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Research Article

A functional Jak2 tyrosine kinase domain is essential for mouse development

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ABSTRACT

Jak2 is a member of the Janus family of tyrosine kinases and is involved in cytokine signaling. As a part of a study to determine biological functions of Jak2, we used molecular modeling to identify W1038 as a residue that is critical for tyrosine kinase function. Mutation of W1038, in tandem with E1046, generates a dominant-negative form of the Jak2 protein. Mice that were engineered to express two copies of this dominant-negative Jak2 protein died in utero. Additionally, heterozygous mice expressing Jak2 with kinase activity that is moderately reduced when compared to wild-type activity appear phenotypically normal. Collectively, these data suggest that Jak2 kinase activity is essential for normal mammalian development.

Introduction

Jak2 is a member of the *Janus* family of tyrosine kinases, which also includes Jak1, Jak3 and Tyk2. Jak proteins interact with a variety of receptors, including cytokine receptors, growth factor receptors and G-protein-coupled receptors [1]. Functionally, Jak2 has been implicated in several critical biological activities, including proliferation, cell survival and developmental processes. For example, deletion of the murine Jak2 gene by homologous recombination results in embryonic lethality due to a lack of fetal hematopoiesis [2,3]. Jak2 kinase activity is also reported to play a causative role in several myeloid and lymphoid leukemias. Chromosomal translocation of the Jak2 kinase domain to the ETS transcription factor protein TEL generates a hyperactive kinase that is critical in pathogenesis of these cancers [4–6].

Structurally, Jak family members contain seven conserved Jak homology (JH) domains. JH 1 and 2 encode the kinase and pseudokinase domains, respectively [7]. JH 3–7 comprise the N-terminal region of Jaks, the site(s) of interaction with various cytokine receptors. JH1, the kinase domain, has been the focus of numerous mutagenesis studies, which have identified several residues essential for kinase activity [8–11]. Recent structure–function studies have focused new attention on potential functions for the pseudokinase and the N-terminal domain. The pseudokinase domain, which is conserved within the Jak family, likely plays a role in regulating the kinase domain [12–14], while physical interaction with various cognate receptors was mapped to regions within the N-terminal domain [15–19]. In addition to mediating interactions with cognate receptors, the N-terminal domain contains a FERM domain, an approximately 300 amino acid protein

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interaction domain. Initially, this domain was identified in band 4.1 protein, ezrin, radixin and moesin and is thought to play a role in membrane–cytoskeletal interactions [20,21]. The FERM domain of Jak2 acts as a "chaperone" for the Golgi processing and cell surface expression of the Epo receptor (EpoR). Huang et al. demonstrated that wild-type Jak2 enhances membrane expression of EpoR and that this does not require an active kinase domain since in vitro expression of the N-terminal domain alone was sufficient for enhancing the cell surface expression [22]. These data are the first functional data to suggest that the N-terminal domain of Jak2 may have critical biological functions, independent of the kinase activity.

The idea that a cytoplasmic kinase has important biological function independent of catalytic activity is supported by a recently published study by Middendorp and colleagues [23]. Bruton's tyrosine kinase (Btk) is an important kinase in B cell development and also functions as a tumor suppressor [24,25]. Approximately 44% of mice lacking Btk and its adaptor protein SLP-65 develop pre-B cell lymphoma, a much higher frequency than in wild-type mice. When a kinase-inactive form of Btk was expressed in the double-deficient background, the observed lymphoma frequency was reduced to ~14%. Thus, the tumor suppressing function of Btk is independent of its catalytic activity.

In this study, we wanted to identify in vivo functions for Jak2 that were dependent on the presence of the Jak2 protein, but independent of Jak2 kinase activity. Our goal was to generate mice expressing a dominant-negative form of Jak2 in place of the wild-type allele. In a system in which signal transduction is dependent upon dimerization, such as dimerization of cytokine receptors and subsequent activation of Jak2 [26–29], a dominant-negative Jak2 could potentially reduce the activity of the dimers. Presumably, mice with two dominant-negative Jak2 alleles (i.e., a DN/DN genotype) would identify signaling properties of Jak2 that were independent of the kinase domain. In addition, a knock down of Jak2 signaling would be expected in heterozygous mice (i.e., a +/DN genotype). Here, we report that inactivation of the kinase domain on both alleles of the Jak2 gene in mice is embryonic lethal and that heterozygous mice appear phenotypically normal when compared to wild-type littermate controls. Collectively, the data suggest that, in vivo, the tyrosine kinase function of Jak2 is essential for life.

Materials and methods

Molecular modeling

Murine Jak2 amino acid sequence spanning from glutamic acid 814 to the stop codon (position 1129) was used to generate a structural model of the Jak2 kinase domain. The model was based on five known kinase crystal structures; Hck, Lck, insulin receptor, fibroblast growth factor receptor and the carboxyl terminus of c-Src [30–34]. The modeling algorithm weighted the model in a manner that was proportional to the amino acid sequence homology against each of the five known structures using the program Modeller as described [35].

In vitro Jak2 autophosphorylation assay

Point mutations were introduced into Jak2 via the QuikChange site directed mutagenesis system (Stratagene) in the context of a pRC-CMV plasmid containing the full-length murine Jak2 cDNA. Mutations were confirmed by DNA sequence analysis. The Jak2 constructs were expressed and activated using the vaccinia virus-mediated transfection/infection protocol [36,37]. Briefly, 100-mm dishes of nearly confluent BSC-40 cells were transfected with the indicated Jak2 DNA expression plasmids using Lipofectin and following the manufacturer's instructions (Invitrogen). After a 4-h incubation, vaccinia virus clone vTF7-3 was added at a multiplicity of infection of 1.0 and incubated for 1 h. The medium was then removed, and the cells were incubated overnight in DMEM with 10% newborn calf serum. At 18 to 20 h post-infection, the cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 0.8 ml ice-cold RIPA buffer containing protease inhibitors. The samples were sonicated, incubated on ice for 1 h and spun at 16,000×*g* for 5 min at 4°C. The protein supernatants were then immunoprecipitated for 4 h at 4°C with either 2 μg of anti-Jak2 antibody (Santa Cruz Biotechnology, HR-758) or 2 μg of anti-HA antibody (Santa Cruz Biotechnology, F-7) and 20 μl of Protein A/G Plus agarose beads (Santa Cruz Biotechnology, SC-2003). After centrifugation, the beads were washed 3 times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl and 0.1% Triton X-100) and resuspended in SDS-containing sample buffer. Bound proteins were boiled, separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then Western blotted with either anti-phosphotyrosine antibody (Santa Cruz Biotechnology, PY99) or anti-Jak2 antibody (Upstate Biotechnology Inc., 06-255).

Creation of kinase-inactive Jak2 mice

A BAC library containing SV129 mouse genomic DNA was screened using a 300 bp cDNA encoding a portion of the Jak2 kinase domain (Genome Systems, Inc.). Three putative clones were identified and subsequently digested with restriction enzymes, and the resulting fragments were Southern blotted using a PCR-generated Jak2 cDNA probe encoding the kinase domain at nucleotide positions 3149–3312 (GenBank accession number L16956). A positive 5.9 kb *Xba*I/*Bam*HI fragment was subcloned into the pBlueScript vector. A total of 4033 nucleotides of DNA sequence were obtained from the 5′ end of the fragment and deposited in GenBank (accession number AY785782). A determination of intron/exon organization was made by comparing our sequence with that of known Jak2 genomic and cDNA sequence. Site-directed mutagenesis (QuikChange) was then used to generate the W1038G and E1046R mutations. An exogenous *Bam*HI site was generated via redundant nucleotide substitution to aid in the verification of the targeted allele. The 3.8 kb testis ACE/cre-neomycin resistance cassette [38] was inserted into an exogenous *Bcl*I site created in the intron just 5′ of the active site mutations. The 1.8 kb thymidine kinase cassette was inserted into the *Sal*I site located in the multiple cloning region of pBlueScript. The targeting vector (14.4 kb) was linearized with *Not*I and electroporated into R1 ES cells. These cells were derived

from a 129/SV×129/SvJ F1 embryo [39]. After positive and negative selection, individual ES clones were isolated and screened for the targeted allele by Southern blot hybridization and PCR. Generation of chimeric mice and subsequent breeding was performed as previously described [40]. All studies were done using mice of F2 generation from crosses of F1 heterozygous mice. All animal care and practices were performed according to Emory University Institutional Animal Care and Use Committee (IACUC).

Primers

The primers using in screening the ES clones were as follows: 5′ ACGAAGTTATGAATTCATCG 3′ (forward) and 5′ GTGTAG-TACAACTGTAAG 3′ (reverse). The primers used in genotyping the mice were as follows: 5′ GTTAGGAGGACCCACTGTCAG 3′ (forward) and 5′ TAAGAAGCGCACTGACCACGG 3′ (reverse).

Analysis of Jak2 mutant mice

Day 12.5 embryos were isolated in cold PBS, and each embryo was homogenized in 500 μl of HEPES lysis buffer (10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM NaCl, 1 mM DTT 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitors) using a tight fitting Dounce homogenizer and 30 strokes on ice. After 60 min of incubation on ice, the lysate was clarified by centrifugation at 10,000×*g* for 10 min in a microfuge. The supernatant was removed, and samples (25 μg) were combined with SDS loading buffer, heated to 95°C for 5 min and run on a 7.5% SDS-PAGE gel. Western blot analysis was performed as described above.

Vascular aortic smooth muscle cell isolation from wild-type and heterozygous mice

Vascular aortic smooth muscle cell (VSMC) isolation was adapted from a previously published work [41]. Briefly, Jak2 +/+ and Jak2 +/DN mice (8–12 weeks) were used for smooth muscle isolation from thoracic aorta. Ketamine:xylazine: heparin at a ratio of 2:1:1 was administered by intraperitoneal injection at a dose of 2.5 μl/g body weight. After anesthesia, the thoracic cavity was opened, the aorta was cut at the iliac bifurcation and 3 ml of PBS was slowly perfused through the left ventricle using a 25-gauge needle. Fat and adventitia were removed, and the aorta transferred from the mouse to a dish containing DMEM with 25 ng/ml Fungizone solution (Invitrogen). The aorta was cut into rings and incubated in a solution of 1.4 mg/ml collagenase (type II, Worthington Biochemical Corp.) in complete media for 5 h at 37°C. The dissociated cells were rinsed two times in complete media and plated in 24-well plates. Typically, cells were >80% smooth muscle cells as assessed by staining with anti-smooth muscle alpha-actin (clone 1A4, Sigma-Aldrich).

In vitro characterization of kinase activity in vascular smooth muscle cells

Primary cultures of quiescent Jak2+/+ and Jak2+/DN vascular smooth muscle cells were pretreated for 1 h with 0.1 mM Na_3VO_4 and then stimulated with 600 ng/ml growth hormone (GH) as indicated. The cells were lysed, and Jak2 tyrosine phosphorylation levels were measured via immunoprecipitation and Western blot as described above. Densitometry was performed on the resulting chemiluminescent-derived protein bands using the UN-SCAN-IT scientific software package (Silk Scientific).

Hematocrit measurements and treatment with phenylhydrazine

Wild-type (+/+, $n = 6$) and heterozygous (+/DN, $n = 6$) mice were injected interperitoneally with phenylhydrazine (5 mg/ml in 0.9% saline) at a dose of 60 mg/kg body weight. Injections were performed on day 1 and day 2. On days 0, 3, 5, 7 and 9, a drop of tail blood was collected in a capillary tube, centrifuged at 12,000×*g* for 4 min and loaded into a manual microcapillary reader for the determination of the hematocrit. The data shown are an average (±SEM) of the hematocrit taken on each day for each genotype.

Results

Molecular modeling of Jak2 active site predicts new residues important for kinase activity

Previous structure–function studies have identified several residues in the activation loop and the ATP binding sites that are critical for the kinase activity. These include K882E, Y1007F and the double mutation of W1020G/E1024A [9–11]. This double mutation is catalytically inactive and can function as a dominant-negative [10]. Molecular modeling of the Jak2 active site has identified a new residue, E1046, which is also critical for enzymatic activity. The negative charge of E1046 forms a hydrogen bond with the ring nitrogen of W1020, stabilizing the activation loop in a proper tertiary structure [42]. Here, we investigated a second potentially important residue, W1038, identified by that model. The location of the tryptophan side chain in three-dimensional space suggests that this residue may be critical for the tertiary structure and interior packing of the activation loop (Fig. 1).

In vitro evidence suggests the E1046 and W1038 residues are critical for kinase function

To determine the importance of the W1038 and E1046 residues on Jak2 kinase function, we measured the ability of Jak2 to autophosphorylate when these residues were mutated. Wild-type Jak2 and a W1038G point mutant were overexpressed in BSC-40 cells using vaccinia virus transfection/infection protocol. Jak2 immunoprecipitates were subsequently immunoblotted with anti-phosphotyrosine antibody. Wild-type Jak2 was found to readily tyrosine autophosphorylate whereas the W1038G mutant was not, even though both were expressed at roughly equivalent levels (Fig. 2A). This is the first in vitro evidence to suggest that the W1038 residue is critical for Jak2 tyrosine autophosphorylation.

To determine whether a W1038G/E1046R double mutant was similarly incapable of tyrosine autophosphorylation, cells were transfected with the indicated plasmids and Jak2 tyrosine

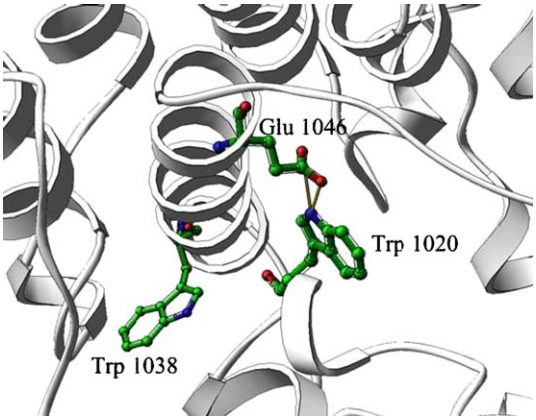

Fig. 1 – Molecular modeling of the Jak2 active site. The active site of murine Jak2 from AA 814 to the stop codon is represented in the ribbon model. Tryptophan 1020, tryptophan 1038 and glutamic acid 1046 are shown as ball-and-stick figures. The oxygen of Glu 1046 forms a hydrogen bond (0.18 nm, shown in yellow) with the ring nitrogen of Trp 1020. Trp 1038 interacts with an adjacent α-helix via a helix/helix interaction.

autophosphorylation levels were again measured by Jak2 immunoprecipitation followed by anti-phosphotyrosine immunoblotting. We found that, while wild-type Jak2 was able to tyrosine autophosphorylate, both the W1038G/E1046R and the well-characterized kinase-inactive W1020G/E1024A mutant were completely unable to tyrosine autophosphorylate (Fig. 2B).

To demonstrate that expression of the W1038G/E1046R mutant protein acts as a true dominant-negative, we co-expressed an HA-tagged wild-type Jak2 protein along with increasing amounts of the W1038G/E1046R mutant and measured wild-type Jak2 tyrosine phosphorylation levels via immunoprecipitating with anti-HA antibody and immunoblotting with anti-phosphotyrosine antibody. We found that significantly increasing the expression of the W1038G/E1046R mutant protein in cells inhibited HA-tagged wild-type Jak2 autophosphorylation in a dose-dependent manner (Fig. 2C). Furthermore, expression of the W1038G/E1046R mutant had no affect on the expression of the wild-type protein. As such, these data suggest that the reduction in autophosphorylation of wild-type Jak2 protein is due to the dominant-negative activity of the mutant and not due to a simple reduction in wild-type Jak2 protein expression.

Next, we compared the dominant-negative activity of W1038G/E1046R to that of the well-characterized W1020G/E1024A dominant-negative mutant. To do this, an HA-tagged wild-type Jak2 protein was co-expressed with increasing amounts of either the W1038G/E1046R mutant or the W1020G/E1024A mutant. The tyrosine phosphorylation levels were again determined via anti-HA immunoprecipitation and anti-phosphotyrosine Western blot analysis (Fig. 2D). We found that the W1038G/E1046R double mutant possessed some dominant-negative properties as it reduced the levels of wild-type Jak2 tyrosine autophosphorylation. However, the dominant-negative activity was not as potent as that seen for the W1020G/E1024A mutant (~4-fold less). These data suggested that, in vitro, the W1038G/E1046R mutant possessed a "mild to moderate" dominant-negative phenotype, when compared to the well-characterized W1020G/E1024A mutant.

Collectively, the data in Fig. 2 demonstrate that conversion of W1038G completely abolishes Jak2 kinase activity. Furthermore, a Jak2 molecule expressing a W1038G/E1046R double mutation is not only kinase inactive, but also possesses a mild to moderate dominant-negative phenotype.

Generation of kinase-inactive Jak2-expressing mice

The previous in vitro evidence suggests that residues E1046 and W1038 are important for Jak2 kinase activity and that mutation of these residues results in a Jak2 protein that is both kinase-inactive and dominant-negative. To answer the central question of whether Jak2 protein has important biological roles independent of its kinase activity, we generated genetically modified mice in which the Jak2 allele contained the W1038G and E1046R point mutations. In making these dominant-negative mice, we chose to mutate the W1038/E1046 residues, and not the W1020/E1024 residues, because residue W1020 is at an exon/intron splice junction, and our concern was that mutating this residue would possibly disrupt the splicing machinery and potentially generate a truncated molecule. The exon/intron organization of this region was determined by DNA sequence analysis of a 5.9 kb BAC clone that spans the active site of Jak2 (GenBank accession number AY785782).

Fig. 3A shows the DNA sequence for the intron/exon region at the end of the Jak2 activation loop. A *BclI* site was introduced into the intron sequence so that the ACE/Neo cassette could be inserted within close proximity to the modified allele. Fig. 3B shows that nucleotide and protein sequence for the modified exon. The two point mutations at positions W1038 and E1046 and the exogenous *Bam*HI restriction site were generated by redundant nucleotide substitution. The *Bam*HI site aided in the confirmation of the targeted allele.

An overview of the targeting construct is shown as Fig. 4A. The targeting construct was generated from a 5.9 kb genomic DNA piece containing 3 exons from the Jak2 kinase domain. The testis ACE promoter-neomycin cassette, used to screen for positive integrations into the Jak2 locus, was subsequently self-excised during germline transmission of the targeted locus, and the only remaining exogenous sequence in the locus was a single loxP site [38]. To identify properly targeted ES clones, genomic DNA was digested with *Bam*HI and Southern blotted with a fragment from the Jak2 cDNA (Fig. 4B). To verify that the genetic modification had occurred using an alternate approach, genomic DNA was amplified by PCR using primers that flank the remaining loxP site (Fig. 4C). Finally, the point mutations were verified by DNA sequence analysis (data not shown). Collectively, the data indicated that the Jak2 allele was modified as intended.

F1 generation mice were intercrossed, and the genotypes of subsequent pups were analyzed by PCR. The only progeny that were born were either wild-type or mice that were heterozygous, i.e., one copy of the wild-type Jak2 allele and one copy of the modified dominant-negative allele (+/DN). Of 177 mice, 67 were wild-type, 110 were +/DN and none was DN/DN. Analysis of the embryonic phenotype of the DN/DN mice at day 12.5 postcoitum (p.c.) is shown in Fig. 5. To understand why no DN/DN mice were born, pregnant females were sacrificed at day

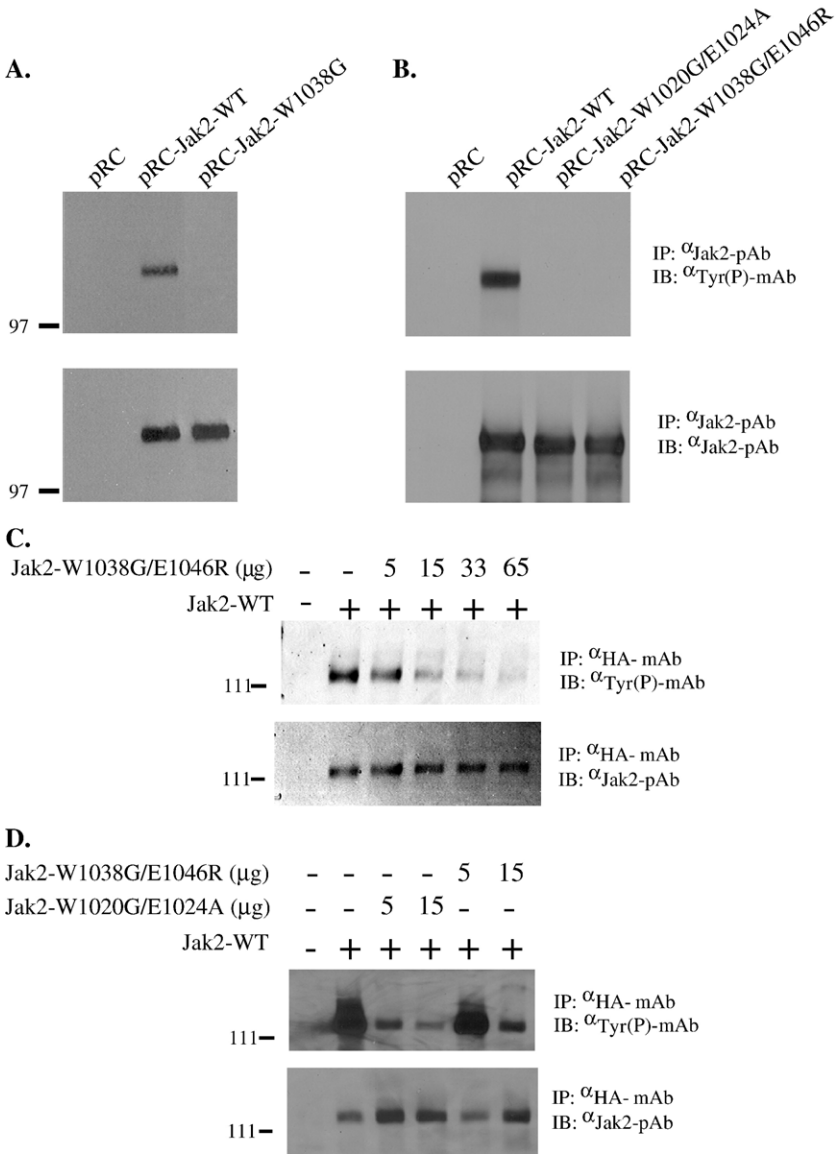

Fig. 2 – Mutations at W1038G and E1046R act as a dominant-negative. Wild-type and mutant Jak2 constructs were overexpressed in BSC-40 cells. Autophosphorylation of wild-type and the single mutant W1038G (A) and the double mutants W1020G/E1024A and W1038G/E1046R (B) was assessed by immunoprecipitation (IP) with anti-Jak2 antibody and then immunoblotting (IB) with anti-phosphotyrosine antibody. Equivalent levels of protein expression were verified by immunoblotting the same membranes with anti-Jak2 antibody. In (C), increasing amounts of W1038G/E1046R construct and a constant amount of the HA-tagged wild-type Jak2 construct were added to BSC-40 cells. Autophosphorylation of the HA-Jak2 construct was assessed by anti-HA immunoprecipitation and immunoblotting with anti-phosphotyrosine antibody. In (D), increasing amounts of each double mutant construct were added to BSC-40 cells in addition to a constant amount of HA-tagged wild-type Jak2. The ability of each double mutant to act as a dominant-negative protein was assessed by immunoprecipitation with anti-HA antibody and immunoblotting with anti-phosphotyrosine antibody. Equivalent level of protein expression was verified by immunoblotting the same membrane with anti-Jak2 antibody. The Western blots shown are a representative example of 3 independent experiments. The molecular size markers are in kilodaltons (kDa).

12.5, embryos were isolated, examined and genotyped by PCR. Wild-type embryos consistently had a bright red appearance in the area of the fetal liver, indicative of the active fetal erythropoiesis that is typical of this stage of development (Fig. 5A). In contrast, all the DN/DN embryos (12 separate embryos) were somewhat smaller, quite pale and lacked any evidence of the red coloration typical of liver erythropoiesis. This phenotypic appearance was identical to the phenotype reported for mice lacking all expression of the Jak2 gene (i.e., Jak2 knockout mice) [2,3]. To rule out the possibility that, in our DN/DN embryos, the modified Jak2 protein was degraded in utero, we analyzed lysates from +/+, +/DN and DN/DN embryos at day 12.5 by Western blot analysis for the presence of Jak2 protein (Fig. 5B). This showed that, in DN/DN embryos, Jak2

A: 5'gttaggaggacccactgtcagcatgtgtcagattcatagctgtcgttcatcactggggagaacagcagag
caaaggccagatgggaaaggtctgtggagc**tgatca**caggccttctgtccccttttgttcccctgggtgttagt
cattngnttgtttcccccctccaatataactgttcattccatgaagtaactcttctaaatgattgcaatattgcagGT
ACGCACCTGAATCCTTGACGGAGAGCAAGTTTTCTGTGGCCTCAG
ATGTGTGGAGCTTTGGAGTGGTTCTATACGAACTTTTCACATACA
TCGAGAAGAGTAAAAGTCCACCCGTGGtcagtgcgcttctta 3'

B: nt 3152-3271; AA 1021-1059
GTACGCACCTGAATCCTTGACGGAGAGCAAGTTTTCTGTGGCCTC
Y A P E S L T E S K F S V A S
AGATGTG***GGATCC***TTTGGAGTGGTTCTATAC***AGA***CTTTTCACATA
D V **G** S F G V V L Y **R** L F T Y
CATCGAGAAGAGTAAAAGTCCACCCGTGG
I E K S K S P P V

Fig. 3 – Sequence data. (A) The genomic DNA sequence encoding the modified portion of the murine Jak2 kinase domain is shown. The intron sequence is in lower case letters and exon sequence (nt 3152–3271, AA 1021–1059) is in upper case letters. The *BclI* site (tgatca) added to the intron sequence for the insertion of the self-excising Neo cassette is indicated in bold. The sequence of the primers used to genotype pups is underlined. (B) The DNA and protein sequence corresponding to the modified exon is shown. Both the W1038G and E1046R substitution mutations are in boldface. Additionally, creation of the silent *Bam*HI mutation used for detection of the modified allele is also shown in boldface.

protein is present at levels equivalent to those of the other two genotypes. These data eliminate the possibility that the phenotype seen in DN/DN embryos is due to the lack of expressed Jak2 protein. Taken together, our data show that DN/DN mice express a mutant form of Jak2 protein and that the mutations in this protein result in an embryonic lethal phenotype equivalent to the defect observed in the total Jak2 knockout.

With respect to the heterozygous animals, the mice were grossly normal when compared to their +/+ littermates. Their life span and body weights were not significantly different from +/+ mice. Finally, heterozygous offspring mated normally and produced litters that were not significantly different from their +/+ littermates.

Collectively, our data demonstrate that mice harboring two copies of a modified Jak2 allele, which lacks any tyrosine kinase function, die during embryonic development. In contrast, mice harboring a single copy of a W1038G/E1046R modified allele (+/DN) are grossly normal when compared to their +/+ littermates.

The level of Jak2 activity in +/DN cells is slightly lowered compared to wild-type control cells

We now wanted to determine whether, in vivo, the W1038G/E1046R mutation yielded a Jak2 protein with a dominant-

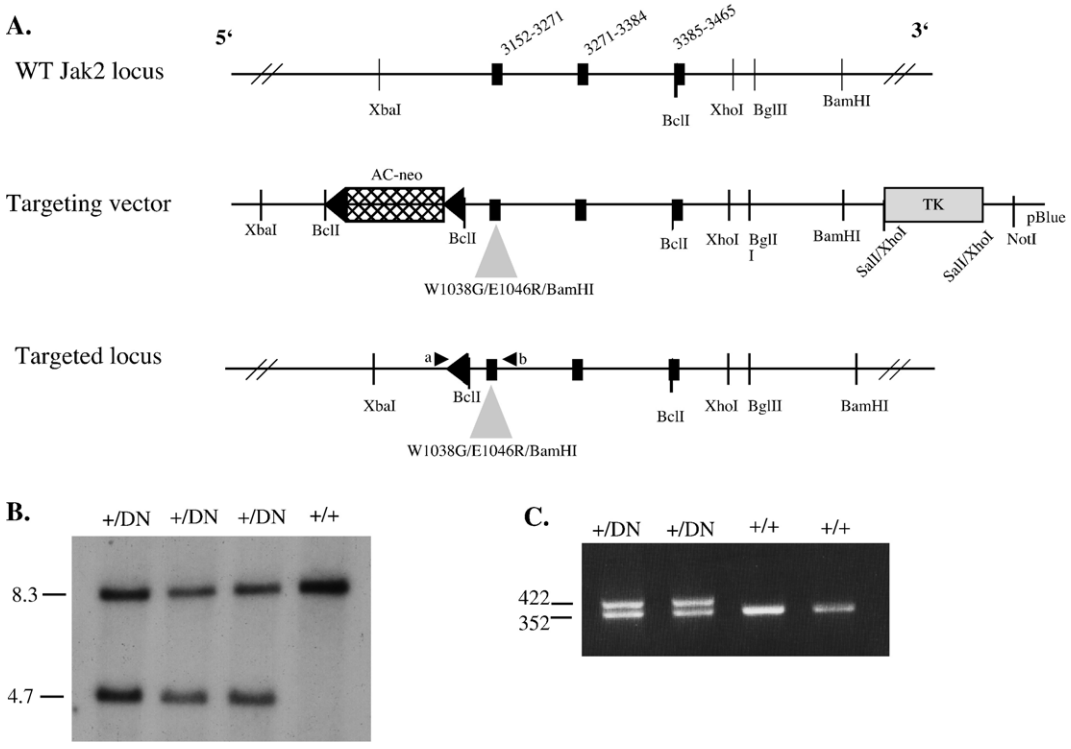

Fig. 4 – Generation of dominant-negative Jak2 expressing mice. (A) The black boxes represent 3 exons present in the 5.9 kb *Xba*I/*Bam*HI BAC clone, which spans from *Xba*I to *Bam*HI. The numbering is based on accession # L16956. The 3.8 kb self-excising testis ACE cre-neomycin cassette (AC-neo, hatched box) with flanking loxP sites (black triangles) was inserted into the intronic sequence at an exogenous *BclI* site. The 1.8 kb thymidine kinase cassette was inserted into a *SalI* site. After germline transmission, the AC-neo cassette self-excised, leaving a single loxP site in the modified allele. (B) Southern blot analysis using a 500 bp Jak2 probe was used to identify correctly targeted ES cell clones. The ladder is in kilobase (kb). (C) Genotyping of pups from F1 intercrossing was performed by PCR using primers a and b which flank the loxP site. The ladder is in basepairs (bp).

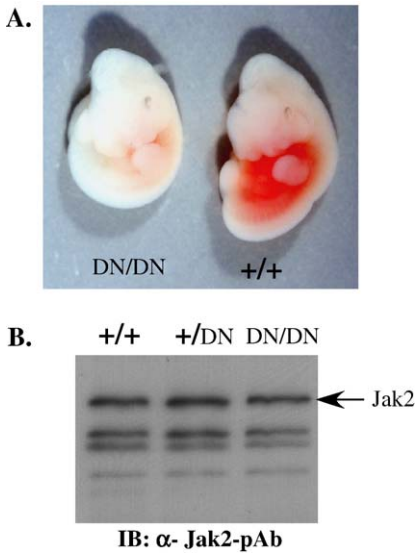

Fig. 5 – Analysis of mutant Jak2 embryos. (A) Day 12.5 p.c. embryos were removed from the embryo sacks and photographed. (B) Expression of Jak2 protein in lysates from +/+, +/DN and DN/DN embryos (day 12.5) was assessed by immunoblot using anti-Jak2 antibody (*n* = 4 individual DN/DN embryos). The arrow indicates the mature form of Jak2 at 120 kDa.

negative phenotype. For this, vascular aortic smooth muscle cells were first isolated from +/+ and +/DN mice and then cultured. The cells were subsequently treated with growth hormone (GH), and Jak2 tyrosine autophosphorylation levels were measured by immunoprecipitation and Western blot analysis (Fig. 6A). Jak2 autophosphorylation in response to GH was reduced in the +/DN cells when compared to the +/+ cells. To determine the relative levels of Jak2 autophosphorylation as a function of genotype, densitometric analysis was performed on the blots representing Fig. 6A. The data are expressed as the ratio of GH-treated tyrosine phosphorylated Jak2:total Jak2 protein (Fig. 6B). We found that there was a higher ratio of tyrosine phosphorylated Jak2:total Jak2 in +/+ cells than in +/DN cells, suggesting that the +/DN cells have a marked reduction in their ability to activate Jak2. Similar to the in vitro data presented in Fig. 2, these data suggest that the targeted locus does produce a Jak2 protein with mild dominant-negative phenotype.

+/DN mice have normal hematopoiesis

Hematopoiesis is intimately tied to Jak2 expression. The deletion of the entire Jak2 gene results in embryonic death due to a lack of definitive erythropoiesis [2,3]. To determine if the dominant-negative activity of the Jak2 mutant protein exerted a physiological effect, we examined hematopoietic function by measuring the hematocrit of +/+ and +/DN adult mice. As shown in Fig. 7A, the hematocrit of the +/DN mice did not differ significantly from +/+ controls.

Since Jak2 is involved in maturation of red blood cells (RBC) precursors, we examined the role of kinase-inactive Jak2 in de novo synthesis of red blood cells (RBC). Acute hydrolysis of RBCs was induced by phenylhydrazine injection, and the hematocrit was measured over time. The data in Fig. 7B illustrate a decrease in the hematocrit after phenylhydrazine treatment and subsequent increase of hematocrit several days later. We observed no statistical difference in the measured hematocrit between the +/+ mice and the +/DN mice. We conclude from these data that, although the level of ligand-activated Jak2 tyrosine kinase activity in the +/DN mouse reaches only ~40% that of wild-type, it is sufficient to support normal hematopoiesis.

Discussion

Jak2 is a protein critical to numerous cytokine and growth signaling pathways as it facilitates signals from cell surface receptors to the nucleus. The molecular modeling of the Jak2

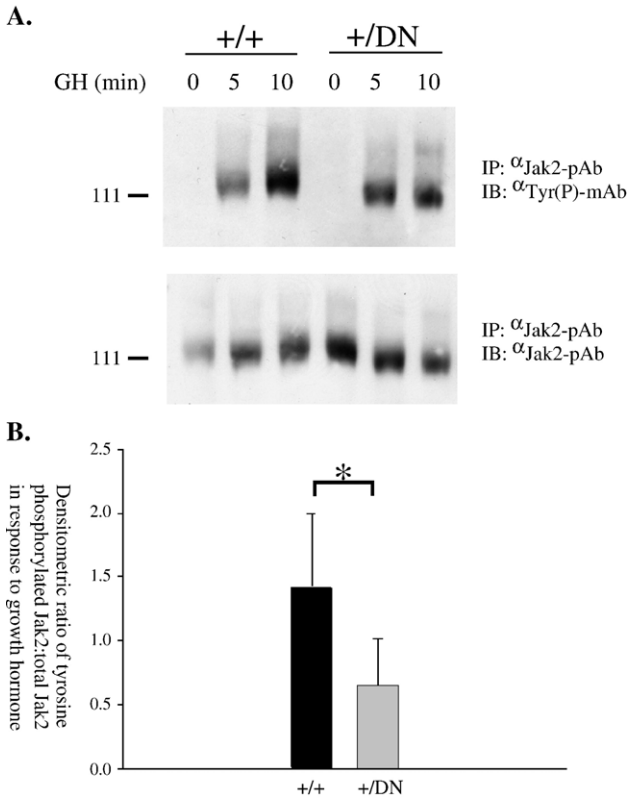

Fig. 6 – Mutated Jak2 protein acts as a dominant-negative in vivo. (A) VSMCs were isolated and cultured from wild-type (+/+) and heterozygous (+/DN) Jak2 mice. Quiescent cells were stimulated with growth hormone (GH) for 0, 5, or 10 min then lysed, and the Jak2 tyrosine phosphorylation levels were detected as described in Fig. 2. The upper panel indicates the level of Jak2 autophosphorylation in vivo, and the lower panel demonstrates the level of expression of Jak2 protein. The Western blots shown are a representative example of 5 independent experiments. (B) Quantitation of the Western blots was performed using UN-SCAN-IT scientific software package. A statistically significant reduction of Jak2 autophosphorylation was seen in the +/DN VSMCs when compared to wild-type controls ($P < 0.05$, Student's *t* test).

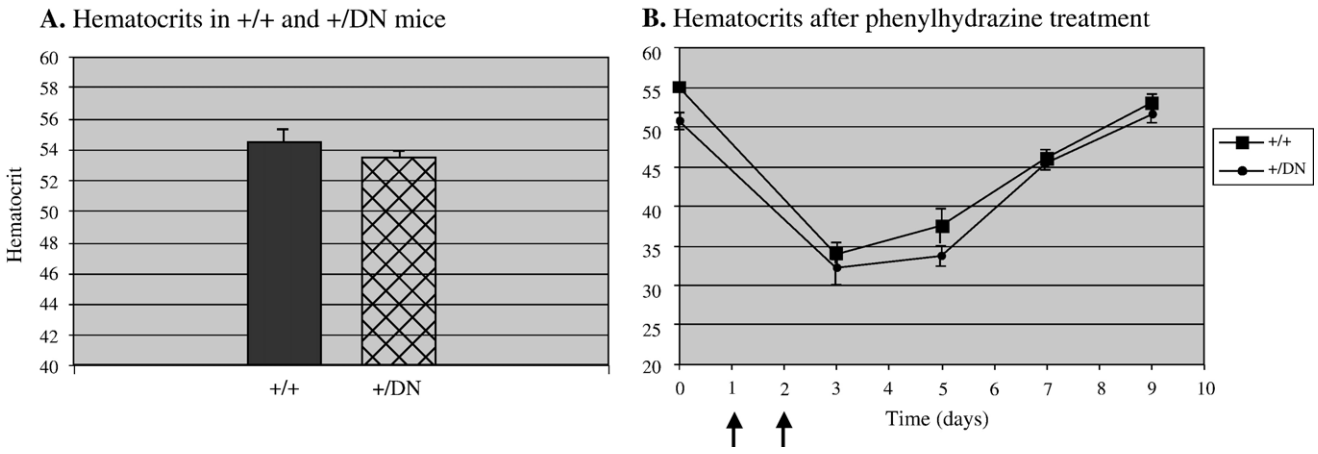

Fig. 7 – Heterozygous mice have normal red blood cell production. (A) Hematocrit levels in wild-type (+/+) and dominant-negative (+/DN) mice. (B) Baseline hematocrit was measured in these animals at day 0. Mice were then treated with phenylhydrazine on days 1 and 2 (arrows) in order to promote red blood cell lysis. Hematocrits were then measured on the indicated days for 9 days total. The data shown are the average value of each genotype on each day ± SEM. There was no statistical difference observed between the two genotypes.

kinase domain has identified a novel amino acid that disrupts kinase function; namely, W1038. We found that the side chain of this amino acid occupies a space between two adjacent α-helices. We inferred that the side chain of W1038 stabilizes the tertiary structure of Jak2 by maintaining an appropriate helix/helix interaction at the position of the activation loop. In vitro experiments demonstrated that the W1038G mutation rendered Jak2 catalytically inactive, thus confirming the predictions made from the molecular model. This report therefore identifies an amino acid adjacent to the activation loop that clearly is critical for tyrosine kinase activity, yet whose role is largely structural via a helix/helix interaction.

The overall biological question addressed in this study was: does Jak2 tyrosine kinase exist solely for its catalytic function or are there important structural roles for it, independent of its tyrosine kinase activity? Clearly, Jak2 tyrosine kinase activity is the predominant *raison d'être*, but this does not preclude it from having other cellular functions. We genetically modified the Jak2 allele to contain the W1038G/E1046R mutations that inactivated the kinase domain and conferred a mild dominant-negative phenotype onto the protein. Mice which have two copies of the mutated allele expressed the mutated Jak2 protein at wild-type levels, as measured by Western blot analysis. However, the phenotype of these mice was embryonic lethal (day 12.5) due to a deficiency in fetal hematopoiesis. In contrast to our work, Neubauer et al. and Parganas et al. both generated mice that completely lacked Jak2 protein, as opposed to production of Jak2 protein which lacks tyrosine kinase function. Both groups found that complete loss of Jak2 protein resulted in an embryonic lethal phenotype (day 12.5) and that there was a concomitant loss of Jak2-dependent signaling [2,3]. One could infer from their data that the lethality was due to the loss of kinase activity but that was not directly tested. Here, we supply evidence that it is, in fact, Jak2 kinase activity that is essential for normal mammalian development.

Analysis of Jak2 activation in the +/DN mice indicated that, overall, Jak2 is unable to activate to levels similar to +/+ littermate controls. These data suggest that the dominant-negative phenotype of the W1038G/E1046R mutant seen in vitro also exists in vivo. However, we were unable to identify a grossly observable phenotype in these animals. The mechanism by which Jak2 functions in hematopoiesis has been well documented, and it is clear that, in these heterozygous mice, red blood cell production is quite normal. Thus, despite a modest reduction of Jak2 kinase activity, the RBC production was unaffected. We therefore conclude that the level of Jak2 kinase activity that remains in these mice is sufficient to sustain hematopoiesis.

In conclusion, we have identified W1038 as an amino acid within Jak2 that is critical for its tyrosine kinase function. We subsequently created mice that expressed a kinase-inactive form of Jak2 and found that it is the tyrosine kinase function of Jak2 that is essential for normal mouse development. Additionally, mice expressing Jak2 with kinase levels that are significantly reduced compared to that of wild-type mice appear normal. Collectively, these data suggest that the regions of Jak2 that are independent of its tyrosine kinase function (i.e., FERM, pseudokinase, SH2-like) are unable to support Jak2-dependent processes. Furthermore, mice expressing a reduced level of Jak2 tyrosine kinase activity appear to mediate normal Jak2-dependent responses.

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