The death domain-containing kinase RIP1 regulates p27^{Kip1} levels through the PI3K–Akt–forkhead pathway

Seongmi Park¹, Deepti B. Ramnarain¹, Kimmo J. Hatanpaa^{2,4}, Bruce E. Mickey^{3,4}, Debabrata Saha⁵, Ramasamy Paulmurugan⁶, Christopher J. Madden^{3,4}, Paul S. Wright¹, Salman Bhairi¹, M. Aktar Ali⁵, Krishna Puttaparthi¹, Wei Hu¹, Jeffrey L. Elliott¹, Olaf Stuve¹ & Aryn A. Habib^{1,4,7+}

¹Department of Neurology, ²Department of Pathology, ³Department of Neurosurgery, ⁴Annette G. Strauss Center for Neuro-Oncology, ⁵Department of Radiation Oncology, and ⁷Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, Texas, USA, and ⁶Molecular Imaging Program at Stanford, Department of Radiology and the Bio-X Program, Stanford University School of Medicine, Stanford, California, USA

Elucidating the cross-talk between inflammatory and cell proliferation pathways might provide important insights into the pathogenesis of inflammation-induced cancer. Here, we show that the receptor-interacting protein 1 (RIP1)—an essential mediator of inflammation-induced nuclear factor- κ B (NF- κ B) activation—regulates p27^{Kip1} levels and cell-cycle progression. RIP1 regulates p27^{Kip1} levels by an NF- κ B-independent signal that involves activation of the phosphatidylinositol 3-kinase (PI3K)–Akt–forkhead pathway. Mouse embryonic fibroblasts (MEFs) from RIP1-knockout mice express high levels of p27^{Kip1}. Reconstitution of MEFs with RIP1 downregulates p27^{Kip1} levels in a PI3K-dependent manner. RIP1 regulates p27^{Kip1} at the messenger RNA level by regulating the p27^{Kip1} promoter through the forkhead transcription factors. RIP1 expression blocks accumulation of cells in G₁ in response to serum starvation and favours cell-cycle progression. Finally, we show that overexpression of p27^{Kip1} blocks the effects of RIP1 on the cell cycle. Thus, our study provides a new insight into how components of inflammatory and immune signalling pathways regulate cell-cycle progression.

Keywords: receptor-interacting protein; RIP1; cell cycle; p27^{Kip1}; PI3K; Akt

EMBO reports (2008) 9, 766–773. doi:10.1038/embor.2008.102

INTRODUCTION

Inflammatory and immune responses induce cellular proliferation by various mechanisms, which include the release of cytokines and growth factors (Karin & Greten, 2005). Chronic inflammation has an important role in the development and progression of cancer, affecting various organs such as lung, colon, stomach and liver (Balkwill & Mantovani, 2001). The transcription factor nuclear factor- κ B (NF- κ B) is known to be a crucial component of the inflammation and might also have an important role in the pathogenesis of inflammation-induced cancer (Karin, 2006). Cross-talk between inflammatory and oncogenic signalling pathways might be important in cancer (Albanese *et al*, 2003; Hu *et al*, 2004; Chien *et al*, 2006; Schneider *et al*, 2006; Perkins, 2007).

The death domain-containing kinase receptor-interacting protein 1 (RIP1, RIPK1) is an essential component of the signalling cascade that leads to NF- κ B activation in response to cellular stress (Festjens *et al*, 2007). RIP1 is composed of a kinase, intermediate and death domain (Stanger *et al*, 1995), and is expressed both constitutively and inducibly in response to cytokines (Meylan & Tschopp, 2005).

The phosphatidylinositol 3-kinase (PI3K)–Akt pathway has a crucial role in cell-cycle progression and the regulation of p27^{Kip1}. PI3K–Akt-mediated regulation of p27^{Kip1} seems to occur at various levels and includes control of p27^{Kip1} expression as well as a decrease in protein stability (Liang & Slingerland, 2003). The forkhead/FoxO transcription factors regulate p27^{Kip1} transcription (Tran *et al*, 2003) and Akt has been reported to inhibit the transcription of p27^{Kip1} by inactivating the forkhead transcription factors (Medema *et al*, 2000).

Here, we show that RIP1 is important in the regulation of p27^{Kip1} and cell-cycle progression. We show that RIP1 expression is sufficient to downregulate p27^{Kip1}, phosphorylate retinoblastoma protein (Rb) and inhibit the accumulation of cells in G₁ by activation of the PI3K–Akt–forkhead pathway. As RIP1 is known to have an important role in inflammatory responses, our findings

¹Department of Neurology, ²Department of Pathology, ³Department of Neurosurgery, ⁴Annette G. Strauss Center for Neuro-Oncology, ⁵Department of Radiation Oncology, and ⁷Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Mail Code 8813, Forest Park Drive, ND4.136, Dallas, Texas 75390-8813, USA

⁶Molecular Imaging Program at Stanford, Department of Radiology and the Bio-X Program, Stanford University School of Medicine, Stanford, California, USA

⁺Corresponding author. Tel: +1 214 645 6237; Fax: +1 214 645 6240;

E-mail: amyn.habib@utsouthwestern.edu

Received 1 April 2008; revised 7 May 2008; accepted 7 May 2008; published online 20 June 2008

Fig 1 | Receptor-interacting protein 1 regulates p27^{Kip1} levels. (A) Western blot shows that p27^{Kip1} levels are increased in primary MEFs from RIP1^{-/-} mice compared with wild-type controls (RIP1^{+/+} cells). Cells were cultured in DMEM with 10% FBS. (B) RIP1^{+/+} and RIP1^{-/-} immortalized cells were serum starved for 72 h followed by western blot. (C) Introduction of RIP1 (using adenovirus) into RIP1^{-/-} MEFs prevents p27^{Kip1} accumulation in response to serum starvation (48 h). In addition, RIP1 downregulates p27^{Kip1} accumulated in response to serum starvation (96 h, RIP1 was added after 48 h of serum starvation). In RIP1 (-) lanes, tetracycline was added to prevent the expression of RIP1. (D) Overexpression of RIP1 in U87MG cells prevents the accumulation of p27^{Kip1} in response to serum starvation for 48 h. RIP1 was introduced into cells at the onset of serum starvation. Densitometry values show relative signal intensity normalized for ERK2 (loading) signal. (E,F) Expression of RIP1 for 48 h does not affect (E) p21^{Cip1} or (F) CDK4 levels in RIP1^{-/-} MEFs. (G) Expression of RIP1 in RIP1^{-/-} MEFs results in phosphorylation of Rb. (H) RIP1 influences p27^{Kip1} at the mRNA level. Northern blot probed with a mouse probe for p27^{Kip1} and also with a GAPDH probe to check loading. RIP1^{-/-} MEFs were infected with Ad-RIP1 overnight followed by RNA extraction and northern blot. Three independent experiments were performed and a representative experiment is shown. CDK4, cyclin-dependent kinase 4; ERK2, extracellular signal-regulated kinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MEF, mouse embryonic fibroblast; pRb, phosphorylated Rb; Rb, retinoblastoma protein; RIP1, receptor-interacting protein 1.

suggest that RIP1 might participate in mechanisms involved in cellular proliferation during immune and inflammatory states.

RESULTS

RIP1 regulates expression of p27^{Kip1}

We observed a higher level of p27^{Kip1} in RIP1^{-/-} mouse embryonic fibroblasts (MEFs) compared with RIP1^{+/+} MEFs, both in cells growing in DMEM with 10% FBS and in those growing under serum-starved conditions (Fig 1A,B). Next, by using a tetracycline-inducible adenoviral vector (tet-off), we introduced RIP1 into RIP1^{-/-} MEFs that had been serum starved, either at the onset of serum starvation or 48 h after serum starvation. Introduction of RIP1 resulted in downregulation of p27^{Kip1} under both conditions (Fig 1C). Similarly, the introduction of RIP1 into many other cell lines such as U87MG (Fig 1D), T98G and Rat-1 cells (data not shown) downregulated p27^{Kip1} levels, showing that RIP1 is a potent negative regulator of p27^{Kip1} levels. The effect of RIP1 on p27^{Kip1} was also detected in the presence of serum (supplementary Fig 5C online); however, RIP1 expression did not change the levels of two other cell-cycle proteins, p21^{Cip1} and cyclin-dependent kinase 4 (CDK4; Fig 1E,F).

p27^{Kip1} is an inhibitor of several cyclin-dependent kinases, particularly cyclin E/CDK2, and one of the outcomes of p27^{Kip1} downregulation is phosphorylation of the Rb. Introduction of RIP1 into cells resulted in Rb phosphorylation (Fig 1G), as shown by a mobility shift in the Rb blot and by an increased signal on the pRb blot.

Next, we compared the levels of p27^{Kip1} messenger RNA in RIP1^{-/-} MEFs with those in RIP1^{-/-} MEFs reconstituted with RIP1 (RIP1^{-/-} MEFs + RIP1) by using northern blot and found that RIP1

downregulated p27^{Kip1} mRNA levels (Fig 1H) but not its protein stability (supplementary Fig 1 online).

RIP1 regulates p27^{Kip1} through PI3K

To investigate the signalling pathway used by RIP1 to downregulate p27^{Kip1}, we introduced RIP1 into RIP1^{-/-} MEFs and studied the effect of inhibition of either PI3K or MAP-kinase kinase on RIP1-induced p27^{Kip1} downregulation. Cells were serum starved for 72 h to induce p27^{Kip1}. RIP1 was introduced into cells along with the inhibitors at the onset of serum starvation. RIP1 efficiently downregulated p27^{Kip1} levels under all conditions except when PI3K was inhibited with LY294002 (Fig 2A). PI3K inhibition did not have a significant effect on RIP1 expression. This suggests that RIP1 downregulates p27^{Kip1} through the PI3K pathway, whereas MEK inhibition (U0126) has no effect.

RIP1 activates the PI3K-Akt pathway

A previous study has shown that RIP1 is essential for lipopolysaccharide-induced Akt activation by toll-like receptor 4 (TLR4; Vivarelli et al, 2004); however, whether RIP1 has a direct effect on

Fig 2 | Receptor-interacting protein 1 induces activation of Akt and downregulates p27^{Kip1} through a phosphatidylinositol 3-kinase-dependent pathway. (A) Western blot showing that LY294002 (LY; 10 μ M) prevents RIP1-mediated downregulation of p27^{Kip1} in RIP1^{-/-} MEFs. The lower panel shows RIP1 expression and shows that RIP1 expression is similar in LY294002-treated cells compared with DMSO-treated cells. Cells were cultured in DMEM with 10% FBS in experiments B–G. (B) Western blot showing that introduction of RIP1 adenovirus into an RIP1^{-/-} immortalized MEF cell line for 24 h induces phosphorylation of Akt (S473), whereas introduction of GFP or p27^{Kip1} adenovirus does not. (C) Introduction of RIP1 into RIP1^{-/-} cells also results in phosphorylation of Akt on Thr 308 (T308). (D) Akt kinase assay using Akt immunoprecipitated from RIP1^{-/-} immortalized MEFs with or without RIP1 reconstitution. Akt was immunoprecipitated 48 h after RIP1 infection. (E) The kinase domain of RIP1 is not required for activation of pAkt or downregulation of p27^{Kip1}. Wild-type RIP1 (WT) or a kinase domain deletion mutant (DKD) was introduced into RIP1^{-/-} cells followed by western blot. The middle panel shows expression of wild-type RIP1 and the DKD mutant. Densitometry values show relative signal intensity normalized for ERK2 (loading) signal. (F) RIP1-induced phosphorylation of Akt blocked by LY294002 (10 μ M). (G) RIP1 expression induces phosphorylation of GSK3. (H) RIP1 expression induces phosphorylation of p70S6K (Thr 389). Three independent experiments were performed and a representative experiment is shown. ERK2, extracellular signal-regulated kinase 2; GFP, green fluorescent protein; GSK3, glycogen synthase kinase-3; MEF, mouse embryonic fibroblast; pAkt, phosphorylated Akt; RIP1, receptor-interacting protein 1.

PI3K–Akt activation is not known. It was found that introduction of adenoviral RIP1 into RIP1^{-/-} MEFs resulted in a robust phosphorylation of Akt as tested by phospho-Akt (S473 and T308) antibodies (Fig 2B,C). As a control, we infected RIP1^{-/-} MEFs with green fluorescent protein or p27^{Kip1} in adenoviral vectors, but did not detect any Akt activation with either (Fig 2B). The level of Akt was not altered by RIP1. Ectopic expression of RIP1 into a variety of other cell types also resulted in Akt phosphorylation (supplementary Fig 2 online). We also confirmed RIP1-induced PI3K–Akt activation by performing immune complex kinase assays (Fig 2D). The kinase domain of RIP1 was not required for Akt activation or

p27^{Kip1} downregulation, as deletion of the kinase domain (DKD) did not impair either (Fig 2E). Use of a PI3K inhibitor (LY294002) resulted in a block of RIP1-mediated Akt phosphorylation (Fig 2F). Finally, we found that introduction of RIP1 into cells also resulted in phosphorylation of cellular glycogen synthase kinase-3 (GSK3; Fig 2G) and of p70 S6K, which is a widely used indicator of mTOR (mammalian target of rapamycin) activation (Fig 2H).

RIP1 influences the activity of the p27^{Kip1} promoter

From the observation that RIP1 regulates p27^{Kip1} through a PI3K-dependent pathway and regulates p27^{Kip1} at the mRNA

Fig 3 | Receptor-interacting protein 1 suppresses the p27^{Kip1} promoter by inhibiting the forkhead transcription factors. (A) RIP1 inhibits the p27^{Kip1} promoter in reporter assays in a dose-dependent manner. 293 cells were transfected with wild-type RIP1 along with p27^{Kip1}-Luc (p27-Luc). Luciferase activity was measured after 24 h. (B) 293 cells were transfected with wild-type RIP1 or mutant RIP1 along with p27^{Kip1}-Luc. The RIP1 mutants used here are deletions of the kinase domain (RIP1-DKD), intermediate domain (RIP1-DID) or death domain (RIP1-DDD). (C) RIP1 inhibits the activity of forkhead transcription factors in reporter assays. An FHRE-Luc (pGL3-E4-DBEx6) plasmid was co-transfected into 293 cells with either RIP1 or empty vector and luciferase activity was measured after 24 h. (D) The effect of RIP1 on the p27^{Kip1} promoter is rescued by either wild-type FoxO3a or a constitutively active (AAA) mutant FoxO3a. The experiment was conducted in 293 cells by transfecting cells with RIP1 plus p27^{Kip1}-Luc and either empty vector or wild-type or mutant FoxO3a. (E) Mutation of a FoxO response element in the p27^{Kip1}-Luc promoter abolishes the ability of RIP1 to suppress the p27^{Kip1} promoter. In this experiment, RIP1 was co-transfected into 293 cells with either wild-type or a mutant p27^{Kip1}-Luc promoter followed by luciferase assays. In all reporter assays, *Renilla* luciferase activity was measured as an internal control. Three independent experiments were performed in triplicate and a representative experiment is shown. Expression of RIP1 was confirmed by western blot (data not shown). RIP1, receptor-interacting protein 1; RLU, RLU, relative luciferase unit.

level, we examined the effect of RIP1 expression on the p27^{Kip1} promoter by using reporter assays. RIP1 strongly inhibited the activity of the p27^{Kip1} promoter linked to luciferase in a dose-dependent manner ($P < 0.05$; Fig 3A). Deletion of the death domain of RIP1 led to a significant loss in the ability of RIP1 to suppress the p27^{Kip1} promoter ($P < 0.001$), whereas deletion of the kinase domain had the least effect (Fig 3B). Expression of the Flag-tagged RIP1 mutants was detected by western blotting (supplementary Fig 3 online). In general, death domains act as protein interaction domains with other death domains, and a loss of the inhibitory effect of RIP1 on p27^{Kip1} in the death domain

deletion mutant might reflect the need for interaction with another death domain protein.

RIP1 inhibits the forkhead transcription factors

We tested the ability of RIP1 to inhibit the activity of a forkhead-Luc reporter (pGL3-E4-DBEx6) in 293 cells. As shown in Fig 3C, RIP1 produced a significant repression of forkhead activity ($P < 0.024$). Furthermore, expression of a wild-type FoxO3a or a constitutively active mutant FoxO3a rescued RIP1-mediated repression of the p27^{Kip1} promoter ($P < 0.05$; Fig 3D). Expression of the Myc-tagged wild-type and constitutively active mutant

Fig 4 | Receptor-interacting protein 1 blocks accumulation in G₁. (A) Addition of RIP1 prevents serum starvation-induced cell-cycle arrest. RIP1^{-/-} MEFs were cultured in serum-depleted conditions (72 h) with or without RIP1. RIP1 adenovirus was introduced into cells at the onset of serum starvation. After treatment of cells with 10% serum for 6 or 24 h, cells were collected and analysed by FACS. Serum starvation induces cell-cycle arrest in cells in G₀/G₁, whereas expression of RIP1 prevents cell-cycle arrest. (B) RIP1 expression is sufficient to induce cell-cycle progression. RIP1^{-/-} MEFs were cultured in serum-depleted conditions for 3 days. RIP1 was added to cells on day 3 for 24 h. As a positive control, cells were treated with 10% serum (for 24 h). Cells were collected for FACS analysis. (C) Increased expression of p27^{Kip1} blocks the effect of RIP1 on cell-cycle progression, whereas GFP has no effect. U87MG cells were serum starved for 72 h. After 48 h of serum starvation, RIP1 was added with either GFP or p27^{Kip1} adenovirus for 24 h. RIP1 blocks the accumulation of cells in G₁ in GFP-infected cells but not in p27^{Kip1}-overexpressing cells. (D) Expression of p27^{Kip1} and RIP1 was tested by western blot in parallel. (E) U87MG cells were serum starved for 48 h followed by introduction of RIP1 for 24 h. At 24 h after RIP1 addition, nocodazole was added for a further 16 h followed by FACS analysis. As RIP1 favours cell-cycle progression, in the RIP1-expressing cohort, more cells are trapped in the G₂/M phase as a result of nocodazole treatment. (F) A BrdU incorporation assay shows that the addition of RIP1 to RIP1^{-/-} cells results in significantly increased BrdU incorporation. Three independent experiments were performed with similar results and a representative experiment is shown. BrdU, 5-bromodeoxyuridine; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; RIP1, receptor-interacting protein 1.

FoxO3a was confirmed by western blotting (supplementary Fig 4 online). Finally, mutating a FoxO response element in the p27^{Kip1} promoter resulted in a loss of the ability of RIP1 to inhibit the p27^{Kip1} promoter ($P < 0.05$; Fig 3E). These findings suggest that RIP1 downregulates p27^{Kip1} by inhibition of the forkhead transcription factors.

NF- κ B is not required for downregulation of p27^{Kip1}
LY294002 has only a minor inhibitory effect on RIP1-induced activation of NF- κ B-Luc and fails to block RIP1-mediated downregulation of I κ B α (supplementary Fig 5A,B online), arguing against a requirement for PI3K-Akt activation in RIP1-induced NF- κ B activation. Next, we investigated whether RIP1

Fig 5 | Receptor-interacting protein-mediated downregulation of p27^{Kip1} and FoxO does not require JNK activity. (A) Transduction of RIP1^{-/-} cells with RIP1 adenovirus results in JNK activation. JNK activity was assessed by measuring cJun phosphorylation. (B) Transduction of JNK1^{+/+} or JNK1^{-/-} MEFs with RIP1 adenovirus. The basal level of p27^{Kip1} is higher in the JNK1^{-/-} MEFs compared with the wild-type MEFs (WT), but introduction of RIP1 results in a sharp decrease in p27^{Kip1} levels. (C) cJun is not phosphorylated in JNK1^{-/-} cells after TNF-α stimulation. (D) Phosphorylation of cJun after transduction of JNK2^{+/+} or JNK2^{-/-} MEFs with RIP1 adenovirus. (E) RIP1-mediated downregulation of p27^{Kip1} is blocked by PI3K inhibition but not by JNK inhibition. Treatment of RIP1^{-/-} cells with RIP1 and inhibitors was as described in Fig 2A. SP600125 and LY294002 concentration was 10 μM. (F) SP600125 inhibits anisomycin-induced JNK activation in RIP1^{-/-} cells. SP600125 was added 10 min before the addition of anisomycin. (G) RIP1-mediated downregulation of FoxO activity is blocked by PI3K inhibition but not by JNK inhibition. An FHRE-Luc plasmid was co-transfected into 293 cells with either RIP1 or empty vector, and DMSO, SP600125 or LY294002 was added at the time of transfection. Luciferase activity after 24 h is shown normalized to *Renilla* luciferase activity. (H) Flag-RIP1 in the experiment described in (G). (I) SP600125 inhibits anisomycin-induced JNK activation in 293 cells. Throughout this figure, detection of ERK2 was used as a protein loading control. Three independent experiments were conducted and a representative experiment is shown. ERK2, extracellular signal-regulated kinase 2; JNK, Jun amino-terminal kinase; MEF, mouse embryonic fibroblast; PI3K, phosphatidylinositol 3-kinase; RIP1, receptor-interacting protein 1; TNF-α, tumour necrosis factor α.

downregulates p27^{Kip1} by activation of NF-κB. The introduction of RIP1 adenovirus into AT3 cells stably expressing the IκBα super-repressor mutant that does not allow NF-κB activation (Lin *et al*, 1998) failed to block RIP1-mediated downregulation of p27^{Kip1} (supplementary Fig 5C online).

RIP1 regulates cell-cycle progression

RIP1^{-/-} MEFs were serum starved for 72 h followed by the addition of serum for 6 or 24 h, which was followed by cell-cycle analysis. RIP1 was introduced into cells at the onset of serum starvation. As can be seen in Fig 4A (lower panel), introduction of RIP1 into cells prevented the reconstituted cells from arresting in G₁ in response to serum starvation. Next, cells were serum starved

for 48 h and then adenoviral RIP1 was introduced into cells. We found that RIP1 expression was sufficient to induce cell-cycle progression in serum-starved and growth-arrested cells (Fig 4B). Furthermore, RIP1 expression increased 5-bromodeoxyuridine (BrdU) incorporation in serum-starved RIP1^{-/-} MEFs, confirming the effect of RIP1 on S-phase progression (Fig 4F). Next, we showed that p27^{Kip1} overexpression inhibited the effect of RIP1 on the cell cycle (Fig 4C), suggesting that RIP1-mediated cell-cycle progression might be mediated by downregulation of p27^{Kip1}. Expression of p27^{Kip1} and RIP1 is shown in Fig 4D. Finally, the addition of nocodazole, which arrests cells in the G₂/M phase of the cell cycle, to cells grown under serum starvation and then exposed to RIP1, resulted in a greater accumulation of

RIP1-expressing cells in the G₂/M phase of the cell cycle (Fig 4E) compared with control cells, again indicating the effect of RIP1 on the cell cycle.

JNK is not required for downregulation of p27^{Kip1}

As RIP1 is known to activate Jun amino-terminal kinases (JNKs), we investigated whether RIP1 can downregulate p27^{Kip1} in JNK^{-/-} fibroblasts. We confirmed that RIP1 activated JNK in RIP1^{-/-} cells (Fig 5A); however, RIP1 efficiently downregulated p27^{Kip1} in both JNK1^{-/-} and JNK2^{-/-} fibroblasts (Fig 5B,D). Furthermore, inhibiting JNK with SP600125 failed to block both RIP1-mediated downregulation of p27^{Kip1} and inhibition of FoxO activity (Fig 5E,G).

DISCUSSION

The main finding of this study is that RIP1, an essential component of NF- κ B activation pathways, regulates expression of p27^{Kip1} and cell-cycle progression through an NF- κ B-independent pathway involving PI3K–Akt–forkhead. We identified a signalling pathway triggered by RIP1 that led to cell-cycle progression, as shown in the schematic diagram in supplementary Fig 6 online. We have presented genetic evidence that RIP1 regulates p27^{Kip1} levels. RIP1-knockout MEFs express high levels of p27^{Kip1}, and reconstitution of RIP1^{-/-} cells with RIP1 results in a lowering of p27^{Kip1} levels. In addition, phosphorylation of Rb is increased in response to RIP1 expression. Thus, RIP1 influences crucial regulators of G₁-to-S transition and blocks accumulation of cells in G₁.

RIP1 regulates p27^{Kip1} mRNA levels by repressing the p27^{Kip1} promoter, and regulation of p27^{Kip1} by RIP1 is blocked by inhibition of PI3K. Expression of RIP1 is sufficient to induce a potent activation of the PI3K–Akt pathway; however, the kinase activity of RIP1 is not required for activation of PI3K–Akt, as an RIP1 mutant lacking the kinase domain activates Akt and downregulates p27^{Kip1}. This is analogous to the lack of a requirement for the kinase activity of RIP1 in NF- κ B activation. It has been proposed that RIP1 acts as an adaptor in NF- κ B activation (Meylan & Tschopp, 2005), and we propose a similar mechanism for RIP1 in the activation of PI3K, with the death domain having a crucial role. It is known that Akt negatively regulates the expression of p27^{Kip1} by inactivation of forkhead transcription factors. We found that RIP1 suppressed the activity of forkhead transcription factors in reporter assays. Overexpression of a wild-type FoxO3a or a constitutively active mutant FoxO3a inhibits the RIP-mediated suppression of p27^{Kip1} transcription. Finally, mutation of a forkhead-binding site in the p27^{Kip1} promoter abolishes the ability of RIP1 to downregulate p27^{Kip1}. These experiments show that RIP1 negatively regulates p27^{Kip1} expression by activating a PI3K–Akt–forkhead pathway, whereas RIP1-mediated JNK activation does not seem to be important for RIP1-mediated p27^{Kip1} or FoxO regulation (Fig 5).

As RIP1 expression favours cell-cycle progression, RIP1 could contribute to cellular proliferation during states of inflammation. From our recent work, we propose that RIP1 is an important node in the cross-talk between inflammatory and growth factor signalling and cell-cycle progression pathways. Here, we have shown that RIP1 activates the PI3K–Akt pathway and promotes cell-cycle progression. However, consistent with the known complexity of inflammatory and NF- κ B signalling pathways, RIP1 might have antiproliferative/apoptotic or proliferative effects

depending on the cellular context. For example, we have recently shown that RIP1 negatively regulates the expression of EGFR in fibroblasts (Ramnarain et al, 2008). Future studies will investigate the role of RIP1 in cancer.

METHODS

Plasmids and cell lines. Wild-type RIP and deletion mutants of RIP domains were cloned into pcDNA3.1 with a carboxy-terminal Flag tag. Immortalized wild-type and *rip1*^{-/-} 3T3 cell lines were prepared according to established protocols. A forkhead response element linked to Luc (pGL3-E4-BBEx6), FoxO3a wild type and FoxO3aA3 were provided by Dr Diego Castrillon. A putative FoxO transcription factor-binding site (GGTTTGTG) around –683 relative to the translation start site was mutated with primer 5'-GCAAGCCAGAGCAGGTGGGTGGCAGCAGTACC-3' according to the manufacturer's protocol (QuikChangeMultiSite-Directed Mutagenesis Kit, Stratagene, La Jolla, CA, USA).

Antibodies. The antibodies used in this study are listed in the supplementary information online.

Northern blot. RIP^{-/-} MEFs or RIP^{-/-} MEFs reconstituted with RIP for 16 h were used in this experiment and northern blot was conducted using standard protocols (supplementary information online).

Transfection. 293 cells were transfected using the calcium phosphate technique and expression of transfected genes was confirmed by western blot. For transient transfection experiments, cells were collected 24–48 h after transfection.

Luciferase assays. A total of 2×10^5 293 cells were transfected with either p27^{Kip1}-Luc or FHRE-Luc (pGL3-E4-DBEx6) along with RIP plasmids or empty vector, or FoxO plasmids. A dual-luciferase reporter assay system was used according to the instructions of the manufacturer (Promega, Madison, WI, USA). Firefly luciferase activity was measured in a luminometer and normalized on the basis of *Renilla* luciferase activity.

Production of adenovirus expressing RIP1. RIP1 wild type or DKD mutant with a deletion of the kinase domain (deletion of amino acids 1–303) was cloned into an adenoviral vector. This resulted in a Tet operon-minimal CMV promoter-driven cassette in place of the AdE1 region; Ad-tTA (tetracycline-controlled transactivator) was also prepared. A multiplicity of infection of 50 was used in the experiments. A p27^{Kip1} adenovirus was obtained from Vector Biolabs (Philadelphia, PA, USA). Cells were exposed to RIP1 adenovirus in the presence or absence of tetracycline in this Tet-off system.

Cell-cycle analysis. Cell-cycle analysis was performed according to standard protocols. For nocodazole experiments, cells were serum starved for 48 h followed by the addition of RIP1 for 24 h. At 24 h after RIP1 addition, nocodazole (100 ng/ml) was added for a further 16 h followed by cell-cycle analysis.

BrdU incorporation assay. RIP^{-/-} cells were plated in a 96-well plate. Cells were serum starved for 48 h followed by the addition of RIP1 for 24 h and later subjected to a BrdU incorporation assay using a kit obtained from EMD Biosciences (no. QIA58, Gibbstown, NJ, USA) according to the manufacturer's instructions. BrdU was added for 2 h before starting the assay.

Statistical analysis. Error bars represent the means $\pm$ s.d. of three independent experiments. To determine the statistical significance between different samples, we used Student's *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank M. Kelliher for RIP-knockout MEFs, M. Karin for JNK-knockout MEF cell lines, D. Castrillon for FoxO plasmids and T. Sakai for p27^{Kip1} luciferase construct. This work was supported in part by the National Institutes of Health grant CA 78741 to A.A.H. and by funding from the Annette Strauss Center for Neuro-oncology at the University of Texas Southwestern Medical Center.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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