten solulinjojen erilaistumisessa. JAK2:ssa esiintyvien mutaatioiden on todettu aiheuttavan rakenteellisia ja toiminnallisia muutoksia proteiinissa sekä johtavan erilaisiin myeloproliferatiivisiin sairauksiin. JAK2:n biologista ja rakenteellista tutkimusta on hidastanut sen epästabiili rakenne, minkä vuoksi proteiinista haluttiin mutaatioiden avulla inaktiivinen. Opinnäytetyön tarkoituksena oli luoda JAK2:n pseudokinaasiosaan eli JH2-domeeniin inaktivoiva K581A-mutaatio ja tuottaa rekombinanttia proteiinia bakulovirussysteemissä. Työn tavoitteena oli analysoida tuotettujen JAK2-domeenien liukoisuutta sekä tutkia niiden kolmiulotteista rakennetta ja toimintaa. Proteiinia tuotettiin *Bac-to-Bac® Baculovirus Expression System* –tuottosysteemillä. Kompetentit *E. coli*-solut transformoitiin mutatoidulla plasmidilla rekombinantin bakmidin aikaansaamiseksi, minkä jälkeen bakmidi-DNA eristettiin. Sf9-hyönteissolulinja transfektoitiin eristetyllä ja puhdistetulla bakmidi-DNA:lla, ja solut alkoivat tuottaa haluttua rekombinanttiproteiinia.

Hyönteissoluissa tuotetut proteiinit analysoitiin Western blot -menetelmällä. Solut kykenivät ilmentämään haluttua proteiinia, mutta molemmat JAK2-konstruktit osoittautuivat liukenemattomiksi. Liukoisten ja inaktiivisten proteiini-osien tuottaminen laboratorioissa on osoittautunut haastavaksi. Ilmeisesti K581A- ja D976N-mutaatioiden samanaikainen läsnäolo häiritsevi tutkittavan proteiinin stabiilisuutta ja aiheutti sen liukenemattomuuden. Mutatoituja konstruktia voidaan jatkossa tuottaa uudelleen esimerkiksi jollakin toisella tuottosysteemillä, minkä jälkeen proteiinia voidaan puhdistaa rakenteellista tutkimusta varten.

Avainsanat: rekombinanttiproteiini, JAK2, K581A, fosforylaatio, DNA-yhdistelmätekniikka, bakulovirussysteemi

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ABBREVIATIONS

AB	Antibody
AcNPV	<i>Autographa californica</i> nucleopolyhedrosis virus
ADP	Adenosine diphosphate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Amp ^r	Ampicillin resistance selection gene)
Asp	Aspartic acid
Asn	Asparagine
ATP	Adenosine triphosphate
BEVS	Baculovirus Expression Vector System
BSA	Bovine serum albumin
C	Carboxyl
D976N	mutation in JH1-JH2 domains
D-F-G	Aspartic acid-Phenylalanine-Glycine
D-P-G	Aspartic acid-Proline-Glycine
DH10Bac	Supercompetent cell
DNA	Deoxyribonucleic acid
<i>Dpn-I</i>	Restriction endonuclease
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
EDTA	EthyleneDiamineTetraAcetic acid
ET	Essential thrombocythemia (disorder in MPN)
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FERM	Domain containing kinase domains JH4-JH7
Gen ^r	Gentamycin (a selection gene)
GTP	Guanosine triphosphate
GV	<i>Granulovirus</i> genus
H-R-D	Histidine-Arginine-Aspartic acid
IBT	Institute of Biomedical Technology
IgG	Immunoglobulin G, antibody isotype
IgG-HRP	IgG conjugated to Horseradish peroxidase
IPTG	Isopropyl beta-D-thiogalactopyranoside

JAK1	Janus Kinase 1
JAK2	Janus Kinase 2
JAK3	Janus Kinase 3
JH1-JH7	JAK homology domains
JH1	Kinase domain (homology domain)
JH2	Pseudokinase domain (homology domain)
kDa	Kilodalton
K581A	Kinase-inactivating point mutation in domain JH2
K882	Mutation in domain JH2
LB	Luria broth medium
MCS	Multiple cloning site
mini-attTn7	Mini Tn7
MPN	Myeloproliferative neoplasms
N	Amino
NPV	<i>Nucleopolyhedrovirus</i>
NRTK	Non-receptor tyrosine kinase
NZY	Reagent containing NZ Amine-A (casein hydrolysate)
ORI	Origin for DNA replication
P0	Passage zero virus
P1	Passage one viruses
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pFastBac1™	Donor vector
PK	Protein kinase
PMF	Primary myelofibrosis (disorder in MPN)
PMSF	Phenylmethylsulphonyl fluoride
PTK	Protein tyrosine kinase
pUC19	Plasmid vector
PV	Polycythemia vera (disorder in MPN)
RNAse	Ribonuclease
RTK	Receptor tyrosine kinase
S.O.C	Nutrient media
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser523	Amino acid serine, residue 523

Sf9	<i>Spodoptera frugiperda</i> , insect
SH2	Src homology-2
Sol	Solution
ssDNA	single stranded DNA
STAT	Signal Transducer and Activators of Transcription
TAE	Buffer containing a mixture of Tris base, acetic acid and EDTA
Taq	Thermostable DNA polymerase named after bacterium <i>Thermus aquaticus</i>
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
Tn7L, Tn7R	Left and right ends of transposon Tn7
Tris	Tris (hydroxymethyl) aminomethane
TYK2	Tyrosine kinase 2
Tyr570	Amino acid tyrosine, residue 570
V671F	Mutation in JAK2
V-A-I-K	Valine-Alanine-Isoleucine-Lysine
Y1007, Y1008	Tyrosine residues
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
XL1-Blue	Supercompetent cell

1 INTRODUCTION

This Bachelor's thesis was carried out in the research group of Professor Olli Silvennoinen at the Institute of Biomedical Technology (IBT), University of Tampere. The thesis was supervised by Daniela Ungureanu, PhD. Olli Silvennoinen's group is focused on cytokine signalling research, trying to understand the mechanism of cellular and molecular regulation of the JAK-STAT pathway.

JAK2 is a key protein involved in erythropoiesis and myeloid differentiation. Expressing and purifying JAKs has been difficult and mutations in JAK2 that are gain-of-function mutations, lead to many forms of myeloproliferative disorders. One of the most known is V617F mutation that induces polycythemia vera. Therefore, understanding the mechanism of JAK2 regulation is very important, offering the right platform for developing disease treatments.

The objective of this thesis is to analyze the solubility and biological function of recombinant JAK2 protein produced in Baculo system. The purpose is to mutate JH1-JH2 domains of JAK2 protein and express the mutated recombinant protein in insect cell line.

2 THEORETICAL BACKGROUND

2.1. Protein kinases

Protein kinases (PKs) are a large group of enzymes which catalyze transfer of a phosphate group, usually from ATP, to the hydroxyl group of tyrosine, threonine or serine residue of their substrates (*Choreschi K. et al. 2009*). Vertebrate genome consists of approximately 2000 different protein kinase enzymes. Protein kinases play an inevitable role in regulating signalling processes, DNA replication and transcription, cell differentiation and carbohydrate and lipid metabolism. Protein kinases are divided in two major classes based on which substrate they phosphorylate (Figure 1, p. 11). Protein kinases phosphorylating serine or threonine residues are called serine protein kinases (PSKs), and protein kinases phosphorylating tyrosine residues form a group of tyrosine protein kinases (PTKs). In addition to these two classes, a smaller group called dual-specific proteins kinases are able to phosphorylate serine and threonine residues of their substrates. (*Adams J. A. 2001*)

During the recent years especially the conserved kinase domain has been intensively studied for its nature to stay as conserved domain through the evolution. Despite the fact that protein kinases have structural and regulatory differences, they all contain a highly conserved kinase domain which conveys the phosphotransfer reaction (*Engh R.A. & Bossemeyer D., 2001*). All kinases have a catalytic domain or a kinase domain which is approximately 300 amino acids long. There are 518 kinases in human genome, 48 kinases which contain a pseudokinase and only 4 kinases containing both a pseudokinase and a tyrosine kinase domain. (*Haan C. et al., 2010*)

2.1.1 Protein tyrosine kinases

Protein tyrosine kinases (PTKs) are enzymes that catalyze the transfer of the terminal phosphate of ATP to a specific tyrosine residue in a protein. Protein tyrosine kinases form two subfamilies; *receptor tyrosine kinases (RTKs)* and *non-receptor tyrosine kinases (NRTKs)*. RTKs consist of an intracellular tyrosine domain, a transmembrane domain and an extracellular ligand-binding domain. RTKs are transmembrane

glycoproteins that become activated as a result of the binding of their ligands. The binding ligand can for instance be a cytokine or a hormone. NRTKs are soluble, cytoplasmic kinases. They do not contain any transmembrane domains to act as receptors, but they interact *via* SH2 or SH3 domains. The JAKs belong to NRTKs and are non-covalently associated with the cytoplasmic domain of their corresponding receptor. (Hubbard S.R. *et al.*, 2000)

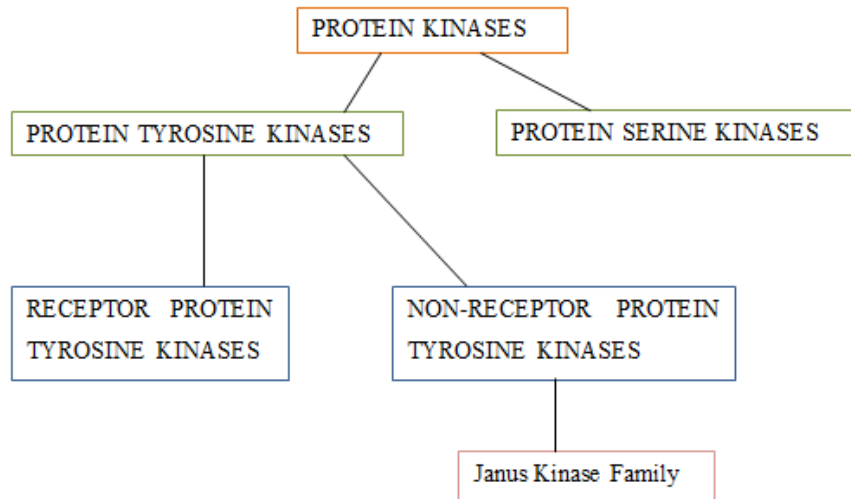


FIGURE 1. A Simple depiction of the protein kinases. (Derived; Adams J. A. 2001)

2.2. Protein phosphorylation

Amino acids serine, threonine and tyrosine can form phosphate esters, where the phosphate binds covalently to a hydroxyl group of the residues. Phosphorylation (Figure 2, p.12) affects the structure and catalytic activity of an enzyme and binding of the substrate. When a phosphate is removed by protein phosphatase, dephosphorylation occurs and on the contrary when a phosphate is attached by protein kinases, phosphorylation takes place. The energy for phosphorylation is usually obtained from ATP which donates the phosphate and forms ADP. (Campbell M.K. & Farrel S.O., 2008)

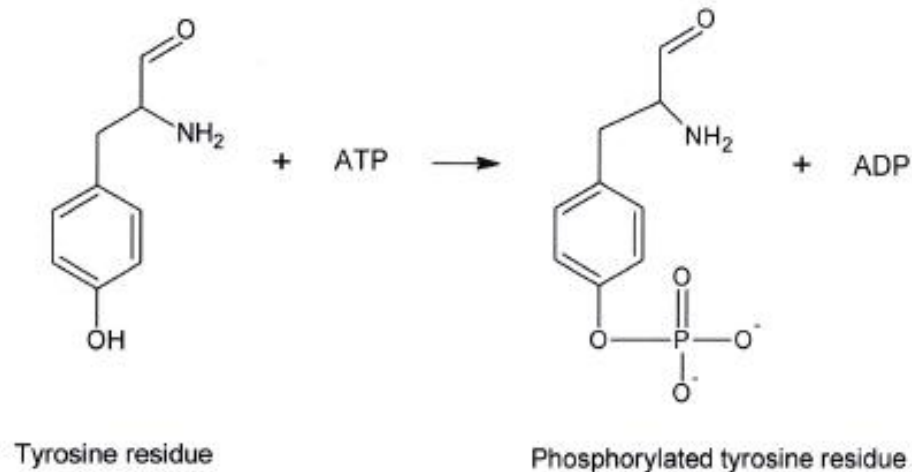


FIGURE 2. Amino acid tyrosine becomes phosphorylated when a phosphate binds to a hydroxyl group on the tyrosine residue (Modified; Campbell M.K. & Farrel S.O., 2008)

Phosphorylation plays an essential role in transferring signals from the outside to the inside of a cell and regulates various cellular processes such as growth, motility, metabolism, proliferation and differentiation (*Walker J.M., 2009*). The phosphorylation of the protein kinases requires a divalent metal ion (Mg^{2+}) which helps the binding of ATP (*Adams, J. A. 2001*). The metal ion acts as one of the enzyme substrates and binds either with ATP or GTP and chelates the β - and γ -phosphoryl groups (*Matte A. et al., 1998*).

2.3. The JAK-STAT pathway

The JAK-STAT signaling pathway (Figure 3, p.14) regulates gene expression by conveying extracellular signals to nucleus *via* cell-surface receptors. STATs (signal transducers and activators of transcription) are latent gene regulatory proteins which are normally inactive and located in the cytosol. (*Alberts, B., et al., 2008*) Seven mammalian STATs are known (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) (*Paukku K. & Silvennoinen O, 2004*). STATs are 700-900 amino acids long and their structure contains a SH-domain which has two functions (*Kisseleva T. et al., 2001*). First, to mediate the STAT protein's binding on the cytokine receptor and

secondly, to mediate the STAT protein's binding to another STAT molecule (*Alberts, B., et al., 2008*) The cytokine receptors consist of an extracellular domain, a single transmembrane domain and intracellular domains which mediate the signaling function (*Saharinen P., 2002*).

In the JAK-STAT pathway, a ligand (cytokine, growth hormone, interleukin or interferon) binds to its receptor leading the receptors to oligomerize. This induces the JAKs trans-phosphorylation on the tyrosine residues. Activated JAKs phosphorylate the cytokine receptors and form specific phosphotyrosine docking sites for STATs. The JAKs phosphorylate the STATs and the cytokine receptors activate the STATs which dissociate from the cytokine receptors and form a homodimer or heterodimer. (*Kisseleva K.,2001*) STAT dimer translocates to the nucleus and with the help of other gene regulatory proteins start to stimulate the target genes (*Imada K. & Leonard W.J., 2000*).Several cytokines and hormones activate the JAK-STAT pathway and its dysfunction affects the cell's ability to differentiate, proliferate, develop and survive (*Paukku K. & Silvennoinen O., 2004*).

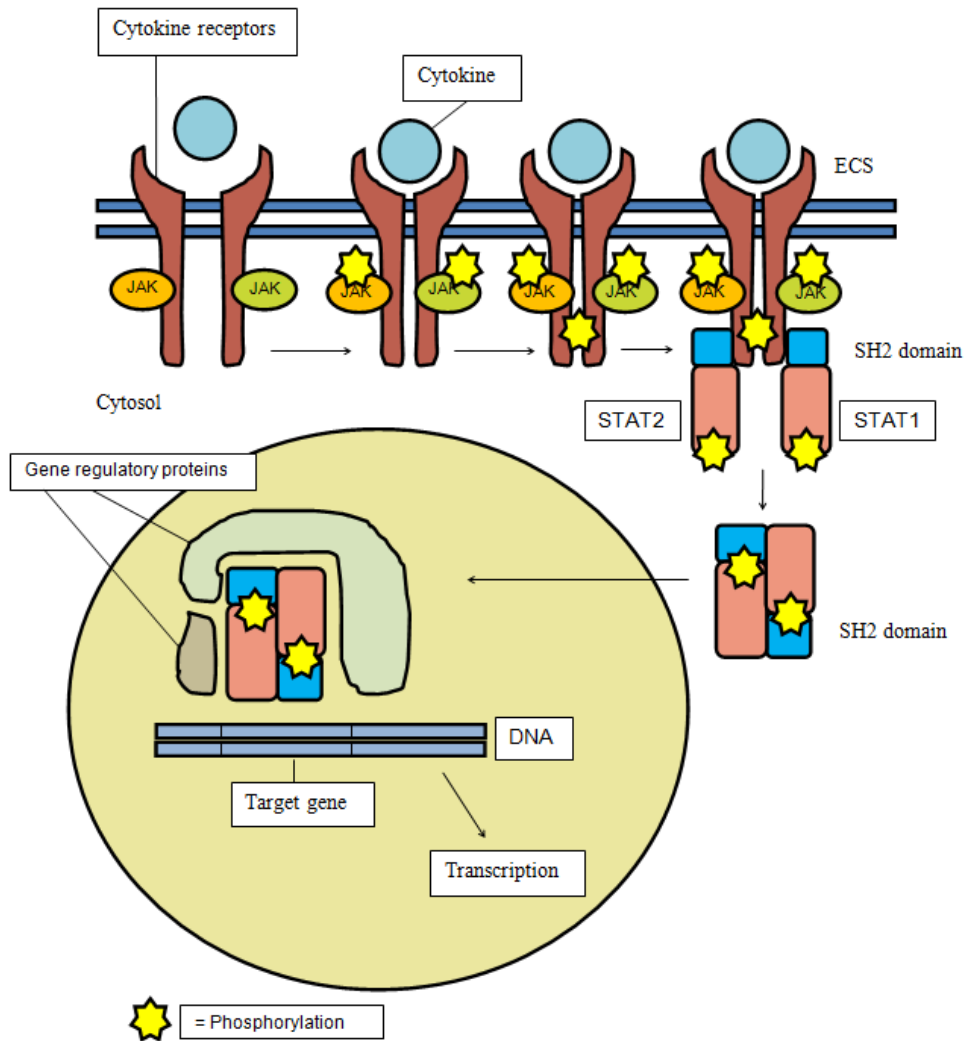


FIGURE 3. *The JAK-STAT signalling pathway. A cytokine binds to its receptors and induces the receptors to converge each other which trigger the JAKs to trans-phosphorylate one another. As a result the activated JAKs phosphorylate the cytokine receptors which recruit the STATs. Phosphorylated STATs migrate to the nucleus and with the help of other gene regulatory proteins bind to a specific DNA sequences and start to stimulate the transcription of target genes. (Modified; Alberts B. et al., 2007)*

2.4. The family of Janus kinases

The family of Janus kinases (JAKs) consists of four mammalian members; JAK1, JAK2, JAK3 and TYK2. JAKs have also been found in birds, fish and insects. Human JAK1 gene is located on chromosome 1p31.3, the JAK2 gene on chromosome 9p24, the JAK3 gene on chromosome 19p13.1 and TYK2 gene on chromosome 19p13.2. (Firmbach-Kraft I. et al. 1990)

JAK structure comprises seven conserved homology domains, JH1-JH7 (Figure 4). The major feature of the JAK kinases structure is that they consist of a tyrosine kinase domain (JH1) and pseudokinase domain (JH2) which comprise approximately half of the entire JAK molecule and are located at C-terminal side of the JAK. FERM domain forms the other half of JAK at the N-terminal side. SH2-like domain is located in the middle between pseudokinase domain and FERM domain. (*Hubbard et al., 2000*)

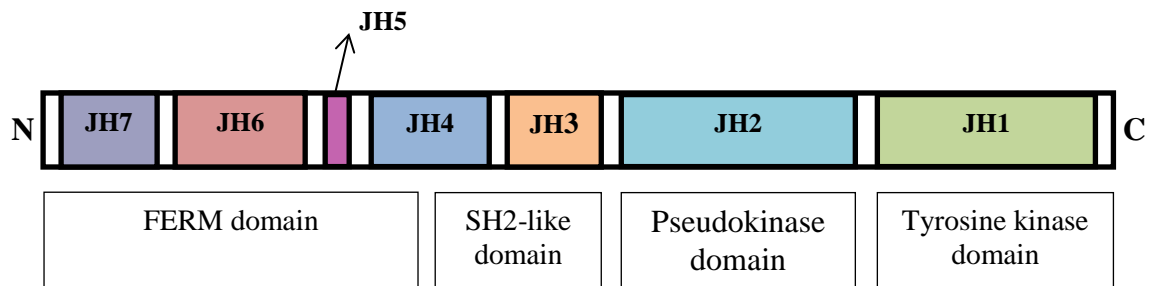


FIGURE 4. Schematic domain structure of JAK kinases (Modified; Ungureanu, D. 2005)

The JAKs kinases are relatively large molecules and their molecular weight ranges between 120-140 kDa (*Kawamura et al., 1994*). The FERM domain (30 kDa) binds to JAK's receptor and the SH2-like domain structurally supports and stabilizes the FERM domain by binding phosphotyrosine residues in the next protein in the signaling pathway (*Leonard & O'Shea, 1998*). The SH-like domain does not act as a typical phosphotyrosine-binding domain and its exact role is still unknown (*Bandaranayake et al., 2012*)

The tyrosine kinase domain (JH1) is approximately 300 amino acids long domain and has a nature of a typical eukaryotic tyrosine kinase domain. It has an important role as a catalytically active domain. (*Briscoe et al., 1996, Gurniak & Berg, 1996, Sanz et al., 2011*) JH1 domain contains the active site (A-loop) and binds the ATP during the phosphorylation. The pseudokinase domain (JH2) is conserved in all JAK kinases, referring that the JH2 domain may have a major role for the function of JAK protein (*Wilks A.F. et al., 1991*). The JH2 domain was assumed to be catalytically inactive domain but was discovered to be an active domain. The JH2 domain plays an essential role in regulating the activation of the whole JAK protein. (*Sanz et al., 2011*).

2.4.1 The structure of JH1-JH2 domains of JAK2

The whole JAK2 protein is composed of 1132 amino acids (*European Bioinformatics Institute, 2012*). Only the crystal structures of JH1 and JH2 domains of JAK2 have been resolved. Despite few differences, they both share a structure of a typical folding of a eukaryotic protein kinase. (*Bandaranayake et al., 2012*) The phosphotransfer reaction occurs in the active site (A-site) that is located between the amino-terminal lobe (N-lobe) and carboxyl-terminal lobe (C-lobe). These two lobes are further divided into twelve subdomains, and between those are the activation loop and the catalytic loop. The N-lobe consists of five β -strands and single α -helix called α -C. The C-lobe is mainly α -helical. (*Hanks S.K. & Hunter T., 1995*) The first subdomain (I) including β 1– β 2 strands in the N-lobe contains an amino acid sequence with several conserved glycines (Gly) (*Saharinen P. et al., 2000*). This motif is often called a nucleotide binding pocket where the ATP binds (*Huse M. & Kuriyan J., 2002*). The activation loop of JH2 (Figure 5, p.17) domain is shorter than in JH1 domain (*Bandaranayake et al., 2012*).

The second subdomain (II) which is a β 3-strand in the N-lobe contains also a conserved motif, V-A-I-K (Valine-Alanine-Isoleucine-Lysine). In the JH2 domain of JAK2, the conserved glutamic acid is changed to alanine. The subdomain VIb is called the catalytic loop (β 6-7 strands) containing aspartic acid (Asp), H-R-D (Histidine-Arginine-Aspartic acid) role is to receive a proton for the phosphotransfer reaction. In the JH2 domain the aspartic acid is replaced with asparagine (Asn). β 8-9 strands in subdomain VII contain a motif D–F–G (Aspartic acid-Phenylalanine-Glycine) which is located at the start of the activation loop and is able to align easily with other tyrosine kinases. (*Huse and Kuriyan, 2002*) The similar motif in the JH2 domain is D-P-G (Aspartic acid-Proline-Glycine) (*Bandaranayake et al., 2012*).

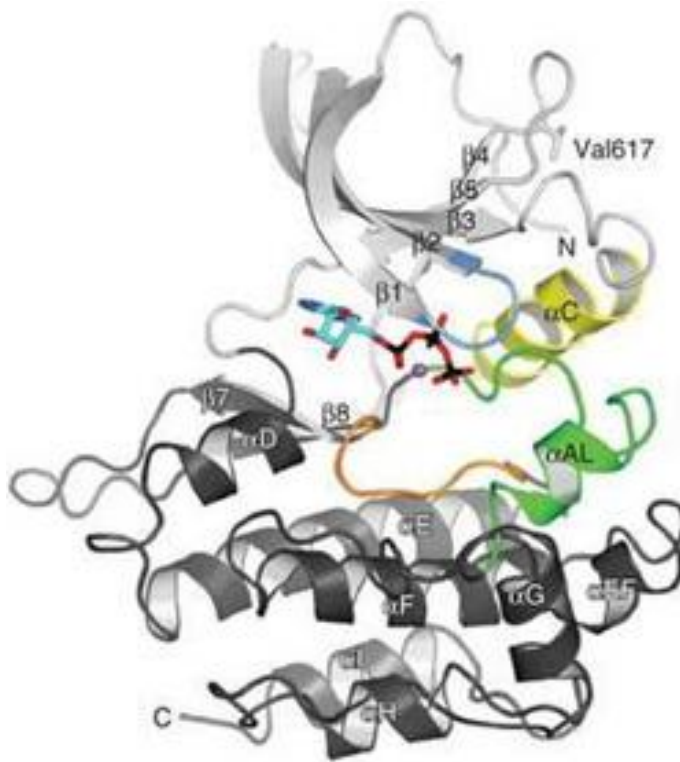


FIGURE 5. Crystal structure of JH2 domain of JAK2 protein. N- and -C terminals are described with light grey color, yellow color presents the N-lobe except nucleotide-binding is colored blue and α C yellow. The activation loop has a green color and the Mg^{2+} between the green and orange colors is labeled with purple. Orange color presents the catalytic loop. ATP is shown in the middle and is colored with red, blue and black. The α -helices and β -strands are also marked in the figure. (Bandaranayake et al., 2012)

2.4.2 Mutations in JAK2

There have been several structural studies performed to investigate the function of JAKs, including point mutations, partial deletions or deletion of a whole domain. It has been indicated that JAKs lacking the JH3-JH7 domains, are not able to bind their receptors. While a mutant TYK2 with erased JH2 domain inhibits the catalytic activity and function of the protein, in contrast a similar JAK2 is capable to function and actually has shown to increase its catalytic activity. (Lindauer K. et al., 2001)

A number of studies indicate that mutations in human JAK genes cause myeloproliferative neoplasms (MPNs) affecting myeloid lineages (Bandaranayake et al., 2012). MPNs include three main disorders, polycythemia vera (PV), essential

thrombocythemia (ET) and primary myelofibrosis (PMF) which cannot be cured for the present (*MPN Research Foundation, 2012*).

Also mutations in JH2 domain of JAK1, JAK2 and JAK3 are proved to be associated with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (*Bandaranayake et al., 2012*).

Many mutations in JH2 of JAK2 have been analyzed and more than thirty mutations are proved to be linked to hematological diseases (*Haan et al., 2010*). Deletion of JH2 domain induces functional changes in JAKs (*Saharinen P., 2002*). Proposed role for JH2 domain is to regulate the kinase domain activity in absence of cytokine signalling. It has been suggested that mutations in JH2 domain disrupt the inhibitory interaction between JH1-JH2 domains of JAK2 or may even induce gain-of-function constitutive activity. Activating mutations such as V617F may lead to myeloproliferative diseases. (*Boudeau J. et al., 2006*)

Though the structure of JH2 domain shares similarity to JH1 domain, mutations in JH2 domain induce the lack of kinase-activity (*Ungureanu D. et al., 2011*). For example, K882 mutation in the JH2 domain has shown to generate deficient kinases and to inactivate the JAK2 (*Briscoe J., 1996*). K581A is a kinase-inactivating point mutation and inhibits binding of ATP to the active site of the JH2 domain. In the mutation, a large and positively charged lysine is converted to a small uncharged alanine. K581A mutation is located in the (β -3 strand) in the N-lobe of the JH2 domain (Figure 6, p.19). K581A mutation participates the binding of ATP by coordinating the α - and β -phosphates. This is assumed to inhibit the efficient binding of ATP into the nucleotide binding pocket. (*Ungureanu D. et al., 2011*)

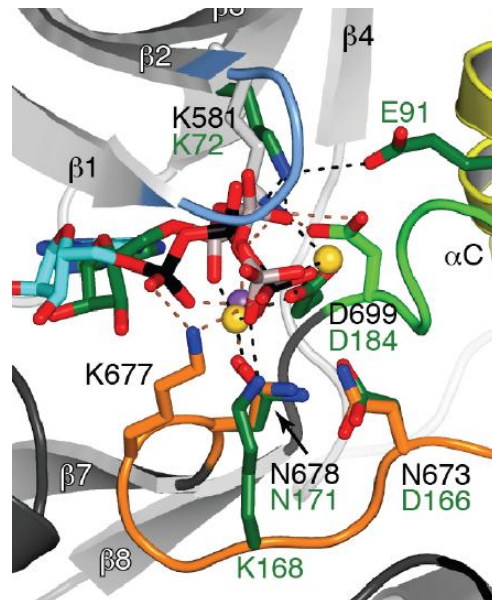


FIGURE 6. ATP binding in JH2 domain of JAK2. Orange color describes the catalytic loop, red color is marked for oxygen, blue for nitrogen and black for phosphorus. Mg^{2+} is colored purple. The residue K581 is also shown in the figure. (Bandaranayake *et al.*, 2012)

2.4.3 Regulation of JAK2 protein

Several tyrosines are phosphorylated as a result of the activation of JAK2. There are two tyrosine residues, Y1007 and Y1008 which become phosphorylated in the A-loop (Bandaranayake *et al.*, 2012). The precise mechanism remains unknown, but it has been proposed that when Y1007/Y1008 phosphorylate, it leads to a conformational change and provides space for JH1 activation. This action induces ATP to bind into the nucleotide binding pocket. (Ungureanu D. *et al.*, 2002)

The pseudokinase domain, JH2, phosphorylates two negatively regulatory sites in JAK2, Ser523 (*in cis*) and Tyr570 (*in trans*) which conserve low basal activity of JAK2 and do not occur in other JAKs. This leads to an assumption that JAK2 is the only JAK which has an additional negative regulatory mechanism. (Ungureanu D., *et al.*, 2011)

2.5. Production of recombinant proteins

Recombinant protein is a protein whose amino acid sequence is encoded by a cloned gene (Glick B.R. *et al.*, 2010). Heterologous protein production is discussed when the cells express recombinant proteins which the cells do not naturally produce (Walsh G., 2002). These proteins are produced artificially by ligating the protein coding gene of interest into an appropriate vector using recombinant DNA techniques. Recombinant proteins are widely used in medicaments, vaccines or as biotechnological enzymes in industrial processes. Recombinant proteins can also be used to analyze their biological activity, to study their three dimensional structure and interactions with other molecules, to develop site-specific drugs or to produce therapeutic proteins. (Florian M.W. 2004)

After ligating the protein coding gene and the vector together, the recombinant DNA is introduced to a host cell. Bacteria, yeast and mammalian cells such as an insect cell can act as a host organism. Cells that are able to take DNA in from outside environment are called *competent cells*. The process of introducing the desired DNA sequence into the host cell is *transformation* when the host organism is a bacterial or a yeast, and *transfection* when the host organism is a mammalian cell. After transformation or transfection of the host organism, the cell starts to produce recombinant proteins. (Baltimore D. *et al.*, 1999)

2.6. Molecular cloning

Molecular cloning (Figure 8, p. 22) is a process where genetic information is transferred from one organism to another. A specific DNA fragment is first isolated and then inserted to a plasmid vector. To obtain the gene of interest out from the cell, the cell membrane and lipid bilayer of nucleus need to be broken down. The cells can be lysed with mechanical, chemical, physical or enzymatic way. The isolated DNA is purified and fragmented using restriction endonucleases which cut the DNA at a specific sequence of nucleotides. These restriction enzymes such as *EcoRI*, are naturally found in bacteria where they use the enzymes as a defense of a viral infection. The restriction enzymes are able to cut the DNA into so called “*blunt ends*” or “*sticky ends*” which is more often used in molecular cloning (Figure 7). The sticky ends leave longer overhangs which are ssDNA left over after the cleaving. (Glick B.R., 2010)

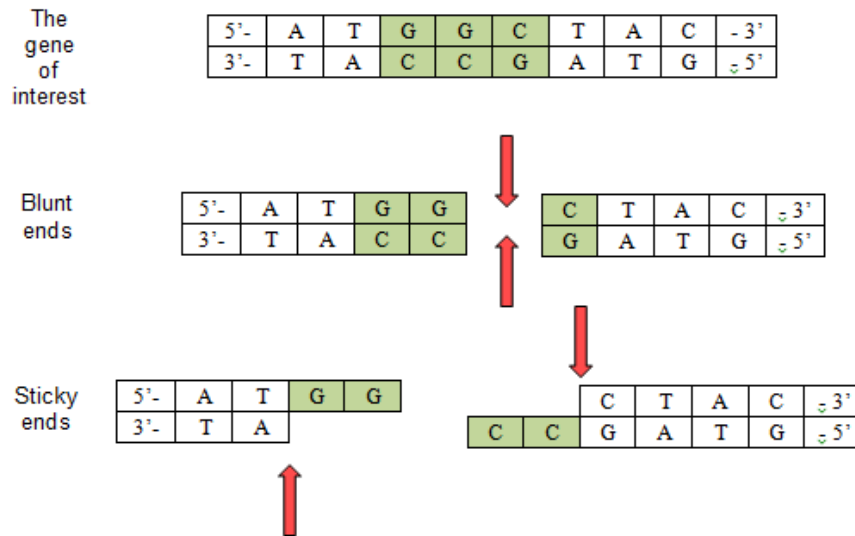


FIGURE 7. *DsDNA* sequence of the desired gene is cut with restriction endonuclease(s) as a result of blunt ends or sticky ends. The restriction site is colored green.

The vector used in the cloning need to be cut with the same restriction enzyme than the desired DNA fragment to achieve the exact recombination of the base pairs. Many small and identical DNA fragments are produced and circular plasmid DNAs are linearized into smaller fragments by the treatment of restriction endonuclease. These DNA fragments are joined together with assistance of an enzyme *ligase*. The recombinant molecule is introduced to a host organism that will express the recombinant proteins. There are few types of vectors which can be used in molecular cloning. One of them is bacmid, a baculovirus shuttle vector that can replicate in both, *E.coli* and insect cells. (Glick B.R. et al., 2010)

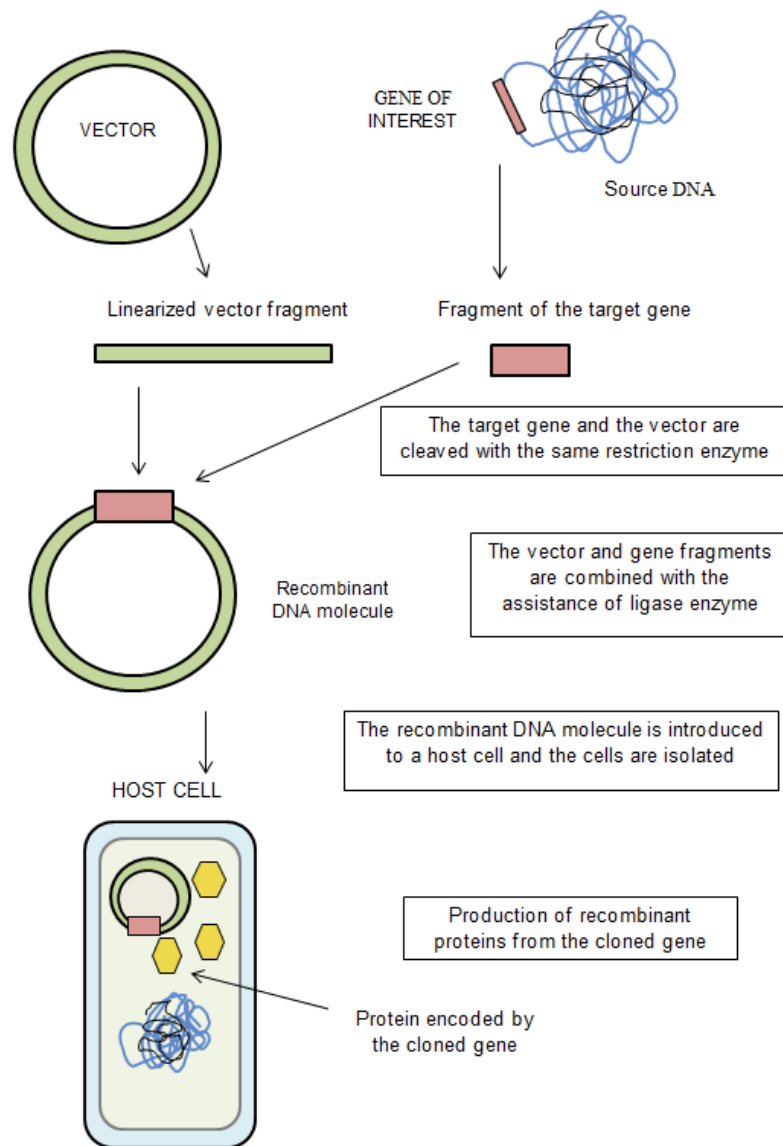


FIGURE 8. Molecular cloning procedure. The target gene and the vector are cleaved with the same restriction endonuclease. The fragments are combined and introduced to a host cell which will produce recombinant proteins from the cloned gene. (Modified; Glick B.R. et al., 2010)

2.6.1 Plasmid vectors and blue-white selection

Plasmids are widely used as cloning vectors whose gene regulation areas enable the gene expression and efficient protein production in selected hosts. (Garret R.H. & Grisham C.M., 2005) Plasmid is a small, circular, extrachromosomal dsDNA and has an ability to self-replicate. Plasmids occur naturally in bacteria, yeast and some higher eukaryotic cells. Recombinant DNA technology utilizes plasmids which replicate in *E.coli*. (Baltimore D. et al., 1999)

Three major features are required for plasmid to function as a cloning vector; a *replicator*, an origin of replication (*ori*), a *selectable marker* which is usually an antibiotic resistance gene and a *cloning site*. The selectable marker helps to select the cells containing recombinant plasmid, and separate them from the cloning vector which will grow in the presence of the antibiotic. The desired gene is inserted into the cloning site. (Garret R.H. & Grisham C.M., 2005)

Identification of the *E.coli* colonies containing the recombinant plasmid is performed with blue-white selection. The transformed competent cells are incubated in medium in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), IPTG (isopropyl beta-D-thiogalactopyranoside) and ampicillin. A plasmid vector (pUC19) contains a gene called *lacZ* that encodes the production of β -galactosidase. The plasmid also contains a resistance gene for ampicillin (Amp^r), a *lacI* gene that produces a repressor protein regulating the expression of the *lacZ* gene, a multiple cloning site (MCS) and an origin for DNA replication (ORI). (Glick B.R. et al., 2010)

When the cells carrying the vector are grown in the medium described earlier, the protein product of *lacI* gene cannot bind to the promoter-operator region of *lacZ* gene (Glick B.R. et al., 2010). *Promoter* is a short DNA sequence which enables the RNA-polymerase to attach the DNA. Promoter is located close to the gene and determines the site of transcription initiation for an RNA polymerase. (Baltimore D. et al., 1999) The lack of the repressor binding induces the *lacZ* gene to become transcribed and translated. The cell is now able to produce β -galactosidase which appears with blue colonies on the plate. The blue color is a result of reaction where the X-Gal is hydrolyzed by β -galactosidase. Nontransformed cells cannot grow at all in the presence of ampicillin. The recombinant plasmid containing DNA from other source is inserted into the MCS region, leading to *lacZ* gene to be disrupted. Since the recombinant plasmid is not able to produce β -galactosidase, it appears as white colonies. Positive colonies can be selected from the plate, grown afresh and used for further analysis. (Glick B.R. et al., 2010)

2.6.2 Site-directed mutagenesis

Mutagenesis is a process where the nucleotides of DNA are changed or deleted. There are several different methods to generate mutations into the DNA sequence. In random mutagenesis a variety amount of unspecific mutations are generated into different sites of the DNA sequence. (*Glick B.R. et al., 2010*) In contrast, oligonucleotide-directed (site-directed mutagenesis) can be performed in a bacteriophage M13 or in a plasmid producing specific point mutations. In this type of mutations, the exact mutated nucleotide and the site of mutation need to be known beforehand. (*Sawano A. & Miyawaki A., 2000*) A commercial test kit called QuikChange® site-directed mutagenesis (Stratagene) can be used for oligonucleotide-directed mutagenesis.

QuikChange® site-directed mutagenesis test kit can be used to generate point mutations, switch amino acids and erase or insert single or multiple amino acids and does not require specialized vectors, unique restriction sites or multiple transformations. The procedure requires a supercoiled dsDNA vector with an insert of interest and two synthetic oligonucleotide primers which contain the desired mutation. (*Stratagene, 2005*)

First, the plasmid is denatured, the reverse and forward primers are annealed. The primers are extended by the presence of *PfuTurbo* DNA polymerase and incorporation of the primers generates a mutated plasmid containing staggered nicks. The PCR product is treated with *Dpn* I –endonuclease which is used to digest the parental DNA template and to select for synthesized mutated DNA. It is relevant to use plasmid DNA isolated from a *dam*⁺ *E.coli* strain. Dam (DNA adenine methylase) is an enzyme which helps the cell to separate the template strand and newly synthesized strand by adding a methyl group to the adenine in newly synthesized DNA. Secondly, the XL1-Blue supercompetent cells (*E.coli*) are transformed with the nicked vector DNA containing the mutations. After transformation the supercompetent cells repair the nicks in the mutated plasmid (Figure 9, p.25). (*Stratagene, 2005*)

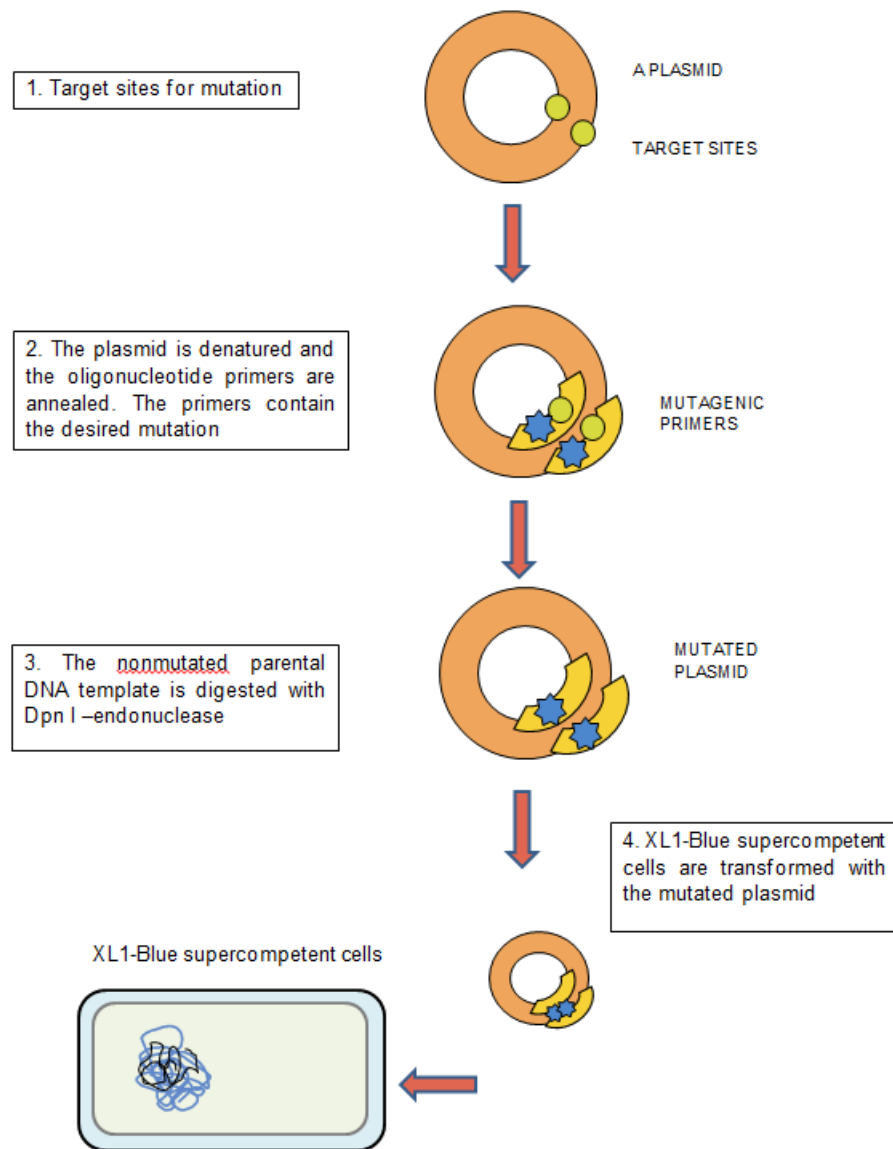


FIGURE 9. Site-directed mutagenesis. The mutation is generated with PCR. The plasmid is denatured and the primers containing the desired mutation are annealed into the plasmid. Dpn I is used in digestion. XL1-Blue supercompetent cells are transformed with the mutated plasmid. (Modified; Stratagene, 2005)

2.6.3 Protein production in baculovirus system

Baculoviruses are rod-shaped viruses and contain a large circular dsDNA genome. These pathogen viruses infect insect cells or insect larvae. Baculoviruses are safe to work with since they are not infectious to vertebrates. (*Invitrogen, instruction manual, 2010*) They are divided into two genera, *Granulovirus* genus (GV) and *Nucleopolyhedrovirus* (NPV) (*Sherwood L.M. et al., 2009*).

A baculovirus particle contains viral DNA surrounded by a cylindrical nucleocapsid. The particles are often located in an occlusion body called *polyhedron* inside the nucleus of an infected cell. (*Glick B.R. et al., 2010*) Polyhedron is mainly made up of a protein called *polyhedrin* which protects the virus from the outside environment such as heat, low pH or chemicals (*Sherwood L.M. et al., 2009*). After the infected insect dies, large amount of polyhedron is released and migrated to the midgut of the insect where an alkaline environment forces the polyhedrin protein coat to dissolve. This induces the infected nucleocapsids to release and migrate to the nucleus where the nucleocapsid is removed. (*Glick B.R. et al., 2010*)

The viral replication occurs in the nucleus of the midgut cells whereof the infection can spread from cell to cell through the cell membrane (*Sherwood L.M. et al., 2009*). This form of the virus is called a budding form including a single nucleocapsid. While the virus particles become separated from the insect cell, the infected cell membrane envelopes the nucleocapsid. The insect dies during one week after the infection and as a result, a quarter of the dry weight of the insect consists of polyhedron. (*Glick B.R. et al., 2010*)

Baculovirus system can be utilized in the production of heterologous proteins. One example of such system is a commercial Bac-to-Bac® Baculovirus expression system (*Invitrogen*) where the insect cells are transfected with the recombinant bacmid DNA.

2.6.4 Overview of Bac-to-Bac® Baculovirus expression system

Bac-to-Bac® Baculovirus Expression System (Invitrogen) is an efficient method to produce high levels (up to 1000 mg/mL), post-translationally modified, biologically active and functional recombinant proteins. The reason why Baculovirus Expression Vector System (BEVS) has been widely used is its ability to express high levels of heterologous genes especially for intracellular proteins. The produced recombinant proteins are often soluble and are easily collected from infected cells in the late phase of infection. Baculovirus system are also faster and cheaper to use than other mammalian cell system. (*Invitrogen, instruction manual, 2010*)

The BEVs utilizes *Autographa californica* nucleopolyhedrosis virus (AcNPV), a subgroup of NPV, which often propagates in cell lines derived from the fall armyworm, *Spodoptera frugiperda*. This subgroup of *Nucleopolyhedrovirus* has a polyhedrin promoter. (*Invitrogen, instruction manual, 2010*) The polyhedron promoter is very strong and therefore polyhedrin genes are highly expressed. Replacement of these genes with foreign genes will promote the baculovirus to express recombinant proteins (Figure 10, p. 28). (*Invitrogen, instruction manual, 2010*)

pFastBac1 is a donor vector containing the gene of interest, two selection genes, Amp^r and Gen^r, two flanking sites (Tn7R and Tn7L) and a polyhedron promoter. The vector is transformed into supercompetent DH10Bac cells which contain bacmid DNA with a mini-attTn7 target site and helper plasmid. The cells are engineered to contain viral genome and after transformation the gene of interest is incorporated into the viral genome and becomes a part of the viral genome. Flanking sites allow the recombination of the bacmid DNA and the gene of interest, and the helper plasmid provides proteins for the transposition. The recombinant bacmid DNA is selected using blue-white selection. The mini-attTn7 target site on the bacmid is attached by the Tn7 insertions, leading the expression of lacZ α peptide to disrupt. As a result, the recombinant bacmid will appear as white colonies, while blue colonies contain only the vector. (*Invitrogen, instruction manual, 2010*)

The recombinant bacmid DNA is isolated and purified from a small-scale *E.coli* culture and Sf9 insect cells are transfected with the recombinant bacmid DNA. After transfection, the viable virus begins budding into the media. The viral particles are

harvested from the media and then used to infect fresh insect cells. The insect cells express the desired protein which can be purified and analyzed. (*Invitrogen, instruction manual, 2010*)

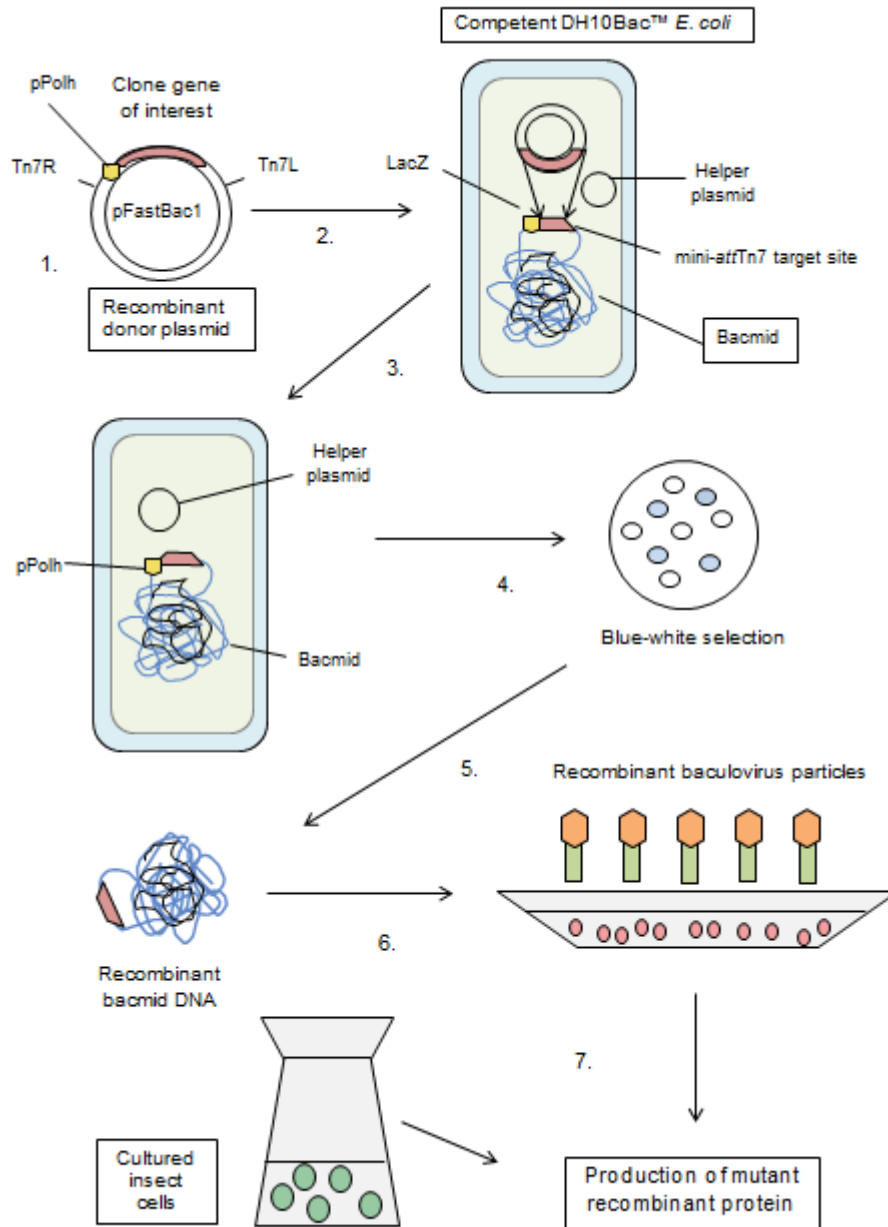


FIGURE 10. Production of mutant recombinant protein with Bac-to-Bac® Expression System using pFastBac™1 donor vector (*Invitrogen*). 1) The gene of interest is first cloned into the pFastBac™1 donor vector 2) Transformation of the competent DH10Bac™ E.coli cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid 3)The pFastBac™1 donor vector exists after the mini-Tn7 element on the plasmid has transposed to the mini-attTn7 target site on bacmid 4) The absence of the recombinant bacmid is identified with blue-white selection 5) The recombinant bacmid DNA is isolated from selected E.coli colonies 6)Viral replication of the recombinant baculovirus particles 7) The insect cells are cultured and transfected with the recombinant bacmid DNA and as a result, the insect cells will produce mutant recombinant protein. (*Modified; Invitrogen, instruction manual, 2010*)

3 AIM OF THE STUDY

The objective of the study is to investigate the solubility, structure and function of JH1-JH2 domains of JAK2 recombinant protein. Following phosphorylation, the both domains become catalytically active and therefore producing inactive protein components have shown to be challenging. By mutating the K581A in the JH2 domain, the protein would have a catalytically inactive JH2 domain. This can be used in analysing to the autophosphorylation of JH1-JH2 proteins and the phosphorylation of other substrates.

The purpose of the thesis is to produce soluble protein product that consists of mutated JH1-JH2 domains of JAK2. The JH1 domain contains a D976N mutation and an additional K581A mutation will be introduced into the JH2 domain. The D976N mutation inhibits the hydrolyzation of ATP and kills kinase activity in JH1 without interfering with the binding of ATP. The mutated DNA construct will be transformed into DH10Bac cells for generating bacmid DNA, which will be isolated and analysed by agarose gel electrophoresis and PCR. Insect cells will be transfected with the recombinant bacmid DNA to produce mutant proteins which will further be purified and analysed.

4 MATERIALS AND METHODS

4.1. Generating K581A mutation into JH1-JH2 domains of JAK2 protein

4.1.1 JAK2 constructs

The JAK2 JH1-JH2 constructs (Figure 11) were cloned into the pFastBac™1 (Invitrogen) donor vector (Figure 12, p.32). JAK2 gene constructs contained D976N mutation in JH1-JH2 domains but additional K581A mutations were generated with QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The pseudokinase domain, JH2 was mutated into two different JAK2 constructs, aa536-1132 and aa513-1132.

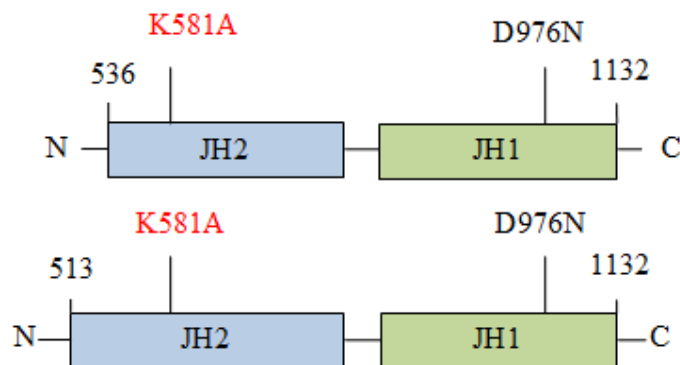


FIGURE 11. Schematic presentation of the JAK2 constructs; JH2 is pseudokinase domain and JH1 is kinase domain which contained D976N mutation. K581A mutations were generated into wild types aa536-1132 and aa513-1132.

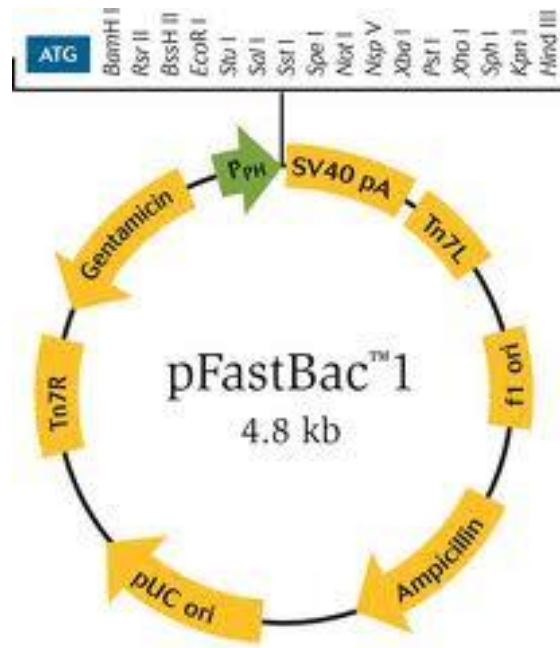


FIGURE 12. *pFastBac™1* vector (Invitrogen). The K581A mutations were cloned into the *pFastBac™1* donor vector. *pFastBac™1* contains the origin of replication (ORI), two selection genes, *Amp^r* and *Gen^r*, two flanking sites (Tn7R and Tn7L), restriction site for several restriction endonucleases and a polyhedron promoter.

4.1.2 Mutagenesis

Mutagenesis was performed with the reagents presented in table 2 and the PCR program used in PCR is shown in table 3. Purified primers used for generating the K581A mutation are shown in table 1. In this particular mutation nucleotides encoding lysine (AAA) were converted to nucleotides encoding alanine (GCA). Samples were prepared on ice and *PfuTurbo* DNA polymerase was added last.

TABLE 1. Primers for generating K581A mutation in JH1-JH2 JAK2 protein

F	5'-	ACA	GAA	GTT	CTT	TTA	GCA	GTT	CTG	GAT	AAA	GCA	-3'
R	5'-	TGC	TTT	ATC	CAG	AAC	TGC	TAA	AAG	AAC	TTC	TGT	-3'

TABLE 2. Reagents for mutagenesis

Reagent	Amount (one sample)
10 x Reaction Mix	5 μ l
Template DNA	1 μ l
Primer (forward)	1 μ l
Primer (reverse)	1 μ l
dNTP Mix	1 μ l
Sterile Water	41 μ l
Total	50 μ l
<i>PfuTurbo</i> DNA polymerase	1 μ l

TABLE 3. PCR program for mutagenesis

Step	Temperature	Time
1	95 °C	30 s
2	95 °C	30 s
3	55 °C	1 min
4	68 °C	6,5 min
5	68 °C	5 min

} 16 cycles

4.1.3 Sequencing

The sequences of mutated JAK2 constructs were analyzed and the nucleotide sequence of both JAK2 constructs were compared to mRNA of JAK2. The reagents for sequencing are shown in table 4. Each PCR product (2 μ l) was prepared for sequencing using optimized instructions (*Koskenalho E., 2011*). Master mix was added into the PCR tubes on ice and run by a program shown in table 5. The DNAs were precipitated by adding sodium acetate and absolute ethanol after which the tubes were incubated at room temperature and centrifuged for 20 minutes. Supernatants from both tubes were discarded and 70 % ethanol was added into the PCR tubes. The tubes were dried and Hi-Di™ formamide was added. The actual sequences were resolved using genetic analyzer at separated sequencing core laboratory at the University of Tampere, IBT.

TABLE 4. Reagents for sequencing

Step	Reagent	Amount (one sample)
PCR	3,3 pmol of Primer	1 μ l
	Sequencing buffer	1 μ l
	Big Dye	1 μ l
	H ₂ O	5 μ l
	Total	8 μl
Precipitation	Sodium acetate	1 μ l
	100 % Ethanol	22 μ l
	70 % Ethanol	200 μ l
Resuspension	Hi-Di™ Formamide	15 μ l

TABLE 5. PCR program for preparing the mutated DNA samples for sequencing.

Step	Temperature	Time
1	96 °C	1 min
2	96 °C	10 s
3	50 °C	10 s
4	60 °C	4 min
5	60 °C	10 min
6	4 °C	cooling/stop

} 33 cycles

4.1.4 Transformation

After generating the mutations, the circular and nicked dsDNA was transformed into XL1-Blue supercompetent cells which repair the nicks in the mutated plasmid. From both mutated gene constructs, 1 μ l of the *Dpn* I-treated PCR product was added into the supercompetent cells on ice. The samples were kept on ice for 30 minutes and treated with a heat shock at 42 °C for 45 seconds. The reagents (Agilent Technologies) for transformation are presented in table 6.

TABLE 6: Reagents for transforming the XL1-Blue supercompetent cells

Reagent	Amount (one sample)
<i>Dpn</i> I endonuclease	1 μ l
XL1-Blue supercompetent cells	50 μ l
PCR-product	1 μ l
NZY broth -medium	500 μ l
LB-agar plates + ampicillin	1 plate

Each transformation reaction was incubated in preheated NZY broth -medium on a shaker at 37 °C for one hour. 250 μ l of the transformation mixture was spread on LB-agar plates containing ampicillin and incubated at 37 °C overnight to obtain recombinant *E.coli* cultures. Five colonies from both plates were selected and used for plasmid DNA extraction and for the plasmid DNA concentration measurement.

4.1.5 Isolation of plasmid DNA

The plasmid DNA was extracted from recombinant *E.coli* cultures by using GenElute™ HP Plasmid Miniprep Kit (Sigma). The reagents provided by the kit are presented in table 7. Harvested bacterial cells were resuspended into resuspension solution containing RNase. The cells were lysed with lysis buffer. The cell debris was precipitated by adding neutralization buffer and centrifuging at 16,000 g.

Possible contaminants were removed using column preparation buffer and the cells were washed by wash solution 1 and 2. The purified plasmid DNA was eluted by transferring the column into a fresh eppendorf-tube and adding TE into the column. The column was erased and DNA concentration measured.

TABLE 7: Reagents for plasmid DNA isolation

Reagent	Amount for one sample
Resuspension solution (RNase added)	200 μ l
Lysis buffer	200 μ l
Neutralization buffer	350 μ l
Column preparation buffer	500 μ l
Wash solution 1	500 μ l
Wash solution 2 (diluted with ethanol)	750 μ l
Tris-EDTA buffer	50 μ l

4.2. Preparation and isolation of recombinant bacmid

4.2.1 Transforming DH10Bac *E.coli*

Reagents for transformation of DH10Bac -cells are presented in table 8. The DH10Bac -cells and the purified plasmid DNA were mixed together in an eppendorf-tube. They were incubated on ice for 30 minutes, treated with a heat shock at 42 °C for 45 seconds and transferred back on the ice for 2 minutes. The samples were incubated in S.O.C -media on a shaker at 37 °C for 6 hours. IPTG and X-Gal were added on the bacmid-plates to achieve blue-white selection. 50 μ l of the incubated mixture was spread on the bacmid-plate containing antibiotics, IPTG and X-Gal. The plates were incubated at 37 °C for 2 days to achieve the blue and white colonies.

TABLE 8: Reagents for transforming DH10Bac *E.coli*

Reagents	Concentration	Amount
DH10Bac competent cells	10^9 /ml	100 μ l
Isolated plasmid DNA	(measured)	1 μ l
Bacmid–plates	LB-agar	500 ml
	Kanamycin 50 μ g/ml	1 ml
	Gentamycin 7 μ g/ml	0,5 ml
	Tetracycline 10 μ g/ml	0,5 ml
Blue-white selection	IPTG 100 mM	25 μ l (one plate)
	X-Gal 20 mg/ml	25 μ l (one plate)
S.O.C –media	2 % Tryptone	900 μ l
	0,5 % Yeast Extract	
	10 mM NaCl	
	2,5 mM KCl	
	10 mM MgCl ₂	
	10 mM MgSO ₄	
	20 mM Glucose	

4.2.2 Isolation of recombinant bacmid DNA

Two positive colonies were selected for restreaking to ensure they are truly white. Restreaking was done by spreading two white, large and separated colonies on a fresh bacmid-plate containing X-Gal and IPTG (table 8). The plates were incubated at 37 °C over night.

A single colony was selected from the plate to produce a liquid *E.coli* culture containing the antibiotics shown in table 8. Reagents for recombinant bacmid DNA isolation are presented in table 9. Bacterial suspension (3 ml) was transferred into an eppendorf-tube and centrifuged at 8,000 rpm for 3 minutes. Supernatant was discarded and pellet was resuspended with Sol I containing RNase A to degrade RNA. Sol II was added to the tubes and they were inverted six times. The tubes were placed in room temperature for 2 minutes and lids open 30 seconds. Sol III was added and the tubes were inverted 8 times

and placed on ice for 10 minutes. The tubes were centrifuged at 16,000 g for 20 minutes.

The supernatant was inserted to a new tube containing isopropanol and placed on ice for 10 minutes inverting them a few times for precipitation. The tubes were centrifuged at 16,000 g and the supernatants were carefully discarded. Small pellets were washed with ethanol and centrifuged at 16,000 g for 5 minutes. The supernatants were removed in sterile environment. The pellets were air dried lids open for 10 minutes after which the DNA was dissolved in TE for 30 minutes.

TABLE 9: Reagents for isolation of recombinant bacmid DNA

Reagents	Contents	Amount for one sample
Sol I + RNase A	50 mM Glucose	300 μ l of Sol I + 9 μ l of RNase A
	25 mM Tris-HCl pH 8	
	10 mM EDTA pH 8	
Sol II	0,2 M NaOH	300 μ l
	1 % SDS	
Sol III	3 M K-Acetate	300 μ l
	Acetic Acid	
Isopropanol		800 μ l
70 % EtOH		500 μ l
TE	10 mM Tris-HCl pH 8	40 μ l
	1 mM EDTA pH 8	

4.2.3 Analysis of recombinant bacmid DNA

The bacmid DNAs were analyzed by agarose gel electrophoresis to confirm the presence of high molecular weight DNA. PCR analysis was done to verify successful transposition to the bacmid. The reagents for agarose gel electrophoresis are presented in table 10. The agarose gel (0.5 %) was prepared using 1x TAE-buffer and 1 kb DNA as a marker. The gel was run at 80 V with 400 mA. A thicker, 0.7 % gel was prepared for agarose gel electrophoresis after the PCR analysis which was run by a program shown in table 13. The reagents for PCR analysis are presented in table 11, the primers

are shown in table 12, and the reagents used for the thicker gel are shown in table 14. The gel was run at 80 V with 400 mA.

TABLE 10: Reagents for agarose gel electrophoresis

	Reagents	Amounts
0,5 % gel	Agarose	0,2 g
	1x TAE	40 ml
	EtBr 0,16 mg/ml	10 µg
Samples	10x loading buffer	2 µl
	Sterile water	12 µl
	Bacmid DNA	6 µl
Marker (GeneRuler)	1 kb DNA	5 µl

TABLE 11: Reagents for PCR analysis of recombinant bacmid DNA

Reagents		Amount (one sample)
10x PCR Buffer (contains MgCl ₂)		5 µl
dNTP mix 10 mM		1 µl
Primer I	10 µM	1,25 µl
Primer II		1,25 µl
Template DNA		1 µl
Taq polymerase 2,5 U		0,5 µl
Sterile water		40 µl

TABLE 12. M13/pUC Amplification primers for the analysis of recombinant bacmid DNA

F	5'-	GGT	TTT	CCC	AGT	CAC	GAC		-3'
R	5'-	AGC	GGA	TAA	CAA	TTT	CAC	AC	-3'

TABLE 13. PCR -program for bacmid DNA analysis

Temperature	Time	
94 °C	5 min	} 31 cycles
94 °C	45 s	
55 °C	45 s	
72 °C	5 min	
94 °C	45 s	

TABLE14: Reagents for agarose gel electrophoresis for bacmid DNA transposition

	Reagents	Amounts
0,7 % gel	Agarose	0,28 g
	1x TAE	40 ml
	EtBr 0,16 mg/ml	10 µg
Samples	10x loading buffer	2 µl
	PCR product	18 µl
Marker (GeneRuler)	1 kb DNA	5 µl

4.3. Recombinant protein production in Sf9 insect cells

4.3.1 Transfection

The Sf9 (*Spodoptera frugiperda*) insect cells (Invitrogen) were transfected for generating P0. P1 –virus is the virus harvested after infecting the insect cells with P0 –virus. The cells were first cultured in Grace’s Insect Medium free from any antibiotics or serum. The cell density was 1×10^6 /ml and viability over 95 %. The insect cells were allowed to attach for 15 minutes at room temperature after which they were transfected with baculovirus DNA in a 6-well plate. A- and B-solutions were prepared using the reagents shown in table 15. The solutions were combined (transfection mixture) and incubated at room temperature for 20 minutes. The mixed solution was added onto the cells and incubated at 27 °C for 5 hours. The transfection mixture was discarded and replaced with a fresh Grace’s Insect Medium, containing 10 % FBS. The cells were

incubated at 27 °C for 72 hours to obtain viral infection in the cells to obtain the P0 -virus. 100 µl of the P0 stage of infected virus was cultured in 10 ml petri dish to obtain the P1 -virus. Both viral stages (P0 and P1) were analyzed with Western blot.

TABLE 15. The Reagents for culturing insect cells and transfecting the cells with baculovirus DNA

Step		Reagent	Amount
Culturing the Sf9 cells		Sf9 insect cells	
		Grace's Insect Medium, containing 10 % FBS (Fetal Bovine Serum)	2 ml (one well)
Transfection	A	Cellfectin® II	8 µl
		Grace's Medium	100 µl
	B	Purified Bacmid DNA	1 µg
		Grace's Insect Medium	100 µl
		Grace's Insect Medium, containing 10 % FBS	2 ml (one well)

4.3.2 Cell lysis, SDS-PAGE and Western Blot

The transfected cells and control cells were lysed on ice with 200 µl of lysis buffer shown in table 16. The cells were scrapped and left on ice for 15 minutes after which they were centrifuged at 4 °C for 15 minutes. Proteins were separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) with reagents shown in table 15. Each lysate (15 µl) was mixed with 5 µl of 4x SDS and loaded onto 10 % gel. The non-infected cells were used as a control sample. The gels were run at 180 V with 400 mA for 45 minutes and transferred onto a nitrocellulose membranes (Whatman® PROTRAN®) using a Trans-blot® SD semi-dry transfer cell apparatus (BIO-RAD).

TABLE16: Reagents for cell lysis

Reagents	Contents	Amounts
Lysis Buffer	1 % Triton X-100	5 ml
	Sodium vanadate	100 μ l
	Proteinase (approtinin)	40 μ l
	PMSF (phenylmethysulphonyl fluoride)	50 μ l

The reagents used in the blotting are shown in table 17. Each membrane was blocked in 5 % BSA for 1 hour at room temperature on a shaker to prevent the unspecific binding of the antibody. The membranes were incubated in the specific primary antibody on a shaker at 4 °C overnight, after which the membranes were washed 10 minutes with each (0,05-0,5 %) Tween TBS washing buffers. Both membranes were incubated in the secondary antibody for 1 hour on a shaker to achieve binding between the primary and secondary antibodies. The membranes were visualized using autoradiography (BioMax MR film, Kodak).

TABLE 17: Reagents for SDS-PAGE and Western blot

Step	Reagents	Contents
Gel run	Running Buffer	25 mM Tris
		192 mM Glycine
		0,1 % SDS
	4x SDS (Sodium Dodecyl Sulphate)	10 ml Glycerol
		5 ml Tris pH 6,8
		5 ml 2-mercaptoethanol
		10 mg Bromophenol blue
		3 g SDS
Marker	PageRuler™ Plus Prestained protein ladder (Fermentas)	
Transfer	Transfer Buffer	30,8 mM Tris
		0,24 mM Glycine
		20 % methanol
Blocking	Blocking Buffer	0,05 % Tween TBS
		5 % BSA (bovine serum albumin)
Antibodies	Primary AB	α JAK2rabbit1:1000 (<i>Upstate Biotech</i>)
	Secondary AB	anti-rabbit IgG-HRP (<i>Santa Cruz Biotech</i>) + 0,05 % Tween TBS 1:15 000
Washing	Tween + 1x TBS	0,05 % Tween TBS
		0,1 % Tween TBS
		0,5 % Tween TBS
Detection	Detection reagent 1.	luminol substrate
	Detection reagent 2.	peroxide solution

5 RESULTS

5.1. Mutagenesis and plasmid DNA analysis

After generating the mutants in both, aa513-1132 and aa536-1132 residues, the constructs were sequenced to confirm the successful mutagenesis of K581A. The mutations were generated into pFastBacTM1 plasmid vector (Invitrogen). Nucleotide sequence of both JAK2 constructs was compared to mRNA of JAK2. Sequencing results showed that the mutation K581A was achieved in the pseudokinase domain (JH2). XL1-Blue supercompetent cells were transformed with the particular mutated pFastBac1 vector and grown in a small scale *E.coli* culture. Plasmid DNA concentration and purity were measured to verify the quality of the desired plasmids. Five colonies from both recombinant *E.coli* cultures were measured (table 18). Samples 3 and 10 were selected for plasmid DNA isolation.

TABLE 18: Concentration and purity of the isolated plasmid DNA

	aa513 – aa1132					aa536 – aa1132				
	1	2	3	4	5	6	7	8	9	10
Conc. ng/μl	187,8	264,9	60,0	247,8	224,9	160,2	273,0	260,5	-	269,1
Purity	1,88	1,90	1,94	1,88	1,86	1,91	1,87	1,87	-	1,89

5.2. Verifying the quality of bacmid DNA

The mutated pFastBacTM1 vectors were transformed into the DH10Bac cells. Recombinant DNAs were selected by blue-white selection. Two white positive colonies from both constructs were selected for restreaking whereof a single colony was selected for the production of liquid *E.coli* cultures. The recombinant bacmid DNAs were isolated from both cultures.

The bacmid DNAs were analyzed by agarose gel electrophoresis to confirm the presence of high molecular weight DNA. Bacmids are large molecules and therefore they migrate slowly on the gel (Figure 13). Bands 1-2 present the wild types aa536-1132 and bands 3-4 the wild types aa513-1132. Band three shows that the bacmid DNA purification was not achieved in one of the aa513-1132 wild type in contrast with the wild type aa536-1132.

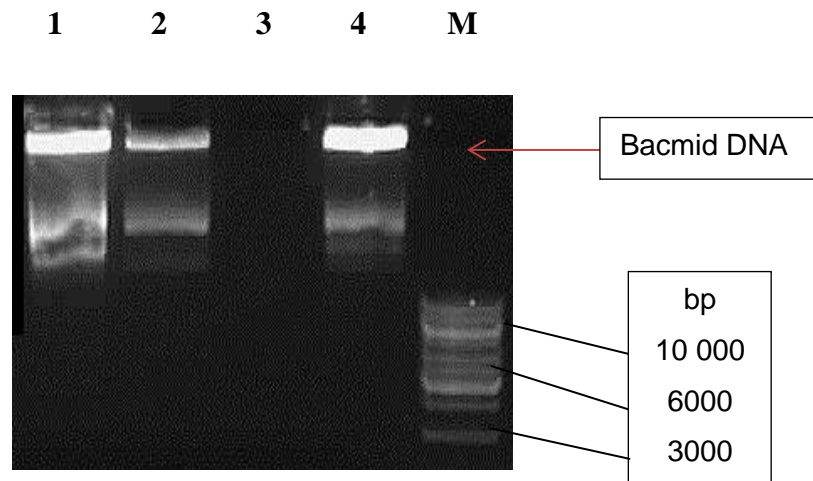


FIGURE 13. Purified bacmid DNA run by agarose gel electrophoresis. Bands 1-2 present the wild types aa536-aa1132 and bands 3-4 the wild types aa513-aa1132

The PCR analysis was performed to confirm that the gene of interest has transposed to the bacmid. When the gene of interest has transposed with pFastBacTM1, the amplified size is 2300 bp plus the size of insert (843 bp). Bands 1-2 (Figure 14) present the wild types aa536-aa1132 and bands 3-4 the wild types aa513-aa1132. All samples migrated on the gel near the size of 3143 bp which indicates successful transposition.

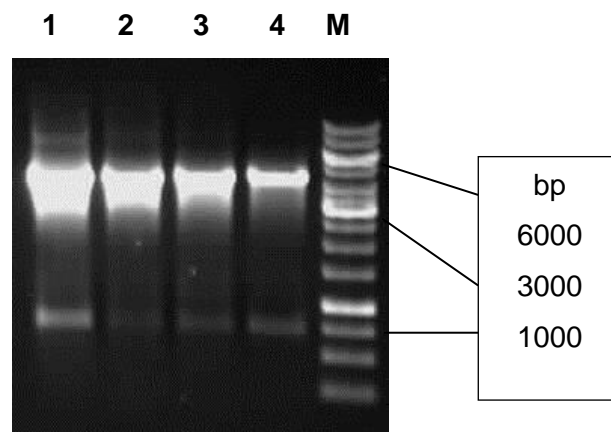


FIGURE 14. Agarose gel electrophoresis was performed to confirm the transposition of the desired gene to the bacmid.

5.3. Validation of high protein expression

The Western blot results (Figure 15) confirm the high protein expression of the recombinant JAK2 proteins in Sf9 cells. The left figure (15a.) shows the protein expression at P0 stage as done by using anti-JAK2 antibody. The molecular weight of JAK is approximately 130 kDa and the JH1-JH2 domains comprise half of the whole molecule which is 65 kDa (Figure 15a.–b.). The aa513-1132 is a larger construct than aa536-1132, and therefore it migrates more slowly on the gel (Figure 15a.). The right panel (15b.) presents the expression at P1 stage infection, which shows a very robust expression for aa513-1132 construct.

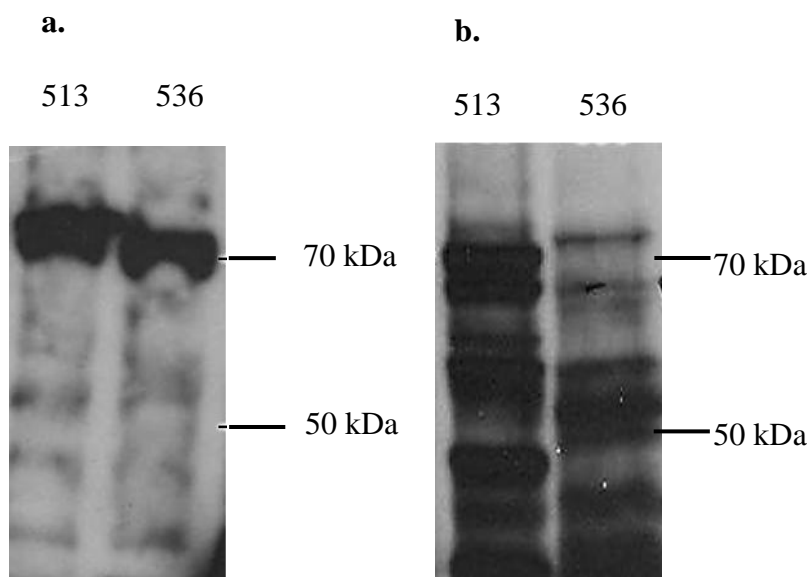


FIGURE 15. High protein expression of recombinant JH1-JH2 of JAK2 in Sf9 cells
a) P0 stage virus infection and protein expression
b) P1 stage virus infection and protein expression

On the contrary, aa536-1132 construct shows weak bands due to its insolubility. The insolubility of a protein can be found during the infection stages P1-P2 when the viral amplification occurs. The construct aa513-aa1132 is soluble (Figure 15b.) appearing with the clear bands. This construct was used in protein production stage P3, but also revealed to be insoluble.

6 DISCUSSION

The purpose of this study was to generate K581A kinase-inactivating point mutation into JH1-JH2 domains of JAK2 protein and produce mutant JAK2 in baculovirus system. The objective of the study was to analyze the solubility and biological function of recombinant JAK2 protein. Two different sizes of constructs were mutated and transformed into competent DH10Bac cells containing bacmid DNA. Recombinant bacmid DNA was isolated and introduced to Sf9 (insect) cells which expressed the desired proteins.

Producing soluble protein components have been shown to be a difficult task when it comes to protein kinases which are highly phosphorylated, leading them to become unstable *in vitro*. In contrast, many kinases cannot be produced as unphosphorylated proteins, obviously due to highly hydrophobic residues on their surface that interfere with their solubility. Stabilizing the structure of JAKs is required to obtain a stable and soluble protein. The previous structural studies of JH1 domain of JAKs were achieved only after the JH1 domain was coupled with a specific inhibitor or compound.

The results indicate that the used baculoviruses were able to encode recombinant JH1-JH2 JAK2 protein with residues 536-1132 and 513-1132. The results also show that the Sf9 (insect) -cells were able to express the recombinant JAK2 protein but not in soluble form, referring that the objective of the thesis was not achieved.

The domains JH1 and JH2 are both catalytically active domains which complicates the biological and structural studies of the protein. Before this thesis project began, a mutation D976N was introduced in the JH1 domain. D976N mutation kills the ATP-binding activity of JH1 domain but does not harm the protein structure. This mutant protein JH1-JH2 D976N was shown to be highly soluble in Sf9 -cells, but at same time it was structurally highly unstable. During this thesis, another mutation K581A was generated in the JH2 domain. The K581A mutation removes the ATP-binding activity of JH2 domain but also keeps the domain structure unharmed. When this protein with two different mutations was produced in Sf9 cells, the protein was insoluble referring that the two mutations (K581A and D976N) together, could interfere with the stability of the protein.

This study was a part of a wider ongoing research, and more mutation studies where a stable two-domain structure for JAK2 kinase is produced, are required. Especially, the JH2 domain as a regulator of the whole JAK2 protein is essential for the function of JAK2. The recent studies show that JH2 domain phosphorylates negatively in two sites, Ser523 and Tyr570 which are conserved only in JAK2. Previous structural studies also show that for example MPN mutation V617F impairs the catalytic activity of the JH2 domain. As a result, JAK2 loses its negative regulatory mechanism and the JH2 domain becomes constitutively activated.

Several inhibitors as therapeutical drugs for JAK2 have been tested but the results have not been satisfying. Producing JAK2 mutation specific inhibitors is the most desirable solution for drug development, and structural information obtained from crystallographic data on soluble proteins is essential for drug development. Therefore constructing soluble recombinant proteins and being able to harvest them is of interest for researchers.

The mutations were generated using the QuikChange® site-directed mutagenesis and the Bac-to-bac® Baculovirus Expression System was used to produce desired recombinant proteins. Both assays included several smaller steps such as DNA isolation, transformation, sequencing, transfection, blue-white selection, agarose gel electrophoresis, PCR analysis and Western blotting. In this thesis, the different steps were repeated if necessary but the methods were entirely used only once. Only the crystal structures of pseudokinase (JH2) and kinase (JH1) domain have been resolved. More recombinant protein production is required for X-ray crystallography studies which will give more information on the three-dimensional structure and catalytic activity of JAK2 and allow the development of new therapeutic drugs for a trial.

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