

Receptor specific downregulation of cytokine signaling by autophosphorylation in the FERM domain of Jak2

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The tyrosine kinase, Janus kinase-2 (Jak2), plays a pivotal role in signal transduction through a variety of cytokine receptors, including the receptor for erythropoietin (Epo). Although the physiological relevance of Jak2 has been definitively established, less is known about its regulation. In studies assessing the roles of sites of tyrosine phosphorylation, we identified Y¹¹⁹ in the FERM (band 4.1, Ezrin, radixin and moesin) domain as a phosphorylation site. In these studies, we demonstrate that the phosphorylation of Y¹¹⁹ in response to Epo downregulates Jak2 kinase activity. Using a phosphorylation mimic mutation (Y¹¹⁹E), downregulation is shown to involve dissociation of Jak2 from the receptor complex. Conversely, a Y¹¹⁹F mutant is more stably associated with the receptor complex. Thus, in cytokine responses, ligand binding induces activation of receptor associated Jak2, autophosphorylation of Y¹¹⁹ in the FERM domain and the subsequent dissociation of the activated Jak2 from the receptor and degradation. This regulation occurs with the receptors for Epo, thrombopoietin and growth hormone but not with the receptor for interferon- γ .

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Introduction

A variety of cytokines regulate the proliferation and differentiation of cells by binding to hematopoietic cytokine receptors (Ihle, 1995; O'Shea *et al*, 1997; Leonard and O'Shea, 1998). Cytokine receptors lack intrinsic catalytic activity and rely on receptor-associated cytoplasmic Janus kinase (Jak) tyrosine kinases for signal transduction. The Jak family of protein tyrosine kinases consists of four different members (Jak1, Jak2, Jak3 and Tyk2), which mediate essential and nonredundant functions in cytokine signaling for selective cytokine receptors (Ihle, 1996; Leonard and O'Shea, 1998).

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The Jak kinases are characterized by the presence of seven regions of sequence similarity found between Jak kinases and designated as Janus homology (JH) domains. JH1 contains the catalytically active tyrosine kinase domain located in the C terminus of Jak kinases. A centrally located domain, JH2, displays high sequence identity with JH1 but lacks catalytic activity and is termed the pseudokinase domain. The JH2 domain has been proposed to negatively regulate Jak kinase activity (Saharinen *et al*, 2000, 2003; Saharinen and Silvennoinen, 2002). More recently, mutations in this domain have been shown to activate Jak2 activity resulting in polycythemia vera, essential thrombocythemia and myeloid metaplasia with myelofibrosis (Baxter *et al*, 2005; James *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Steensma *et al*, 2005; Zhao *et al*, 2005). Sequence-based predictions have also indicated the existence of an SH2-like domain in JH3–JH4 regions of Jak kinases; however, its function has been unknown (Kampa and Burnside, 2000).

The amino terminal JH4–JH7 domains of Jaks have been shown to be the region that is involved in receptor association in all Jak/receptor interactions studied. This region contains a band 4.1, Ezrin, radixin and moesin (FERM) domain, a feature shared with only one additional kinase family, the Fak family (Girault *et al*, 1998; Hilkens *et al*, 2001; Zhou *et al*, 2001). The importance of the FERM domain has been best illustrated by the observation that several mutations within the FERM domain of JAK3 were found in severe combined immunodeficiency (SCID) patients (Cacalano *et al*, 1999; Zhou *et al*, 2001). These mutations inhibit receptor association and concomitantly abrogate Jak3's ability to be activated in response to ligand binding. Binding of the FERM domains of Jak1 and Jak2 to cytokine receptors have also been proposed to assist in cell surface expression of the receptor (Hilkens *et al*, 2001; He *et al*, 2003).

Among the Jaks, mice lacking the Jak2 gene have revealed a critical role for Jak2 in signaling through several receptors including the erythropoietin receptor (EpoR) (Neubauer *et al*, 1998; Parganas *et al*, 1998). Jak2-deficient embryos are profoundly anemic and die at around 12.5 dpc. Although primitive erythrocytes are unaffected, definitive erythroid progenitors including the less committed burst-forming unit-erythroid (BFU-E) and the more committed colony-forming unit-erythroid (CFU-E) are completely absent in the mutant mice. Epo binds to Epo receptor, which leads to activation of the associated Jak2, resulting in rapid autophosphorylation of multiple tyrosines of the 49 tyrosines in Jak2 (Witthuhn *et al*, 1993; Miura *et al*, 1994). However, to date, little is known regarding the consequences of phosphorylation of these tyrosines. Tyrosines at 1007 and 1008 in the activation loop of the kinase domain of Jak2 have been shown to be phosphorylated, and mutagenesis studies have demonstrated that activation of Jak2 kinase activity requires Y¹⁰⁰⁷ but not Y¹⁰⁰⁸ phosphorylation (Feng *et al*, 1997).

Recently, several groups reported that tyrosines at 221, 570 and 813 in Jak2 are also autophosphorylated (Argetsinger *et al*, 2004; Feener *et al*, 2004; Kurzer *et al*, 2004). Based on mutagenesis studies, phosphorylation of Y²²¹ slightly increased its kinase activity; on the other hand, phosphorylation of Y⁵⁷⁰ decreased kinase activity. Although phosphorylation of Y⁸¹³ does not affect the intrinsic activity of Jak2 in contrast to these tyrosines, this site is required for Jak2 to bind the β splicing variant of SH2-B and for the ability of SH2-B β to enhance Jak2 activation (Kurzer *et al*, 2004). The significance to Jak2 signaling *in vivo*, however, is unclear since deletion of the gene for SH2-B in mice does not affect Jak2 signaling (Duan *et al*, 2004). Collectively, the data suggest the possibility that the activation of Jak2 and its ability to initiate signal transduction is dependent upon phosphorylation of numerous tyrosine residues.

In studies to further assess the roles of individual sites of tyrosine phosphorylation, we observed that Y¹¹⁹ in the FERM domain of Jak2 is phosphorylated following Epo stimulation. To assess the potential role of Y¹¹⁹, mutations to phenylalanine (Y¹¹⁹F) or glutamate (Y¹¹⁹E) were examined for their ability to reconstitute Epo signaling in Jak2-deficient cells. Interestingly, the Y¹¹⁹F mutation was associated with a more stable association of Jak2 with EpoR and prolonged activation. In contrast, the Y¹¹⁹E mutation abrogated the ability of Jak2 to bind with EpoR, resulting in inhibition of Jak2 activation. Thus, this work suggests a novel role for phosphorylation of Y¹¹⁹ within Jak2 FERM domain in regulation of Epo signal transduction.

Results

The highly conserved Y¹¹⁹ in Jak2 is phosphorylated in response to Epo

Jak2 is activated in the context of several cytokine receptors. With activation, 10 tyrosine residues are phosphorylated as assessed with baculovirus-produced Jak2 using 2-D peptide

mapping (Matsuda *et al*, 2004). One of the major autophosphorylation sites was potentially identified as Y¹¹⁹, a highly conserved residue in Jak kinases (Figure 1A), in JH7 within the FERM domain. To confirm the phosphorylation of Y¹¹⁹, we did additional phosphopeptide mapping studies with Y¹¹⁹ mutants. As shown in Figure 2, autophosphorylation of wild-type Jak2 results in a major phosphopeptide that comigrates with a tryptic peptide containing phospho-Y¹¹⁹. In contrast, autophosphorylation of the mutant Jak2, Y¹¹⁹F, resulted in the loss of that peptide without a gain of another phosphopeptide. Since the tryptic peptide also contains Y¹²⁴, a second potential site of phosphorylation, we examined the extent of autophosphorylation of Jak2 mutants for this tyrosine. As illustrated, with the mutation Y¹²⁴F, a phosphopeptide was evident that migrated slightly below the Y¹¹⁹ phosphorylated peptide (arrow). This phosphopeptide was not detected with the Y¹¹⁹F/Y¹²⁴F mutated Jak2. These results establish that Y¹¹⁹ is a major site of autophosphorylation.

To verify that Y¹¹⁹ is phosphorylated in response to Epo stimulation under physiological conditions, we generated antibodies specific for the phosphorylated form of Y¹¹⁹ (α -P-Y¹¹⁹). Wild-type murine embryonic fibroblasts expressing the EpoR were treated with Epo for the times indicated (Figure 1B) and cell lysates were immunoblotted with α -P-Y¹¹⁹ Jak2 antibody. Phosphorylation at Y¹¹⁹ was readily detected in 2 min and persisted through 60 min (Figure 1B), and showed a comparable temporal pattern of phosphorylation to that of Y^{1007/1008} within the activation loop.

The specificity of the α -P-Y¹¹⁹ is demonstrated in Figure 1C by the lack of detection of a Y¹¹⁹F mutant following Epo stimulation although this mutant is still capable of being activated as assessed by phosphorylation of Y^{1007/8} (Figure 1C; WB:P-Y^{1007/8}) or by more global tyrosine phosphorylation (Figure 1C; WB:PY). The requirement for Jak2 kinase activity for phosphorylation of Y¹¹⁹ is also illustrated in Figure 1C by the lack of detectable phosphorylation of this site with a kinase-inactive mutant (KD) containing a K⁸⁸²R

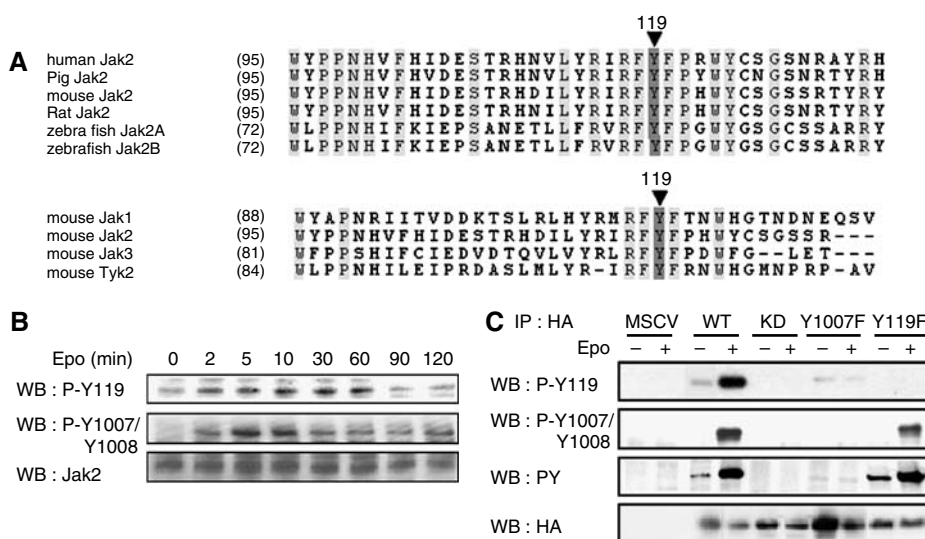


Figure 1 Jak2 Y¹¹⁹, a conserved tyrosine, is phosphorylated in response to Epo. (A) Sequence alignment of Jak2 sequences from various species Jak family kinases. (B) MEFs were infected with EpoR and stimulated with Epo (10U/ml) for indicated periods. Cell lysates were immunoblotted with antiphospho-Y¹¹⁹ Jak2 antibody, antiphospho-Y^{1007/1008} Jak2 antibody or anti-Jak2 antibody. (C) Jak2-deficient MEFs were coinfecting with EpoR and Jak2-HA mutants. Cells were stimulated with Epo (10U/ml) for 15 min. Cell lysates were subjected to immunoprecipitation using an anti-HA antibody and immunoblotted with antibodies to anti-phospho-Y¹¹⁹ Jak2, anti-phospho-Y^{1007/1008} Jak2, anti-phosphotyrosine or anti-Jak2.

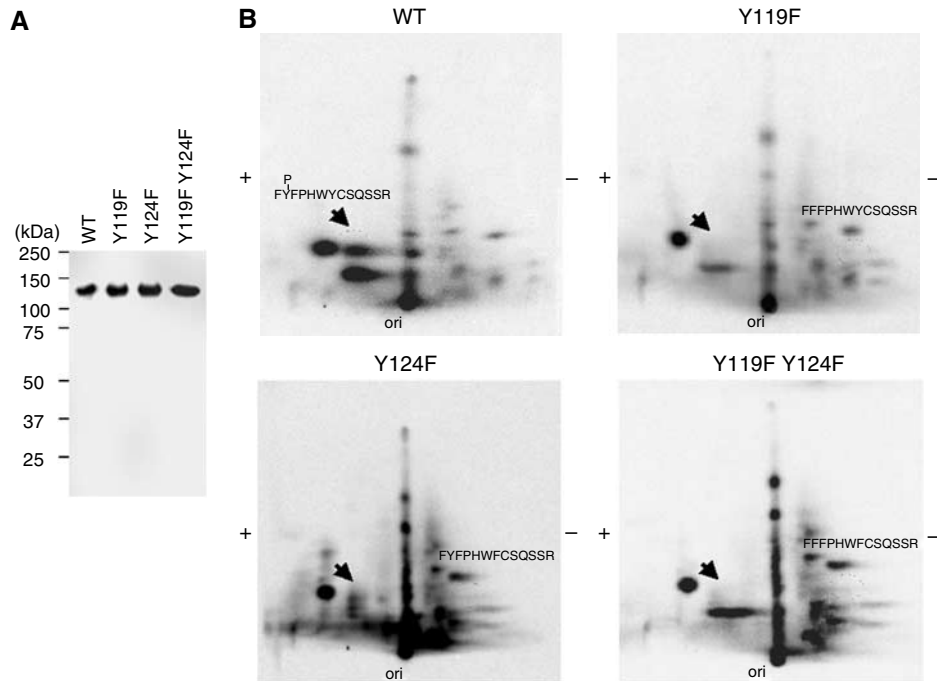


Figure 2 Phosphopeptide mapping of Jak2. 293T cells were transfected with pRK5-Jak2 Flag, pRK5-Jak2 Y¹¹⁹F, pRK5-Jak2 Y¹²⁴F or pRK5-Jak2 Y¹¹⁹F Y¹²⁴F with MSCV-Puro EpoR. The cells were incubated with [³²P]orthophosphate for 3 h and stimulated with Epo for 15 min. The lysates were immunoprecipitated with Flag antibody and eluted with Flag peptide. The eluted proteins were visualized by autoradiography (A). The digest of the eluted proteins was resolved in two dimensions on thin-layer cellulose plates with synthetic peptides (B). The ³²P-labeled spots were visualized using a phosphoimager. The positions of synthetic peptides were determined by ninhydrin staining and are indicated by broken circles. The position of phosphopeptide (FY¹¹⁹FPHWYCSQSSR) is indicated by an arrow.

mutant in the ATP-binding pocket or by a mutant containing a Y¹⁰⁰⁷F mutation in the activation loop. The specificity of the antibody was also established by peptide competitions (Supplementary Figure 1). The ability of the antibody to detect Epo induced tyrosine phosphorylation was blocked by the phosphopeptide but not by an unphosphorylated peptide. Together, the results demonstrate that Y¹¹⁹ is a site of autophosphorylation of Jak2 during Epo induced activation.

Phosphorylation of Y¹¹⁹ abrogates activation of Jak2 catalytic activity by Epo

To investigate the potential roles of Y¹¹⁹, two mutants were constructed in which Y¹¹⁹ was mutated to either phenylalanine or to glutamic acid (Figure 3A). Glutamic acid mutations have been used to mimic the consequences of phosphorylation in several studies and, for example, were shown to mimic the consequences of phosphorylation within the juxtamembrane domain of the EphB2 receptor tyrosine kinase (Wybenga-Groot *et al*, 2001). Each mutant was expressed in Jak2-deficient MEFs. The expression level of the Y¹¹⁹E mutant was consistently lower than either wild type or the Y¹¹⁹F mutant (Figure 3B). When coinfecting with EpoR, Epo stimulation induced the activation of kinase activity of the wild-type enzyme as measured by *in vitro* autophosphorylation (Figure 3C) or by phosphorylation of a peptide substrate (Figure 3D). As expected, no activation of kinase activity was seen with either the KD mutant or the Y¹⁰⁰⁷F mutant. Epo stimulation activated the Y¹¹⁹F mutant and the enzyme was more active than the wild-type enzyme. In addition, the activated kinase persisted longer (Figures 3C,

4A and B). Strikingly, and unexpectedly, although expressed, there was no activation of the Y¹¹⁹E mutant.

To further explore the properties of the Y¹¹⁹ mutants, activation of a downstream signaling event was examined (Figure 4C and D). Epo stimulation induces the tyrosine phosphorylation of Stat5 (Figure 4C) and activation of its transcriptional activity (Figure 4D). The Y¹¹⁹F mutant was associated with a higher level of and more persistent phosphorylation (Figure 4C) as well as by a higher level of transcriptional activity of Stat5 (Figure 4D). Consistent with the lack of activation of the Y¹¹⁹E, Epo stimulation of cells expressing this mutant resulted in no Stat5 activation.

Unexpectedly, we noticed that the Y¹¹⁹E mutant was uniquely tyrosine phosphorylated in unstimulated cells although this phosphorylation did not include the activation loop tyrosines (Figure 4A). A series of mutants were used to further explore the basis of this phosphorylation. Tyrosine phosphorylation of the Y¹¹⁹E mutant was not detected with a Jak2 containing both the Y¹¹⁹E and KD mutations, demonstrating that the phosphorylation is dependent upon Jak2 kinase activity. However, tyrosine phosphorylation was still evident with a double mutant containing the Y¹¹⁹E and Y¹⁰⁰⁷F mutations, indicating that this phosphorylation was only dependent upon the basal activity of Jak2. The results suggest that the Y¹¹⁹E mutation in the FERM domain is influencing the basal activity of the kinase and suggests that, like Jak3 (Zhou *et al*, 2001), the FERM domain interacts with the kinase domain to influence kinase activity.

To assess the specificity of the properties of the Y¹¹⁹ mutants, we compared the Y¹¹⁹ mutants with mutants of tyrosine Y¹²⁴ in close proximity (Supplementary Figure 2). In contrast to the Y¹¹⁹E, the Y¹²⁴E mutation had no effect on

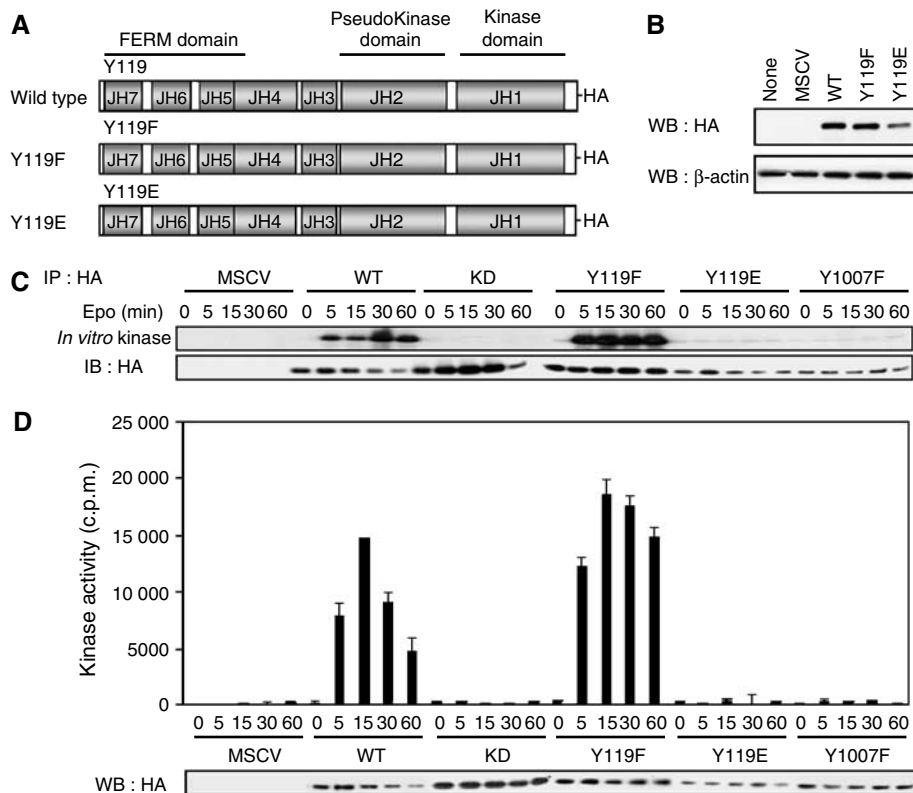


Figure 3 Phosphorylation of Y¹¹⁹ abrogates Epo-induced Jak2 activation. (A) Schematic structure of Jak2 mutants. The tyrosine residue at 119 was altered to phenylalanine (Y¹¹⁹F) or glutamic acid (Y¹¹⁹E). (B) Jak2-deficient MEFs were infected with Jak2-HA mutants. Cell lysates were immunoblotted with anti-HA antibody (upper panel) and anti- β -actin antibody (lower panel). (C) Jak2-deficient MEFs were coinfecting with retroviruses encoding EpoR and Jak2-HA mutants. Cells were stimulated with Epo (10 U/ml) for indicated periods. The cells were lysed and immunoprecipitated with anti-HA antibody. *In vitro* kinase activity of Jak2 was measured by autophosphorylation of Jak2. Samples were then separated by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography (upper panel) and blotting with anti-HA antibody (bottom panel). (D) *In vitro* kinase activity of Jak2 was measured in the presence of a synthetic peptide derived from the Y^{1007/Y1008} region of Jak2 (VLPQDKEYYKVKEPGES). Phosphorylated peptides were measured by scintillation counter (upper panel). Immunoprecipitated samples were separated by SDS-PAGE and subjected to blotting with anti-HA antibody (bottom panel).

the ability to be activated in response to Epo stimulation nor did the Y¹²⁴ mutant lose the ability to activate Stat5.

Mutation of Y¹¹⁹ affects the association and stability of Jak2 interaction with the EpoR

Since Y¹¹⁹ resides in the amino-terminal FERM domain, a region that is known to be required for interaction with the box1/2 region of cytokine receptor cytoplasmic domains, the possibility existed that the Y¹¹⁹ mutations affected receptor association. For these studies, Jak2-deficient MEFs were coinfecting with FLAG-tagged EpoR and HA-tagged Jak2 mutant. Immunoprecipitation of EpoR followed by Western blotting for various Jak2 mutants (Figure 5) demonstrated that wild-type Jak2 associates with the receptor even in the absence of Epo stimulation. Kinase activity is not required for Jak2 association with the receptor since both the K⁸⁸²R and Y¹⁰⁰⁷F mutants also associate with the receptor. Importantly, stimulation with Epo results in a time dependent loss of association of wild-type Jak2 with the receptor. The time-dependent loss of association requires kinase activity since neither mutant shows the loss of association seen with the wild-type receptor.

The Y¹¹⁹ mutants had distinctly different properties with regard to their interaction with the EpoR (Figure 5). The Y¹¹⁹F associated with the EpoR but unlike the wild-type enzyme, the Y¹¹⁹F mutant did not undergo the time-dependent dis-

sociation. In contrast, the Y¹¹⁹E mutant was unable to associate with the EpoR. Consistent with a requirement for receptor association, Jak2 activation, as measured by phosphorylation of the EpoR, Jak2 or Y^{1007/8}, was only detected with the wild-type enzyme or the Y¹¹⁹F mutant. It should be noted that the activation of the Y¹¹⁹F mutant was stronger and more sustained than the wild-type enzyme by any of the parameters examined. Importantly, although Y¹¹⁹ phosphorylation was detected following Epo stimulation in cell lysates (bottom panel), no Y¹¹⁹ phosphorylated protein was detected in receptor complexes (top panels).

Mutation of Y¹¹⁹ affects the stability of the kinase

The above results demonstrated that the Y¹¹⁹F mutant is more strongly activated and its activation is sustained longer relative to the wild-type enzyme while the Y¹¹⁹E mutant was consistently expressed at lower levels than the wild-type enzyme. We therefore assessed whether these differences were due to the *in vivo* stability of the mutants. For this, Jak2-deficient MEFs were infected with various Jak2 mutants and wild-type enzyme, pulse-labeled with ³⁵S and chased for various times with or without Epo stimulation (Figure 6). The wild-type enzyme shows a time-dependent loss in the absence of Epo stimulation, and this time-dependent loss is dramatically increased with Epo stimulation. In contrast, the kinase inactive mutants (K⁸⁸²R, Y¹⁰⁰⁷F) are significantly more

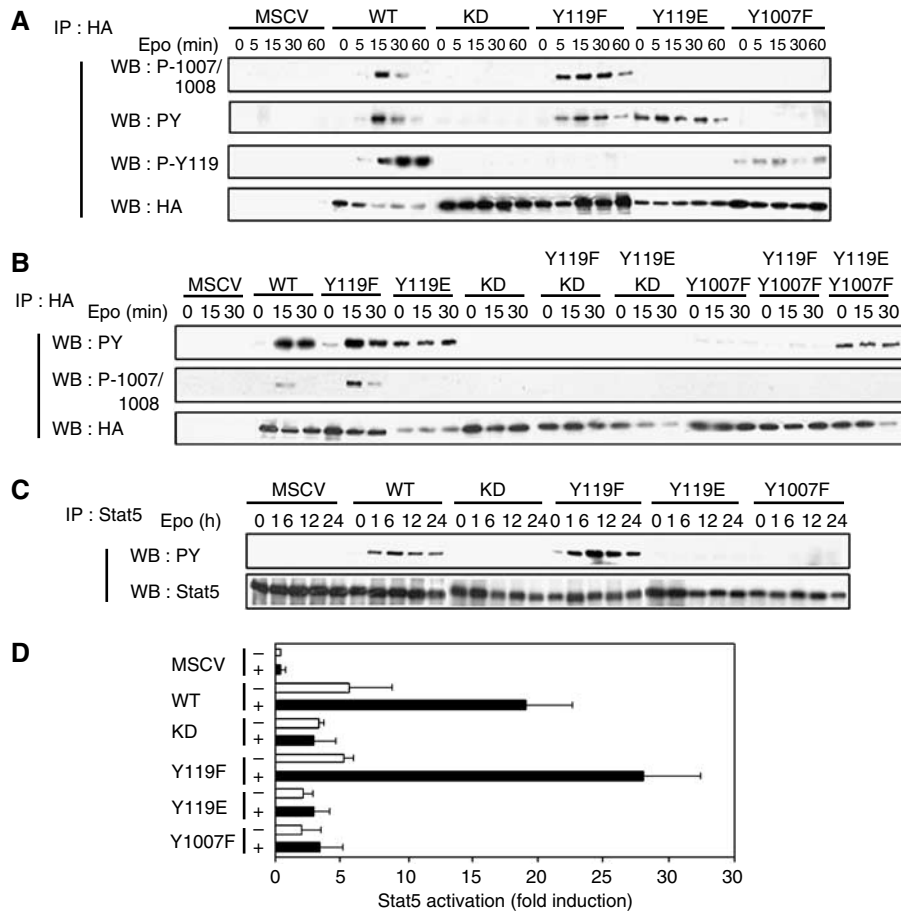


Figure 4 Phosphorylation of Y^{119} abrogates Epo-induced Jak2 phosphorylation at Y^{1007}/Y^{1008} and STAT5 activation. Jak2-deficient MEFs were coinfecting with EpoR and Jak2-HA mutants. Cells were stimulated with Epo (10 U/ml) for indicated periods. (**A**, **B**) The cell lysates were immunoprecipitated with anti-HA antibody and blotted with the antibodies antiphospho- Y^{1007}/Y^{1008} Jak2, antiphosphotyrosine, phospho- Y^{119} Jak2 or anti-HA. (**C**) Cell lysates were immunoprecipitated with anti-Stat5 antibody and blotted with antiphospho tyrosine antibody (upper) or anti-Stat5 antibody (bottom). (**D**) Jak2-deficient MEFs were cotransfected with EpoR, Jak2 mutants and a Stat5-dependent luciferase reporter construct. Stat5 dependent luciferase activity was normalized to the activity of a constitutive, TK promoter driven *Renilla* luciferase construct.

stable than the wild-type enzyme, even in Epo stimulated cells. These results are consistent with the hypothesis that Jak2 activation induces an increased turnover of the enzyme. The increased turnover with activation may occur within the receptor complex or be a consequence of dissociation from the receptor complex. The later is supported by the observation that the $Y^{119}F$ mutant is as stable as the kinase inactive mutants. This is further supported by the observation that the $Y^{119}E$ mutant, which fails to associate with the receptor but has an increased basal kinase activity, is considerably less stable than the wild-type enzyme.

Receptor specificity of Y^{119} mutations

Jak2 is required for signal transduction through a number of cytokine receptors, including the receptors for thrombopoietin (Tpo), growth hormone (GH) and prolactin (PRL), which are structurally closely related to the Epo receptor. In contrast, the receptor for interferon gamma ($IFN-\gamma$) is more distantly related and while requiring Jak2, also requires Jak1 for function (Briscoe *et al*, 1996). We therefore assessed whether role of Y^{119} played a comparable role in all the receptors. It should be noted that previous studies (Kohlhuber *et al*, 1997) demonstrated that the $Y^{119}F$ mutant was fully capable of restoring $IFN-\gamma$ signaling in Jak2-deficient cells. Consistent with the results with the Epo receptor,

there was no activation of the $Y^{119}E$ mutant in the context of PRL or GH signaling (Supplementary Figure 4, PRL and 5, GH). Also, note that in each case, the $Y^{119}E$ mutant is expressed at lower levels than the wild-type Jak2 or the other mutants. In contrast, the $Y^{119}E$ mutant was fully activated in the context of the $IFN-\gamma$ receptor complex. As illustrated, endogenous Jak2 is phosphorylated on Y^{119} in response to $IFN\gamma$ (Figure 7A). Introduction of the $Y^{119}E$ mutant into Jak2-deficient fibroblasts and stimulation with $IFN\gamma$ resulted in activation of kinase activity comparable to that seen with wild-type Jak2. Lastly, we examined the ability of the Jak2 mutants to associate with the various receptor complexes. Jak2 $Y^{119}E$ did not associate with either the PRL receptor (Supplementary Figure 4D) or the GH receptor (Supplementary Figure 5D), but it did associate with the $IFN-\gamma R2$ receptor comparable with wild-type Jak2 or the $Y^{119}F$ mutant (Figure 7D).

Mutation of Y^{119} inhibits erythroid colony formation

The above studies relied on the transfection of various mutant constructs into Jak2-deficient MEFs. It was therefore important to determine whether the Y^{119} mutations had comparable consequences in primary erythroid lineage progenitors. We therefore expressed various Jak2 mutants in the primary culture of the fetal liver cells from 12.5-day-old

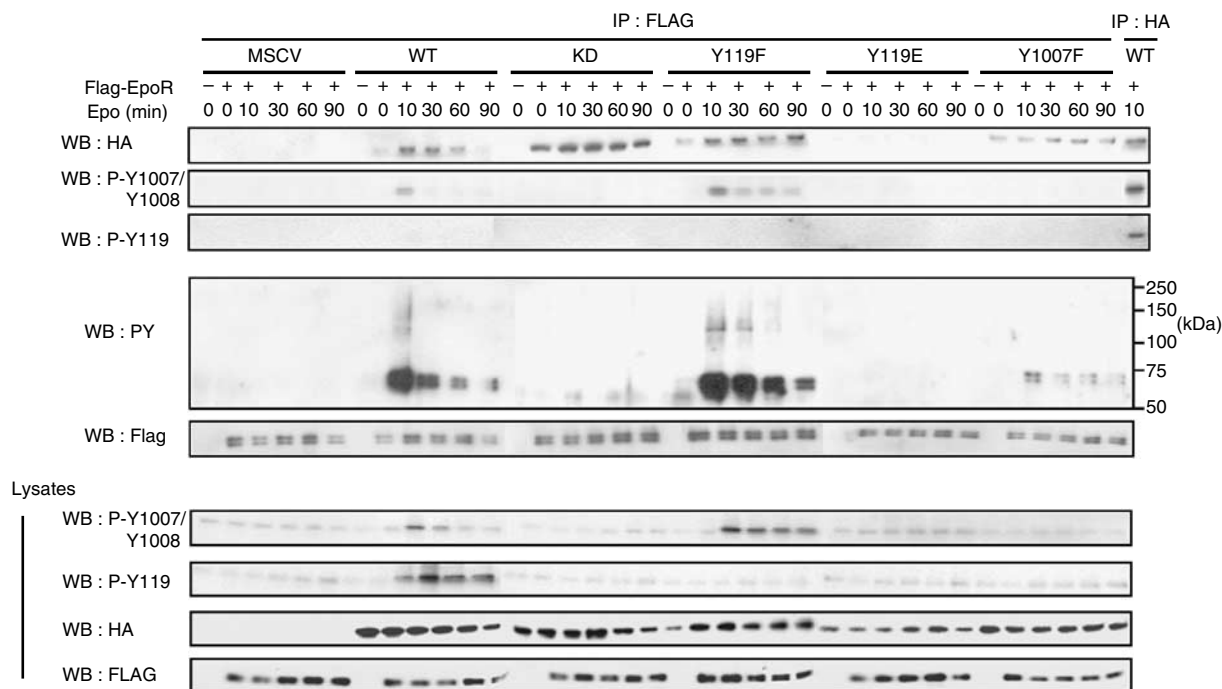


Figure 5 Phosphorylation of Y¹¹⁹ disrupts the interaction between Jak2 and EpoR. Jak2-deficient MEFs were coinfecting with EpoR-FLAG and Jak2-HA mutants. Cells were stimulated with Epo (10 U/ml) for indicated periods. The cells were lysed and immunoprecipitated with anti-FLAG antibody and blotted with the antibodies anti-HA, phospho-Y^{1007/1008} Jak2, antiphospho tyrosine or anti-FLAG. Whole-cell lysates were immunoblotted with anti-HA antibody or anti-FLAG antibody.

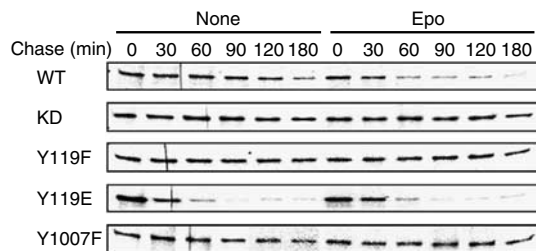


Figure 6 Phosphorylation at Y¹¹⁹ reduces the stability of Jak2. Jak2-deficient MEFs were coinfecting with EpoR and Jak2-HA mutants. Cells were pulse-labeled with ³⁵S-methionine for 40 min and chased for the indicated periods in the absence or presence of Epo (10 U/ml). Jak2 protein levels were analyzed by HA immunoprecipitation followed by autoradiographic exposure.

Jak2-deficient embryos by retroviral transduction. In the absence of Jak2, neither CFU-E (Figure 8A) nor BFU-E (Figure 8C) is detectable. However, Jak2-deficient fetal liver cells transduced with the Y¹¹⁹F mutant generated as many mature erythroid CFU-E (Figure 8B) and BFU-E (Figure 8D) progenitors *in vitro* as did cells transduced with wild type of Jak2. In contrast, no BFU-E and CFU-E colonies were formed from Jak2-deficient fetal liver cells infected with the Y¹¹⁹E mutant. As expected, the catalytically inactive mutants, K⁸⁸²R and Y¹⁰⁰⁷F, were not capable of inducing either BFU-E or CFU-E from Jak2-deficient fetal liver cells.

Discussion

Our results demonstrate that Jak2 autophosphorylation in the FERM domain plays an unexpected role in promoting dissociation of the kinase from receptor complexes and thereby mediates the receptor-specific, time-dependent downregulation of cytokine signaling. The FERM domain of the Jak

kinases has been shown to be the critical site of interaction with cytokine receptors (Frank *et al*, 1995; Chen *et al*, 1997; Kohlhuber *et al*, 1997; Zhou *et al*, 2001). In the case of Jak3, a number of naturally occurring mutations in the FERM domain are found in humans. These affect the interaction of Jak3 with the common γ -chain of the IL-2 subfamily of cytokine receptors and result in SCID (Cacalano *et al*, 1999). Studies of interaction of Jak2 with the GH receptor (He *et al*, 2003) and the IFN γ R2 subunit have similarly demonstrated the essential role for elements of the FERM domain in receptor association (Kohlhuber *et al*, 1997).

In addition to receptor association, the FERM domain of Jak3 has been proposed to be required for the maintenance of kinase integrity (Zhou *et al*, 2001) and a model has been proposed in which interactions of the FERM domain with the receptor may affect the structure of the kinase and pseudo-kinase domains and thereby control the ability of the kinase to be activated. Consistent with this, we have recently identified an activating mutation of Jak2 that is only manifested when the kinase is tethered to the Epo receptor (unpublished data). The role of the FERM domain in regulating kinase activity has also been suggested by the existence of a single amino-acid mutation (G³⁴¹E) in the FERM domain of the *Drosophila* Jak kinase that causes a leukemia-like pathology (Harrison *et al*, 1995; Luo *et al*, 1995).

The structure of the full-length Jak2 is not known although a recent study (Boggon *et al*, 2005) has reported the crystal structure of the kinase domain, which has the anticipated structural properties of tyrosine kinase domains. However, a theoretical model of Jak2 has been described that was generated by application of homology modeling approaches (Giordanetto and Kroemer, 2002). In this model, the FERM domain assumes a three lobed structure (F1, aa 37–115; F2, aa 146–258 and F3, aa 269–387). Interestingly, Y¹¹⁹ lies in a

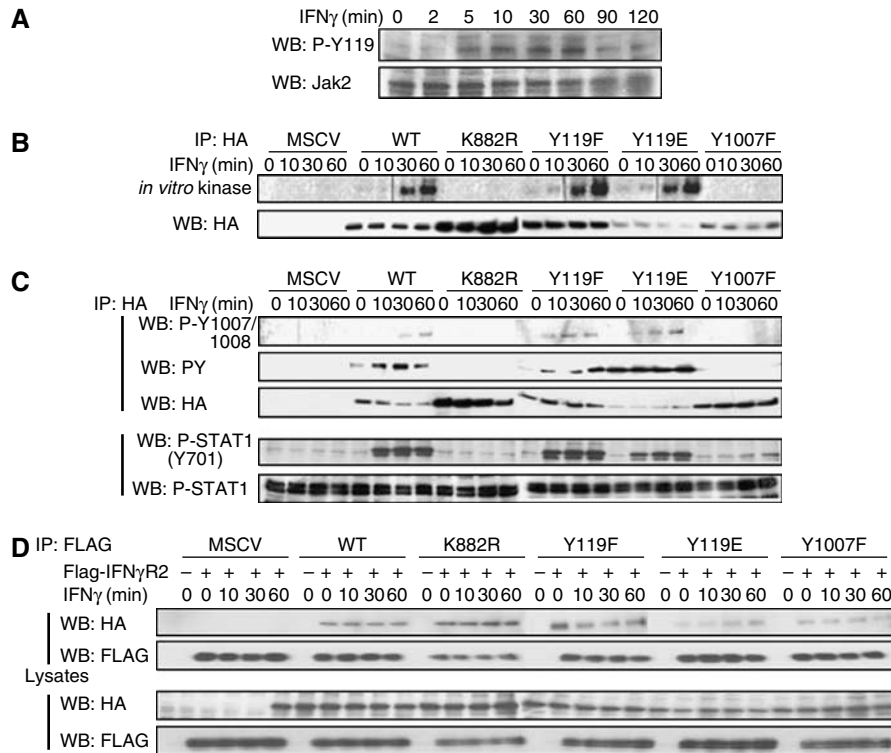


Figure 7 Receptor specificity of Y¹¹⁹ mutation. (A) Mouse embryonic fibroblasts were stimulated with IFN γ for the indicated times. Cell lysates were immunoblotted with anti-phospho-Y119 Jak2 antibody or with anti-Jak2 antibody. (B) Jak2-deficient fibroblasts were infected with wild type Jak2 or the indicated mutants. Cells were stimulated with IFN γ (5 ng/ml) for the indicated times. The cells were lysed and immunoprecipitated with anti-HA antibody. *In vitro* Jak2 kinase activity was measured by autophosphorylation of Jak2. Samples were separated by SDS-PAGE, transferred onto nitrocellulose and subjected to autoradiography (upper) or blotted with anti-HA antibody (bottom). (C) Cells were stimulated as above and cell lysates were immunoblotted and probed with the indicated antibodies to assess Jak2 activation (P-Y1007/1008; total tyrosine phosphorylation (PY), protein levels (HA) and activation of Stat1 (P-Stat1 Y701). (D) Jak2-deficient MEFs were coinfecting with IFN γ R2-FLAG and Jak2-HA mutants. Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-HA antibody or anti-FLAG.

hydrophobic linker region between the F1 and F2 lobes and therefore is in a position that may influence the overall structure of the domain. In the proposed model, Y¹¹⁹ is almost completely buried in a hydrophobic pocket and it was suggested that this residue would not be phosphorylated. However, our data clearly demonstrate that this residue is phosphorylated during normal receptor activation of Jak2 and that this phosphorylation is likely an auto- or transphosphorylation site.

A role for phosphorylations within FERM domains in regulating function has recently obtained interest from structural studies. In particular, phosphorylation of Y¹⁴⁶ in the FERM domain of ezrin has been described in response to epidermal growth factor stimulation (Krieg and Hunter, 1992). Initially, this phosphorylation was unexpected since Y¹⁴⁶ was fully buried in the crystal structure of moesin. However, structures of the active FERM domain of Ezrin (Smith *et al*, 2003) allowed the identification of structural changes that occur with activation that would allow the phosphorylation to occur. It was proposed that such phosphorylations within the FERM domain could change the binding specificity in a model similar to what we propose for the FERM domain of Jak2.

Previous studies demonstrated that the mutant Y¹⁰⁰C in human Jak3 results in a reduced ability of the kinase to associate with the common γ -chain (Cacalano *et al*, 1999). This is interesting since Y¹⁰⁰ is equivalent to Y¹¹⁴ in Jak2 and

thus is near Y¹¹⁹. However, in our studies, we have not detected phosphorylation of Y¹¹⁴ (data not shown) and consequently have not studied mutants of this site. In human Jak3, the equivalent position to Y¹¹⁹ is Y¹⁰⁵. Although not studied in detail, mutation of Y¹⁰⁵ to an alanine or phenylalanine (Zhou *et al*, 2001) had no effect on receptor association or kinase activity. Since the mutations Y¹¹⁹F in Jak2 as well as Y¹⁰⁰F, Y¹⁰⁵F or Y¹⁰⁵A in Jak3 have no consequences on receptor association, it can be proposed that it is the introduction of a charged residue in the hinge region of the FERM domain that disrupts the ability to associate with the receptor. There is specificity to this effect since introduction of a charged residue at a neighboring tyrosine (Y¹²⁴E) in the linker region between the FERM domain lobes F1 and F2 did not have the same consequences. We have also examined the FERM domain mutations Y³⁷²F and Y³⁸²F, neither of which had properties that differed from wild-type enzyme.

The FERM domain of Jak2 has been generally implicated in receptor associations, including the highly related receptors for Epo, TPO, PRL and GH as well as the most distantly related receptor for IFN- γ . One of the striking results was that while the Y¹¹⁹E mutant affected the interaction of Jak2 with the receptors for Epo, GH and PRL, there was no consequence of the mutation on the ability of Jak2 to associate with the IFN- γ R2 although Y¹¹⁹ is phosphorylated in response to IFN- γ . These observations provide two important insights into Jak2 function. First, the results demonstrate that there are

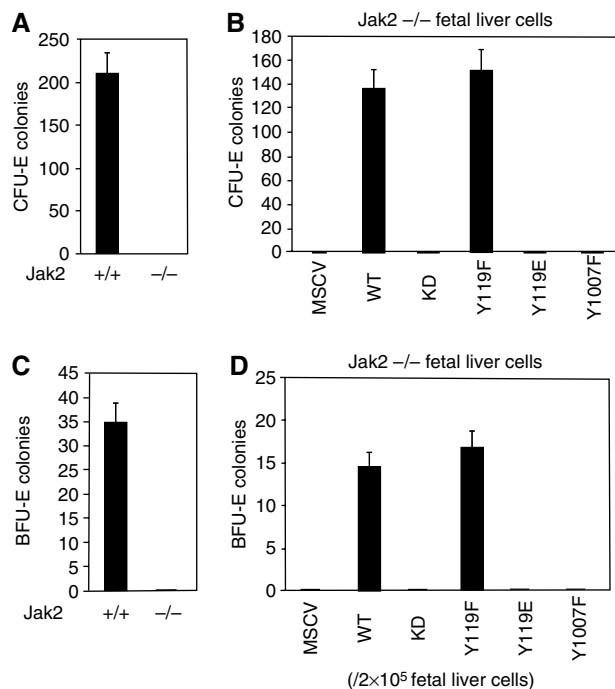


Figure 8 Phosphorylation at Y¹¹⁹ inhibits erythroid colony formation. (A, C) Wild type and Jak2-deficient fetal liver cells were subjected to *in vitro* colony assays with Epo and/or IL-3. (B, D) Fetal liver cells from Jak2-deficient embryos were infected with retrovirus encoding various Jak2 mutants and subjected to *in vitro* colony assays with Epo and/or IL-3. The benzidine-positive CFU-E colonies and BFU-E colonies were scored at day 3 and 8, respectively.

fundamental differences in the manner in which Jak2 interacts with the Epo/GH/PRL receptors and the IFN- γ receptor that would not have been anticipated. Second, as a consequence, the downregulation of receptor signaling between the two groups of receptors has fundamental differences.

Two groups previously identified Y²²¹ within the FERM domain of Jak2 as a site of tyrosine phosphorylation (Argetsinger *et al*, 2004; Feener *et al*, 2004). In transient transfection assays into 293T, the mutant Y²²¹F was found to have decreased autophosphorylation (Argetsinger *et al*, 2004) while the same mutant was found to mediate signal transduction normally in HEK293 cells transfected with the mutant and a Epo-leptin receptor chimeric protein (Feener *et al*, 2004). However, in neither study was the consequence of mutating the tyrosine to an amino acid other than phenylalanine examined.

We identified the Y¹¹⁹E mutant in a series of studies to explore the individual roles of the multiple tyrosines that are phosphorylated on Jak2 as a consequence of Epo receptor engagement. The importance of exploring the role of Jak2 tyrosine phosphorylation has been emphasized by the demonstration that a minimal receptor cytoplasmic domain, containing no tyrosines, is nearly as functional as the full-length receptor (Zang *et al*, 2001). Therefore, if Epo receptor signal transduction involves recruitment of phosphotyrosine binding proteins, they are most likely to be recruited to sites of tyrosine phosphorylation on Jak2. We have identified 10 sites of phosphorylation (Matsuda *et al*, 2004), however, with the exception of the Y¹⁰⁰⁷F mutation, none of the Y^F mutations individually affects the ability of the kinase to rescue

Epo dependent, erythroid differentiation of Jak2-deficient fetal liver cells. Recent studies have also identified Y⁵⁷⁰, in the pseudokinase domain, as a site of phosphorylation (Argetsinger *et al*, 2004; Feener *et al*, 2004). The mutant Y⁵⁷⁰F was more active than the wild-type enzyme in studies involving transfection of mutant genes into cell lines, suggesting a role of phosphorylation of Y⁵⁷⁰ in downregulating kinase activity. However, in our studies, involving the transfection of Jak2-deficient fetal liver cells, the Y⁵⁷⁰F mutant is equivalent to the wild-type enzyme.

The importance of the regulation of Jak2 kinase activity has been illustrated by the observations of several groups that a point mutation in the Jak2 pseudokinase domain is associated with a variety of myeloproliferative disorders, including polycythemia vera, essential thrombocythemia and myeloid metaplasia (Jones *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Steensma *et al*, 2005; Zhao *et al*, 2005). Since Jak2 is required for signaling through the receptors for Tpo, interleukin-3 as well as Epo, it might be anticipated that mutations that increase the ability of Jak2 to be activated would have implications in a variety of hematopoietic lineages. The autophosphorylation of Jak2 Y¹¹⁹ that results in dissociation of the kinase from the receptor complex is another unexpected mechanism for controlling the extent of signaling that may be a target for mutations.

Materials and methods

Antibodies and reagents

Anti-FLAG M2 monoclonal antibody and antiphosphotyrosine monoclonal antibody (4G10) were purchased from SIGMA and Upstate Biotechnology, respectively. Anti-HA antibody (3F10) and anti-HA-peroxidase antibody (3F10) were purchased from Roche. Rabbit polyclonal antisera were raised against STAT5A as previously described (Quelle *et al*, 1996). Phospho-Jak2 (Y^{1007/1008}) antibody and Phospho-Stat1 (Y⁷⁰¹) were from Cell Signaling Technology. Anti- β -actin antibody was purchased from Abcam. Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were from Amersham Life Science. Antibodies recognizing phosphorylated Y¹¹⁹ of Jak2 were raised in rabbits by injecting a keyhole limpet hemocyanin-coupled synthetic 12-amino-acid phosphorylated peptide centered on Y¹¹⁹. Antisera was affinity purified on the antigen peptide coupled to NHS-activated sepharose (Amersham Bioscience), followed by passage over NHS-activated sepharose coupled to nonphosphorylated antigen peptide (LYRIR FYFPHWY) and Y¹¹⁹F mutated peptide (LYRIRFFFPHWY) to remove antibodies directed against the nonphosphorylated form and YF mutated form of the site. Recombinant Epo was purchased from AMGEN and recombinant SCF, IL-6, IL-3, IFN- γ , PRL and GH were purchased from R&D system.

Cell culture

Heterozygote Jak2^{+/-} (129J \times C57BL/6J background) mice were screened by polymerase chain reaction as previously described (Parganas *et al*, 1998). Embryonic fibroblasts from Jak2 wild type or Jak2-deficient embryos at day 12.5 and human kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine and 100 U each of penicillin and streptomycin (Life Technologies). The cells were incubated for 14–16 h in DMEM containing 0.1% FBS and 0.4% BSA before the addition of Epo (10 U/ml), PRL (1 μ g/ml), GH (10 ng/ml) or IFN- γ (5 ng/ml) for the indicated periods. Individual E12.5 Jak2^{-/-} fetal livers were isolated, dissected and disaggregated into single-cell suspensions in alpha-modified minimum essential media (α -MEM, Stem Cell Technologies, Vancouver, BC, Canada) containing 15% fetal calf serum (FCS). They were passed through a 21-gauge needle and 25-gauge needle, and washed twice in the same medium. The cells were strained through a 70 μ m cell filter (Becton Dickinson, San Jose, CA).

Phosphopeptide mapping

Analysis of phosphopeptide mapping was performed with some modification as previously described (Matsuda *et al*, 2004). Briefly, 293T cells were transfected with pRK5-Jak2 Flag, pRK5-Jak2 Y¹¹⁹F, pRK5-Jak2 Y¹²⁴F or pRK5-Jak2 Y¹¹⁹F Y¹²⁴F with MSCV-Puro EpoR construct and cultured for 48 h before being transferred to phosphate-free DMEM medium and incubated with 1 mCi of [³²P]orthophosphate (ICN) per ml for 3 h. Cells were stimulated with Epo for 15 min and lysed as described above. The lysates were immunoprecipitated with Flag antibody for 2 h and eluted with Flag peptide. The eluted proteins were digested with TPCK-treated trypsin (Sigma) and lysilendopeptidase (Lys-C) (Sigma) at 37°C for 18 h and oxidized with performic acid. The synthetic peptides were treated comparably with performic acid. Peptides were separated by thin-layer electrophoresis at pH 1.9 followed by thin-layer chromatography using phosphochromatography buffer. The ³²P-labeled spots were visualized using a phosphoimager (Bio-Rad model 505). The positions of synthetic peptides were visualized by ninhydrin staining.

Plasmids

Jak2-HA was subcloned into MSCV-IRES-GFP. Mutagenesis of amino-acid residues in Jak2: K⁸⁸²R, Y¹¹⁹F, Y¹¹⁹E and Y¹⁰⁰⁷F were performed using Site-Directed Mutagenesis Kit, according to the manufacturer's instruction (STRATAGENE). The full-length EpoR, PRLR and GHR containing C-terminal FLAG were subcloned into MSCV-IRES-Puro. JAK2-Flag or Jak2-HA was subcloned into pRK5 or MSCV-IRES-GFP, respectively. Mutagenesis of amino-acid residues in Jak2: K⁸⁸²R, Y¹¹⁹F, Y¹¹⁹E, Y¹²⁴F, Y¹²⁴E and Y¹⁰⁰⁷F were performed using Site-Directed Mutagenesis Kit, according to the manufacturer's instruction (Clontech, San Francisco, CA).

Retrovirus production and infection

Human kidney 293T cells were transfected with helper retrovirus plasmid together with retroviral plasmids by FUGENE6 Transfection Reagent, according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Viruses were harvested 24–60 h post-transfection, pooled and stored on ice. Exponentially growing Jak2-deficient MEFs, in 100 mm culture dishes, were infected three times at 3 h intervals with 2 ml of fresh virus-containing supernatant in complete medium containing 8 µg/ml polybrene (Sigma Chemicals). Jak2^{-/-} fetal liver cells (2 × 10⁵ cells/ml) were incubated with IL-6 (10 ng/ml) and SCF (100 ng/ml) prior to the infection and replaced into the precoated plates by RetroNectine (TAKARA), according to the manufacturer's instructions. The cells were collected and analyzed for GFP expression by FACS analysis to monitor the infection efficiency.

In vitro colony assays

Retrovirally transduced cells were washed three times with α-MEM medium (GIBCO/BRL) containing 2% FBS. Diluted cell suspensions and recombinant cytokines specific for each of the assays (see below) were mixed with MethoCult M3234 (StemCell Technologies). This gave a final concentration of 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine. The cells were plated in 35 mm culture dishes in duplicate and cultured at 37°C, 5% CO₂. For the CFU-E assay, 2 × 10⁵ cells/dish were cultured in 0.2 U/ml recombinant human Epo, rhEpo (Amgen) and benzidine-positive CFU-E colonies were scored at day 3. For the BFU-E assay, 2 × 10⁵ cells/dish were cultured in 3 U/ml rhEpo and 10 ng/ml recombinant murine IL-3, rmlL-3 (R&D Systems) and benzidine-positive BFU-E colonies were scored at day 8.

Immunoprecipitation and Western blotting

The cells were harvested in ice-cold PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM NaF, 0.2 mM Na₃VO₄) supplemented with protease inhibitors. Cell lysates were centrifuged at 12 000 g for 15 min to remove debris, and the supernatants

were incubated with the indicated antisera for 4 h. Immune complexes were precipitated with protein G-Sepharose (Zymed Laboratory), washed three times in lysis buffer, and then eluted with sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). Eluted proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were probed by using the designated antibodies and visualized with the ECL detection system (Amersham). For co-immunoprecipitation with EpoR, the immunoprecipitates were washed with lysis buffer described above, then eluted from the beads by elution buffer (10 mM Tris (pH 8.0), 150 mM NaCl) containing FLAG peptide (Sigma) to avoid the nonspecific binding of Jak2 with EpoR.

In vitro kinase assay

The cells were lysed in kinase lysis buffer (10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 20% glycerol, 5 mM EDTA, 50 mM NaCl, 50 mM NaF and 1 mM Na₃VO₄) supplemented with protease inhibitors. Jak2 was immunoprecipitated using anti-HA antibody and washed three times with kinase lysis buffer and twice with kinase assay buffer (10 mM HEPES (pH 7.4), 50 mM NaCl, 5 mM MnCl₂, 5 mM MgCl₂, 50 mM NaF, 1 mM Na₃VO₄). The immunocomplex was resuspended in 50 µl of kinase buffer containing 1 µCi of [γ-³²P]ATP (Amersham) and incubated at 30°C for 30 min. Reactions were terminated by addition of 1 ml of cold kinase lysis buffer, and the complexes were washed three times with lysis buffer. The proteins were eluted in sample buffer and separated by SDS-PAGE (7.5% polyacrylamide). ³²P-labeled proteins were detected by autoradiography. For reaction using Jak2 peptide (VLPQDKEYKVKKEPGES), 1 mg of the peptide per ml was added to the reaction mixture as described above. An aliquot of reaction mixture was transferred onto p81 phosphocellulose paper (Whatman) and then the p81 phosphocellulose paper was washed three times for 5 min each with 0.75% phosphoric acid and once for 5 min with acetone. The radioactivity was measured using scintillation counter. The counts detected in the absence of peptide were used as a back ground control.

Luciferase assay

Jak2-deficient MEFs were transfected with MSCV-FLAG-EpoR, MSCV-IRES-GFP-Jak2 and STAT5-dependent luciferase reporter construct together with a pRL-TK control vector constitutively expressing *Renilla luciferase* (Promega). Cells were stimulated with Epo for 12 h and lysed in passive lysis buffer (Promega), and the luciferase activity of the lysates was determined using the dual luciferase reporter assay system (Promega), according to the manufacturer's instructions. STAT5-dependent luciferase activity was normalized to the activity of the constitutively expressed *Renilla Luciferase*.

Pulse-chase experiments

Infected Jak2-deficient MEFs were washed twice with PBS and starved by adding methionine-free and cysteine-free DMEM (Gibco-BRL) for 30 min. Cells were then pulsed with 0.1 µCi of [³⁵S] methionine and [³⁵S] cysteine (ProMix; Amersham) for 40 min. Then, cells were washed with PBS and chased in complete medium containing 2 mM methionine and 2 mM cysteine. Jak2 protein was immunoprecipitated using anti-HA-antibody and the labeled protein was detected by autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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