

The N-Terminal Domain of Janus Kinase 2 Is Required for Golgi Processing and Cell Surface Expression of Erythropoietin Receptor

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Summary

We show that Janus kinase 2 (JAK2), and more specifically just its intact N-terminal domain, binds to the erythropoietin receptor (EpoR) in the endoplasmic reticulum and promotes its cell surface expression. This interaction is specific as JAK1 has no effect. Residues 32 to 58 of the JAK2 JH7 domain are required for EpoR surface expression. Alanine scanning mutagenesis of the EpoR membrane proximal region reveals two modes of EpoR-JAK2 interaction. A continuous block of EpoR residues is required for functional, ligand-independent binding to JAK2 and cell surface receptor expression, whereas four specific residues are essential in switching on prebound JAK2 after ligand binding. Thus, in addition to its kinase activity required for cytokine receptor signaling, JAK is also an essential subunit required for surface expression of cytokine receptors.

Introduction

The erythropoietin receptor (EpoR) belongs to the cytokine receptor family and is the primary regulator of mammalian erythropoiesis (Constantinescu et al., 1999a; D'Andrea et al., 1989). Cytokine receptors lack intrinsic catalytic activities and rely on the Janus (JAK) family of cytosolic protein tyrosine kinases for signal transduction (Ihle et al., 1995). The EpoR and PrIR bind and activate JAK2, but JAK1, JAK3, and TYK2 participate in signaling by other receptors. Homodimerization of the EpoR in response to erythropoietin (Epo) activates JAK2, which in turn phosphorylates tyrosine residues in the EpoR cytoplasmic domain (Ihle et al., 1995; Witthuhn et al., 1993). These phosphorylated tyrosines then serve as docking sites for SH2-containing signaling molecules, including STAT5 (signal transducer and activator of transcription 5), that ultimately cause proliferation, differentiation, and survival of erythroid progenitor cells (Darnell, 1997; Ihle, 1996).

The number of EpoRs present on the cell surface is critical for downstream signaling events. In normal erythroid cells, the level of surface EpoR is extremely low (D'Andrea and Zon, 1990), and even in cell lines that overexpress the EpoR only a minor fraction is expressed

on the cell surface (Neumann et al., 1993, 1996; Supino-Rosin et al., 1999; Yoshimura et al., 1990). A mutant EpoR with a mutation in the conserved WSXWS motif in the extracellular domain is processed more efficiently in the endoplasmic reticulum than the wild-type receptor, suggesting that normally the wild-type EpoR is folded inefficiently (Hilton et al., 1995). Moreover, overexpression of murine EpoR in human UT-7 cells does not increase total cell surface expression of the EpoR but leads to the complete replacement of the endogenous human EpoR by a similar number of murine EpoRs (Hermine et al., 1996). These results strongly suggest that an unidentified cellular factor may control or limit cell surface expression of the EpoR. We hypothesize that this factor is JAK2.

JAK2, like other JAK family kinases, contains seven regions of sequence similarity found among JAK family members, designated as JAK homology (JH) domains. The catalytically active tyrosine kinase domain (JH1) at the C terminus is adjacent to a pseudokinase domain (JH2), and the N-terminal region of JAK kinases is further dissected into JH3 to JH7 domains (Harpur et al., 1992). JH3 to JH7 domains of JAK kinases have been proposed to be the site of binding to multiple cytokine receptors (Cacalano et al., 1999; Yeh and Pellegrini, 1999). Recent computational studies identified a potential FERM domain between JH4 and JH7 in all JAK kinases (Girault et al., 1999). This 300 amino acid domain was initially identified in erythrocyte band 4.1 protein, ezrin, radixin, and moesin and mediates the attachment of these proteins to transmembrane and cytoskeletal proteins (Chishti et al., 1998). Its function in JAKs is unclear.

Here we show that cell surface expression of the EpoR is JAK2 dependent. Specifically, the N-terminal region (JH3 to JH7) but not the pseudokinase or kinase domain binds to the EpoR in the endoplasmic reticulum and promotes its cell surface expression, probably by enhancing EpoR folding. Further, we define two discrete modes of productive interaction of the EpoR with JAK2. One long block of continuous amino acids is required for functional binding to JAK2 and EpoR surface expression. Four specific residues, three of them highly conserved among cytokine receptors, are essential for Epo-triggered activation of prebound JAK2 but are not required for JAK2 stimulation of EpoR surface expression. Thus, in addition to its kinase activity required for cytokine receptor signaling, JAK is also an essential subunit required for surface expression of cytokine receptors.

Results

JAK2 but Not JAK1 Induces Expression of the EpoR on the Plasma Membrane

We developed a FACS-based assay to measure EpoR surface expression in the presence or absence of JAK2 using γ 2A, a JAK2 deficient human fibrosarcoma cell line (Kohlhuber et al., 1997; Pellegrini et al., 1989). First, we transiently expressed HA-EpoR, which contains an HA epitope tag in the extracellular domain, using a bicis-

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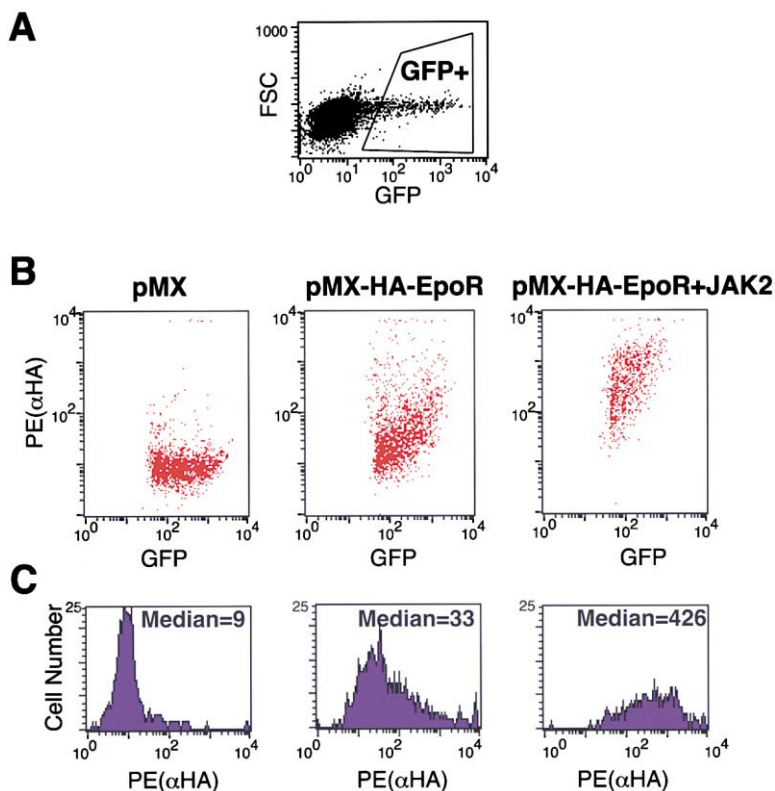


Figure 1. JAK2 Induces Expression of the HA-EpoR on the Plasma Membrane

Surface expression of HA-EpoR was measured in γ 2A cells transiently transfected with the bicistronic vector pMX-HA-EpoR-IRES-GFP with or without coexpression of JAK2. Control cells transfected with an empty pMX vector (left panel) were used as a negative control. GFP positive cells (shown in [A]; FSC, forward scatter), indicating the expression of HA-EpoR, were gated for analysis of PE fluorescence (B). Median fluorescence intensities of PE were used for quantitation of surface expression of HA-EpoR since this measure of central tendency is least sensitive to outliers (C).

tronic vector (pMX-IRES-GFP) that also expresses GFP (Liu et al., 2000). Since GFP and HA-EpoR are expressed simultaneously and proportionally in individual cells, GFP fluorescence was used to identify cells expressing the HA-EpoR (Figure 1A). We then used flow cytometry on a cell-by-cell basis to measure the expression level of the HA-EpoR on the plasma membrane using antibodies against the HA tag. HA-EpoR was detected on the cell surface at a low level (Figure 1B, middle panel), when compared to the level of phycoerythrin (PE) fluorescence (against the HA tag) of cells transfected only with the “empty” vector pMX-IRES-GFP (Figure 1B, left). Importantly, when cotransfected with JAK2, over a 50-fold range of HA-EpoR expression levels (monitored by the level of GFP expression), the level of HA-EpoR on the surface of all transfected cells increased dramatically (Figure 1B, right). To quantify the relative amounts of HA-EpoR at the cell surface, the median fluorescence intensity of PE staining against the HA tag was used. As shown in Figure 1C, JAK2 induced a 12-fold increase of HA-EpoR expression on the plasma membrane, whereas the median fluorescence of GFP (i.e., total cellular EpoR expression level) remained unaffected.

Since JAK2 but not JAK1 is normally required for EpoR signaling, we next examined JAK1 for its ability to promote surface expression of the HA-EpoR in γ 2A. In γ 2A cells, the endogenous level of JAK1 is low (Figure 2B), and overexpression of JAK1 did not affect surface expression of HA-EpoR (Figure 2A). The ability of JAK2 to enhance EpoR surface expression is not specific to γ 2A cells since we found JAK2-dependent EpoR surface expression in JAK2-deficient (JAK2^{-/-}) embryonic fibro-

blasts (Figure 3). Therefore, the ability of JAK2 to promote EpoR surface expression is specific, which correlates with its function in EpoR signaling. This specificity of JAK2 versus JAK1 is remarkable in light of the fact that Epo does also activate JAK1. However, no role could be found for JAK1 in EpoR signaling (Miura et al., 1994; Witthuhn et al., 1993). In addition, a mutant JAK2 with a point mutation in the kinase domain (Lys 882 to Asp) that abolishes kinase activity retained full ability to induce cell surface expression of HA-EpoR, indicating that kinase activity is not required for promoting EpoR cell surface expression (Figure 5A, JAK2 [K882D]).

Together, these results suggested that the number of EpoR expressed on the cell surface is JAK2 dependent and therefore we have defined a novel function of JAK2. The low level of HA-EpoR on the plasma membrane in wild-type (JAK2^{+/+}) embryonic fibroblast cells may be due to the low level of expression of JAK2 in these cells.

JAK2 Increases the Golgi-Processed Form of the EpoR

In both transfected and normal hematopoietic cells, the majority of the newly made EpoRs is retained in the endoplasmic reticulum (ER). Approximately 20% of the newly made EpoR is processed to a mature form with a Golgi-processed Endo H resistant oligosaccharide, of which only a minor fraction is expressed at the cell surface (Hilton et al., 1996; Neumann et al., 1993). To examine if JAK2 affects the processing of the HA-EpoR, the glycosylation state of HA-EpoRs was examined in γ 2A cells. Populations of cells stably expressing the HA-EpoR together with either vector alone, JAK2, or kinase-deficient JAK2

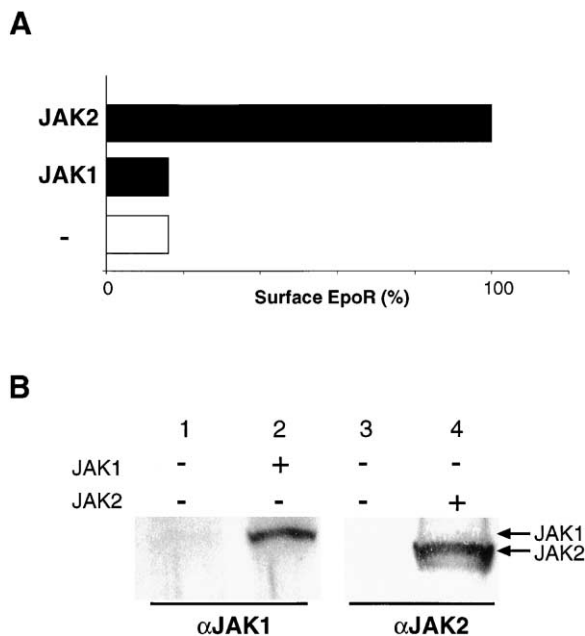


Figure 2. JAK2 but Not JAK1 Promotes Cell Surface Expression of HA-EpoR

(A) Surface expression of HA-EpoR was measured in γ 2A cells transiently cotransfected with vectors expressing JAK1 or JAK2. Median PE fluorescence was measured for each sample as described in Figure 1 and then normalized against the sample cotransfected with JAK2. (B) Expression of JAK1 and JAK2 was detected in the transiently transfected γ 2A cells. γ 2A transfected with vector alone (-), JAK1, or JAK2 were lysed in PBS containing 1% NP40 and protease inhibitors. Proteins from these lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-JAK1 (lanes 1 and 2) or anti-JAK2 (lanes 3 and 4) antibodies.

were selected by FACS sorting for GFP and CD4 and examined. Endo H sensitive species of the HA-EpoR were identified in cell lines with or without coexpression of JAK2, whereas the Endo H resistant form was only identified in cell lines coexpressing either JAK2 or kinase-deficient JAK2 (JAK2 [K882D], lanes 8 and 11).

These Endo H resistant species were sensitive to treatment with PNGase F and neuraminidase (lanes 9 and 12) and were absent in nontransfected cells (lanes 1–3), indicating that they are glycosylated EpoRs. These results suggested that the existence of the Golgi-processed (Endo H resistant) form of the HA-EpoR depends on expression of either JAK2 or kinase-deficient JAK2. The overall expression level of HA-EpoR in these cells did not increase in the presence of JAK2 (Figures 4B and 5B). Consequently, the JAK2-dependent increase in HA-EpoR surface expression was due to an increase in the amount of the mature HA-EpoRs.

To test if the cytoplasmic domain of the EpoR contains an ER retention signal that is masked by its interaction with JAK2, two cytoplasmic truncation mutants of the EpoR were generated. As shown in Figure 4C, HA-EpoR (1–304), which includes Box 1 and the membrane proximal region, was not expressed on the cell surface unless JAK2 was coexpressed. In contrast, HA-EpoR (1–249), which includes only two amino acids in the cytoplasmic domain, was expressed on the surface with or without the expression of JAK2. These experiments indicated that if there were a retention signal in the cytoplasmic domain of the EpoR that becomes unmasked by JAK2, it must be located between residues 249 and 304. As we show later in Figure 7, almost all of the amino acids in this region were replaced in groups with alanines, and no EpoR mutant in this segment was expressed on the surface without the coexpression of JAK2. These results, together with the fact that no di-lysine motif was identified in the cytoplasmic domain of the EpoR (our unpublished observation), strongly indicated that there is no ER retention signal in the cytoplasmic domain of the EpoR that is responsible for its low surface expression in the absence of JAK2 and that becomes unmasked upon JAK2 binding.

By examining the glycosylation state of EpoRs associated with JAK2, we could demonstrate that JAK2 binds directly to the ER form of the EpoR. We transiently expressed HA-EpoR in 293T cells which express endogenous JAK2 and used antibodies to JAK2 in a coimmunoprecipitation assay to isolate the EpoR/JAK2 complex.

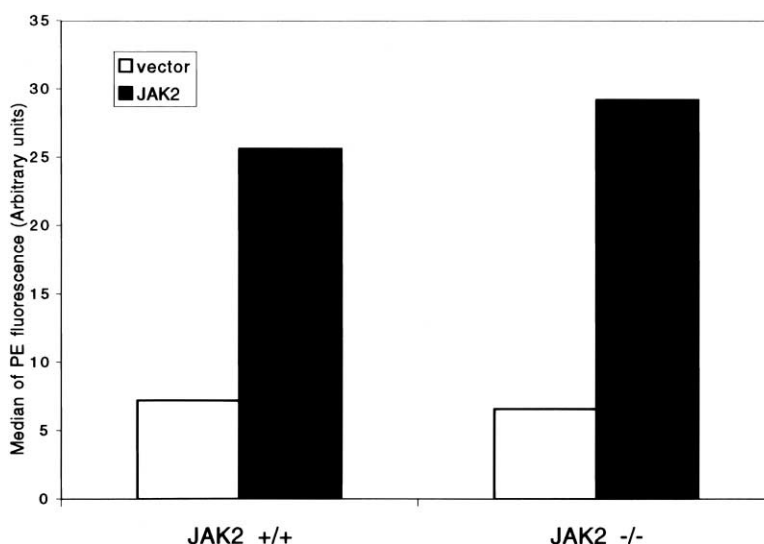


Figure 3. JAK2 Promotes Surface Expression of HA-EpoR in JAK2-Deficient Embryonic Fibroblasts

Surface expression of HA-EpoR was measured in embryonic fibroblasts derived from JAK2^{+/+} or JAK2^{-/-} embryos and infected with the retrovirus vector encoding HA-EpoR-IRES-GFP with or without coexpression of JAK2 in a pMX-JAK2-IRES-CD4 vector. GFP and APC double positive cells, indicating the coexpression of HA-EpoR and JAK2 (measured by an APC-conjugated anti-CD4 antibody), were gated for analysis of PE fluorescence as described in Figure 1.

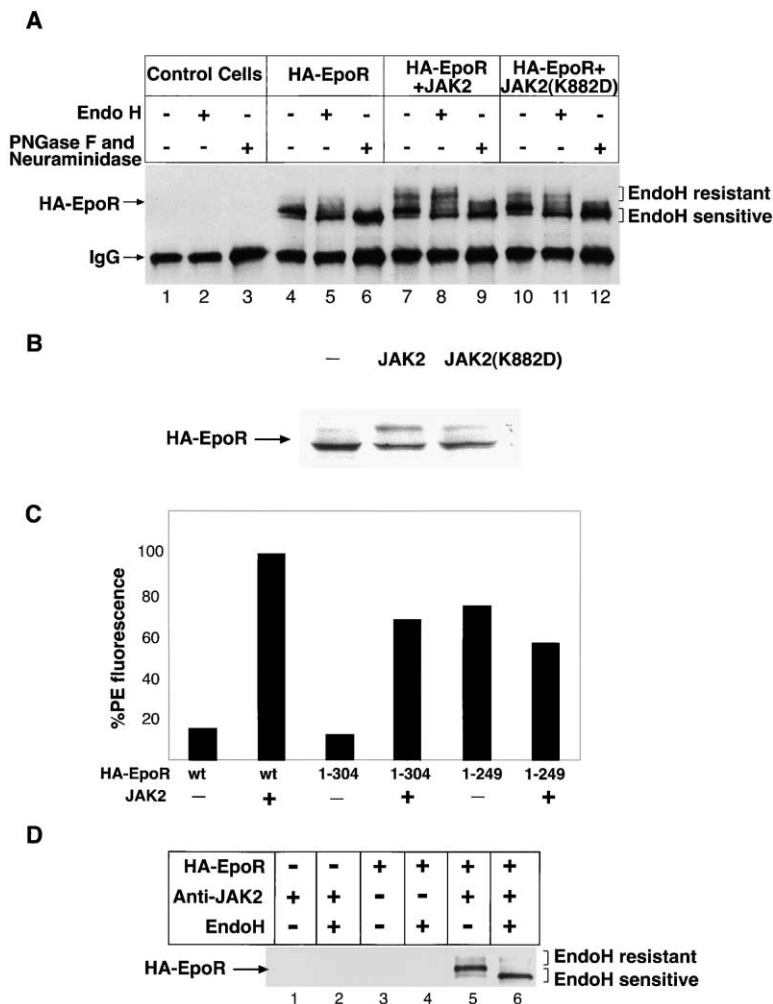


Figure 4. JAK2 or Kinase-Deficient JAK2 Enhances Golgi-Processed EpoR

(A) The glycosylation state of the HA-EpoR was examined in γ 2A cells stably expressing HA-EpoR together with vector alone, JAK2, or JAK2 (K882D). HA-EpoR was first immunoprecipitated by anti-HA antibodies, treated either with Endo H or with PNGase F and neuraminidase, run on SDS-PAGE, then Western blotted with anti-HA antibodies. EndoH resistant HA-EpoR and EndoH sensitive HA-EpoR are indicated.

(B) Total protein expression level of HA-EpoR in γ 2A cells used in (A). Lysates of γ 2A cells stably expressing HA-EpoR together with vector alone, JAK2, or JAK2 (K882D) were Western blotted using anti-HA antibodies.

(C) Surface expression of truncated EpoRs. Surface expression of HA-EpoR(1-304) and HA-EpoR(1-249) were measured in the presence and absence of JAK2 in transiently transfected γ 2A cells. The signal from cells expressing JAK2 and the full-length EpoR was set at 100%.

(D) Endogenous JAK2 interacts with Endo H sensitive and Endo H resistant HA-EpoR. Lysates from 293T cells transiently expressing HA-EpoR were immunoprecipitated by anti-JAK2 antibodies, treated with Endo H, run on SDS-PAGE, then Western blotted with anti-HA antibodies (lanes 5 and 6). Lysates without the expression of HA-EpoR (lanes 1 and 2) and lysates immunoprecipitated with normal rabbit serum (lanes 3 and 4) were treated similarly and served as controls.

As shown in Figure 4D, the majority of the HA-EpoRs associated with JAK2 was Endo H sensitive (ER form), while a minor portion was Endo H resistant. These results are consistent with the notion that the initial interaction of the EpoR and JAK2 takes place when the EpoR is in the ER, followed by the movement of the EpoR/JAK2 complex to the plasma membrane.

The N-Terminal JH3 to JH7 Domains of JAK2 Are Sufficient to Promote Surface Expression of HA-EpoR

To define the domain(s) of JAK2 that is responsible for the enhancement of surface HA-EpoR expression, various domains of JAK2 were expressed as GST-fusion proteins and tested for their ability to promote HA-EpoR surface expression in γ 2A cells. As shown in Figure 5A, a construct containing domains JH3 to JH7 was sufficient for normal JAK2-dependent induction of HA-EpoR surface expression, while neither the kinase domain nor the pseudokinase domain had any effect. Furthermore, the increase in cell surface HA-EpoR expression was not due to an increase in the overall protein level of the HA-EpoR (Figure 5B). Interestingly, this result is different from those showing that the N-terminal domain of TYK2 is necessary for maintaining the steady-state protein

level (and presumably its surface expression) of the IFNAR1 subunit of the interferon α/β receptor (Richter et al., 1998).

A direct interaction between domains JH3 to JH7 of JAK2 and the EpoR was further demonstrated in an in vitro coprecipitation assay using JAK2 or JAK2 (JH3-JH7) translated in a rabbit reticulocyte lysate system. The cytoplasmic domain of the EpoR was bacterially expressed as a GST-fusion protein and incubated with either untagged JAK2 or untagged JAK2 (JH3-JH7). As shown in Figure 5C, GST-EpoR bound directly to both JAK2 and JAK2 (JH3-JH7) (lanes 2 and 5), while no interaction was detected with GST alone (lanes 1 and 4).

Further deletion analysis was then performed to determine the minimal segment of JAK2 necessary for promoting HA-EpoR expression on the plasma membrane. As shown in Figure 5D, loss of either JH7 (i.e., JAK2 [JH3-JH6]) or JH3 (i.e., JAK2 [JH4-JH7]) abolished the ability to enhance surface expression of HA-EpoR, indicating that both the JH3 and the JH7 segments are necessary for this essential JAK2 function. To delineate if these two domains participate directly in binding the EpoR or if they are required to maintain the proper structure for other JH domains to interact with the EpoR, chimeric proteins between JAK1 and JAK2 were engi-

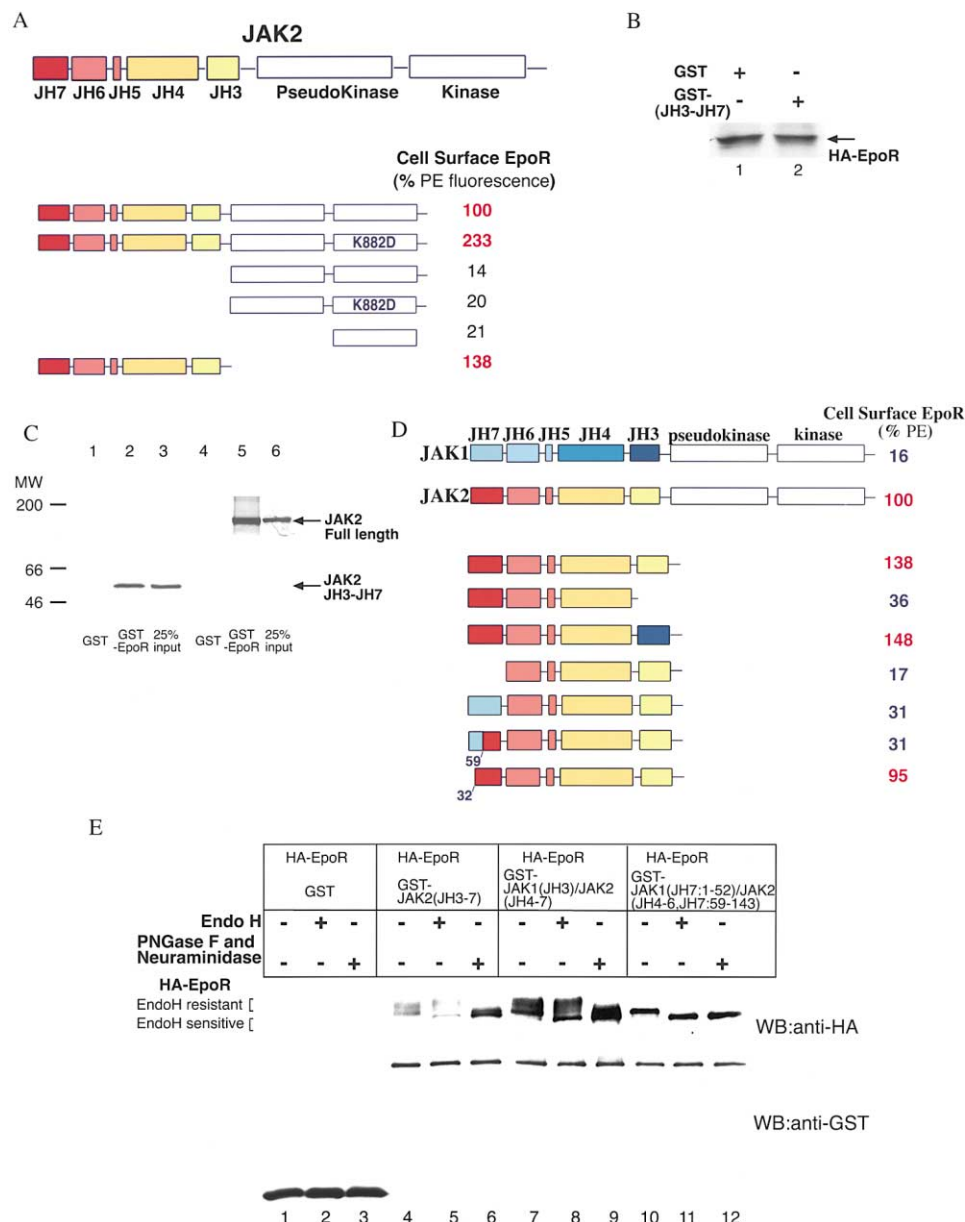


Figure 5. The Intact N-Terminal JH3 to JH7 Domains of JAK2 Bind EpoR Directly and Enhance HA-EpoR Surface Expression

(A) The N-terminal JH3 to JH7 domains of JAK2 are necessary and sufficient for EpoR surface expression. Surface expression of HA-EpoR coexpressed with full-length or various domains of JAK2 fused with GST was analyzed as described in Figure 1. The ability of these constructs to enhance HA-EpoR on the surface was quantified by measuring median PE fluorescence and normalized against that obtained with full-length JAK2 (see legend to Figure 1).

(B) HA-EpoR is expressed to a similar level when cotransfected with GST-JAK2 (JH3-JH7) or GST alone. Cell lysates were prepared from γ 2A cells cotransfected with HA-EpoR and GST-JAK2 (JH3-JH7) (lane 2), or HA-EpoR and GST (lane 1). Proteins from these lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-HA antibodies.

(C) JAK2 and JAK2 (JH3-JH7) bind EpoR directly. The cytoplasmic domain of EpoR was expressed as a GST-fusion protein and purified on glutathione sepharose. In vitro translated JAK2 or JAK2 (JH3-JH7) radiolabeled with ^{35} S-methionine was incubated with GST-EpoR (lanes 2 and 5) or GST alone (lanes 1 and 4). Proteins associated with the glutathione sepharose were eluted by boiling in SDS sample buffer and loaded on SDS-PAGE followed by autoradiography. 25% of the input JAK2 or JAK2 (JH3-JH7) was loaded in parallel for comparison (lanes 3 and 6). Molecular weight markers (in kilodaltons) are shown on the left.

(D) Residues 32 to 58 in the JH7 domain of JAK2 are necessary to promote surface expression of the EpoR. Surface expression of HA-EpoR coexpressed with full-length or various domains of JAK2 fused with GST was analyzed as described in (A). The ability of these constructs to enhance HA-EpoR on the surface was quantified by measuring median PE fluorescence and normalized against that obtained with full-length JAK2.

(E) The N-terminal JH3 to JH7 domains of JAK2 interact with Golgi-processed EpoR. The HA-EpoR was pulled down by GST fusion proteins of JAK2(JH3-JH7), JAK1(JH3)/JAK2(JH4-JH7), and JAK1(JH7:1-52)/JAK2(JH7:59-143, JH3-JH6) from lysates of cotransfected 293T cells. Proteins associated with the glutathione sepharose were eluted and subjected to glycosidase digestions. The digested products were then separated by SDS-PAGE followed by Western blotting with antibodies to the HA tag. The expression levels of these GST fusion proteins were shown in the bottom panel by reprobing the same blot with antibodies to GST.

needed. Since JAK1 could not promote surface expression of the HA-EpoR, the JH3 or the JH7 segments of JAK2 (JH3-JH7) were exchanged with the corresponding domains of JAK1 to generate fusion proteins of the N-terminal segments of JAK1 and JAK2. A chimeric protein where the JH3 domain of JAK1 was fused to the JH4 to JH7 domains of JAK2, JAK1 (JH3)/JAK2 (JH4-7), promotes the same maximal surface expression of HA-EpoR as does JAK2 (JH3-JH7), indicating that the JH3 domains of JAK1 or JAK2 are interchangeable. On the other hand, a chimeric protein where the JAK1 JH7 domain was fused to the JAK2 JH3 to JH6 domains, JAK1 (JH7)/JAK2 (JH3-6), lost the ability to promote surface expression of the HA-EpoR, indicating that specific residues in the JAK2 JH7 domain are required for the essential JAK2 function of allowing the EpoR to reach the plasma membrane. The JH3 domain may be important to maintain a specific conformation to position JH7 for proper interaction with the EpoR.

In addition, the chimera JAK1 (JH7:1-52)/JAK2 (JH7:59-143, JH3-6) lost the ability to promote surface expression of the HA-EpoR. Since the JH7 domain of JAK2 has 143 amino acids and since residues 1 to 58 could not be replaced with the corresponding residues of JAK1, we further dissected the JH7 domain of JAK2 by testing a deletion of JAK2 that lacks the first 32 residues. This deletion mutant, JAK2 (JH7: 32-143, JH3-6), retained full activity. Taken together, our results narrowed down the segment of the JAK2 JH7 domain that is critical to promote HA-EpoR on the surface to residues 32 to 58.

To further test our hypothesis that specific interactions between the EpoR and JAK2 within the ER are necessary for EpoR surface expression, we examined the glycosylation state of the HA-EpoRs associated with the JAK2 or JAK1/JAK2 chimeric N-terminal domains. We coexpressed HA-EpoR with GST-fused JAK2 (JH3-7), JAK1 (JH3)/JAK2 (JH4-7), or JAK1 (JH7:1-52)/JAK2 (JH7:59-143, JH3-6) in 293T cells. HA-EpoRs associated with these fusion proteins were isolated by incubation with glutathione sepharose and subjected to glycosidase digestions. As shown in Figure 5E, the Endo H sensitive form of the HA-EpoR was bound to all GST-JAK fusions. In contrast, Endo H resistant (Golgi-processed) HA-EpoRs were only identified in association with JAK2 (JH3-7) and JAK1 (JH3)/JAK2 (JH4-7) chimeras (lanes 5 and 8), both of which facilitate the surface expression of the EpoR. GST alone did not interact with HA-EpoR (lanes 1-3). These results confirmed our hypothesis that specific interactions between the EpoR and JAK2 in the ER are necessary for EpoR surface expression. Residues 32-58 of JAK2 may be responsible for direct binding to the EpoR, or it may be involved in generating the specific conformation of the EpoR/JAK2 complex that is necessary for its expression on the plasma membrane.

Domains JH3 to JH7 of JAK2 Induce Expression of an EpoR-Prolactin Receptor Chimera on the Plasma Membrane

Like the EpoR, activation of the prolactin receptor (PrIR) leads to the phosphorylation of JAK2 and STAT5 (Dusanter-Fourt et al., 1994; Pallard et al., 1995; Wakao et al., 1994). We constructed a chimeric receptor (HA-EP)

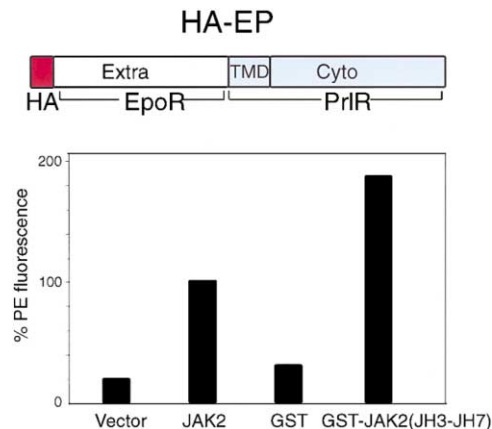


Figure 6. The N-Terminal Domain of JAK2 Promotes Surface Expression of a Chimeric EpoR-PrIR

Surface expression of HA-EP (a chimeric construct with the extracellular domain of EpoR and the transmembrane and cytoplasmic domain of PrIR) was determined by the assay used in Figure 1. γ 2A cells were transiently transfected with the bicistronic vector pMX-HA-EP-IRES-GFP with or without coexpression of JAK2, GST, or a fusion protein of GST with JAK2 (JH3-JH7). Percent PE fluorescence (a measure of cell surface HA-EP) for each sample was normalized against that from cells transfected with wild-type JAK2.

containing the extracellular domain of the EpoR and the transmembrane and cytoplasmic domains of the PrIR. Consistent with our results with the EpoR, both full-length JAK2 and the N-terminal JH3-JH7 domains of JAK2 enhanced the surface expression of HA-EP (Figure 6). Since both the EpoR and the PrIR depend on JAK2 for high surface expression, this may be a general mechanism for cytokine receptors to acquire the necessary conformation for efficient cell surface expression.

Mutant EpoRs Define Specific Residues Important for Productive Binding and Activation of JAK2

To further investigate the interactions between JAK2 and the EpoR, we dissected the membrane proximal region of the EpoR. EpoR (1-304), containing the region between the transmembrane domain and Box 2, interacted functionally with JAK2 in the trafficking assay (Figure 4C). We thus replaced groups of amino acids in this region with alanine residues to generate a set of mutated EpoRs in the bicistronic expression vector pMX-EpoR-IRES-GFP. These mutants were analyzed as HA-tagged receptors for their ability to interact with JAK2 by appearing on the cell surface in transiently transfected γ 2A cells when cotransfected with JAK2 (see Figure 1). To examine the physiological consequences of these mutants, the mutant receptors were analyzed for their signaling ability *in vivo* in Ba/F3, an IL-3-dependent cell line (Liu et al., 2000; Palacios and Steinmetz, 1985). Populations of Ba/F3 cells that stably express specific mutant receptors above a predetermined level, selected by flow cytometry, were depleted of IL-3 and analyzed for their ability to grow in Epo (Constantinescu et al., 1999b). The ability of these mutant receptors to activate JAK2 was further assayed in these stably expressing Ba/F3 cells by tyrosine phosphorylation of JAK2 upon Epo stimulation.

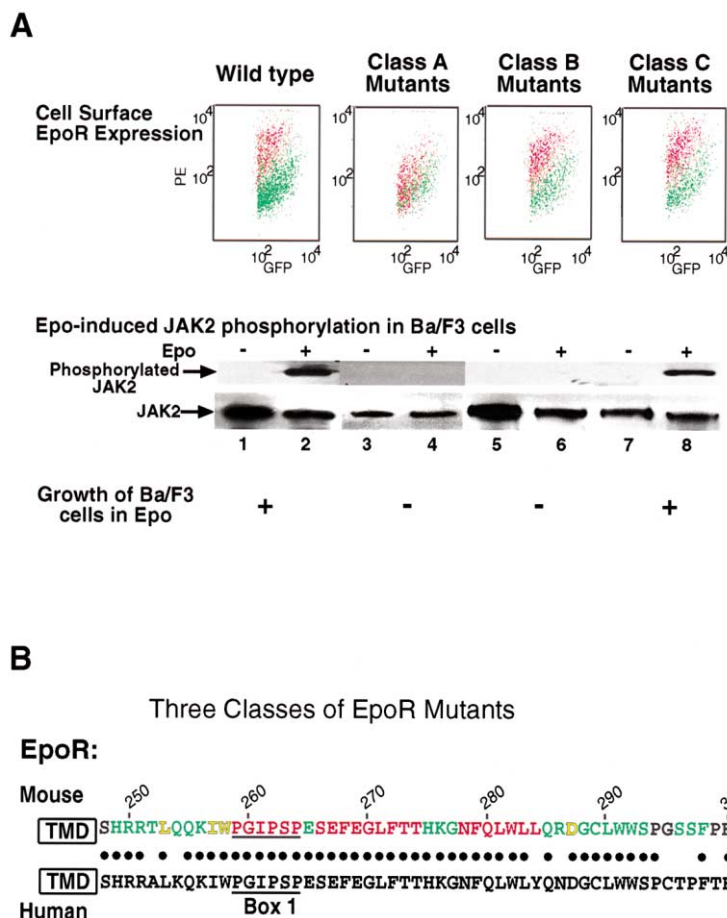


Figure 7. Three Classes of EpoR Mutants

(A) EpoR mutants were analyzed in three ways. The surface expression of wild-type or mutant HA-tagged receptors was analyzed in the trafficking assay as described in Figure 1 with (red dots) or without (green dots) coexpression of JAK2 (top panel). Their ability to activate JAK2 was determined in Ba/F3 cells stably expressing the corresponding wild-type or mutant receptor (middle panel) by measuring tyrosine phosphorylation of JAK2 upon Epo stimulation. To examine the ability of these Ba/F3 mutant cell lines to sustain Epo-dependent growth, these stably expressing cells were washed and depleted of IL-3 and incubated with 1 U/ml Epo for 4 days (bottom panel).

(B) Sequence alignment of the mouse EpoR and the human EpoR. The three classes of mutants were highlighted. TMD indicates the transmembrane domain. The Class A mutants are colored red. These mutants include Box 1²⁵⁹⁻²⁶⁴→AAAAA, S²⁶⁶E²⁶⁷F²⁶⁸→AAA, E²⁶⁹G²⁷⁰→L²⁷¹→AAA, F²⁷²T²⁷³T²⁷⁴→AAA, N²⁷⁸F²⁷⁹Q²⁸⁰→AAA, and L²⁸¹W²⁸²L²⁸³L²⁸⁴→AAAA. The class B mutants are colored yellow. These mutants include L²⁵³A, I²⁵⁷A, W²⁵⁸A, and D²⁸⁷A. The class C mutants are colored green. They include H²⁴⁹R²⁵⁰R²⁵¹→AAA, E²⁶⁵A, H²⁷⁵K²⁷⁶G²⁷⁷→AAA, G²⁸⁸C²⁸⁹I²⁹⁰→AAA, W²⁹¹W²⁹²S²⁹³→AAA, and S²⁹⁶S²⁹⁷F²⁹⁸→AAA.

As summarized in Figure 7A, these mutants can be categorized into three classes. Class A mutants interacted poorly, if at all, with JAK2 in the trafficking assay in that they did not appear on the surface whether or not JAK2 was coexpressed. As expected, they did not activate JAK2. Class A mutant receptors span a continuous segment from amino acid 259 to 284. They consist of Box 1 (residues 259–264) and the region immediately C-terminal to it. This segment may form the surface for interacting with residues 32–58 in the JH7 domain of JAK2. This is consistent with previous results that the Box 1 is necessary for association between JAKs and the PrIR, the growth hormone receptor, the EpoR, and the IL-9 receptor (Tanner et al., 1995; Zhu et al., 1997).

When the cytoplasmic domains of the Class A mutant receptors were expressed and purified as GST fusion proteins and incubated with in vitro translated JAK2 (JH3-JH7), all mutants tested retained their ability to bind this JAK2 N-terminal domain (data not shown). Therefore, although Class A mutant receptors physically bind JAK2, their binding does not lead to productive functional interactions as measured by EpoR appearance at the cell surface.

Class B mutants interacted normally with JAK2 in the trafficking assay in that they appeared on the cell surface with, but not without, JAK2. However, they did not activate JAK2 as measured by Epo-induced tyrosine phosphorylation of JAK2 or by the ability of these mutant

receptors to support proliferation of Ba/F3 cells. These mutants include T²⁵²L²⁵³Q²⁵⁴→AAA, I²⁵⁷A, W²⁵⁸A, and Q²⁸⁵R²⁸⁶D²⁸⁷→AAA. These amino acids do not fall into a linear stretch, unlike the class A mutant receptors. Mutant EpoRs that contain mutation of single residues were then generated to pinpoint the exact amino acids that are required for JAK2 activation. L²⁵³A, I²⁵⁷A, W²⁵⁸A, and D²⁸⁷A failed to activate JAK2 or sustain Epo-dependent growth, indicating that the identity of these amino acids is necessary for EpoR to activate the prebound JAK2. Since these mutants interacted with JAK2 and appeared on the surface but failed to activate it, they may be necessary to trigger a conformational switch either on the receptor or on JAK2 that is necessary for JAK2 activation. These mutants may also be defective in interacting with an unidentified molecule that is necessary for JAK2 activation.

Class C mutants interacted normally with JAK2 and activated it in a manner similar to the wild-type EpoR. The ability to activate JAK2 correlated perfectly with their ability to sustain Epo-dependent growth, reinforcing the apparent essential nature of JAK2 in Epo signaling.

Discussion

Our key finding is that JAK2 binds to the endoplasmic reticulum (ER) immature form of the EpoR, enhances its folding, and facilitates EpoR surface expression. Only

the N-terminal JH3 to JH7 domains of JAK2, not the kinase or pseudokinase domains, are required for this function. JAK2 also stimulates surface expression of an EpoR-prolactin receptor chimera which contains the cytoplasmic domain of the prolactin receptor, attesting to the generality of our findings. Further, JAK1 has no effect on surface expression of the EpoR and we have narrowed the essential JAK2 segment to a piece of the JH7 domain encompassing amino acid residues 32-58. On the EpoR, we identified a continuous block of amino acid residues (residues 259 to 284) that are likely to be the target of binding to the JH7 domain of JAK2. Furthermore, we defined two discrete modes of functional interaction between the EpoR and JAK2: one interaction that takes place before ligand binding which promotes cell surface receptor expression and the other interaction that is involved in switching on JAK2 activity. Thus, in addition to its kinase activity required for cytokine receptor signaling, JAK is also an essential subunit required for surface expression of cytokine receptors.

JAK2 Enhances the Normally Inefficient Folding of the EpoR in the Endoplasmic Reticulum

In both transfected and normal hematopoietic cells, the majority of the newly made EpoRs are retained in the ER, destined for degradation. Only a small fraction exits the ER and is expressed on the plasma membrane (D'Andrea and Zon, 1990; Neumann et al., 1993, 1996; Supino-Rosin et al., 1999; Yoshimura et al., 1990). Here we showed that JAK2 binds to EpoRs localized in the ER, and that both full-length JAK2 and a fragment containing just the N-terminal JH3 to JH7 domains of JAK2 (but not JAK1) enhance EpoR Golgi-processing and surface expression. Because the overall expression level of the EpoR is similar with or without coexpression of JAK2 (JH3-JH7), it is unlikely that the increase of EpoR on the surface is due to a decrease in the rate or extent of internalization or degradation of the EpoR in the presence of JAK2. Indeed, coexpression of JAK2 with the EpoR increases the fraction of EpoRs with Golgi-processed oligosaccharides, consistent with an effect on the extent of EpoR exit from the ER en route to the Golgi. Furthermore, JAK2 normally is a limiting factor in EpoR surface expression, since the amount of the EpoR on the cell surface increased when JAK2 was overexpressed even in cells endogenously expressing JAK2.

JAK2 also facilitates the surface expression of a mutant EpoR whose cytoplasmic domain was severely truncated (EpoR [1-304]). Extensive mutagenesis of residues corresponding to this severely truncated cytoplasmic domain did not generate a mutant EpoR whose surface expression is independent of JAK2. Therefore, it is unlikely that formation of an EpoR/JAK2 complex masks a hypothetical ER retention signal in the EpoR cytoplasmic domain. We hypothesize that JAK2 is involved in the proper folding of the EpoR cytosolic domain and that only an EpoR/JAK2 complex is allowed to transit to the Golgi or escape retention to the ER from the Golgi. In this sense JAK2 is an essential subunit of the EpoR.

No known ER export signal has been identified in JAK2. However, we cannot rule out the possibility that formation of the EpoR/JAK2 complex generates a novel

ER export signal or this complex binds to yet another factor that facilitates the surface expression of the EpoR/JAK2 complex. Recently, Lavoie et al. (2000) identified JAK2 as the tyrosine kinase that phosphorylates P97 and in turn regulates assembly of the transitional ER. Whether P97 is involved in the processing of the EpoR/JAK2 complex is unknown.

Formation of an EpoR/JAK2 complex in the ER is reminiscent of the assembly of multimeric protein complexes, where individual subunits or partial multimeric complexes are retained in the ER to await proper assembly of all the subunits. This mechanism serves as a quality control to prevent cell surface expression of partially assembled or misfolded complexes (Zerangue et al., 1999). Proper assembly of all of its subunits is required for the secretion of immunoglobulins (Reddy and Corley, 1998) and cell surface expression of major histocompatibility (MHC) proteins (Diedrich et al., 2001), T cell antigen receptors (Alarcon et al., 1988; Geisler, 1992; Sussman et al., 1988), ATP-sensitive potassium channels (Zerangue et al., 1999), nicotinic receptors (Blount and Merlie, 1990; Blount et al., 1990), and GABA_B receptors (Margeta-Mitrovic et al., 2000).

We consider that JAK2 is an essential subunit of the EpoR/JAK2 complex and that this complex should be viewed as one functional entity. The ability of JAK2 to promote EpoR surface expression does not require JAK2 kinase activity, consistent with the fact that formation of the EpoR/JAK2 complexes precedes Epo-induced JAK2 activation.

The JAK Kinase JH7 Domain Confers Specificity between JAK1 and JAK2

In JAK2, both its JH3 and JH7 domains are necessary to promote EpoR surface expression. A JAK2 chimera containing only the JH7 domain of JAK1 lost the ability to promote EpoR surface expression, whereas a chimera with only the JH3 domain of JAK1 retained the ability. Therefore, we hypothesize that the JH7 domain of JAK2 is the main site for specific interaction with the EpoR and that the JH3 domain is conformationally important for JH7 orientation.

A FERM domain was predicted in the region between JH4 to JH7 in all JAK kinases. A truncated JAK2 lacking the first 32 residues, JAK2 (JH7: 32-143, JH4-6), functioned like wild-type JAK2 in enhancing surface HA-EpoR expression. Since residue 32 corresponds to the first residue of the predicted FERM domain, this predicted FERM domain might in fact be crucial for this JAK2 function — enhancement of EpoR movement to the plasma membrane. Consistent with this, the JH3 domain, interchangeable between JAK1 and JAK2 for EpoR surface expression, is not included in the predicted FERM domain.

Within the JH7 domain, we found that a segment encompassing residues 32 to 58 is critical for promoting EpoR surface expression. Based on the crystallographic models of FERM domains from moesin, radixin, and protein 4.1R, the analogous region of this segment constitutes the first two β sheets in the ubiquitin-like subdomain and is suggested to be a potential protein interaction site (Hamada et al., 2000; Han et al., 2000; Pearson et al., 2000). Thus, the segment corresponding to residues 32 to 58

of JAK2 may be one of the primary sites of interaction with cytokine receptors. Our alanine scanning mutagenesis of the EpoR points to a continuous stretch of residues, located between Box 1 and residue 284, which are involved in functional binding to JAK2. We hypothesize that the 27 amino acids of the JAK2 JH7 domain may bind to this region of the EpoR.

The N-terminal domain of JAKs has been demonstrated to interact with other cytokine receptors. A mutation in the JH7 region of JAK3, discovered in a patient with severe combined immunodeficiency, disrupted JAK3 interaction with the common receptor γ chain (γ c) (Cacalano et al., 1999). Therefore, the N-terminal domains of JAKs may be the principal sites of interaction with all cytokine receptors. Different combinations of the JH regions may generate distinctive interaction surfaces to enforce specificity between JAKs and their corresponding receptors.

Two Modes of Interaction of JAK2 with the EpoR

The proper activation of JAK kinase activity upon Epo stimulation depends on an intricate array of interactions between the EpoR and JAK2. As an example, all of the Class A EpoR mutants tested still bound specifically to JAK2 in vitro when expressed as GST fusion proteins (data not shown and Witthuhn et al., 1993) even though they did not mature to the cell surface in the presence of JAK2. In our in vitro binding assays (unpublished data) several segments of JAK2 could bind tightly and specifically to at least two different segments of the EpoR; while we have not completed these studies, it is apparent that simple binding of JAK2 to a cytokine receptor is insufficient for the receptor to fold properly in the ER or to activate the JAK kinase activity.

A truncated EpoR (residues 1 to 304) containing the membrane proximal region interacted functionally with JAK2 and appeared on the membrane when coexpressed with JAK2, indicating that this membrane proximal region is sufficient for functional binding to JAK2 and cell surface expression. Class A mutants mapped to a continuous region between Box 1 and residue 284. These residues may be directly interacting with residues on JAK2, in particular residues 32 to 58 in the JH7 domain. Alternatively, they may be required for the EpoR to adopt a specific conformation important for functional JAK2 binding that results in EpoR surface expression.

The Class B mutants interacted functionally with JAK2 in the trafficking assay; however, they were unable to induce tyrosine phosphorylation of JAK2 upon Epo addition or sustain Epo-dependent growth of Ba/F3 cells. Therefore, the Class B mutants defined four amino acids in the EpoR cytosolic domain essential for JAK2 activation but not for productive JAK2 binding: L²⁵³, I²⁵⁷, W²⁵⁸, and D²⁸⁷. Residues L²⁵³, I²⁵⁷, and W²⁵⁸ constitute a highly conserved juxtamembrane hydrophobic motif among various cytokine receptors (Constantinescu et al., 2001). The orientation of this motif and not its distance from the membrane bilayer is critical for signaling (Constantinescu et al., 2001). Residue D²⁸⁷ lies C-terminal of the region required for functional binding between the EpoR and JAK2. Sequence similarity among different cytokine receptors in this region is limited. These four Class B residues may function as molecular switches through

which ligand-induced conformational changes in the receptor are coupled to intracellular activation and function of JAK2. They may not interact with the JH3 to JH7 region of JAK2 but rather with the pseudokinase or the kinase domains to switch the kinase to an "on" state. Alternatively, these residues may trigger a specific conformational change between the pseudokinase and the kinase domains to activate JAK2.

Our results thus define a novel function for JAK2 as an essential subunit of the EpoR and characterize the molecular mechanism used by the EpoR to activate JAK2. Molecular characterization of the communication between the EpoR and JAK2 will be crucial for understanding the mechanisms of cytokine receptor signal transduction. A detailed understanding of the precise molecular interactions between the EpoR and JAK2 awaits the crystallographic structure of the EpoR/JAK2 complex.

Experimental Procedures

cDNA Constructs

The wild-type and HA-tagged murine EpoR cDNA was subcloned in the pMX-IRES-GFP bicistronic retroviral vector upstream of the IRES as described previously (Constantinescu et al., 1999b; Liu et al., 2000). Mutant EpoR cDNAs were generated by overlap extension using PCR with synthetic oligonucleotides encoding the desired amino acid substitutions. All constructs were verified by sequencing. The wild-type and kinase-deficient JAK2 in the pMX-IRES-CD4 bicistronic retroviral vector were made by subcloning of the corresponding genes generated by PCR. Various domains of JAK2 (Figure 5) were generated by PCR and subcloned into the pEBG vector that expresses GST upstream of and in frame with the JAK2 domains.

Generation of Cell Lines

To obtain γ 2A cell lines stably expressing HA-EpoR and JAK2, or HA-EpoR and JAK2 (K882D), γ 2A cells stably expressing the ecotropic murine leukemia virus receptor (Albritton et al., 1989) were first generated. After transfection of the pMX-ecotropic receptor-IRES-puromycin vector, clonal γ 2A cells expressing the ecotropic receptor were selected by puromycin. High-titer ecotropic replication-defective retroviral supernatants were generated by transient transfection of the BOSC23 packaging cell line with bicistronic vectors encoding HA-EpoR in the pMX-IRES-GFP vector, or JAK2 or JAK2 (K882D) in the pMX-IRES-CD4 vector (Liu et al., 2000). Viral supernatants collected at 48 hr after transfection were used to infect γ 2A cells stably expressing the ecotropic receptor. All infections were performed in the presence of 4 μ g/ml polybrene (Sigma). Populations of γ 2A cells expressing above a predetermined level of both GFP and CD4 (top 25%) were isolated by FACS sorting. γ 2A cells were grown in DMEM medium supplemented with 10% fetal bovine serum and antibiotics.

Ba/F3 cell lines stably expressing wild-type or mutant EpoRs were obtained as described (Constantinescu et al., 2001). Populations of cells expressing GFP above a predetermined level were isolated by FACS sorting.

Isolation of Primary Mouse Embryonic Fibroblasts

E12.5 wild-type and JAK2^{-/-} embryos (Neubauer et al., 1998) were kindly provided by Dr. S. Ghaffari. After removal of the head, tail, and fetal liver for genotyping, each embryo was minced with a sterile razor blade and incubated with 2 ml trypsin solution (0.25% trypsin and 1 mM EDTA) at 37°C for 5 min. The embryos were then passed through an 18G needle and incubated with DMEM for 3 days. Primary embryonic fibroblasts were further cultured in DMEM medium with 10% fetal bovine serum and antibiotics.

Surface Expression of HA-EpoR

Surface expression of HA-EpoR was measured in γ 2A cells with or without coexpression of JAK2. pMX-HA-EpoR-IRES-GFP was transiently transfected alone into γ 2A cells or was cotransfected

with a vector expressing JAK2. Forty-eight hours after transfection, cells were incubated for 30 min in cold phosphate buffered saline (PBS) containing 2% bovine serum albumin and 4% donkey serum (Buffer A). Cells were then incubated for 1 hr with mouse monoclonal anti-HA antibodies (10 μ g/ml, diluted in Buffer A, Babco), washed three times with PBS containing 0.2% bovine serum albumin and 0.4% donkey serum (Buffer B), and incubated with R-phycoerythrin (PE)-conjugated donkey F(ab')₂ anti-mouse IgG secondary antibody (12.5 μ g/ml in Buffer A) at 4°C. Cells were washed three times with Buffer B and were analyzed by flow cytometry on a FACScan cytometer (Becton-Dickinson). GFP positive cells, indicating the expression of HA-EpoR, were gated for analysis of PE fluorescence. Median fluorescence intensities of PE were used for quantitation since this measure of central tendency is least sensitive to outliers. For each sample, 15,000 cells were collected. To measure surface expression of HA-EpoR in JAK2^{+/+} or JAK2^{-/-} embryonic fibroblasts, infected cells were incubated in cold phosphate buffered saline containing 4% BSA and 200 μ g/ml rabbit IgG (Buffer C) for 30 min. Subsequently, cells were incubated for 1 hr with mouse monoclonal anti-HA antibodies conjugated with biotin (10 μ g/ml, diluted in Buffer C, Babco), washed three times with cold PBS, and then incubated with streptavidin conjugated PE (1:200, diluted in Buffer C) and allophycocyanin (APC)-conjugated mouse monoclonal anti-CD4 antibodies (1:20, diluted in Buffer C). Cells were washed three times with PBS and were analyzed as described above. Cells expressing a predetermined amount of GFP and APC were gated for analysis of PE fluorescence.

Glycosidase Digestion of the EpoR4

γ 2A cells stably expressing HA-EpoR with vector alone, with JAK2, or with JAK2 (K882D) were lysed in lysis buffer (150 mM NaCl, 1% NP40, and 50 mM Tris [pH 7.4]) with protease inhibitors at 4°C. Supernatants from these lysates were subjected to immunoprecipitation by monoclonal antibodies to the HA tag and binding to protein-A and protein-G sepharose. Proteins associated with the resin were eluted with 0.5% SDS and 1% β -mercaptoethanol followed by digestions with either Endo H (New England Biolabs, 500 U) alone, or with PNGase F (New England Biolabs, 500 U) and neuraminidase (New England Biolabs, 50 U) at 37°C for 16 hr. For digestions with PNGase F and neuraminidase, NP-40 was added to the final concentration of 1%. The digested products were then separated on SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted with the same HA tag antibodies.

Assay for Epo-Dependent Proliferation

Ba/F3 cells expressing wild-type or mutant EpoRs and growing in IL-3 were washed extensively in RPMI medium with 1% BSA and then placed in RPMI medium containing Epo (1 U/ml). Cell numbers were counted using a Coulter Counter after 3 to 6 days.

Immunoprecipitation and Immunoblotting

To detect JAK1 and JAK2 expression in transfected γ 2A cells, γ 2A transfected with vector alone, JAK1, or JAK2 were lysed in PBS containing 1% NP40 and protease inhibitors. Proteins from these lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-JAK1 or anti-JAK2 (both from Upstate Biotechnology, 1:1000) antibodies. Bound antibodies were detected by incubation with horseradish peroxidase-coupled secondary antibodies (1:2000, Amersham) and the enhanced chemiluminescence system.

To coimmunoprecipitate HA-EpoR and endogenous JAK2, 293T cells transiently expressing HA-EpoR were lysed in 1% NP40 lysis buffer with protease inhibitors. The lysates were incubated with antibodies against JAK2 (Upstate Biotechnology) and immune complexes were recovered by binding to protein-A sepharose beads. Proteins associated with protein-A sepharose were eluted with 0.5% SDS and 1% β -mercaptoethanol and subjected to Endo H digestion. The digested products were then separated on SDS-PAGE and Western blotted with antibodies to the HA tag. Ba/F3 cells stably expressing wild-type or mutant EpoRs were lysed in 1% NP40 lysis buffer with sodium vanadate and protease inhibitors. These lysates were then immunoprecipitated with antibodies against JAK2 (Upstate Biotechnology) and Western blotted with antibodies specific

for phospho-tyrosine (4G10, Upstate Biotechnology) or JAK2 as described (Constantinescu et al., 2001).

In Vitro Binding of GST-EpoR and JAK2

The cytoplasmic domain of EpoR was expressed as a GST-fusion protein and purified on glutathione sepharose (Frangioni and Neel, 1993). In vitro translated JAK2 or JAK2 (JH3-JH7) radiolabeled with 35S-methionine (TnT coupled reticulocyte lysate systems, Promega) was incubated with GST-EpoR or GST alone in phosphate buffered saline with 1% NP40 for 2 hr and washed three times with the same buffer. Proteins associated with the glutathione sepharose were eluted by boiling in SDS sample buffer and loaded on SDS-PAGE followed by autoradiography.

GST Pull-Down of HA-EpoR and JAK2 Domains

293T cells transiently cotransfected with HA-EpoR and GST-JAK2 (JH3-7), GST-JAK1(JH3)/JAK2(JH4-7), or GST-JAK1(JH7:1-52)/JAK2(JH7:559-143, JH3-6) were lysed in NP40 lysis buffer with protease inhibitors. Supernatants from these lysates were incubated with glutathione Sepharose 4B (Amersham Pharmacia Biotech.) at 4°C for 16 hr, and washed three times with lysis buffer. Proteins associated with the resin were eluted with 0.5% SDS and 1% β -mercaptoethanol and then subjected to glycosidase digestions as described. The digested products were then separated on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antibodies to the HA tag.

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