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PHOSPHORYLATION OF Y372 IS CRITICAL FOR JAK2 TYROSINE KINASE ACTIVATION

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Abstract

Jak2 tyrosine kinase plays an important role in cytokine mediated signal transduction. There are 49 tyrosine residues in Jak2 and phosphorylation of some of these are known to play important roles in the regulation of Jak2 kinase activity. Here, using mass spectrometry, we identified tyrosine residues Y372 and Y373 as novel sites of Jak2 phosphorylation. Mutation of Y372 to F (Y372F) significantly inhibited Jak2 phosphorylation, including that of Y1007, whereas the Jak2-Y373F mutant displayed only modest reduction in phosphorylation. Relative to Jak2-WT, the ability of Jak2-Y372F to bind to and phosphorylate STAT1 was decreased, resulting in reduced Jak2-mediated downstream gene transcription. While the Y372F mutation had no effect on receptor-independent, hydrogen peroxide-mediated Jak2 activation, it impaired interferon-gamma (IFNγ) and epidermal growth factor (EGF)-dependent Jak2 activation. Interestingly however, the Y372F mutant exhibited normal receptor binding properties. Finally, co-expression of SH2-Bβ only partially restored the activation of the Jak2-Y372F mutant suggesting that the mechanism whereby phosphorylation of Y372 is important for Jak2 activation is via dimerization. As such, our results indicate that Y372 plays a critical yet differential role in Jak2 activation and function via a mechanism involving Jak2 dimerization and stabilization of the active conformation.

Keywords

Janus Kinase 2; tyrosine phosphorylation; Y372; Jak2 regulation

1. Introduction

Jak2 is a non-receptor tyrosine kinase belonging to the *Janus* family of tyrosine kinases that also includes Jak1, Jak3 and Tyk2. A key cellular role of Jak2 is to phosphorylate and hence activate the STAT family of latent cytoplasmic transcription factors. Once activated, the

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dimerized STAT proteins translocate to the nucleus, bind DNA promoter elements and modulate gene expression. Jak2 is activated extrinsically by a variety of cytokine and growth factor receptor ligands as well as by reactive oxygen species resulting in signaling cascades that are involved in the regulation of cell growth, proliferation and death. Jak2 activity is also intrinsically regulated through the specific phosphorylation/dephosphorylation of some of its tyrosine residues.

The Jak kinases are structurally composed of seven Jak homology (JH) domains. The JH1 domain, located at the C terminal end of the protein, corresponds to the catalytically active tyrosine kinase domain [1]. The JH2 domain that lacks catalytic activity, but has sequence similarity with the JH1 domain, is termed as the pseudokinase domain. The pseudokinase domain has been shown to negatively regulate Jak kinase activity [2; 3; 4]. The JH3 and JH4 regions represent the SH2-like domain whose function is not fully defined. At the amino terminus, the JH4-JH7 regions of Jaks comprise the FERM domain. The FERM domain has been shown to be involved in Jak association with receptors [5; 6; 7]. The significance of the FERM domain was understood when mutations in the Jak3 FERM domain that resulted in the loss of its kinase activity were identified in severe combined immunodeficiency (SCID) patients [5; 8]. Specifically it was reported that these mutations reduced the interaction of Jak3 with the common γ-chain of the IL-2 receptors and in parallel, inhibited its activation in response to ligand binding [5; 8]. Therefore, it appears that structural changes in the FERM domain brought about by point mutations can alter the activity of Jak kinases.

Of the 49 Jak2 tyrosine residues encoded in murine Jak2, phosphorylation of some of them have been shown to play important roles in overall Jak2 tyrosine kinase regulation. Interestingly, many of these characterized tyrosine residues are situated at the C terminus of Jak2, where the pseudokinase and kinase domains reside. For example, phosphorylation of Y1007 in the activation loop of the kinase domain is required for maximal Jak2 activation [9]. Additionally, phosphorylation of Y868, Y966 and Y972 has been shown to enhance Jak2 activation [10; 11]. However, auto phosphorylation at Y913 in the kinase domain negatively regulates Jak2 by suppressing erythropoietin-induced Jak2 activation [12]. Finally, phosphorylation of Y570 situated in the pseudokinase domain of Jak2 has been shown to suppress Jak2 tyrosine kinase activity, while that of Y637 enhances Jak2 activation [13; 14; 15]. Collectively, these data suggest that the activation or inhibition of Jak2 tyrosine kinase can be influenced by the phosphorylation status of its numerous tyrosine residues.

Fewer phosphorylated tyrosine residues have been characterized in the N terminal region of Jak2, which includes the FERM domain. The characterized phosphorylated tyrosine residues in the FERM domain have different consequences for Jak2 tyrosine kinase regulation, based on the presence or absence of ligand activation and the type of ligand-receptor system involved. For instance, phosphorylation of Y119 has been shown to dissociate Jak2 from the erythropoietin receptor and down-regulate its kinase activity in response to erythropoietin [16]. Conversely, phosphorylation of Y119 has no effect on Jak2 regulation in the presence of the interferon-gamma receptor. Our laboratory has shown that phosphorylation of Y201 facilitated Jak2/SHP-2 interaction, which allowed for the recruitment of Jak2 to the angiotensin II type-1 receptor signaling complex [17]. In addition, phosphorylation of Y221 increases ligand-independent Jak2 tyrosine kinase activity [15]. However, it has no effect on Jak2-dependent signaling in the presence of an erythropoietin-leptin receptor chimera [14]. Further, phosphorylation of Y317 has been shown to play an important role in the feedback inhibition of Jak2 kinase activity following ligand-mediated activation [13]. Given the limited knowledge of how the FERM domain regulates Jak2 function, identification of novel Jak2 tyrosine phosphorylation sites in this region is important.

In this study, we identified Y372 as a novel Jak2 phosphorylation site in the FERM domain of Jak2. We found that phosphorylation of Y372 is critical for maximal Jak2 phosphorylation, STAT1 activation and Jak2-dependent gene transcription. In addition, Y372 phosphorylation has an important and differential role on Jak2-dependent signal transduction in response to ligand. In particular, loss of Y372 phosphorylation reduces interferon-γ and epidermal growth factor-mediated Jak2 activation, but has no effect on hydrogen peroxide-mediated Jak2 activation. Interestingly, Y372 does not contribute to Jak2 receptor association, despite its impaired catalytic activity. Lastly, co-expression of SH2B-β partially restored the activation of the Jak2-Y372F mutant. As such, this work demonstrates a critical yet differential role of Y372 phosphorylation in the regulation of the Jak2 kinase activity and subsequent downstream signaling.

2. Materials and Methods

2.1 Cell Culture

BSC-40, COS-7, and Jak2 deficient mouse embryonic fibroblasts (Jak2−/− MEF) were cultured at 37°C in a 5% CO_2 humidified atmosphere. BSC-40 cells were maintained in high glucose (4.5 g/L) DMEM supplemented with 10% newborn calf serum. COS-7 and MEF cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum. The Jak2 null MEFs were kindly provided by Dr. James Ihle [18]. BSC-40 cells were used for vaccinia virus mediated transfection-infection experiments. COS7 cells were used for experiments involving co-transfection of luciferase or SH2B-β plasmids. MEFs were used to examine Jak2 activation in response to ligand stimulation. MEFs treated with interferon-gamma, epidermal growth factor or hydrogen peroxide were growth-arrested with serum-free DMEM containing 0.5% BSA for 18 hours prior to stimulation.

2.2 Mass Spectrometry

Recombinant Jak2 protein was over-expressed in BSC-40 cells using a vaccinia virus expression system similar to how we have done previously [19]. The expressed protein was resolved via SDS-PAGE, coomassie stained, excised from the gel and subjected to nano-HPLC/μESI ionization on an LTQ mass spectrometer as previously described [17].

2.3 Site-Directed Mutagenesis

The Y372F and Y373F Jak2 mutations were created in the murine Jak2 cDNA using the QuikChange Mutagenesis protocol (Stratagene, La Jolla, CA). The sense primer sequence used to create the Jak2-Y372F mutation was 5′-TTAATTGACGGGTTT TACAGACTAACT and the antisense primer sequence was 5′-AGTTAGTCTGTAAAACCCG TCAATTAA. The sense primer sequence for the Jak2-Y373F mutation was 5′-ATTGACGGGTAT TTTAGACTAACTGCG and the antisense primer sequence was 5′CGCAGTTAGTCTAAA ATACCCGTCAAT. All the mutations were verified by DNA sequencing.

2.4 Transient Cell Transfections

For vaccinia virus mediated transient Jak2 expression, BSC-40 cells were transfected with 10 μg of pRC-CMV plasmids encoding the murine Jak2-WT, Jak2-Y372F or Jak2-Y373F cDNA using Lipofectin (Invitrogen, Carlsbad, CA). After 4 hours of transfection, the cells were infected with the recombinant vaccinia virus, vTF7-3, at a multiplicity of infection (MOI) of 1.0 for 1 hour. The media containing Lipofectin/DNA/vTF7-3 was then removed from the cells, replaced with fresh serum-containing media and the cells were allowed to recover for 16 hours at which time the protein lysates were prepared. For Targefect-mediated transfections of Jak2−/− MEF cells, plasmid DNA, Targefect and virofect

enhancer (Targeting Systems, El Cajon, CA) were combined in a total volume of 1 mL of serum-free DMEM and incubated at 37°C for 20 minutes. 1 mL of serum-containing DMEM was mixed with the transfection complex and added onto the cells in 100 mm dishes. The cells were incubated with the transfection complex for 3 hours at 37°C and then allowed to recover for 48 hours in serum-containing media. For, SuperFect-mediated co-transfections, the respective plasmid DNA and SuperFect (Qiagen, Valencia, CA) were incubated in 300 μL of serum free-DMEM for 10 minutes at room temperature. Later, 3 mL of serum containing DMEM was mixed with the transfection complexes and added to the MEFs in 100 mm dishes. The cells were then allowed to recover for 48 hours.

2.5 Cell Lysis and Immunoprecipitation

Cells were washed with two volumes of ice-cold PBS containing 1 mM Na_3VO_4 and lysed with 0.8 mL of ice cold RIPA buffer (20 mM Tris pH 7.5, 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, 4 mM benzamidine, and 10 μg/mL aprotinin). Cleared protein lysates were incubated with 2 μg of antibody and 20 μL of protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 4° C. Protein complexes were washed three times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS sample buffer. Immunoprecipitated proteins were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Anti-Jak2 and anti-STAT1 antibodies for immunoprecipitation were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.6 Western Blotting

Nitrocellulose membranes were blocked in either 5% BSA/TBST or 5% milk/TBST at room temperature. The membranes were then incubated with the primary antibody, followed by the respective secondary antibody (1:4000, GE Healthcare), with TBST washes in-between. A mixture of PY99, PY20 and 4G10 monoclonal antibodies from Santa Cruz Biotechnology, BD Transduction Labs, and Millipore, respectively, were used to detect phosphotyrosine. Anti-Jak2 pY1007/pY1008 and anti-STAT1 pY701 antibodies were from BioSource and Santa Cruz Biotechnology, respectively. A cocktail of antibodies from Millipore and BioSource were used to detect Jak2. The anti-STAT1 antibody was from Santa Cruz Biotechnology and the anti-myc antibody was from Cell Signaling. The blots were visualized using Western Lightning-*Plus* or *Ultra* enhanced chemiluminescence system (Perkin Elmer, Waltham, MA). Densitometry was performed using the automated digitizing software, Un-Scan-It, Version 5.1 (Silk Scientific, Orem, Utah). All phosphorylation levels were normalized to total protein levels.

2.7 Luciferase Assay

COS-7 cells were transiently transfected with the appropriate Jak2 expression plasmid and 2 μg of a luciferase reporter plasmid consisting of four tandem copies of the interferon-γ activating sequence (pLuc-GAS) using Lipofectin. Following 5 hours of transfection, approximately 7×10^5 cells were seeded onto six well culture dishes. The cells were allowed to recover in serum containing DMEM for 48 hours and then lysed in 1X Reporter Lysis buffer (Promega) for 5 hours. During lysis, the lysates were exposed to one freeze-thaw cycle between 23°C and −80°C. 20 μL sample of lysate was combined with 100 μL of luciferase substrate and relative light units were assessed by a Monolight 3010 luminometer.

2.8 Immunofluorescence

Jak2−/− MEFs were cultured in 2-well chamber slides where they were transfected with 2 μg of either pRK5-FLAG-Jak2-WT or Y372F using Targefect and allowed to recover for 48 hours. The cells were then serum starved for 18 hours and then treated with 500 IU/ml (final

concentration) of interferon-gamma for either 0 or 15 minutes. Following interferon-gamma treatment, the cells were fixed at −20°C in a mixture of 50% methanol and 50% acetone for 10 minutes. The fixed cells were blocked with 10% BSA for 30 minutes at room temperature. They were then incubated overnight with a primary antibody mixture of rabbit anti-FLAG and mouse anti-IFNGR at 4°C. Next day, the cells were washed with PBS for four times at room temperature. Following this, they were incubated with a secondary antibody mixture of anti-rabbit conjugated to TR (Red) and anti-mouse conjugated to FITC (Green) for one hour at room temperature. The cells were again washed with PBS, mounted with UltraCruz DAPI containing mounting media (Santa Cruz) and sealed with a cover slip. These cells were imaged using a 60x objective on an inverted fluorescence microscope (Olympus). The anti-FLAG antibody was from Sigma and the anti-IFNGR antibody was from Abcam. The secondary antibodies were from Santa Cruz.

2.9 Statistical Analysis

Statistical significance between groups was analyzed using Student's t-test. Significance was set at * = $p < 0.05$ and ** = $p < 0.005$.

3. Results

3.1 Y372 is a Conserved Site of Jak2 Phosphorylation

Jak2-WT was over expressed in BSC-40 cells using a vaccinia virus system and then purified to homogeneity as previously described [19]. The purified Jak2 protein was then subjected to a combination of nano-HPLC/μESI ionization on a LTQ mass spectrometer. Peptide fragment analysis corresponding to Y372 and Y373 indicated that they were both phosphorylated (Fig. 1A).

To further characterize the significance of Y372 and Y373 phosphorylation, we determined whether these amino acid residues were conserved evolutionarily. Comparison of the amino acid sequence of Jak2 from diverse species revealed that tyrosines 372 and 373 are conserved (Fig. 1B). However, evaluation of the amino acid sequence of the different Jak family members in mouse revealed that Y372 is highly conserved, while tyrosine at position 373 is replaced with phenylalanine in murine Jak1, Tyk2, and Jak3 (Fig. 1C). This indicates that the aromaticity of 373 is conserved, but not its phosphorylation.

Thus, the data in Fig. 1 indicate that Y372 and Y373 in murine Jak2 are sites of phosphorylation. The Y372 residue is highly conserved across various species and also in other Jak family members while Y373 is conserved only across species. The highly conserved nature of Y372 relative to Y373 suggests that phosphorylation of Y372 could play an important role in Jak2 function.

3.2 Loss of Y372 and Y373 Phosphorylation Reduces Jak2 Phosphorylation

Our next step was to determine if these tyrosines were important for total Jak2 tyrosine phosphorylation as well as for phosphorylation of Y1007, a residue whose phosphorylation is essential for maximal Jak2 activation [9]. For this, Y372 and Y373 were mutated to phenylalanine in order to disrupt phosphorylation at these sites, while preserving the protein structure. BSC-40 cells were transfected with empty vector, Jak2-WT, Jak2-Y372F, or Jak2-Y373F. Overexpressed Jak2 protein was immunoprecipitated with a Jak2 antibody and the precipitates were serially probed with antibodies to detect overall tyrosine phosphorylation (Fig. 2A), tyrosine 1007 phosphorylation (Fig. 2B), and total Jak2 protein (Fig. 2C). Three independent blots were then quantified and the average levels of total Jak2 tyrosine phosphorylation (Fig. 2D) and Y1007 phosphorylation (Fig. 2E) were plotted as a function of Jak2 status. The cumulative results show that total Jak2 phosphorylation was significantly

reduced for both Jak2-Y372F and Jak2-Y373F, although the reduction with Jak2-Y372 was much greater (Fig. 2D). With respect to Jak2 Y1007 phosphorylation levels, we found that the loss of phosphorylation at Y372 completely eliminated Jak2 Y1007 phosphorylation, when compared to wild-type Jak2 protein. The Y373 mutant also exhibited significantly reduced Y1007 phosphorylation when compared to Jak2-WT, but to a lesser extent than that of the Y372F mutant.

Collectively, the data in Fig. 2 demonstrates the significant yet differential roles of Y372 and Y373 in regulating Jak2 autophosphorylation. Specifically, Y372 appears to be more important for the catalytic activity of Jak2 when compared to Y373, since Y372F mutation more effectively reduced Y1007 and total Jak2 phosphorylation relative to Y373F. Furthermore, this observation is in accordance with the sequence conservation of tyrosine at 372 when compared to the mere conservation of aromaticity at position 373 (Fig. 1C).

3.3 Mutation of Jak2 at Y372 Suppresses the Ability of Jak2 to Phosphorylate STAT1

Given its greater effect on Jak2 phosphorylation and activation (Fig. 2), moving forward, we chose to focus our efforts on investigating the effects of Y372 phosphorylation on overall Jak2 kinase function and signaling. Since Jak2 activation is significantly impaired in Jak2-Y372F, we hypothesized that it would also have an impaired ability to phosphorylate its substrate. To test this, we examined the effect of the Jak2-Y372F mutant on STAT1 phosphorylation. STAT1 is a signaling protein that is phosphorylated on tyrosine 701 in the presence of active Jak2 [20]. Here, BSC-40 cells that endogenously express STAT1 were transiently transfected with empty vector, Jak2-WT, or Jak2-Y372F plasmids. Whole cell lysates were prepared and samples were serially blotted with a phospho-STAT1 antibody (Fig. 3A), an anti-STAT1 antibody to detect total STAT1 protein levels (Fig. 3B), and an anti-Jak2 antibody to detect expressed Jak2 protein (Fig. 3C). After normalizing the phospho-STAT1 levels to total STAT1 protein, the aggregate data was plotted as a function of Jak2 status (Fig. 3D). We found that the loss of Y372 phosphorylation significantly reduced the ability of Jak2 to phosphorylate STAT1 at tyrosine 701 when compared to wild-type Jak2 (Fig. 4D). In summary, the data in Fig. 3 indicate the importance of phosphorylation at Y372 for Jak2-dependent STAT1 phosphorylation.

3.4 Loss of Y372 Phosphorylation Impairs the Co-association between Jak2 and STAT1

Given the impaired ability of Jak2-Y372F to phosphorylate STAT1, we now wanted to determine whether this was due to an impaired ability of these two proteins to co-associate with one another. The general Jak-STAT signaling paradigm suggests that STATs dock to phosphorylation sites on the receptor and are then phosphorylated by the proximal Jak2. Additionally, several results in the literature have shown that Jak2 co-associates with STAT1 during this process [21; 22; 23]. To determine this, BSC-40 cells were transiently transfected with empty vector, Jak2-WT or Jak2-Y372F plasmids. The following day, protein lysates were immunoprecipitated with anti-STAT1 antibody and immunoblotted with anti-Jak2 antibody to detect STAT1/Jak2 co-association (Fig. 4A). We found that elimination of Y372 phosphorylation significantly hindered the ability of Jak2 to co-associate with STAT1. To demonstrate equal STAT1 precipitation across all samples, the membrane was stripped and re-blotted with anti-STAT1 antibody (Fig. 4B). In addition, whole cell lysates were blotted with anti-Jak2 antibody to determine Jak2 expression levels (Fig. 4C).

To demonstrate this in another way, the inverse experiment was performed whereby protein lysates were immunoprecipitated with anti-Jak2 antibody and then immunoblotted with anti-STAT1 antibody (Fig 4D). Similar to the experiment above, mutation at Y372 significantly decreased Jak2/STAT1 co-association. To demonstrate equal Jak2 precipitation among

samples, the membrane was stripped and re-probed with an anti-Jak2 antibody (Fig. 4E). Whole cell lysates from these samples were blotted with an anti-STAT1 antibody to assess the levels of expressed STAT1 (Fig. 4F).

Statistical analysis of the aggregate data from both the experiments revealed that the Jak2-Y372F mutant exhibited significantly impaired STAT1/Jak2 (Fig. 4G) and Jak2/STAT1 co-association (Fig. 4H) when compared to Jak2-WT.

All together, the results in Figure 4 show that the loss of Y372 phosphorylation reduces the ability of Jak2 to co-associate with its downstream substrate, STAT1.

3.5 The Jak2-Y372F Mutation Abrogates Jak2-mediated Gene Expression

It is known that Jak2 is capable of driving a basal level of gene expression even in the absence of ligand stimulation [24; 25]. This ligand-independent gene expression corresponds to the intrinsic functional activity of Jak2. Here, we sought to determine whether loss of Y372 phosphorylation affects the intrinsic functional capacity of Jak2 to drive gene expression. For this, COS-7 cells were transiently transfected with a plasmid encoding the firefly luciferase cDNA under the control of four tandem Jak/STAT-binding promoter elements [24]. In addition, the cells were co-transfected with empty vector, Jak2-WT, or Jak2-Y372F plasmids. Two days later, luciferase activity was determined and graphed as a function of Jak2 status (Fig. 5A). We found that the Jak2-Y372F mutation significantly reduced the ability of Jak2 to drive luciferase gene expression when compared to Jak2-WT. To confirm equal expression levels of both Jak2-WT and Jak2-Y372F, a portion of the transfected protein lysates were immunoblotted with a Jak2 antibody (Fig. 5B).

Thus, the results from the luciferase assay reveal the importance of Y372 phosphorylation in Jak2 dependent activation of downstream gene transcription. This is in agreement with the previously observed reduction in STAT1 phosphorylation and co-association by the Jak2-Y372F mutant (Figures 3 and 4).

3.6 Loss of Y372 Phosphorylation Prevents Interferon-Gamma- and Epidermal Growth Factor-, but not Hydrogen Peroxide-Mediated Jak2 Activation

Next, we wanted to examine the functional significance of Y372 phosphorylation in the context of ligand-dependent Jak2 signaling. Interferon-gamma is a known and potent activator of Jak2 tyrosine kinase [18; 26]. We therefore sought to determine the effect of Y372 phosphorylation on interferon-gamma-mediated Jak2 activation. Here, Jak2−/− MEFs that endogenously express the interferon-gamma receptor were transiently transfected with empty-vector, Jak2-WT or Jak2-Y372F expressing plasmids. The cells were treated with interferon-gamma as indicated and then lysed. Jak2 protein was immunoprecipitated from the lysate and Jak2 Y1007 phosphorylation levels were measured by immunoblotting with an anti-Jak2 pY1007/pY1008 antibody. In agreement with published reports [16; 18], we found that Jak2-WT was activated in a ligand-dependent manner (Fig. 6A, top). However, loss of Y372 phosphorylation blocked the interferon-gamma-mediated increase in Jak2 tyrosine 1007 phosphorylation. The membrane was subsequently stripped and equal Jak2 protein expression was verified via Western blot with an anti-Jak2 antibody (Fig. 6A, bottom).

Jak2 can also be activated in response to mitogenic growth factors such as epidermal growth factor [27; 28]. Thus, we sought to determine the effect of Jak2 Y372 phosphorylation on the ability of Jak2 to respond to tyrosine kinase growth factor stimulation as opposed to cytokine stimulation. Cells were again transfected with empty vector, Jak2-WT or Jak2-Y372F plasmids. We found that in agreement with previously published reports [27; 28], Jak2-WT was activated in response to epidermal growth factor stimulation (Fig. 6B, top).

On the other hand, loss of Y372 phosphorylation completely inhibited the epidermal growth factor-mediated increase in Jak2 kinase activity as determined by the complete lack of Y1007 phosphorylation on the Y372F mutant. The membrane was stripped and re-probed with an anti-Jak2 antibody to verify equal protein loading (Fig. 6B, bottom).

Lastly, Jak2 can be activated by hydrogen peroxide induced oxidative stress [29; 30; 31]. This mechanism of activation is uniquely different from that of interferon-gamma and epidermal growth factor, in that it involves oxidation of inhibitory phosphatases rather than oligomerization of receptor bound Jak2 molecules as in the former case [29; 32]. Therefore, we wanted to determine the impact of Y372 phosphorylation on receptor independent Jak2 activation in response to hydrogen peroxide. For this, Jak2−/− MEFs were transiently transfected with empty vector, Jak2-WT or Jak2-Y372F expressing plasmids. After serum starvation, the cells were treated as shown. Consistent with previous reports [29; 30; 31], hydrogen peroxide potently activated Jak2-WT (Fig. 6C, top). Unlike interferon-gamma and epidermal growth factor, the loss of Y372 phosphorylation did not have any effect on hydrogen peroxide-mediated Jak2 tyrosine 1007 phosphorylation. To demonstrate equal Jak2 expression amongst all samples, the membrane was stripped and re-blotted with anti-Jak2 antibody (Fig. 6C, bottom).

Collectively, the data in Fig. 6 demonstrate that phosphorylation of Y372 is critical for interferon-gamma- and epidermal growth factor-mediated Jak2 activation, but not for hydrogen peroxide-dependent Jak2 activation.

3.7 Loss of Y372 Phosphorylation does not affect Jak2/receptor co-association

The data in Fig. 6 demonstrate that phosphorylation of Y372 is important for receptor-mediated Jak2 activation through interferon gamma and epidermal growth factor, but not for receptor-independent activation through hydrogen peroxide. Y372 is present in the FERM domain, which is known to play an important role in Jak2/receptor co-association [33; 34]. Therefore, one possible explanation for the observed inability of interferon gamma and epidermal growth factor to activate Jak2 is that the Y372F mutation may disrupt Jak2/receptor co-association. To assess this, Jak2−/− MEFs were transfected with FLAG tagged Jak2 expression plasmids and cellular localization of both Jak2 and the interferon-gamma receptor (IFNGR) was determined using immunofluorescence. We observed that for both Jak2-WT and Jak2-Y372F, Jak2 localized near the membrane and was distributed diffusely in the cytoplasm and this pattern did not change with interferon-gamma treatment (Fig. 7). The IFNGR was located primarily at the plasma membrane. There was considerable overlap in the co-localization pattern of Jak2 and IFNGR in the case of Jak2-WT and this did not change with the introduction of the Y372F mutation. Furthermore, the ability of the Jak2-Y372F mutant to fully co-associate with the IFNGR was also observed in IP/western co-immunoprecipitation assays (data not shown).

In summary, the data in Fig. 7 indicate that the inability of interferon gamma to activate the Y372F mutant is not due to an impaired ability of the protein to bind the IFNGR.

3.8 Co-expression of SH2B-β is capable of partially restoring Jak2-Y372F activation

Following ligand binding, receptor bound Jak2 molecules are brought within close proximity to one another allowing for Jak2 dimerization and subsequent activation [35; 36]. SH2B-β is an adaptor protein that promotes both Jak2 dimerization and stabilization of its active conformation [37; 38; 39]. As the next step in understanding the mechanism of Jak2 regulation through phosphorylation of Y372, we hypothesized that the Y372F mutation may impair Jak2 dimerization and/or stabilization of the activation loop. To test this, cells were transfected with SH2B-β as well as Jak2-WT or Jak2-Y372F plasmids. Two days later, the

cells were lysed and biochemical analysis was performed. Shown are representative blots for the levels of Y1007 phosphorylation (Fig. 8A), Jak2 protein expression (Fig. 8B), and SH2B-β expression (Fig. 8C). Quantification of the phospho-Y1007 levels normalized to total Jak2 protein revealed that in the case of Jak2-Y372F, Jak2 kinase activity was only partially restored in the presence of the strong dimerizing agent, SH2B-β (Fig. 8D).

Taken together, the data in Fig. 8 demonstrate that co-expression of SH2B-β is capable of partially restoring Jak2-Y372F activation. As such, this suggests that the mechanism by which the Y372F mutation affects Jak2 kinase function is via mechanisms that involve Jak2 dimerization and/or stabilization of the activation loop.

4. Discussion

Using mass spectrometry analysis, we report in this study the identification of tyrosines 372 and 373 as novel sites of Jak2 phosphorylation. The data suggest that Y372 has an important role to play in Jak2 activation. Under basal conditions, the Jak2-Y372F mutant had an impaired ability to autophosphorylate, bind STAT1, phosphorylate STAT1, and drive Jak2-dependent gene expression. Interestingly, the Jak2-Y372F mutant was not activated in response to interferon-gamma and epidermal growth factor, but it was fully activated in response to hydrogen peroxide. However, the inability of the Jak2-Y372F mutant to activate in response to interferon-gamma stimulation was not due to a defect in receptor co-association. Lastly, co-expression of SH2B-β partially restored Jak2-Y372F activation suggesting that phosphorylation of Y372 affects Jak2 kinase activity via mechanisms that involve Jak2 dimerization and/or stabilization of the active conformation of the protein.

Of the 49 Jak2 tyrosine residues, some are known to be phosphorylated and play important roles in Jak2 tyrosine kinase function. Interestingly, many of these characterized Jak2 tyrosine phosphorylation sites are situated either in the pseudokinase or kinase domains of the protein. Less is known regarding the consequences of phosphorylation of tyrosines in the N-terminus of Jak2, where the FERM domain resides. Early work demonstrated that the FERM domain is important for cytokine receptor interaction [4; 5; 40; 41]. More recent work however has shown the importance of the FERM domain in Jak2 activation and kinase regulation [16; 42]. Additional evidence comes from the identification of phosphorylation sites in the FERM domain that regulate Jak2 kinase activity.

Our results clearly demonstrate that Jak2-Y372F is defective in activation and kinase activity. Since Y372 is situated in the FERM domain of Jak2, the loss of Jak2-Y372F activity could be explained by one of three possible mechanisms: i) phosphorylation of Y372 is critical for receptor co-association ii) phosphorylation of Y372 initiates conformational changes in the FERM domain that results in changes in kinase activity at the C terminal end of the protein or iii) phosphorylation of Y372 facilitates Jak2 dimerization and subsequent activation. Our results here demonstrate the Y372F mutant binds the IFNGR normally (Fig. 7) and treatment of cells with hydrogen peroxide results in full activation of the Jak2-Y372F mutant protein characterized by a completely stabilized active conformation (Fig. 6C). As such, these results suggest that the inability of the Jak2-Y372F mutant to activate is neither due to impaired receptor co-association nor an inability to stabilize the active conformation of the protein. Thus, our data suggests that the mechanism by which Jak2-Y372F has weakened kinase activity is due to impaired dimerization of the Jak2 molecules. This is supported by the observation that expression of SH2B-β, which strongly promotes dimerization and stabilizes the active conformation of the protein [37; 38; 39], only partially restored Jak2-Y372F activity (Fig. 8). Further, the role of dimerization in Jak2 activation is supported by the observed constitutive activation of Jak2 fusion proteins in cancer, where the Jak2 kinase domain is fused with the oligomerization domains from other

partner genes [43; 44]. Our results also demonstrate that while Y372 is important for Jak2 activation in response to receptor-bound ligand, it has no effect on hydrogen peroxide-dependent Jak2 activation. Reactive oxygen species such as hydrogen peroxide activate Jak2 by inactivating phosphatases through oxidation [29]. Therefore, Y1007 phosphorylation and Jak2 activation happen autonomously simply due to the blockade of phosphatase activity. In the case of ligand binding to cytokine or growth factor receptors, Jak2 dimerizes and activates via auto- and trans-phosphorylation mechanisms. Therefore, an impaired dimerizing ability of the Jak2-Y372F mutant would be consistent with the inability of the protein to activate in response to receptor activation.

A previous work investigated the effect of several conserved Jak2 tyrosine residues, including Y372, on Jak2 function [11]. Although no evidence was provided that Y372 is phosphorylated, it was shown that a Jak2-Y372F mutant had no effect on Jak2-mediated erythroid progenitor colony formation. We show here that Y372 is phosphorylated using mass spectrometry. Further, Y372 phosphorylation is critical for Jak2 regulation as loss of Y372 phosphorylation suppressed Jak2 auto-phosphorylation as well as interferon-gamma and epidermal growth factor-mediated Jak2 activation. The discrepancy between these two findings could be due to several factors. First, our analysis was done at the protein level whereas Funakoshi-Tago *et al.* used cell based assays. Thus, there may be other kinases in the cell that can act in place of the Y372F mutant. Second, while Funakoshi-Tago *et al.* examined the effect of the Jak2-Y372F mutation in the context of erythropoietin signaling, we studied Jak2-Y372F activation in response to interferon-gamma and epidermal growth factor signaling. As such, Y372 may play a differential role in response to different stimuli. This concept is supported by the observation that a Jak2-Y119F mutant protein exhibited impaired erythropoietin receptor binding, but had no consequence on interferon-gamma receptor binding [16]. Collectively, these results suggest that there are underlying differences in how individual FERM domain tyrosines interact with cytokine receptors.

5. Conclusion

We have identified a novel site of Jak2 phosphorylation and shown that phosphorylation of this site is critical for Jak2 kinase function. Furthermore, our results advance the overall understanding of Jak2 tyrosine kinase regulation via mechanisms that involve the FERM domain.

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Abbreviations

Jak	Janus Kinase
STAT	Signal Transducers and Activators of Transcription
FERM	4.1 protein/ezrin/radixin/moesin
IFN	interferon
SH2	Src Homology 2

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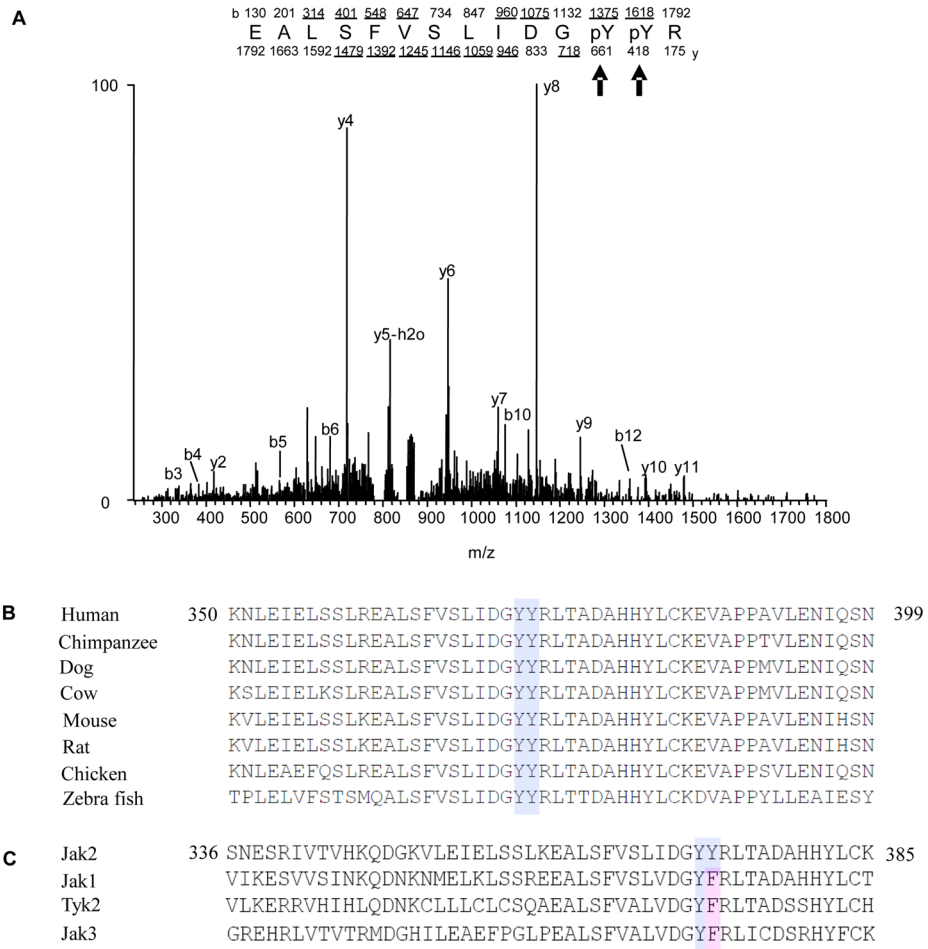

FIGURE 1. Y372 and Y373 are Jak2 phosphorylation sites that are conserved across varying species and among Jak family members

A) Jak 2 was overexpressed in BSC-40 cells using vaccinia virus system and purified as previously described. Purified Jak2 protein was then subjected to nano-HPLC micro ESI analysis on an LTQ mass spectrometer. The tryptic peptide containing Y372 and Y373 was found to be doubly phosphorylated based upon mass of the peptide. Phosphorylation sites were located to the tyrosine residues by MS/MS sequencing, specifically the b10 ion, which is labeled in the spectra. Jak2 sequences from different species (B) and Jak family members (C) were aligned using ClustalW. Conservation of residues 372 and 373 is highlighted in both the alignments.

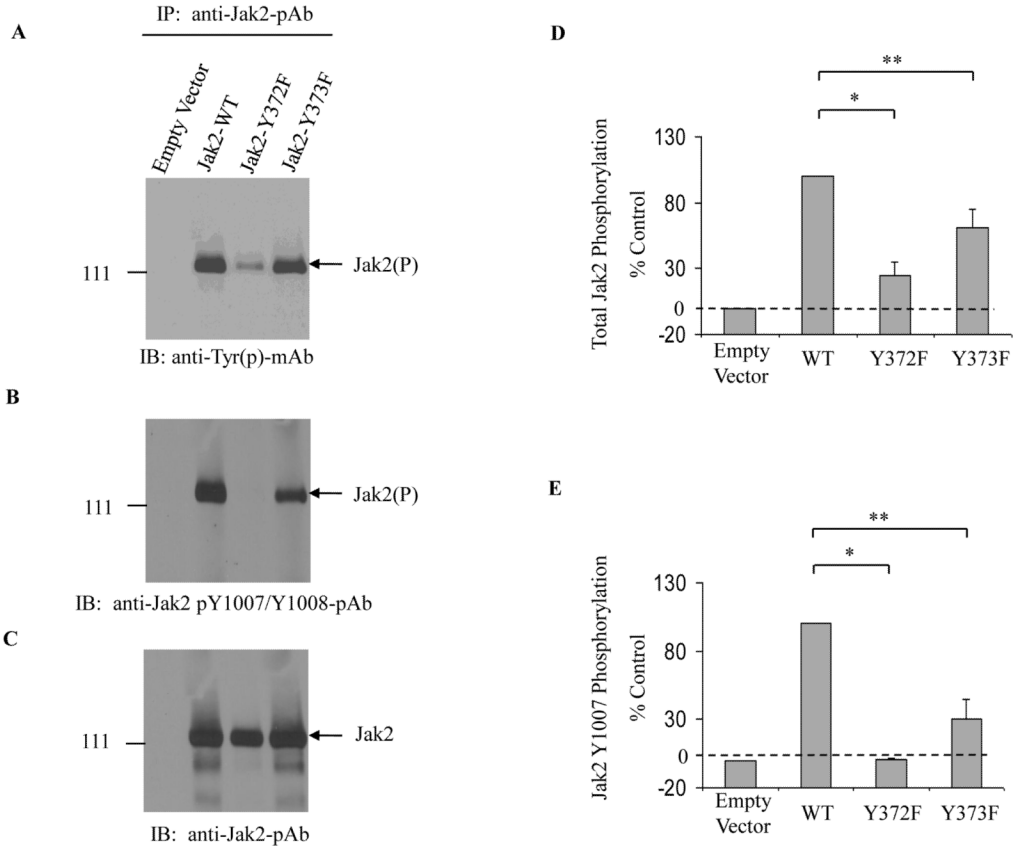

FIGURE 2. Loss of phosphorylation at Y372 and Y373 decreased the ability of Jak2 to autophosphorylate

A) BSC-40 cells were transfected with 10 μg of empty vector, Jak2-WT or Jak2-Y372F plasmid. Jak2 was immunoprecipitated, resolved by SDS-PAGE and immunoblotted for phosphotyrosine. B) The membrane was stripped and re-probed for phosphorylation at Y1007/Y1008. C) Equal Jak2 expression across all samples was verified. D) Quantification of total Jak2 phosphorylation (n=3) and E) Jak2 Y1007/1008 phosphorylation (n=3) were done using densitometry. Statistical significance was determined via Student's *t*-test, * = $p < 0.05$, ** = $p < 0.005$.

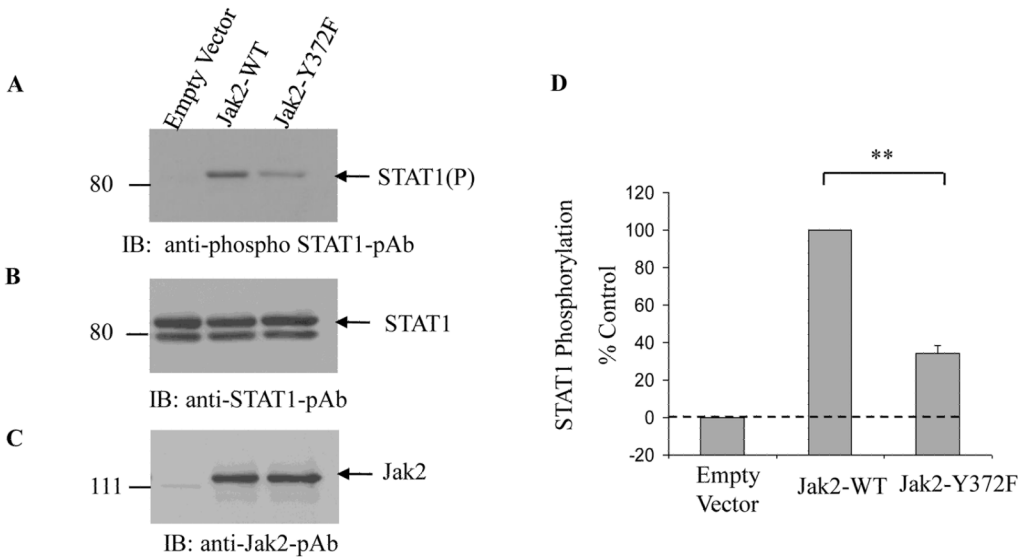

FIGURE 3. Loss of Y372 phosphorylation reduces Jak2-mediated STAT1 phosphorylation

BSC-40 cells were transiently transfected with 10 μg of empty vector, Jak2-WT or Jak2-Y372F plasmid. A) STAT1 phosphorylation at Y701 was determined via western blot analysis of whole cell lysates. Membrane was stripped and equal expression of STAT1 (B) and Jak2 (C) were verified. D) STAT1 phosphorylation was quantified using densitometry (n=3). ** = $p < 0.005$.

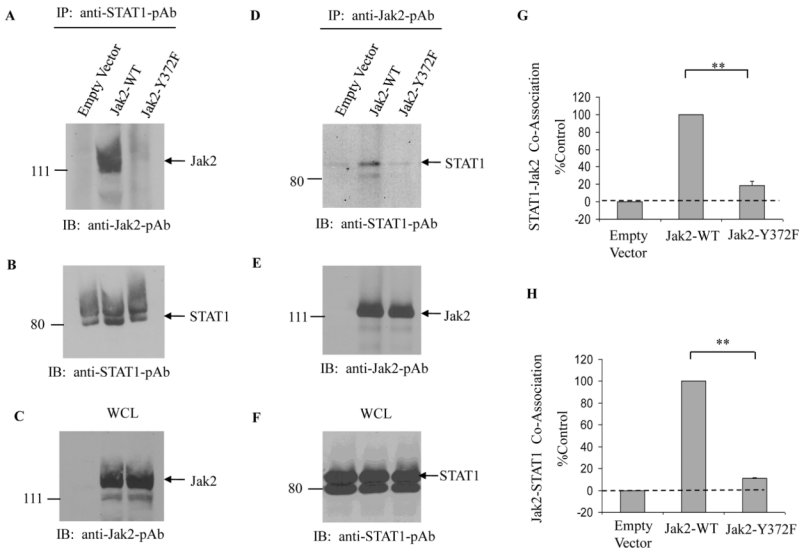

FIGURE 4. The Jak2-Y372F mutant has an impaired ability to co-associate with STAT1

BSC-40 cells were transiently transfected with 10 μg of empty vector, Jak2-WT or Jak2-Y372F plasmid. A) STAT1 was immunoprecipitated and then blotted for Jak2 to determine STAT1/Jak2 association. B) The membrane was stripped and equal STAT1 levels were verified. C) Whole cell lysates were blotted to verify equal Jak2 expression. D) Conversely, Jak2 was immunoprecipitated then blotted for STAT1. E) The membrane was stripped and equal Jak2 levels were verified. F) Whole cell lysates were blotted to verify equal STAT1 expression. STAT1-Jak2 association (G) and Jak2-STAT1 association (H) were quantified, respectively, using densitometry (n=3). ** = $p < 0.005$.

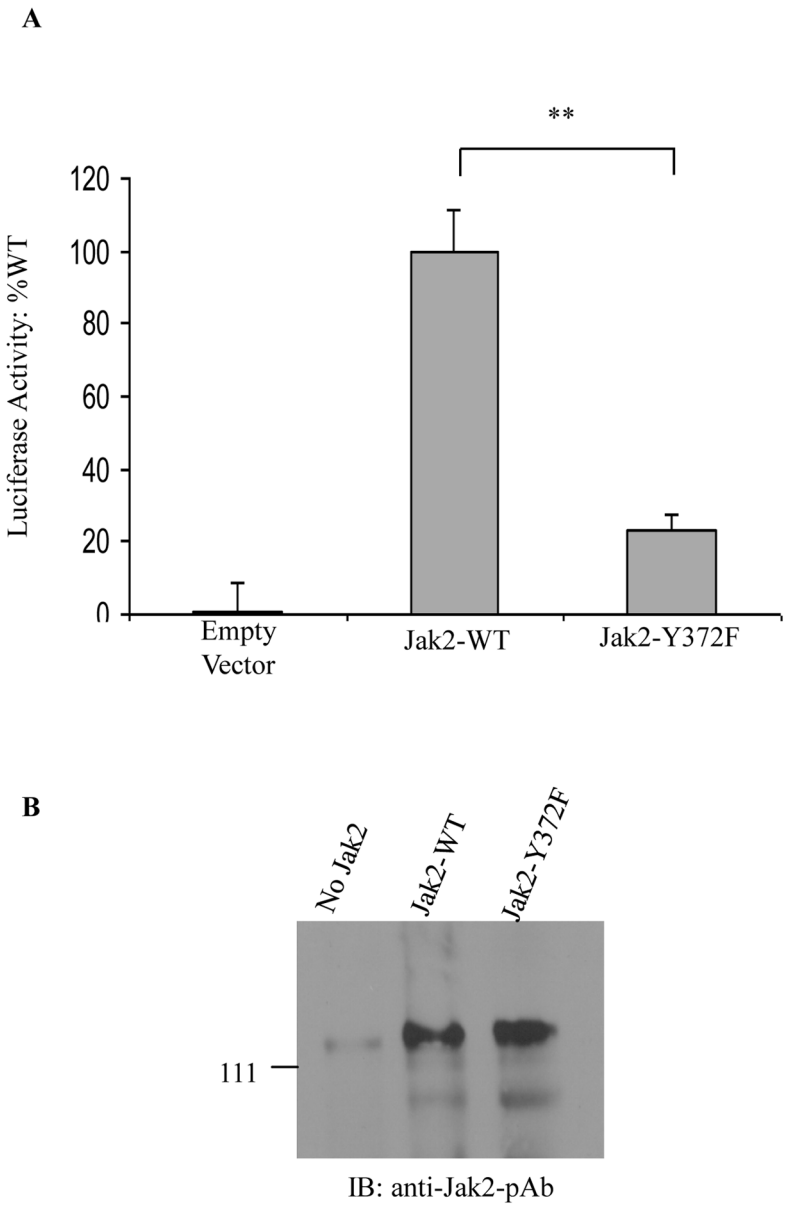

FIGURE 5. Loss of Y372 phosphorylation reduced Jak2-dependent gene transcription

A) COS-7 cells were transiently transfected with 2 μg of luciferase plasmid and 5 μg of empty vector, Jak2-WT or Jak2-Y372F plasmids. Transfected cells were lysed and the relative luminescence units (RLU) were read as a measure of luciferase gene expression using a luminometer. B) Equal Jak2 expression was ascertained via Western blot analysis. ** = $p < 0.005$. Shown is one of three independent results.

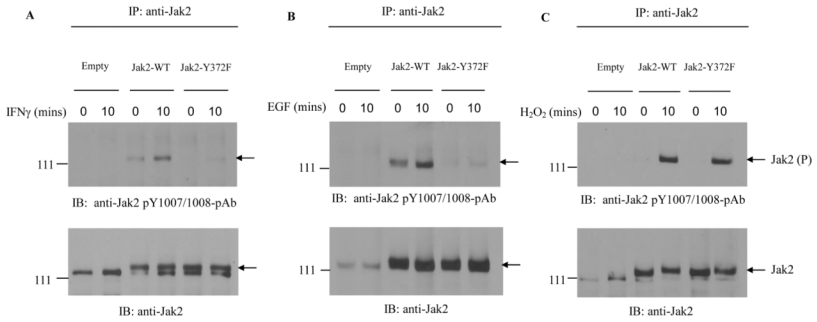

FIGURE 6. Phosphorylation of Y372 is essential for interferon-gamma and epidermal growth factor dependent, but not hydrogen peroxide dependent Jak2 activation

MEF cells were transiently transfected with 10 μg empty vector plasmid, Jak2-WT plasmid or Jak2-Y372F plasmid. Following transfection, the cells were treated with A) 500 IU/mL interferon-gamma (n=3), B) 200 ng/mL epidermal growth factor (n=2), and, C) 0.5 mM hydrogen peroxide (n=2), for 0 or 10 minutes. The cells were subsequently lysed and Jak2 was immunoprecipitated. Jak2 Y1007 phosphorylation was determined via Western blot analysis with an anti-Jak2 pY1007/pY1008 antibody (top). The membrane was stripped and re-blotted with anti-Jak2 antibody to verify equal Jak2 expression among samples (bottom).

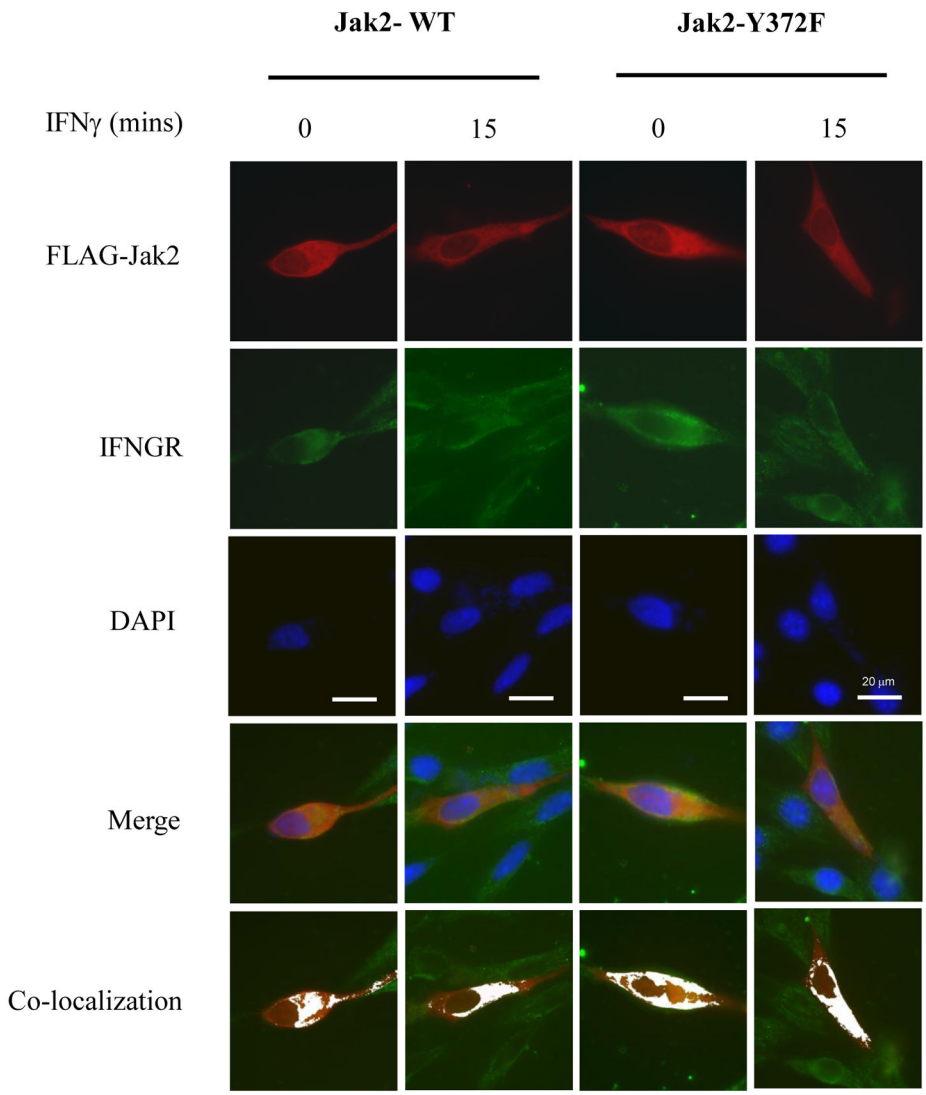

FIGURE 7. Phosphorylation of Y372 is not required for Jak2-IFNGR co-association

After transfection with the indicated Jak2 plasmid and subsequent serum starvation, the cells were treated with interferon-gamma as indicated. The cells were then co-immunostained with anti-Jak2 and anti-IFNGR (β chain) antibodies and imaged using a fluorescence microscope. FLAG-Jak2 was stained using Texas Red (red) and IFNGR with FITC (green). The nuclei were counter stained with DAPI (blue). ImageJ software was used to confirm the co-localization pattern of Jak2 with IFNGR. Shown is one of two independent results.

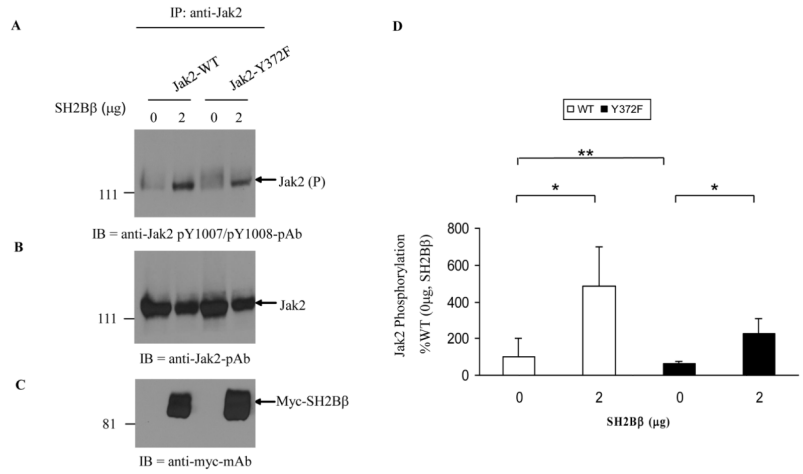

FIGURE 8. Activation of Jak2-Y372F is only partially recovered in the presence of SH2B-β

COS-7 cells were transiently transfected with 2 μg of either empty vector or myc-SH2B-β along with 5 μg of either Jak2-WT or Jak2-Y372F plasmids. A) Transfected cells were lysed and the Jak2 activation was measured by western blot using anti-pY1007/pY1008 Jak2. Expression levels of Jak2 (B) and SH2B-β (C) were ascertained using anti-Jak2 and anti-myc antibodies. D) Densitometry analysis was performed on the western blots to quantify Jak2 phosphorylation and plotted with respect to Jak2-WT without SH2B-β (n=4). * = $p < 0.05$, ** = $p < 0.005$.