Erythropoietin Receptors Associate with a Ubiquitin Ligase, \( p33^{\text{RUL}} \), and Require Its Activity for Erythropoietin-induced Proliferation*

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The proliferation and survival of hematopoietic cells is strictly regulated by cytokine growth factors that act through receptors of the Type I cytokine receptor family, including erythropoietin (Epo) and its receptor, EpoR. Mitogenic signaling by these receptors depends on activation of Jak tyrosine kinases. However, other required components of this pathway have not been fully identified. In a screen for proteins that interact with EpoR and Jak2, we identified a novel member of the U-box family of ubiquitin ligases. This receptor-associated ubiquitin ligase, RUL, co-precipitated with EpoR from mammalian cells and mediated ubiquitination of EpoR. Also, endogenously expressed RUL was rapidly and transiently phosphorylated on serine after cytokine treatment of factor-dependent hematopoietic cells. Expression of ubiquitin ligase-deficient mutants of RUL inhibited Epo-induced expression of c-myc and bcl-2, two immediate-early genes normally associated with Epo-induced cell growth. Consistent with that finding, expression of mutant RUL also inhibited Epo-dependent proliferation and survival of factor-dependent cells. Together, these observations suggest that RUL is a required component of mitogenic signaling by EpoR. We also show that RUL is phosphorylated in response to growth factors that act through non-cytokine receptors, suggesting that RUL may function as a common regulator of mitogenesis.

EpoR, is largely restricted to supporting the growth of developing erythroid cells, whereas IL-3 promotes the growth of a broad range of immature myeloid cell lineages expressing its receptor. Cells of these lineages exhibit an absolute dependence on their corresponding growth factors and undergo rapid G1 phase cell cycle arrest and apoptotic death in the absence of that growth factor. Thus, these cytokine receptors regulate signaling pathways that directly promote cell cycle progression and inhibit apoptosis.

Type I cytokine receptors have universally been shown to mediate their activities through association with, and activation of, Jak family tyrosine kinases (1, 2). EpoR activates Jak2 through an interaction with its cytoplasmic domain proximal to the plasma membrane (3, 4). All Epo-dependent responses, including proliferation, are abrogated by disrupting Jak2 activation (4, 5). EpoR can also activate other well characterized signaling intermediates, including components of the Ras pathway, Stat transcription factors, and PI 3-kinase (1). In several instances, functional outcomes have been assigned to activation of these signaling pathways. For example, Epo-induced activation of Stat5 has been reported to enhance expression of the anti-apoptotic gene, bcl-X\(_L\), and thereby enhance survival of erythroid cells (6, 7). Activation of PI 3-kinase is required to override checkpoints in cell cycle progression, and, as such, it promotes proliferation of cells suffering various forms of stress (8, 9). Despite these identified functions, however, EpoR mutants lacking domains required for Stat5, Ras, and PI 3-kinase activation continue to promote survival and proliferation of factor-dependent hematopoietic cells (10, 11). Similar correlations have been derived from analysis of other cytokine receptors, including the receptor for IL-3 (12–14). Thus, Jak activation represents the only identified cytokine receptor-induced activity that is absolutely required to promote survival and proliferation.

Although the signaling intermediates downstream of Jak2 in Epo-induced growth and survival signals remain to be identified, several immediate-early gene targets have been linked to this signaling pathway. For instance, the membrane-proximal cytoplasmic domain of EpoR required for Jak2 activation and cell growth also mediates induced transcription of the immediate-early genes, c-myc and pim-1 (10). This is consistent with the previous observation that forced expression of both c-myc and pim-1 cooperates to render cells factor-independent for growth (15). Thus, transcriptional activation of c-myc and pim-1 are relevant targets of a growth signal mediated by EpoR. In keeping with its ability to inhibit p53-dependent apoptosis, Epo induces expression of anti-apoptotic genes, bcl-2 and bcl-\(X_L\), through the membrane-proximal domain of EpoR (16, 17). Thus, bcl-2 and bcl-\(X_L\), are likely transcriptional tar-

tol-3 kinase; FBS, fetal bovine serum; wt, wild-type; eNOS, endothelial nitric-oxide synthase; siRNA, small interfering RNA.

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†The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; EGFr, epidermal growth factor; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; G-CSF, granulocyte-colony stimulating factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HA, hemagglutinin epitope; HGF, hepatocyte growth factor; IL, interleukin; RUL, receptor-associated ubiquitin ligase; PI 3-kinase, phosphatidylinositol-3 kinase; FBS, fetal bovine serum; wt, wild-type; eNOS, endothelial nitric-oxide synthase; siRNA, small interfering RNA.

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gets of the survival signal initiated by a minimally active EpoR.

Signaling pathways activated by Type I cytokine receptors are subject to negative regulation by several mechanisms, including inhibition of Jak activity by phosphorylases, and inhibited activation of Stats by PIAS and SOCS proteins (18). Cytokine signaling may also be influenced by protein turnover mediated by proteasome-dependent degradation of receptors, Jak, and Stats (19–22). Targeted degradation of these proteins can, in part, be mediated through recruitment of ubiquitin conjugating complexes associated with the SOCS-box of SOCS proteins (23).

Ubiquitination of cellular proteins is mediated by a multi-enzyme system (24, 25). The first step is the formation of a thiol ester bond between the carboxyl terminus of ubiquitin and a ubiquitin-activating enzyme (E1). This is an ATP-dependent reaction resulting in release of AMP and PPi. The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). Ubiquitin ligase (E3) then mediates transfer of the activated ubiquitin from the E2 to lysine residues on the target protein. In this process, the ubiquitin ligase is principally responsible for selecting the protein target for ubiquitination. In mammalian cells, the identified components of ubiquitination cascades include a single E1, several E2s, and a large and growing number of E3s. Enzymes contributing ubiquitin ligase activity now comprise several protein families defined by retention of conserved domains, including HECT (homologous to EG-AP carboxyl terminal) domains, RING fingers, and the recently identified U-box (26).

The conjugation of ubiquitin to cellular proteins regulates a broad range of cellular functions (25, 27). Among the best characterized of these is the proteasome-dependent degradation of polyubiquitinated proteins. This result is associated with the formation of polyubiquitin chains linked through lysine 48 of the ubiquitin subunits. For example, EGF receptor internalization and degradation is regulated by polyubiquitination mediated by the RING finger protein, c-Cbl (28, 29). Alternatively, polyubiquitin chains linked through lysine 63 of the ubiquitin subunits have been associated with modification of protein function rather than degradation. For example, NF-κB activation by tumor necrosis factor-α receptors is mediated through the RING finger protein, Traf6. This is initiated through a cascade in which Traf6 is polyubiquitinated with chains linked through lysine 63 (30). Polyubiquitinated Traf6 then mediates activation of the serine kinase, Taks1, which then phosphorylates and activates the IκB kinase (31). Phosphorylated IκB is then targeted for lysine 48-linked polyubiquitination and subsequent proteolytic degradation, allowing the activation of the NF-κB transcription factor. Thus, ubiquitination of receptors and signaling proteins can exert either positive or negative influence on the activities of receptor-activated signaling pathways.

In the present study, we identified a novel receptor-associated ubiquitin ligase, p33RUL. We show that RUL interacts with EpoR, mediates ubiquitination of EpoR, and is inducibly phosphorylated on serine in response to Epo treatment. Expression of RUL mutants that lack ubiquitin ligase activity specifically inhibits Epo-induced expression of c-myec and bel-2 and inhibits Epo-dependent proliferation and survival of factor-dependent hematopoietic cells. We also show that RUL is phosphorylated in response to growth factors acting through non-cytokine receptors. Together, these observations suggest that RUL is a required component of Epo-dependent signaling and may function as a common regulator of mitogenic signaling pathways.

**Experimental Procedures**

Cloning of p33RUL—A bait construct for a yeast-3-hybrid screen was prepared consisting of the cytoplasmic domain of wild-type EpoR (32) containing the DNA binding domain of Gal4 in the pgD expression vector (Clontech). A wild-type Jak2 cDNA (33) was cloned into the second expression cassette of this vector, which is under control of the PMet promoter that is repressed in the presence of methionine. Yeast cells (strain Y190) harboring the EpoR-Jak2 bait plasmid were used to screen 1.2 × 10^9 clones from a MATCHMAKER human bone marrow cDNA library (Clontech) for growth on media lacking leucine, tryptophan, histidine, and methionine. Prey plasmids from positive colonies were isolated and screened for growth on deficient media when transformed into fresh yeast with the EpoR-Jak2 bait plasmid but without the bait plasmid. To assess the effect of Jak2 expression on interactions, positive colonies were grown on media lacking leucine and tryptophan with or without methionine. Colonies were transferred to paper filters, permeabilized by freezing, and assayed for expression of the β-galactosidase reporter gene product by formation of blue color when incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). A positive clone (p33RUL) identified through this procedure was sequenced and found to contain a complete open reading frame identical to human NOSIP (GenBank™ accession number 14424549).

**cDNA Expression Constructs—**Wild-type and mutant cDNA constructs were prepared by polymerase chain reaction, as previously described (10). EpoR-HA was prepared by inserting an HA tag within the carboxyl-terminal end of the EpoR cDNA (32) open reading frame in the expression vector, pXM. Mutant forms of the RUL cDNA were prepared that converted codon 49 from serine to alanine (RUL-S49A), or codon 229 from proline to serine (RUL-P229S) from pXM to pRUL-P229S plasmids. A C-terminal truncation of RUL (RUL-NT) was prepared containing a translation start site at codon 122 of the RUL open reading frame. Wild-type and mutant RUL cDNAs were cloned in-frame with the GST tag in the plasmid pACGHLT (BD Pharmingen) for preparation of baculovirus expression vectors, as previously described (12). For expression in mammalian cells, RUL cDNAs were cloned into pCMX or the ponasterone-inducible expression vector, pIND (Invitrogen). An epitope tag was incorporated into RUL-pIND constructs to allow expression of proteins to be distinguished from endogenous RUL. A murine ubiquitin cDNA was obtained by reverse transcription-PCR from myeloid D3 cells. This ubiquitin cDNA was prepared with a myc epitope tag at the amino-terminal end of the open reading frame and was cloned into the pXm expression vector.

**Cell Lines and Culture Conditions—**32D cells expressing EpoR (8) and NFS-60 cells (34) have been described, and both were maintained in RPMI 1640 media plus 10% fetal bovine serum (FBS) and recombinant murine IL-3 (70 pg/ml). Human TF-1 cells (ATCC) were maintained in 10% FBS and human granulocyte-macrophage/colony-stimulating factor (5 ng/ml). COS7 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% FBS. Transient transfection of COS7 cells was achieved using LipofectAMINE 2000 (Invitrogen) as directed by the manufacturer. Stable clones expressing EpoR-HA or RUL constructs were prepared by electroporation of plasmids into 32D cells. 32D-EpoR-HA cells were selected for their ability to grow in 5 units/ml Epo and subcloned by dilution. pIND-RUL plasmids were co-transfected with pVgRXR (Invitrogen). Transfected cells were selected in G418 (1 mg/ml) plus Zeocin (1 mg/ml) and were subcloned by dilution. Clonal cell lines were screened by Western blotting for expression of epitope-tagged RUL proteins when cultured in 6 μM ponasterone for 24 h. Growth analyses were performed on at least three different clones expressing RUL-wt or RUL-S49A expressing clone. It is noteworthy that establishing clones expressing RUL-S49A has been extremely difficult, presumably due to the potent growth inhibitory effect of this construct in combination with low level basal expression from the ponasterone-inducible system.

**Growth Factor Stimulations—**For stimulation experiments, cells were washed free of cytokine and were cultured in media plus 10% FBS for 4 h prior to stimulation with Epo (10 units/ml), IL-3 (2 ng/ml), G-CSF (10 ng/ml), or HGF (200 ng/ml). In experiments assaying RUL phosphorylation, cells were cultured in phosphate-free RPMI 1640 plus 10% FBS and [32P]phosphorous (0.5 μCi/ml) for 2 h prior to growth factor stimulation. Cell lines harboring inducible RUL expression constructs were treated with or without 6 μM ponasterone for 20 h prior to stimulation with growth factors.

**In Vitro Ubiquitination—**To prepare proteins for analysis in ubiquitination reactions, insect SF9 cells were infected with baculovirus encoding GST-RUL fusion proteins containing a thrombin-cleavable linker. Infected cells were lysed in NG buffer (0.5% Nonidet P-40, 10%...
p33RUL, a Novel Receptor-associated Ubiquitin Ligase

A.

human-p33RUL
Ubox-consensus

\[
\begin{array}{c}
\text{p33RUL} \\
\text{Ubox-consensus} \\
\text{gi-24949316} \\
\text{gi-1155698} \\
\text{gi-4452559} \\
\text{gi-4982626} \\
\text{gi-15220457} \\
\text{gi-6523054} \\
\text{gi-6617940}
\end{array}
\]

B.

\[
\text{p33RUL} \\
split U-Box
\]

Fig. 1. p33RUL is homologous to the U-box family of ubiquitin ligases. A, the human p33RUL U-box is aligned with the U-box consensus from the SMART data base and nine nearby neighbors assigned to the U-box family. The 104 amino acids separating the two halves of the human p33 U-box are indicated (\(^{\circ}\)). Identical (black background) and similar (shaded background) amino acids are indicated. Conserved amino acids reported to inactivate U-box domains when mutated (38, 39) are indicated by an arrowhead. The locations of predicted \(\beta\)-sheets (arrows) and an alpha-helix (bar) hypothesized to form the U-box core (26) are shown below the alignment. B, the location of the split U-box and helical domain of p33RUL are shown. Serine residues conserved between mouse and human p33RUL are shown (asterisks).

RESULTS

A Novel EpoR Interacting Protein Is a Member of the U-box Family of Ubiquitin Ligases—To identify novel signaling intermediates interacting with EpoR, a yeast-3-hybrid experiment was performed with a bait construct containing the cytoplasmic domain of EpoR and a separate expression cassette encoding wild-type Jak2. Yeast cells harboring the EpoR-Jak2 bait plasmid were used to screen a human bone marrow cDNA library, and a single clone survived re-screening when transformed into fresh yeast harboring the bait construct. In yeast containing a beta-galactosidase reporter, this clone mediated formation of blue colonies when co-expressed with the EpoR-Jak2 bait plasmid (not shown). Suppression of Jak2 expression in these colonies inhibited, but did not abolish, expression of the EpoR cytoplasmic domain in the presence of Jak2.

When sequenced, the cloned cDNA was found to contain a complete open reading frame predicted to encode a 301-amino acid protein with an expected size of 33 kDa (p33). The sequence was identical to a protein recently identified in a yeast-2-hybrid screen for ENSO-interacting proteins, NOSIP (35). Although not previously identified, we found that the amino acid sequence of this protein contained significant homology to the U-box family of ubiquitin ligases (Fig. 1A). The U-box is a modified form of the RING Finger domain that lacks metal-
lysate (38, 39) or absence (−) of ATP, or rabbit reticulocyte lysate (Fr. II) as the source of E1 and E2 activity. Reaction products were performed in the presence (+) or absence (−) of ATP, or rabbit reticulocyte lysate (Fr. II) as the source of E1 and E2 activity. Reaction products specific to p33RUL activity are indicated (Ub). A nonspecific activity is also indicated (NS). B, to confirm equivalent wild-type and mutant protein in each reaction, 300 ng of each purified protein was separated by SDS-PAGE and Western blotted with antibodies specific for p33RUL.

Because the U-box family has only recently been identified, the structural requirements of this functional domain are not fully defined. Thus, it is not clear how the 104-amino acid residues (Fig. 1B) extend through the first 40 of the 104 intervening residues. Interestingly, secondary structure modeling programs (36) predict that the helix beginning within the first 40 of the 104 intervening residues (Fig. 1B). Otherwise, the p33 U-box shows very high homology to the consensus U-box sequence, with a significant expectation (E) value of $6 \times 10^{-11}$ obtained from the RPS-BLAST search (37).

The p33 RUL domain contains two predicted helices, which would be stabilized by salt bridges instead of chelated metal ions. The p33 U-box appears to be an atypical member of this family, because it is split into two halves separated by 104 amino acids. These intervening amino acids reside within the predicted loop between the helix and the third sheet. Interestingly, secondary structure modeling programs (36) predict that the helix beginning within the first 40 of the 104 intervening residues (Fig. 1B). Otherwise, the p33 U-box shows very high homology to the consensus U-box sequence, with a significant expectation (E) value of $6 \times 10^{-11}$ obtained from the RPS-BLAST search (37).

Because the U-box family has only recently been identified, the structural requirements of this functional domain are not fully defined. Thus, it is not clear how the 104-amino acid intervening domain of the p33 U-box may affect its potential function as a ubiquitin ligase. Importantly, conserved amino acids that were reported to inactivate U-box proteins when mutated (38, 39) are retained in the p33 U-box (Fig. 1A). To confirm the identity of p33 as a ubiquitin ligase, the ability of p33 to ubiquitinate itself in an in vitro reaction was assessed. As specificity controls, p33 point mutations were prepared converting essential U-box residues serine 49 or proline 186 to alanine. Wild-type and mutant cDNAs were expressed as GST fusion proteins in insect Sf9 cells and purified by glutathione-Sepharose precipitation and thrombin cleavage of the GST linker. Equivalent amounts of purified proteins were subjected to ubiquitination reactions in which a rabbit reticulocyte lysate provided ubiquitin activating (E1) and conjugating (E2) activities, and a GST-ubiquitin fusion protein provided the only ubiquitin source. As shown in Fig. 2, ubiquitination of wild-type p33 was demonstrated by the appearance of high molecular weight p33 in the glutathione-Sepharose-precipitated reaction product. Moreover, the presence of p33 conjugates larger than the predicted 67-kDa size resulting from addition of GST-ubiquitin (34 kDa) indicated that p33 became polyubiquitinated, either at multiple sites or through the formation of polyubiquitin chains. As would be expected for a ubiquitin ligase, p33 ubiquitination was ATP-dependent and required activities present in the reticulocyte lysate. In contrast to wild-type p33, neither p33-P186A nor p33-S49A were efficiently ubiquitinated. The dependence of p33 ubiquitination on the retention of a functional U-box demonstrates that p33 itself is responsible for the ubiquitinations observed in these reactions. Thus, these observations support the assignment of p33 as a functional ubiquitin ligase.

p33 Interacts with EpoR in Vivo—To verify the interaction between p33 and EpoR, co-precipitation experiments were performed with wild-type p33 and an HA-tagged EpoR (EpoR-HA). As shown in Fig. 3A, EpoR was efficiently precipitated by p33-specific antibodies but not when competed by the p33 peptide to which the antiseraum was raised. As expected, an irrelevant peptide failed to disrupt immunoprecipitation of the EpoR-p33 complex. To assess whether endogenous p33 interacts with receptors in factor-dependent hematopoietic cells, murine myeloid 32D cells stably expressing EpoR-HA were evaluated for co-precipitation with endogenously expressed p33. EpoR-HA was detected in p33 immunoprecipitations and co-migrated with the EpoR-HA complex that was immunoprecipitated by HA antibodies (Fig. 3B). The p33-precipitated EpoR-HA was also competed by the p33-immunizing peptide. Similarly, anti-HA immunoprecipitates from 32D-EpoR-HA cells contained significant amounts of p33 protein compared with HA immunoprecipitates from parental 32D cells. Interaction between p33 and EpoR was also assessed in human TF-1 cells, which express both proteins endogenously. As shown in Fig. 3C, EpoR was precipitated by p33-specific antiserum from TF-1 cell lysates but not in the absence of the p33-immunizing peptide. Interaction between p33 and EpoR appeared to be constitutive and did not require receptor activation, because co-precipitation was observed from lysates of unstimulated cells. However, in transfected COS7 cells, 32D-EpoR-HA cells, and TF-1 cells, Epo treatment reproducibly increased co-precipitation of EpoR and p33, suggesting that this interaction is enhanced by receptor activation. Based on the demonstrated receptor-association of this ubiquitin ligase, we refer to the cloned protein as p33RUL.

p33RUL Mediates Ubiquitination of EpoR—The association between EpoR and RUL suggests the possibility that EpoR may be targeted for ubiquitin ligation. To assess the potential for ubiquitination of EpoR, COS7 cells were transiently transfected with wild-type p33 and an HA-tagged EpoR (EpoR-HA). As shown in Fig. 4A, significant ubiquitination was detected in HA immunoprecipitates from cells co-expressing EpoR-HA and p33, suggesting that this interaction is enhanced by receptor activation. Based on the demonstrated receptor-association of this ubiquitin ligase, we refer to the cloned protein as p33RUL.
The state of RUL ubiquitination was also assessed in transfected COS7 cells (Fig. 4C). Significant ubiquitinated protein was detected in RUL immunoprecipitates from COS7 cells overexpressing wild-type RUL. Ubiquitination of overexpressed RUL was not significantly enhanced by co-expression with EpoR or by Epo treatment of transfected cells. However, Epo treatment induced detectable ubiquitination in RUL immunoprecipitates from cells expressing only endogenous RUL plus transfected EpoR. Thus, overexpressed RUL is constitutively ubiquitinated in COS7 cells and may mask inducible ubiquitinations that are observable with physiological levels of RUL expression. Whether more physiological levels of EpoR expression will similarly unmask Epo-induced ubiquitination has not been determined.

Expression of p33RUL U-box Mutants Inhibits Epo- and IL-3-dependent Survival, Proliferation, and Expression of Immediate-early Genes—To test the hypothesis that the ubiquitin ligase activity of RUL contributes to growth of factor-dependent hematopoietic cells, we examined the effect of ubiquitin ligase-deficient RUL mutants (RUL-S49A and RUL-NT) on 32D cell growth. Mutant and wild-type RUL cDNAs were prepared with epitope tags and cloned into a ponasterone-inducible vector for stable expression in 32D-EpoR cells. Clonal cell lines expressing each of these constructs were obtained while being cultured in medium containing IL-3, and ponasterone-inducible expression of different RUL forms was confirmed by Western blotting (Fig. 5A). In 32D-EpoR cells expressing wild-type RUL, ponasterone treatment had no significant effect on either Epo- or IL-3-induced proliferation (Fig. 5B). In contrast, cells expressing RUL-NT or RUL-S49A grew significantly slower when cultured in ponasterone as compared with cells cultured in its absence. Ponasterone treatment also resulted in reduced viability of cells expressing RUL-NT (less than 50% viability), whereas RUL-S49A cultures lacked viable cells by 48 h after addition of ponasterone. By comparison, parental 32D-EpoR and RUL-wt cultures remained greater than 90% viable throughout the course of the growth assay. Even in the absence of ponasterone, RUL-NT and RUL-S49A clones grew slower than parental controls or other inducible clones. In fact, in the absence of selection (G418 and Zeocin) cultures with mutant RUL proteins rapidly lost expression of the inducible construct, suggesting that even low levels of uninduced expression inhibit growth (not shown).

To determine whether reduced viability in cells expressing mutant RUL was associated with apoptosis or necrosis, cells were stained with annexin V and propidium iodide. Ponasterone-induced expression of RUL-NT or RUL-S49A increased the apoptotic population in these cultures, as indicated by an increase in the propidium iodide-low, annexin V-high population (lower right quadrant, Fig. 5C). No significant apoptosis was observed in RUL-wt cultures in the presence or absence of ponasterone. The lack of significant effects associated with expression of wild-type RUL, as compared with the considerable inhibition of growth and viability mediated by RUL mutants lacking a functional U-box domain, are consistent with a requirement for RUL U-box function in mediating cytokine-dependent cell growth and survival.

The possibility that expression of RUL mutants might alter the stability of EpoR or associated proteins was assessed in 32D-EpoR cells expressing inducible RUL-NT (Fig. 6). Epo treatment of cells for up to 90 min had no observable effect on expression levels of EpoR, Jak2, or endogenous RUL. Similarly, ponasterone-induced expression of RUL-NT did not alter expression of these proteins prior to or following Epo treatment. This observation is consistent with the lack of effects on EpoR.
The effect of RUL activity on Epo-induced expression of immediate-early genes was also assayed in 32D-EpoR cells expressing inducible RUL-NT or RUL-wt. Epo-induced expressions of c-myc, pim-1, bcl-2, and bcl-XL were each modestly enhanced by ponasterone treatment in RUL-wt cells (Fig. 7, A and B), although these differences were not statistically significant (p > 0.1). By comparison, expression of RUL-NT significantly inhibited Epo-induced expression of c-myc and bcl-2. These findings are consistent with the reduced growth and viability of RUL-NT cultures compared with parental and RUL-wt controls. Epo-induced bcl-XL expression was also reproducibly lower in RUL-NT cells as compared with RUL-wt cells; however, bcl-XL levels were not further reduced by ponasterone-dependent expression of RUL-NT. Thus, in contrast to specific inhibition of c-myc and bcl-2, induced expression of RUL-NT did not significantly inhibit Epo-dependent expression of bcl-XL. Likewise, Epo-induced pim-1 expression did not significantly differ between RUL-NT and RUL-wt cells. Thus, conditional expression of a dominant-negative RUL mutant selectively inhibited Epo-induced expression of some, but not all, immediate-early gene targets associated with survival and proliferation of factor-dependent hematopoietic cells.

Endogenous p33RUL Is Transiently Phosphorylated following Cytokine Stimulation of Factor-dependent Hematopoietic Cells—Because RUL was identified based on interaction with EpoR-Jak2 complexes, we attempted to demonstrate cytokine-dependent tyrosine phosphorylation of endogenous RUL by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies. However, we found no circumstances under which endogenous RUL was detectably phosphorylated on tyrosine (not shown). Thus, we have found no evidence to support a role for direct Jak2 phosphorylation of RUL in hematopoietic cells. However, RUL immunoprecipitations performed from 32P-labeled 32D cells stably expressing wild-type EpoR (32D-EpoR) demonstrated that 32P was incorporated into endogenous RUL following stimulation with either Epo or IL-3 (Fig. 8A). Specifically, a major 32P-labeled protein present in RUL immunoprecipitates from Epo- or IL-3-treated cells completely co-migrated with RUL protein visualized by Western blotting. Moreover, immunoprecipitation of the cytokine-induced phosphoprotein was efficiently competed by the RUL peptide to which the antiserum was raised. Little or no phosphorylated RUL was detectable in immunoprecipitates from untreated cells. To determine the site of cytokine-induced RUL phosphorylation, the Epo-induced 32P-RUL band was excised and subjected to two-dimensional phosphoamino acid analysis. As shown in Fig. 8B, 32P-RUL was phosphorylated on serine residues without any detectable phosphorylation at threonine or tyrosine. Identical results were obtained from a similar analysis of IL-3-induced 32P-RUL (not shown).

Most receptor-activated phosphorylation events occur rapidly and are transient following continuous receptor activation.
The time course of Epo-induced RUL phosphorylation was assessed in 32P-labeled 32D-EpoR cells (Fig. 9). Epo-induced phosphorylation of RUL was maximal 10 min after addition of cytokine. By 30 min of Epo stimulation, all detectable RUL phosphorylation was lost. A similar time course was observed for Epo-stimulated Stat5 tyrosine phosphorylation (lower panels, Fig. 9). Although anti-phosphotyrosine Western blotting detected Stat5 tyrosine phosphorylation 5 min after Epo treatment, maximal phosphorylation of Stat5 occurred between 10 and 15 min of stimulation, identical to the RUL time course. Thus, phosphorylation of RUL and Stat5 phosphorylation follow a nearly identical time course of transient induction after cytokine treatment.

To assess whether inhibited proliferation of 32D cells expressing mutant RUL is associated with defects in the phosphorylation of endogenous RUL, the effect of Epo stimulation was evaluated in these cells (Fig. 10). Epo treatment induced significant RUL phosphorylation in cells expressing RUL-wt, whereas inducible phosphorylation of endogenous RUL was barely detectable in RUL-NT-expressing clones. RUL-NT appeared to be weakly phosphorylated, but its phosphorylation was not induced by Epo treatment. These data suggest that RUL-NT has dominant-negative activity with regard to its ability to inhibit phosphorylation of endogenous RUL and are consistent with the ability of RUL-NT to inhibit endogenous RUL ubiquitin ligase activity in COS7 cells (see Fig. 4B). We have been unable to assess RUL phosphorylation in p33-S49A-expressing cells, because inducible expression of this mutant is repeatedly lost during expansion of cultures to the quantities needed for phosphorylation assays (not shown).

**DISCUSSION**

In this study, we identified a novel member of the U-box family of ubiquitin ligases as an EpoR-interacting protein. This receptor-associated ubiquitin ligase (RUL) interacts with EpoR in factor-dependent hematopoietic cells, mediates ubiquitina-
tion of EpoR, and is inducibly phosphorylated at serine after growth factor treatment. Mutants of RUL lacking ubiquitin ligase activity were shown to inhibit cytokine-induced expression of immediate early genes associated with growth and survival of factor-dependent cells. Indeed expression of mutant RUL was growth inhibitory in a factor-dependent cell line.

Fig. 6. Expression of dominant negative RUL does not alter the stability of EpoR or associated proteins. 32D-EpoR cells harboring inducible RUL-NT were treated in the presence (+) or absence (−) of 6 μM ponasterone for 24 h prior to cytokine stimulation. Additionally, cultures were deprived of all cytokine for 6 h prior to stimulation (0 min). Subsequently, cultures were treated with 20 units/ml Epo. At indicated times before or after addition of Epo, $8 \times 10^5$ cells were collected and directly lysed in sample buffer for SDS-PAGE. Lysates were separated by SDS-PAGE and were probed with antibodies specific for EpoR, Jak2, p33RUL, or GAPDH. Lysates of parental 32D cells were also analyzed as a negative control for detection of transfected EpoR.

Fig. 7. RUL-NT inhibits Epo-induced expression of c-myc and bcl-2. 32D-EpoR cell clones harboring inducible RUL-wt or RUL-NT constructs were induced with 6 μM ponasterone or left uninduced for 24 h. Parallel cultures were treated with 10 units/ml Epo or left untreated for 2 h. RNA was isolated and analyzed for expression of immediate-early genes by Northern blotting, as described under “Experimental Procedures.” Data shown are representative of four experiments (A). The percent change in Epo-induced expression resulting from ponasterone treatment was calculated for each gene transcript (B). Values represent averages from four experiments. Error bars represent the standard error. Significant differences between RUL-NT and RUL-wt cells are indicated (*p* values).

Fig. 8. RUL phosphorylation in Epo- or IL-3-treated hematopoietic cells. A, 32D-EpoR cells were labeled with [32P]phosphate and treated for 12 min with Epo (10 units/ml), IL-3 (2 ng/ml), or left untreated (no factor). Cells were lysed and immunoprecipitated with RUL antibodies in the presence (+) or absence (−) of the RUL-immunizing peptide, or an irrelevant peptide. Precipitated proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. [32P]-Labeled proteins were visualized on a PhosphorImager. The membrane was also Western blotted with RUL antibodies. B, the RUL band precipitated from Epo-treated cells (A) was excised from the membrane and analyzed for phosphoamino acid content by two-dimensional thin layer electrophoresis, as previously described (3). The migratory positions of phosphoamino acid standards are indicated.

Fig. 9. Epo-induced RUL phosphorylation is rapid and transient. 32D-EpoR cells were labeled with [32P]phosphate and treated with 10 units/ml Epo. Samples of cells were taken prior to Epo treatment or at indicated times after addition of Epo. Cells were lysed and immunoprecipitated (IP) with RUL antibodies or a mixture of Stat5A and Stat5B antiserum. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. [32P]-Labeled proteins were visualized on a PhosphorImager. The membranes were also Western blotted with RUL antibodies (RUL IPs) or phosphotyrosine (PY) antibodies (Stat5 IPs). Membranes for Stat5 IPs were stripped and reprobed with Stat5A and Stat5B antiserum.
Epo-wt or Epo-NT were treated with 6 μM ponasterone for 20 h. Cells were then labeled with [32P]phosphate and treated for 15 min with 20 units/ml Epo (++) or left untreated (−). Cells were lysed and immunoprecipitated with RUL antibodies. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. 32P-La-}

suggesting that RUL may function as part of a mitogenic signal pathway.

Recently, the human RUL cDNA (also called NOSIP) was isolated in a yeast-2-hybrid screen for proteins interacting with endothelial nitric-oxide synthase (eNOS) (35, 41). Transient overexpression in Chinese hamster ovary cells was found to relocate overexpressed eNOS from plasma membrane caveolae to the cytoplasm, and endogenous RUL could be co-precipitated with eNOS. However, it remains to be determined if there is a functional consequence to this interaction for endogenously expressed eNOS. RUL did not associate with other forms of NOS, suggesting that this potential interaction may not be relevant to RUL function in hematopoietic cells that do not express eNOS (42). The functional identification of RUL as a ubiquitin ligase was not reported previously. Our data suggest a role for the ubiquitin ligase activity of RUL in the regulated proliferation of hematopoietic cells based on the ability of RUL U-box mutants to inhibit factor-dependent growth of 32D cells.

Assignment of RUL to the ubiquitin ligase family is based on the presence of an atypical form of the U-box homology, which is split by 104 intervening amino acids. The intervening sequence in the RUL U-box occurs within a predicted loop between the α-helix and the third β-sheet and thus may not disrupt the hypothesized core structure of this domain (26) and resulting activity. The in vitro ubiquitination experiment shown in Fig. 2 and the dependence on endogenous RUL for EpoR ubiquitination in COS7 cells support the conclusion that RUL is a functional ubiquitin ligase that is dependent on essential residues conserved within the U-box domain. It is presently unclear whether this activity is dependent on, or influenced by, the 104 residues splitting the RUL U-box. This intervening sequence represents the most significant divergence between the human and murine proteins (not shown). However, this does not preclude a functional role in regulating RUL ubiquitin ligase activity, including specificity for ubiquitin conjugating enzymes and targets of ubiquitination.

It remains to be determined if RUL ubiquitin ligase activity is directly influenced by activation of cytokine receptors. Although overexpressed RUL was constitutively ubiquitinated in COS7 cells, ubiquitination of endogenous RUL was enhanced after EpoR activation. Similarly, we have repeatedly observed Epo-induced increases in high molecular weight forms of RUL in Epo-treated 32D-EpoR cells (not shown). However, we have not been able to detect high molecular weight RUL species from either COS7 or 32D cells with several anti-ubiquitin antibodies. Thus, additional experimentation will be required to determine the effects of cytokine receptor activation on endogenous RUL ubiquitination in factor-dependent cells.

The consequences of protein ubiquitination vary depending on the structure of the ubiquitin conjugate (27). Polyubiquitin chains conjugated through lysine 48 generally target proteins for proteasome-dependent degradation, whereas monoubi-}

FIG. 10. Expression of RUL-NT Inhibits Epo-induced phospho-}

FIG. 11. RUL phosphorylation is mediated by cytokine and non-cytokine receptors. NFS-60 cells were labeled with [32P]phosphate and treated for 12 min with IL-3, G-CSF, HGF, or left untreated (no factor). Cells were lysed and immunoprecipitated (IP) with RUL antibodies or a mixture of Stat5α and Stat5β antiserum. Precipitated proteins or total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. 32P-Labeled proteins in RUL IPs were visualized on a PhosphorImager. Membranes were also probed with antibodies against RUL, phosphotyrosine, or phospho-Erk.

Several studies have implicated ubiquitination in regulating the stability of proteins associated with Epo and IL-3 signaling pathways. EpoR, Jak2, and Stat5 have been reported to be targets of proteasome-dependent degradation following cyto-}

The consequences of protein ubiquitination vary depending on the structure of the ubiquitin conjugate (27). Polyubiquitin chains conjugated through lysine 48 generally target proteins for proteasome-dependent degradation, whereas monoubiquitination or polyubiquitin chains conjugated through other lysines generally result in modified function or altered subcellular localization of targeted proteins. Several reports demonstrating ubiquitin ligase activity of U-box proteins have shown that their activities include formation of polyubiquitin chains linked through multiple lysine residues of ubiquitin, in addition to lysine 48 and lysine 63 (38, 39, 43). We have observed that RUL autoubiquitination and ubiquitination of EpoR consist of either multiple monoubiquitinations or polyubiquitination. It is presently unclear which lysine linkages might be involved in polyubiquitination mediated by RUL. However, we have not observed any detectable changes in expression levels of EpoR or associated proteins, including Jak2, as a function of expression of wild-type or mutant RUL (see Figs. 4 and 6).

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nation directly activates a serine kinase, TAK1, and ultimately leads to activation of the transcription factor, NF-κB (30, 31). A relationship between RUL activity and transcription is suggested by the inhibited expression of c-myc and bcl-2 mRNA in cells expressing truncated RUL (RUL-NT), a mutant lacking a complete U-box domain. Notably, Epo-induced expression of pim-1 and bcl-X₇ were not substantially altered by RUL-NT expression, suggesting that RUL-NT did not result in a general inhibition of cytokine-induced gene expression. Such selective inhibition of certain targets of Epo-induced transcription suggests involvement of RUL in some, but not all, signaling pathways responsible for cytokine-induced expression of immediate-early genes. However, the specific reduction in Epo-induced expression of genes associated with growth and survival (c-myc and bcl-2) is consistent with the reduced proliferation and increased apoptotic death of RUL-NT-expressing cells.

The mechanism leading to cytokine-dependent expression of c-myc and bcl-2 has not been clearly identified. Epo and IL-3 have been reported to activate NF-κB (44, 45), a transcription factor that can be activated through the activity of ubiquitin ligases. Although we have not assessed the activity of NF-κB in cells expressing wild-type or mutant RUL, NF-κB is more usually associated with bcl-2 expression rather than bcl-2 expression (46). Epo-induced expression of these immediate-early genes is Jak2-dependent, because this activity is lost from receptors that do not associate with Jak2, or in cells expressing dominant-negative Jak2 (17, 47–49). Notably, RUL-NT appears to function as a dominant negative, at least with regard to its ability to inhibit RUL-dependent ubiquitination of EpoR as well Epo-induced phosphorylation of endogenous RUL. Thus, along with Jak2, RUL may represent a required component of cytokine-induced signaling pathways regulating expression of c-myc and bcl-2.

The rapid and transient phosphorylation of RUL after cytokine treatment links this protein with the early events regulated by cytokine receptors. Indeed, the kinetics of RUL phosphorylation demonstrate that this event occurs at the same time as other events thought to occur within the receptor complex, including Jak2-mediated phosphorylation of Stat5. Whether RUL phosphorylation is related to or downstream of Jak2-dependent signaling pathways is presently unclear. However, the apparent requirement of both activities in the regulated transcription of c-myc and bcl-2 suggests some relationship between RUL and Jak2. We have found that activated Jak2 can phosphorylate RUL on tyrosine in vitro (not shown). However, we have found no evidence that this occurs in cytokine-treated hematopoietic cells. It remains possible that RUL phosphorylation occurs downstream of Jak2 through subsequent activation of a serine kinase. Importantly, RUL phosphorylation is not dependent on Jak activity, because RUL was inducibly phosphorylated following HGF treatment of NFS-60 cells. HGF acts through the receptor tyrosine kinase, c-Met (40), and regulates proliferation of NFS-60 cells without activating Jak kinases or Stat transcription factors (34). Thus, RUL phosphorylation may represent a common target of growth factor receptors from differing families. Consistent with a potential general role in proliferation regulated by a variety of growth factors, we observed ubiquitous expression of RUL mRNA in both human and mouse tissues (not shown).

The kinase that mediates serine phosphorylation of RUL has not been identified, and serine kinase activity has not generally been observed within cytokine receptor complexes. However, PKA has been reported to mediate IL-3-dependent serine phosphorylation of the IL-3 receptor (50). In this regard, serine 36 of RUL does conform to the consensus sequence for PKA phosphorylation (S/R)ₓ(S/R)ₓ(S/R)ₓ(S/R)ₓ, fitting the consensus sequence for R/Kₓ(S/R)ₓ(S/R). Other reports have suggested a role for protein kinase C in regulating signaling activity at EpoR, including possible involvement in induced expression of c-myc and bcl-2 (51–54). Several protein kinase C consensus sites are present in the carboxyl-terminal half of RUL, although these sites are retained in the truncated RUL-NT that is not phosphorylated in response to cytokine treatment. Whether these or other serine kinases can phosphorylate RUL is currently being evaluated. It is presently unclear how growth factor-induced phosphorylation may affect RUL function. By analogy, EGF receptor ubiquitination by the RING finger E3, Cbl, has been reported to require prior phosphorylation of Cbl by the EGF receptor tyrosine kinase (28). It is therefore possible that growth factor-induced phosphorylation of RUL modifies its activity either through altered ubiquitin ligase activity or altered protein associations. In that regard, one potential site of RUL phosphorylation is at serine 49, a residue required for RUL ubiquitin ligase activity in vitro. We have yet to determine if this site is phosphorylated in growth factor-treated hematopoietic cells. Although EpoR activation in COS7 cells increased ubiquitination of endogenous RUL, we have not determined if this was associated with phosphorylation of RUL in these cells. Clearly, many questions remain concerning the involvement of RUL with mitogenic signaling pathways regulated by cytokine receptors, particularly with regard to the specific role of its ubiquitin ligase activity and the status of ubiquitinated proteins in factor-dependent hematopoietic cells. However, the significance of this role is suggested by the specific inhibitory effects of RUL mutants lacking ubiquitin ligase activity and the association of endogenous RUL with early events activated by growth factor receptors.

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p33RUL, a Novel Receptor-associated Ubiquitin Ligase

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