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Phosphorylation of Bcl-associated death protein (Bad) by erythropoietin-activated c-Jun N-terminal protein kinase 1 contributes to survival of erythropoietin-dependent cells

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Abstract

The glycoprotein erythropoietin (Epo) is a hematopoietic cytokine necessary for the survival of erythrocytes from immature erythroid cells. The mitogen-activated c-Jun N-terminal kinase 1 (JNK1) plays an important role in the proliferation and survival of erythroid cells in response to Epo. However, the precise mechanism of JNK1 activation promoting erythroid cell survival is incompletely understood. Here, we reported that JNK1 is required for Epo-mediated cell survival through phosphorylation and inactivation of the pro-apoptotic, Bcl-2 homology domain 3 (BH3)-only Bcl-associated death protein (Bad). Upon Epo withdrawal, HCD57 cells, a murine Epo-dependent cell line, displayed increased apoptotic cell death that was associated with decreased JNK1 activity. Epo withdrawal-induced apoptosis was promoted by inhibition of JNK1 activity but suppressed by expression of a constitutively active JNK1. Furthermore, Epo-activated JNK1 phosphorylated Bad at threonine 201, thereby inhibiting the association of Bad with the anti-apoptotic molecule B-cell lymphoma-extra large (Bcl-X_L). Replacement of threonine201 by alanine in Bad promoted Epo withdrawal-induced apoptosis. Thus, our results provide a molecular mechanism by which JNK1 contributes to the survival of erythroid cells.

Keywords

Erythropoietin; c-Jun N-terminal protein kinase; bcl-associated death protein; apoptosis; signal transduction

1. Introduction

The glycoprotein erythropoietin (Epo) is a hematopoietic cytokine synthesized in the kidney in response to hypoxia, and it is necessary for the survival and production of mature

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erythroid progenitors. Although it has been demonstrated to effectively promote proliferation, differentiation, and survival of erythroid progenitors, the role of Epo to prevent apoptosis of late erythroid progenitors is now considered the primary effect of Epo action (Noguchi et al., 2008; Zhander and Karsan, 2007). The anti-apoptotic function of Epo has been the subject of many studies performed in the past few years (Abutin et al., 2009; Ghezzi and Brines, 2004). Epo exerts its effects on erythroid progenitors by binding to its cognate receptor, EpoR, and activates pathways that include phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal protein kinase (JNK), the Extracellular signal-regulated kinase (ERK), and Signal transducer and activator of transcription (STAT) pathways (Maiese et al., 2005; Richmond et al., 2005). Recently, it was suggested that Epo could play a role in non-erythroid tissues as numerous other cell types (endothelial- and cancer cells) express EpoR (Acs et al., 2001; Maiese et al., 2005). However, the underlying molecular mechanism of Epo-EpoR signaling on cell survival remains unclear.

The c-Jun N-terminal protein kinase (JNK) has been implicated in the regulation of erythroid proliferation and survival (Jacobs-Helber and Sawyer, 2004; Lee et al., 2010). JNK is a member of the MAPK superfamily and the JNK MAPK subfamily has two ubiquitously expressed isoforms, JNK1 and JNK2, and a tissue-specific isoform, JNK3 (Lin, 2006; Wagner and Nebreda, 2009). Between JNK1 and JNK2, JNK1 is the main c-Jun kinase, whereas JNK2 may interfere with JNK1 activation (Lin, 2006; Liu et al., 2004). JNK is known to be activated by a number of extracellular stimuli including growth factors, cytokines, oncogenes, and environmental stresses (Liu and Lin, 2007). Deregulation of JNK activity has been implicated in many human diseases and certain types of cancer (Liu and Lin, 2007; Wagner and Nebreda, 2009). The activation of JNK was initially reported to be induced by Epo (Nagata et al., 1997), and recent reports have suggested that JNK activation is necessary for the initiation of erythroid proliferation and differentiation (Jacobs-Helber and Sawyer, 2004; Lee et al., 2010). However, the mechanism of JNK1 activation in the Epo survival signals has not yet been elucidated.

Bcl-associated death protein (Bad) is a BH3-only pro-apoptotic Bcl-2 family protein and plays a critical role in the crosstalk between the growth/survival factor signaling pathway and the intrinsic death machinery (Chipuk et al., 2010). The pro-apoptotic activity of Bad is regulated by extracellular stimuli via post-translational modifications including phosphorylation (Deng et al., 2008; Yu et al., 2004; Zhang et al., 2005). In response to the stimulation of growth/survival factors such as interleukin-3 (IL-3) and insulin-like growth factor-I (IGF-I), Bad is phosphorylated at several serine and/or threonine residues (Ser112, Ser136, Ser155, or Thr201) by a group of protein kinases such as PKA, Akt, and JNK (Cieslak and Lazou, 2007; Yu et al., 2004). Phosphorylation of Bad makes it inactive as it is sequestered in the cytoplasm via interaction with 14-3-3, a group of cytoplasmic anchorage proteins that interact with proteins through specific phospho-serine/threonine motifs (Sunayama et al., 2005), and thereby preventing Bad interaction with Bcl-2/Bcl-X_L on the mitochondria membrane (Yu et al., 2004). JNK1 suppresses IL-3 withdrawal-induced apoptosis in hematopoietic FL-5.12 cells via phosphorylation of Bad (Deng et al., 2008; Yu et al., 2004). In the present study, we demonstrated that JNK1 activation is also required for the survival of murine erythroleukemia cells through phosphorylation and inactivation of pro-apoptotic molecule Bad, thus our finding indicates an important role of JNK1 signaling in Epo-mediated cell survival.

2. Materials and methods

2.1 Reagents

Antibodies against Bad (C-20), Bcl-X_L (H5), HA (F7), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phospho-Bad Ser136 was from Cell Signaling

Technology (Danvers, MA, USA). Antibodies against JNK (G151-333 for immunoprecipitation and G151-666 for immunoblotting) were from BD PharMingen (San Diego, CA, USA). The specific JNK inhibitor SP600125 was synthesized and purified (Tang et al., 2002). [γ - 32 P]ATP and [35 S] methionine were from Dupont NEN (Boston, MA, USA). Caspase pan inhibitor Z-VAD-FMK was from Promega (Madison, WI, USA). Anti-phospho-BadT201 antibody was generated by injecting KLH-conjugated phosphopeptide (CRNLGKGGSpTPSQ) into rabbits (Cocalico Biologicals Inc.) and purified (Yu et al., 2004). Propidium iodide (PI) and anti-M2 antibody were from Sigma (St.Louis, MO, USA).

2.2 Plasmids

pCDNA3.1/Hygro (+)-M2-Bad wt or T201A mutant was described previously (Deng et al., 2008). Glutathione S-transferase (GST)-JNK1 and GST-JNK1 (APF), pSR α -hemagglutinin (HA)-MKK7-JNK1, and pSR α -HA-MKK7 (KM)-JNK1 was described previously (Yu et al., 2004; Zheng et al., 1999). Murine Bcl-X_L was subcloned into pCDNA3.1-HisC vector between BamHI and XhoI, HA-MKK7-JNK1 and HA-MKK7 (K149M)-JNK1 were subcloned into pCDNA3.1/Hygro(+) between HindIII and BamHI. All constructs were confirmed by DNA sequencing.

2.3 Cell culture and Transfection

Epo-dependent erythroleukemia cell line HCD57 were cultured in Iscove's modified Dulbecco medium (IMDM, Invitrogen, Carlsbad, CA, USA) supplemented with 25% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 10 μ g/ml gentamicin (Invitrogen, California, USA), and 1 U/ml Epo (Amgen, Thousand Oaks, CA, USA) at 37 °C in 5% CO₂. For Epo deprivation studies, HCD57 cells were washed with IMDM three times and re-cultured in IMDM media supplemented with 25% FCS and 10 μ g/ml gentamicin in the absence of Epo for 18 hours. For Epo stimulation, cells were deprived of Epo and stimulated with 10 U/ml Epo for the indicated times. pCDNA3.1/Hygro (+)- M2-Bad wt or T201A, pCDNA3.1/Hygro (+)-HA-MKK7-JNK1 or pCDNA3.1/Hygro (+)-HA- MKK7(KM)-JNK1 were transfected into HCD57 cells using Gene electroporator (Bio-Rad, Hercules, USA) with 250 V, 950 μ F, and 50 Ω resistance in a 0.4-cm cuvette. Stable clones were selected against Hygromycin B (1 mg/ml).

2.4 Protein kinase assays and Immunoblotting

Immune complex kinase assays and GST-c-Jun pull down kinase assays were performed and quantitated as described previously (Deng et al., 2008). Briefly, kinase assays were carried out for 60 min at 30°C in 20 mM HEPES (pH 7.6), 20 mM MgCl₂, 1 mM DTT, 10 μ M nonradioactive ATP, and 10 μ Ci [γ - 32 P]ATP. Wild type (wt) or mutant GST-Bad or GST-c-Jun (2–4 μ g) was used as substrate as indicated. Reactions were terminated by the addition of 4 $\times$ SDS sample buffer and heating at 95°C for 5 min. The proteins were separated by SDS-PAGE and visualized by coomassie brilliant blue staining. Phosphorylated proteins were detected and quantitated by PhosphorImager (Molecular Dynamics, PA, USA). Active JNK1 was immunoprecipitated from the lysates prepared from cells stimulated by Epo (10U/ml), using anti-JNK1 antibody (antibody G151-333).

Immunoblotting was performed as described previously (Deng et al., 2008).

2.5 In vitro translation

The pCDNA3.1 based Bcl-X_L were translated *in vitro* using the Rabbit Reticulocyte Lysate TNT Coupled Transcription/Translation System (Promega, Madison, WI, USA), following the manufacturer's instructions.

2.6 Apoptosis assays

Apoptosis was determined by propidium iodide (PI) staining using flow cytometry analysis. Following cell treatment, cells were fixed in 70% ethanol overnight at 4 °C. Cells were then washed twice in phosphate-buffered saline and stained for 3 hours in 3 mM sodium citrate, 2 mM PI, and 50 mg/ml RNase A at 4 °C in the dark. Cells were analyzed by flow cytometry (FACS Caliber, BD, USA). Sub-G₀/G₁, indicative of apoptosis, was gated and a percentage of the total number of cells was determined.

2.7 Statistical analysis

All statistical analyses were performed by using the Student's t-test. A probability level of 0.05 or less was considered to be significant. Data are presented as mean values ± standard error.

3. Results

3.1 JNK1 is an Epo-activated protein kinase

To determine if JNK1 is involved in the Epo dependent cell survival, we used HCD57 cells to examine the role of JNK1 in apoptosis. HCD57 cells have successfully been used as a model to study the molecular mechanism underlying Epo withdrawal-induced apoptosis (Jacobs-Helber et al., 2000). As expected, HCD57 cells underwent apoptosis upon Epo withdrawal, which was reversed by Epo readdition (Fig. 1A). To further confirm Epo withdrawal-induced apoptosis in HCD57 cells, cells were pretreated with Z-VAD-FMK, a pan-caspase inhibitor, before Epo withdrawal. Apoptosis assay showed that Z-VAD-FMK pretreatment inhibited HCD57 cells from undergoing apoptosis in a dose-dependent manner (Fig. 1B). On the other hand, Immune complex kinase assays revealed that Epo withdrawal resulted in a decrease in JNK1 kinase activity. Eighteen hours after Epo withdrawal, HCD57 cells exhibited only fifteen percent of original JNK1 activity (Fig. 1C, upper panel). However, Epo readdition induced a significant 5-fold increase in the JNK1 kinase activity (Fig. 1D, upper panel). The stimulation or inhibition of JNK1 activity was not the result of changes in its expression, as demonstrated by immunoblotting with anti-JNK antibody (Figs. 1C and 1D, lower panel). These data suggested that JNK1 is an Epo-activated protein kinase for the survival of HCD57 cells.

3.2 JNK1 activation is required for preventing Epo withdrawal-induced apoptosis

To investigate whether JNK1 activation can suppress Epo withdrawal-induced apoptosis, a specific JNK inhibitor, SP600125, was used to inhibit JNK1 activity in HCD57 cells. Immune complex kinase assays showed that pretreatment of HCD57 cells with SP600125 resulted in a dose-dependent inhibition of JNK1 activity (Supplemental Fig. 1). JNK1 activity induced by Epo readdition was dramatically inhibited by 10μM SP600125 (Supplemental Fig. 1 and Fig. 2A). By contrast, the phosphorylation of related MAP kinase ERK1/2 and p38 was unaffected by the SP600125 inhibitor treatment (data not shown).

We next examined the biologic effects of the SP600125 inhibitor on the HCD57 cells. Apoptotic cell death assay revealed that pretreatment with SP600125 resulted in a dose-dependent induction of apoptosis in HCD57 cells (Figs. 2B and 2C). At sixty-fifth hour, HCD57 cells with 15μM SP600125 pretreatment prior to Epo readdition resulted in forty percent apoptotic cell death, whereas cells without SP600125 pretreatment had only eight percent apoptotic cell death (Fig. 2B). Furthermore, inhibition of JNK1 by SP600125 promoted Epo withdrawal-induced apoptosis (Fig. 2C). To further confirm the role of JNK1 activation in Epo withdrawal-induced apoptosis, HCD57 cells were stably transfected with mammalian expression vector encoding HA-MKK7-JNK1, which has constitutive Jun kinase activity; HA-MKK7(K149M)-JNK1, which is a kinase-deficient mutant (Yu et al.,

2004); or empty vector. Immune complex kinase assays confirmed that MKK7-JNK1 had constitutive JNK1 activity, whereas MKK7 (K149M)-JNK1 had no detectable activity (Fig. 2D). Apoptotic cell death assays revealed that cells expressing the constitutively active MKK7-JNK1 were much less sensitive to Epo withdrawal-induced apoptosis than cells expressing kinase-deficient MKK7 (K149M)-JNK1 mutant (Fig. 2E). Thus, JNK1 plays an important role in preventing Epo withdrawal-induced apoptosis.

3.3 JNK1 is an Epo-activated Bad kinase

The survival effect of growth factors is mainly mediated by phosphorylation and consequent inactivation of the pro-apoptotic molecule Bad (Deng et al., 2008; Yu et al., 2004). The fact that Epo-induced JNK1 activation involved in the survival of HCD57 cells (Figs. 1 and 2) led us to investigate if JNK1 could also exert its survival effect through phosphorylation of Bad in HCD57 cells. Indeed, withdrawal of Epo resulted in dephosphorylation of Bad, whereas Epo readdition induced phosphorylation of Bad at several serine residues, including Ser136 (Fig. 3A). Immune complex kinase assays showed that GST-Bad was phosphorylated by Epo-activated JNK1 in a manner that mirrored the phosphorylation pattern of Bad (compare Fig. 3B to 3A). The ability of JNK1 to phosphorylate GST-Bad was well correlated to its phosphorylation of GST-c-Jun (Fig. 3B). In addition, pretreatment of HCD57 cells with SP600125 resulted in a JNK-dependent inhibition of phosphorylation of Bad (Supplemental Fig. 2).

To test whether JNK1 directly phosphorylates Bad, purified wt GST-JNK1 or kinase-deficient GST-JNK1(APF), in which Thr183 and Tyr185 have been replaced by nonphosphorylatable alanines, was modified in an *in vitro* kinase reaction containing nonradioactive ATP and aliquot of extracts from cells transfected with a constitutively active MEKK1(Yu et al., 2004), isolated by glutathione-Sepharose (GSH) beads, and extensively washed. Under these conditions, wt GST-JNK1 was activated, as it significantly phosphorylated GST-c-Jun (Fig. 3C). Meanwhile, the wt GST-JNK1 dramatically phosphorylated GST-Bad, while the kinase-deficient GST-JNK1 (APF) only has a minor phosphorylation on GST-Bad (Fig. 3C). Thus, these results strongly indicate that Epo-activated JNK1 is a Bad kinase.

3.4 Epo-activated JNK1 phosphorylation of Bad at Thr201

Our previous data indicated that phosphorylation of Bad by JNK1 at threonine 201 contributed to IL-3 mediated cell survival (Deng et al., 2008; Yu et al., 2004). To test whether Epo-activated JNK1 can also phosphorylate Bad at Thr201, basal JNK1 was isolated from Epo-deprived HCD57 cells and active JNK1 was isolated from Epo-stimulated HCD57 cells. Immunoblotting with anti-phospho-Thr201 antibody showed that Epo-activated JNK1 phosphorylated only wt GST-Bad but not the GST-Bad (T201A) mutant (Fig. 4A). Furthermore, in HCD57 cells, expression of the constitutively active MKK7-JNK1 but not the kinase-deficient MKK7 (K149M)-JNK1 resulted in phosphorylation of cotransfected M2-Bad at Thr201 in the absence of Epo (Fig. 4B). To further confirm Epo-activated JNK1 induce Bad phosphorylation at Thr201, HCD57 cells was deprived of Epo for 18 hours, and followed by Epo readdition for 15 minute. Immunoblotting with anti-phospho-Thr201 antibody showed that Bad was specifically phosphorylated at Thr201 after Epo readdition (Fig. 4C, middle panel). The phosphorylation of Bad at Thr201 occurred as early as 15 minutes after Epo readdition, corresponding to the initiation of JNK1 activation by Epo (Fig. 4C, upper panel). Phosphorylation of Bad by JNK1 at Thr201 may reduce Bad association with Bcl-X_L thus inhibiting the pro-apoptotic activity of Bad. To test the Bad and Bcl-X_L interaction in response to Epo stimulation, wt GST-Bad or mutant GST-Bad (T201A) proteins were subjected to phosphorylation by Epo-activated JNK1 in the presence of nonradioactive ATP. GST-pull down assays revealed that phosphorylation by active

JNK1 significantly reduced the binding of wt GST-Bad but not mutant GST-Bad (T201A) to ³⁵S-labeled Bcl-X_L (Fig. 4D).

To further confirm that Bad phosphorylation at Thr201 regulates the pro-apoptotic activity of Bad, HCD57 cells stably expressing wt Bad or the Bad(T201A) mutant were used to determine their susceptibility to Epo withdrawal-induced apoptosis. Immunoblotting showed that the expression of Bad (T201A) mutant was similar to that of wt Bad (Fig. 4E). However, cells expressing the Bad (T201A) mutant were more sensitive to Epo withdrawal-induced apoptosis than cells expressing wt Bad (Fig. 4F). Taken together, these data suggests that Bad phosphorylation by JNK1 at Thr201 is involved in the Epo signaling for cell survival.

4. Discussion

Although first identified as a stress-related kinase that was associated with the function of apoptosis, JNK has recently been shown to play a critical role in many cellular activities, from growth control to programmed cell death. (Liu and Lin, 2007; Wagner and Nebreda, 2009). We have previously demonstrated that JNK1 was involved in growth factor-induced cell survival (Deng et al., 2008). Here we showed that JNK1 activation is also required for the Epo-mediated cell survival through phosphorylation and inactivation of Bad. This conclusion is based on the following observations.

First, JNK1 was activated by Epo, which is a survival cytokine for the production of mature erythroid cells (Figs. 1C and 1D). Second, the JNK inhibitor SP600125 suppressed Epo-mediated cell survival and promoted Epo withdrawal-induced cell death (Figs. 2B and 2C). Third, expression of the constitutively active MKK7-JNK1 but not the kinase-deficient MKK7 (149M)-JNK1 inhibited Epo withdrawal-induced apoptosis (Fig. 2E). Fourth, JNK1 phosphorylated and inactivated the pro-apoptotic molecule Bad (Figs. 3A and 3B). Taken together, our results demonstrate that JNK1 functions as an anti-apoptotic molecule to suppress Epo withdrawal-induced apoptosis in murine erythroleukemia HCD57 cells.

Our finding that Epo-induced JNK1 phosphorylation of Bad at Thr201 as early as 15 minutes followed by Epo readdition is consistent with our previous report of IL-3-induced JNK1 activation (Deng et al., 2008). In our Epo withdrawal experiments, the HCD57 cells were incubated in the absence of Epo for 18 hours, which was 14 hours longer than the previous report in a similar experiment (Jacobs-Helber and Sawyer, 2004). This withdrawal of Epo for the duration of 18 hours resulted in an up-regulation of the cell surface receptors for Epo by 10-fold or more over cells maintained in Epo (Sawyer and Hankins, 1993). In addition, this prolonged absence from Epo also resulted in complete quiescence of Epo signaling and this enabled us to observe higher level of signaling activation upon Epo readdition in HCD57 cells (Jacobs-Helber et al., 1997). Moreover, the HCD57 cells did not undergo significant apoptosis after the withdrawal of Epo for 18 hours (Fig. 1A, Figs. 2B and 2C). Thus, we withdrew Epo for 18 hours to completely silence of the Epo signaling pathway.

The fact that the JNK inhibitor SP600125 promoted Epo withdrawal-induced apoptosis in a dose-dependent manner suggests that JNK1 may play an important role in Epo-dependent cell survival. However, less than 40% apoptosis reduction by 15 μ M SP600125 addition in the presence of Epo indicates that signaling pathways other than JNK may also be involved in regulating the survival of HCD57 cells. Several signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K) (Zhande and Karsan, 2007), nuclear factor (NF)- κ B and Janus kinase 2 pathways (Digicaylioglu and Lipton, 2001) are known to be involved

in the anti-apoptotic functions of Epo. Further studies are needed to investigate the cross talk between JNK and these signaling pathways.

The phosphorylation of Bad provides an important connection between survival signaling pathways and the intrinsic cell death machinery. Withdrawal of growth/survival factors leads to dephosphorylation of Bad, which translocates to the mitochondria, where it interacts with pro-survival Bcl-2 family proteins like Bcl-X_L and thereby allowing the oligomerization of Bax and Bak (Cheng et al., 2001). This results in increased mitochondria permeability, release of cytochrome *c*, caspase activation and ultimately apoptotic cell death. Our results indicating that phosphorylation of Bad at Thr201 by Epo-activated JNK1 significantly reduced the association of Bad with Bcl-X_L *in vitro* (Fig. 4D), thus inhibiting the pro-apoptotic activity of Bad, is consistent with our previous reports (Deng et al., 2008; Yu et al., 2004). Since the surrounding amino acids of Thr201 do not comprise the 14-3-3 binding site (Yu et al., 2004), it is possible that binding to 14-3-3 proteins will not be the only mechanism for sequestering Bad in the cytoplasm. Glucokinase (Danial et al., 2003) and phosphofructokinase-1 (Deng et al., 2008) have been shown to associate with Bad in the cytosol and contribute to the cell survival. Further studies are needed to explore whether glucokinase or phosphofructokinase-1 participated in reducing the binding affinity of Bad to Bcl-X_L and preventing the translocation of Bad to the mitochondria in HCD57 cells.

In conclusion, here we reported that JNK1 activation is required for Epo-mediated cell survival. Epo-activated JNK1 phosphorylated Bad at threonine 201, thereby inhibiting Bad association with Bcl-X_L. Replacement of threonine201 by alanine in Bad promoted Epo withdrawal-induced apoptosis. Thus, our results provide a molecular mechanism by which JNK1 contributes to the survival of erythroid cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Epo	Erythropoietin
Bad	bcl-associated death protein
JNK	c-Jun N-terminal protein kinase
Bcl-X_L	B-cell lymphoma-extra large
KA	kinase assays
IB	immunoblotting
IP	immunoprecipitation
CBB	coomassie brilliant blue staining
GST	glutathione S transferase

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Fig. 1. JNK1 is an Epo-activated protein kinase

A, HCD57 cells were deprived of Epo for 18 hrs (–Epo), followed by Epo readdition (+Epo). Apoptotic cell death was measured with PI staining. The results are presented as means $\pm$ standard error and represent three individual experiments (* $p < 0.01$ compared to 0 h control; # $p < 0.05$ compared to 0 h control). B, HCD57 cells were deprived of Epo for the indicated times (–Epo) and the apoptotic cell death was measured. The specific caspase inhibitor Z-VAD-FMK was added 30 min prior to Epo withdraw (* $p < 0.01$ compared to Z-VAD-FMK untreated control). C, HCD57 cells were deprived of Epo for indicated times (–Epo), and the JNK1 activity was measured by immune complex kinase assays (KA) using GST-c-Jun as a substrate and the expression level of JNK was detected by immunoblotting (IB) using anti-JNK antibody. D, HCD57 cells were deprived of Epo for 18 hrs (–Epo), followed by Epo readdition (+Epo, 10 U/ml) for 5–120 min, and the JNK1 activity and expression level of JNK were measured as described in Fig. 1C.

Fig. 2. JNK1 activation is required for preventing Epo withdrawal-induced apoptosis

A, HCD57 cells were deprived of Epo for 18 hrs (–Epo), followed by Epo readdition (+Epo, 10U/ml) for 5 or 15 min. The specific JNK inhibitor SP600125 (10 μM) was added 30 min prior to Epo readdition. JNK1 activity and expression level of JNK were measured as described in Fig. 1C. B and C, HCD57 cells were deprived of Epo for 18 hrs, followed by Epo readdition (+Epo, B), or left untreated (–Epo, C) in the presence of SP600125 (5, 10, 15 μM, respectively). Apoptotic cell death was measured as described in Fig. 1A (#, $p < 0.05$ compared to SP600125 untreated control; *, $p < 0.01$ compared to SP600125 untreated control). D, HA-MKK7-JNK1 and MKK7 (KM)-JNK1 were immunoprecipitated with anti-HA antibody from HCD57 cells that stably express HA-MKK7-JNK1 or HA-MKK7 (KM)-JNK1, respectively. The activity and expression level of HA-MKK7-JNK1 or HA-MKK7 (KM)-JNK1 were measured as described (Yu et al., 2004). E, HCD57 cells stably express HA-MKK7-JNK1 or HA-MKK7 (KM)-JNK1 were deprived of Epo (–Epo) for the time indicated. Apoptotic cell death was analyzed as described in Fig. 1A (* $p < 0.01$ compared to 0 h control).

Fig. 3. JNK1 is an Epo-activated Bad kinase

HCD57 cells were deprived of Epo for 6 or 18 hrs, followed by Epo readdition (10 U/ml) for 15 or 30 min. A, Ser136 phosphorylation and expression of Bad were detected by immunoblotting (IB) using anti-phospho-Bad-Ser136 antibody (upper panel) or anti-Bad antibody (lower panel). B, Phosphorylation of GST-Bad (upper panel) or GST-c-Jun (middle panel) by Epo-activated JNK1 was measured by immune complex kinase assays (KA). C, Phosphorylation of GST-Bad or GST-c-Jun by activated recombinant GST-JNK1 *in vitro*. Inactive GST-JNK1 (APF) was used as a control.

Fig. 4. Epo-activated JNK1 phosphorylation of Bad at Thr201

A, Purified wt GST-Bad and GST-Bad (T201A) mutant were phosphorylated by Epo-activated JNK1 in the presence of nonradioactive ATP (17 μ M) and analyzed by immunoblotting using anti-phospho-Thr201 antibody (upper panel). The amount of GST-Bad and GST-Bad (T201A) mutant was examined by immunoblotting (IB) using anti-Bad antibody (lower panel). B, HCD57 cells were transfected with expression vector encoding M2-Bad along with HA-MKK7-JNK1, HA-MKK7 (K149M)-JNK1, or empty vector. After 24 hrs, cells were deprived of Epo for 18 hrs. Thr201 phosphorylation and expression of Bad were analyzed as described in Fig. 4A. C, HCD57 cells were deprived of Epo for 18 hrs (-Epo), followed by Epo readdition (+Epo, 10 U/ml) for 15 min. Activation of JNK1 was determined by immune complex kinase assay (KA, upper panel). Thr201 phosphorylation and expression of Bad were analyzed as described in Fig. 4A. D, wt GST-Bad and GST-Bad (T201A) mutant proteins modified with nonradioactive ATP were mixed with *in vitro* translated ³⁵S-labeled Bcl-X_L for 12 hrs and extensively washed. GST-Bad-associated ³⁵S-Bcl-X_L was visualized by radio autography. One-tenth of the input ³⁵S-Bcl-X_L was analyzed by radio autography. The amount of GST-Bad proteins was examined by CBB staining. E,

Immunoblotting (IB) analysis of Bad (wt or T201A) in cell extracts from corresponding stable cell lines with anti-M2 antibody. F, HCD57 cells stably express Bad (T201A) mutant were more sensitive to Epo withdrawal-induced apoptosis. Apoptotic cell death was analyzed as described in Fig. 1(* $p < 0.01$ compared to 0 h control).