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ORIGINAL ARTICLE

AGC kinases regulate phosphorylation and activation of eukaryotic translation initiation factor 4B

AGM van Gorp[1,7,8], KE van der Vos[1,8], AB Brenkman[2], A Bremer[3], N van den Broek[2], F Zwartkruis[4], JW Hershey[5], BMT Burgering[4], CF Calkhoven[3] and PJ Coffer[1,6]

[1]*Molecular Immunology Laboratory, Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands;* [2]*Department of Endocrinology and Metabolic Diseases, University Medical Center Utrecht, Utrecht, The Netherlands;* [3]*Leibniz Institute for Age Research–Fritz Lipmann Institute, Beutenbergstr, Jena, Germany;* [4]*Department of Physiological Chemistry, Centre for Biomedical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands;* [5]*Department of Biological Chemistry, School of Medicine, University of California, Davis, CA, USA and* [6]*Department of Pediatric Immunology, University Medical Center Utrecht, Utrecht, The Netherlands*

Eukaryotic translation initiation factor 4B (eIF4B) plays a critical role during the initiation of protein synthesis and its activity can be regulated by multiple phosphorylation events. In a search for novel protein kinase B (PKB/c-akt) substrates, we identified eIF4B as a potential target. Using an *in vitro* kinase assay, we found that PKB can directly phosphorylate eIF4B on serine 422 (ser422). Activation of a conditional PKB mutant, interleukin-3 (IL-3) or insulin stimulation resulted in PKB-dependent phosphorylation of this residue *in vivo*. This was prevented by pretreatment of cells with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 or pharmacological inhibition of PKB. Pretreatment of cells with rapamycin, inhibiting mTOR or U0126 to inhibit MEK, had little effect on eIF4B ser422 phosphorylation. In contrast, following amino-acid refeeding, eIF4B ser422 phosphorylation was found to be mammalian target of rapamycin (mTOR)-dependent. We further identified eIF4B ser406 as a novel mitogen-regulated phosphorylation site. Insulin-induced phosphorylation of eIF4B ser406 was dependent on both MEK and mTOR activity. Utilizing a novel translational control luciferase assay, we could further demonstrate that phosphorylation of ser406 or ser422 is essential for optimal translational activity of eIF4B. These data provide novel insights into complex multikinase regulation of eIF4B phosphorylation and reveal an important mechanism by which PKB can regulate translation, potentially critical for the transforming capacity of this AGC kinase family member.
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Correspondence: Dr PJ Coffer, Molecular Immunology Laboratory, Department of Immunology (KC.02.085.2), University Medical Center, Lundlaan 6, 3584 CA Utrecht, The Netherlands.
E-mail: P.J.Coffer@umcutrecht.nl
[7]Current address: Netherlands Forensic Institute, the Hague, The Netherlands.
[8]These authors contributed equally to this work.
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Introduction

Regulation of protein translation is crucial for the specific expression of proteins important for development, differentiation, cell growth and apoptosis (Holland *et al*., 2004b; Mamane *et al*., 2006). The ability of cells to regulate this process allows a rapid response to external stimuli without the necessity of mRNA synthesis, processing and transport. In transformed cells, components of the translation machinery are often deregulated or misexpressed. In addition, changes in the nucleolus; the suborganelle of the nucleus which functions as the centre of ribosome biogenesis, have long been recognized as a reliable marker of cellular transformation (Gani, 1976; Pandolfi, 2004).

Translational control mostly occurs at the level of initiation. The initiation phase of translation is regulated by a number of eukaryotic translation initiation factors (eIFs; Gingras *et al*., 1999; Hershey and Merrick, 2000). Initially, eIF4E binds to the cap structure at the 5′ end of the mRNA. eIF4E is part of a trimeric complex, termed eIF4F, together with scaffolding protein eIF4G and ATPase/RNA helicase eIF4A. eIF4A unwinds the secondary structure of the 5′UTR allowing the 40S ribosomal subunit to bind to the mRNA. The helicase activity of eIF4A is significantly increased by the cofactor, eIF4B (Lawson *et al*., 1989; Rozen *et al*., 1990; Rogers *et al*., 2001). eIF4B itself has three functional domains, namely two mRNA-binding domains (Methot *et al*., 1994; Naranda *et al*., 1994) and a region rich in aspartic acid, arginine, tyrosine and glycine (DRYG domain) necessary for the dimerization and binding to eIF3 (Methot *et al*., 1996b). The two RNA-binding domains have distinct affinities for RNA, the arginine-rich motif binds mRNA with higher affinity and is essential for RNA helicase activity (Methot *et al*., 1994). The RNA recognition motif binds with high affinity to 18S rRNA (Methot *et al*., 1996a). Therefore, besides being a cofactor for eIF4A, eIF4B is thought to exhibit a bridge function between mRNA and rRNA (Methot *et al*., 1994).

Translational control is intimately connected to the regulation of intracellular signal transduction pathways.

Phosphorylation of initiation factors provides an important means to control the rate of mRNA binding (Raught *et al*., 2000). The phosphorylation state of eIF4E, eIF4G, eIF4B and eIF3 positively correlates with both translation and growth rates of the cell. Changes in phosphorylation, and thus translation, occur in response to a wide variety of extracellular signals, including viral infection, heat shock and in response to cellular growth factors and cytokines (Hershey and Merrick, 2000; Mamane *et al*., 2006). Global changes in protein synthesis after these events are relatively small, but a subgroup of mRNAs exhibits a dramatic change in their rate of translation. Rajasekhar *et al*. (2003) recently demonstrated that upon protein kinase B (PKB/c-akt) and RAS signaling, the profile of mRNA associated to polysomes was drastically altered, although the underlying mechanism remains unclear. Interestingly, these mRNAs mainly encoded proteins involved in the regulation of growth, transcription, cell–cell interactions and morphology. Thus, by controlling the translation efficiency, general stimuli, such as growth factors and cytokines, can selectively induce or suppress the translation of specific set of genes, and the deregulation of these cellular mechanisms controlling translation can lead to cellular transformation (Holland *et al*., 2004a).

Mammalian target of rapamycin (mTOR) plays a major role in the regulation of global and specific mRNA translation. mTOR is activated by phosphatidylinositol 3-kinase (PI3K) through PKB either by direct phosphorylation (Nave *et al*., 1999), or by phosphorylation of TSC2, which inactivates its GTPase activity for the small G-protein Rheb, a potent activator of mTOR (Inoki *et al*., 2002). The best-studied downstream targets of mTOR activation are those involved in translation regulation, namely p70S6kinase (p70S6K) and eIF4E-binding proteins, (4E-BPs). p70S6K phosphorylates ribosomal protein S6, whose hyperphosphorylation status correlates with translation activity. The phosphorylation of the inhibitory 4E-BPs is required for their release of the proto-oncogene eIF4E resulting in the increased cap-dependent translation (Richter and Sonenberg, 2005; Ruggero *et al*., 2004). Deregulation of activation of the PI3K pathway is found in a large variety of human cancers (Luo *et al*., 2003), and importantly, the inhibition of translation by a specific mTOR inhibitor, rapamycin, can effectively block the transformation initiated by perturbed PI3K signaling (Guertin and Sabatini, 2005). This indicates that PI3K/PKB/mTOR-mediated regulation of translational control is crucial for the maintenance of neoplasia.

Eukaryotic translation initiation factor 4B has long been known as a hyperphosphorylated protein (Duncan and Hershey, 1984), and eIF4B phosphorylation is responsive to extracellular stimuli, including serum, insulin and phorbol esters (Duncan and Hershey, 1985). However, it had remained elusive which kinase(s) is/are responsible for the phosphorylation of eIF4B. Recently, two reports have been published concerning the regulation of phosphorylation of a specific serine residue (Ser422). Raught *et al*. (2004) implicated p70S6K as the specific Ser422 kinase and subsequently Shahbazian *et al*. (2006) proposed that p70S6K and p90 ribosomal S6 kinase (RSK) were both able to phosphorylate this residue. Both p70S6K and RSK are the members of AGC protein kinase family, which also contains PKB (Parker and Parkinson, 2001). This kinase family is defined by the high homology within their catalytic domains, resulting in similar substrate consensus sequences. The activity of these kinases, however, is differentially regulated, whereas PKB and p70S6K are components of the PI3K–mTOR pathway, RSK is activated by signaling through the small GTPase RAS.

Recent evidence that long-term rapamycin treatment can inhibit PKB activity (Sarbassov *et al*., 2006) made us to reexamine the importance of mTOR signaling versus PKB signaling in the regulation of translation initiation. In this study, we show that PKB *in vitro* and *in vivo* can phosphorylate eIF4B within the RNA-binding domain at serine 422 (Ser422). We demonstrate that PKB is the dominant AGC protein kinase family member phosphorylating Ser422 upon insulin stimulation *in vivo*. We also demonstrate the regulation of a novel phosphorylation site (Ser406) and show that the phosphorylation of this residue is regulated by RSK and p70S6K *in vivo*. Utilizing a novel reporter assay, we demonstrate that mutation of these phosphorylation sites in eIF4B results in decreased translation initiation. These data provide novel insights into the complex regulation of eIF4B phosphorylation *in vivo*. In addition, we demonstrate for the first time that eIF4B phosphorylation is a novel mechanism by which PKB can regulate protein translation and may be critical for the transforming potential of this AGC kinase family member.

Results

Identification of eIF4B as a PKB substrate

To identify novel PKB substrates, we made use of cytokine-dependent bone marrow-derived Ba/F3 cells, which are normally dependent on interleukin (IL-) 3 for their survival and proliferation. To specifically study the role of PKB in phosphorylation events following cellular activation by IL-3, a Ba/F3 cell line stably expressing conditionally active PKBα (myrPKB:ER (estrogen receptor)) was generated, as previously described in van Gorp *et al*. (2006). The activation of myrPKB:ER is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused ER hormone-binding domain. In the presence of 4-OHT, these proteins dissociate, allowing PKB to become rapidly phosphorylated and activated.

myrPKB:ER cells were cytokine starved and the phosphorylation patterns of unstimulated cells were compared to those stimulated with 4-OHT for 15 min. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analysed by 2D gel electrophoreses and western blotting, utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T; Obata *et al*., 2000; Zhang *et al*., 2002). However, as other members of the

AGC kinase family have similar substrate consensus sequences, this antibody can perhaps best be viewed as a phospho-AGC kinase substrate antibody. We observed several proteins whose phosphorylation was upregulated upon PKB activation. Tandem mass spectrometry identified one of these proteins as eIF4B (data not shown).

To identify potential PKB phosphorylation sites in eIF4B, we performed *in silico* analysis using Scansite 2.0 (Obenauer *et al*., 2003). A high stringency analysis of eIF4B identified two serines likely to be phosphorylated by PKB, serines 406 (Ser406) and 422 (Ser422) in the arginine-rich motif region (Figure 1a).

Because of the availability of a specific antibody raised against the phosphorylated Ser422 on eIF4B, we analysed whether PKB activation resulted in phosphorylation of this residue. COS cells were transfected with FLAG-tagged eIF4B, or eIF4B, in which Ser422 had been mutated to alanine. eIF4B was immunoprecipitated and incubated with active PKB. PKB was indeed able to directly phosphorylate eIF4B at Ser422 (Figure 1b, compare lane 1 to 3), and mutation of this site abolished the phosphorylation (Figure 1b, lanes 2 and 4). Importantly, analysis of eIF4B phosphorylation sites after PKB-mediated *in vitro* phosphorylation by mass spectrometry unambigously demonstrated Ser422

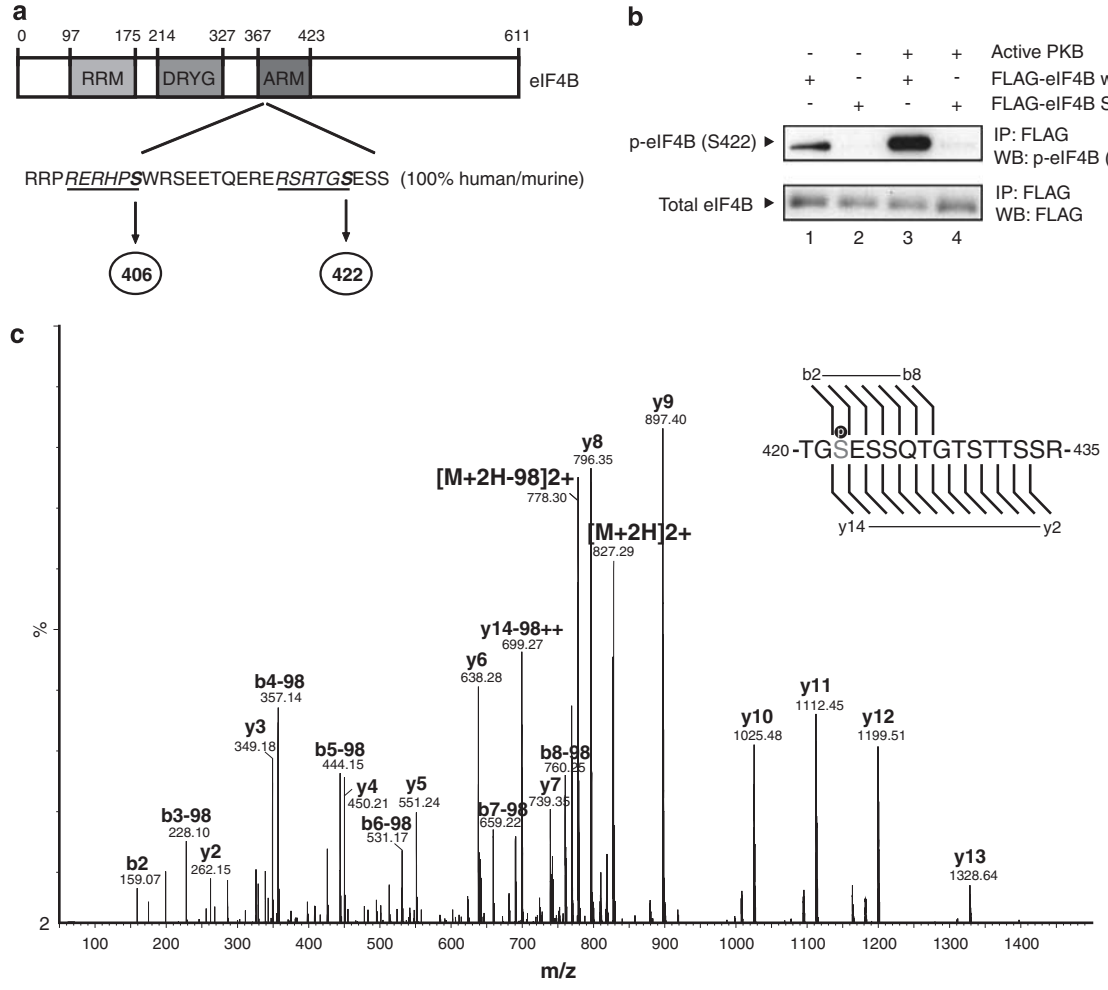

Figure 1 PKB phosphorylates eIF4B on Ser422 *in vitro*. (**a**) Schematic representation of the eIF4B protein. The three functional domains of eIF4B, the RNA recognition motif (RRM), a region rich in aspartic acid, arginine, tyrosine and glycine (DRYG) and the arginine-rich motif (ARM) are shown. Also shown are the two serines (Ser406 and Ser422, bold and italic) within their PKB phosphorylation consensus sequence (underlined) that were identified by *in silico* analysis by Scansite 2.0. Human and mouse eIF4B are 96% homologous. The region in which the two serines are localized is 100% conserved between these two species. (**b**) FLAG-tagged eIF4B or FLAG-tagged eIF4B in which Ser422 was mutated to alanine was phosphorylated by PKB in an *in vitro* kinase assay. Proteins incubated without active PKB were presented as a control. Samples were analysed for levels of phospho-eIF4B (S422) and FLAG. (**c**) Identification of the PKB phosphorylated Ser422 on eIF4B by mass spectrometry. Flag-tagged eIF4B protein was expressed in COS cells and immunoprecipitated protein was phosphorylated *in vitro* by PKB, separated on SDS-PAGE and trypsin digested. The resulting peptides were separated by utilizing TiO_2 phosphopeptide-enrichment columns and subjected to tandem mass spectrometry (LC-MS/MS). MS/MS spectrum and sequence of the Ser422 phosphorylated peptide of eIF4B (AA 420-435) as identified by MASCOT software (see Experimental procedures). Identified b and y ions are indicated. The phosphorylated serine (Ser422) is indicated in gray color. ARM, arginine-rich motif; eIF4B, eukaryotic translation initiation factor 4B; RRM, RNA recognition motif; LC, liquid chromatography; PKB, protein kinase B; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

as the primary phosphorylation site (Figure 1c). Taken together, these data indicate that phosphorylation of Ser422 is an important phosphoacceptor site in eIF4B that can be regulated by PKB *in vitro*.

PKB phosphorylates eIF4B on Ser422 in vivo

To investigate the *in vivo* phosphorylation status of eIF4B, we again made use of the BaF3 myrPKB:ER cell line. BaF3 myrPKB:ER cells were cytokine starved overnight and stimulated with either IL-3 or 4-OHT for the times indicated. Both stimulation with IL-3 and 4-OHT induced phosphorylation of eIF4B on Ser422 as well as that of the PKB substrate forkhead transcription factor, FOXO3 (Dijkers *et al*., 2002b), and the p70S6K substrate ribosomal protein S6 (Figure 2a). As it has been previously demonstrated that the phosphorylation of eIF4B on Ser422 can be mediated by p70S6K activity (Raught *et al*., 2004), we investigated whether the inhibition of its upstream activator, the mTOR/raptor complex, by preincubation of the cells with rapamycin, could abolish phosphorylation on this site. Inhibition of the mTOR/-p70S6K pathway by rapamycin completely

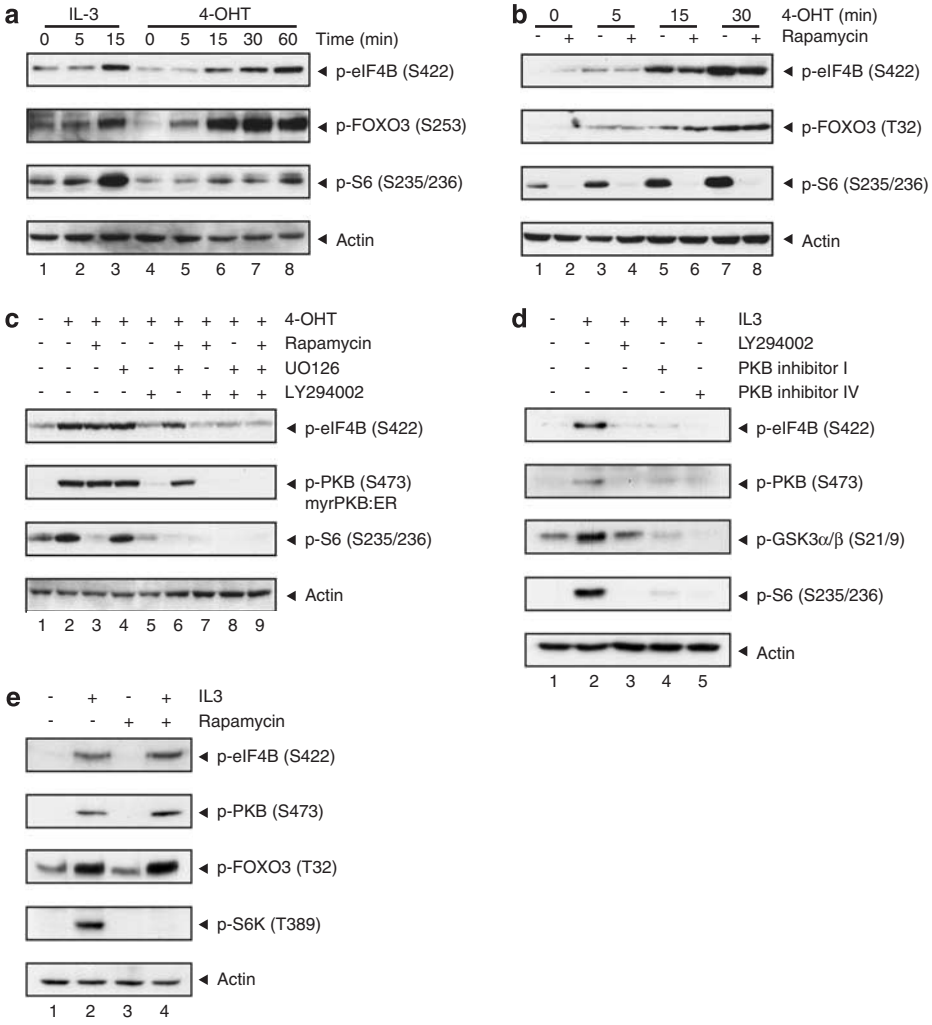

Figure 2 PKB activation is sufficient and necessary for eIF4B phosphorylation of Ser422 in Ba/F3 myrPKB:ER cells. (**a**) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with either IL-3 (10 ng/ml) or 4-OHT (100 nM) for the indicated times; lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phopho-FOXO3 (S253), phospho-S6 (S235/S236) and actin. (**b**) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with 4-OHT (100 nM) for the indicated times after or without pretreatment with rapamycin (20 ng/ml); lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phopho-FOXO3 (T32), phospho-S6 (S235/S236) and actin. (**c**) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with 4-OHT for 15 min after or without pretreatment for 2 h with rapamycin (20 ng/ml), U0126 (15 μM), LY294002 (50 μM) or combinations of these inhibiters; lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and actin. (**d**) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with IL-3 (10 ng/ml) for 15 min after or without pretreatment with LY294002 (25 μM), PKB inhibitor I (20 μM) or PKB inhibitor IV (5 μM); lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-GSK3α/β (S21/9), phospho-S6 (S235/S236) and actin. (**e**) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with IL-3 (10 ng/ml) for 15 min after or without pretreatment with rapamycin (20 ng/ml); lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-FOXO3 (T32), phospho-S6 K (T389) and actin. ER, estrogen receptor; eIF4B, eukaryotic translation initiation factor 4B; IL, interleukin; 4-OHT, 4-hydroxytamoxifen; PKB, protein kinase B.

inhibited phosphorylation of p70S6K target S6, but not the phosphorylation of FOXO3 (Figure 2b). eIF4B phosphorylation was only modestly reduced at later time points by pretreating cells with rapamycin indicating that p70S6K is not responsible for PKB-mediated eIF4B Ser422 phosphorylation (Figure 2b). Previously, Shahbazian *et al*. (2006) proposed that p70S6K and RSK could synergistically regulate eIF4B Ser422 phosphorylation in HeLa cells, when stimulated with serum. We decided to determine whether eIF4B Ser422 phosphorylation was similarly regulated in Ba/F3 cells, when PKB was specifically activated by the addition of 4-OHT. Ba/F3 myrPKB:ER cells were cytokine starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after PKB activation when cells were preincubated with either rapamycin, PI3K inhibitor LY294002, MEK inhibitor U0126 or combinations of these inhibitors. Preincubation with rapamycin abrogated phosphorylation of S6 but again only modestly inhibited eIF4B Ser422 phosphorylation (Figure 2c, lane 3), whereas LY294002 completely abrogated this (Figure 2c, lane 5). LY294002 also inhibited phosphorylation of both PKB and S6 (Figure 2c, lane 5), indicating that the myrPKB:ER protein is still dependent on basal PI3K activity. Pretreatment of cells with U0126 had no effect on the phosphorylation of eIF4B at Ser422 in response to 4-OHT (Figure 2c, lane 4) even when combined with rapamycin (Figure 2c, lane 6). As LY294002 affects several PI3K effectors besides PKB, we cytokine starved Ba/F3 myrPKB:ER cells overnight and analysed IL3-induced eIF4B phosphorylation after pretreatment with LY294002 and two specific PKB inhibitors. Incubation with either LY294002 or the PKB inhibitors completely inhibited the IL3-induced eIF4B Ser422 phosphorylation (Figure 2d). In contrast, pretreatment with rapamycin did not have any effect on the IL3-induced phosphorylation of eIF4B (Figure 2e). Taken together, these data indicate that PKB regulates eIF4B phosphorylation at Ser422, and this is not dependent on either p70S6K or MEK activity.

PKB-mediated phosphorylation of eIF4B in response to insulin

Protein kinase B-β null mutant mice have been shown to be defective in their insulin response and suffer from diabetes (Garofalo *et al*., 2003). This crucial role that PKB plays in mediating the effects of insulin led us to investigate the role of PKB in regulating insulin-stimulated eIF4B phosphorylation. A14 cells were serum starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after stimulation with insulin, when cells were preincubated with rapamycin, LY294002 or U0126. eIF4B Ser422 phosphorylation was increased after insulin stimulation (Figure 3a, lane 2; and Supplementary Figure S1), and pretreatment with either rapamycin or U0126 had no effect on this phosphorylation (Figure 3a, lanes 3 and 4), whereas LY294002 treatment significantly abrogated eIF4B Ser422 phosphorylation (Figure 3a, lane 5). In contrast to LY294002 (Figure 3b, lane 4), a combination of rapamycin and U0126 again did not result in the inhibition of Ser422 phosphorylation (Figure 3b, lane 6). Taken together, these data indicate that insulin also utilizes PKB, and not p70S6K and RSK, to regulate the phosphorylation of eIF4B at Ser422.

mTOR regulates eIF4B phosphorylation in response to amino-acid refeeding

Whereas insulin activates mTOR through class I PI3K and PKB, amino acids can modulate mTOR activity utilizing a distinct pathway, which involves the class III PI3K, hVps34, and this can occur independently of PKB activity (Nobukuni *et al*., 2005). To investigate the role of PKB in eIF4B phosphorylation after amino-acid

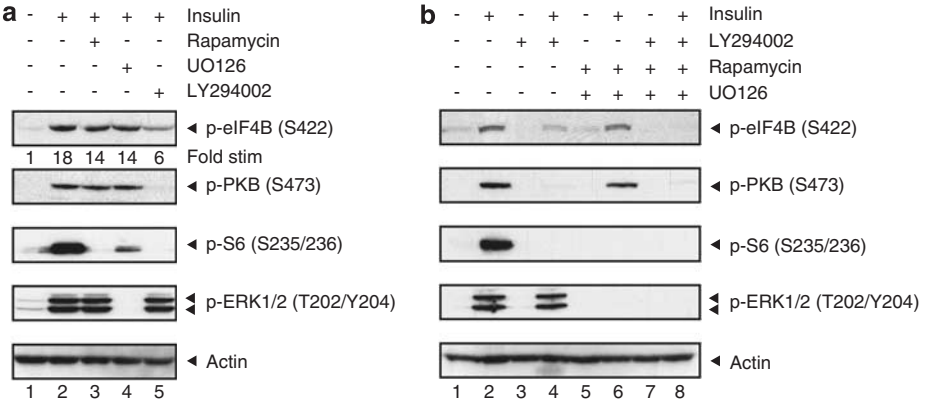

Figure 3 PKB activation is sufficient for eIF4B phosphorylation of Ser422 after insulin stimulation of A14 cells. (**a**) A14 cells were serum starved overnight and left untreated or treated with insulin (1 μg/ml) for 15 min after or without pretreatment for 2 h with rapamycin (20 ng/ml), U0126 (15 μM) or LY294002 (50 μM); lysed and equal amounts of protein were analysed for the levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin. (**b**) A14 cells were serum starved overnight and left untreated or treated with insulin (1 μg/ml) for 15 min after or without pretreatment for 2 h with LY294002 (50 μM), rapamycin (20 ng/ml) and U0126 (15 μM), or all of the above mentioned inhibitors together; lysed and equal amounts of protein were analysed for the levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin. PKB, protein kinase B; eIF4B, eukaryotic translation initiation factor 4B.

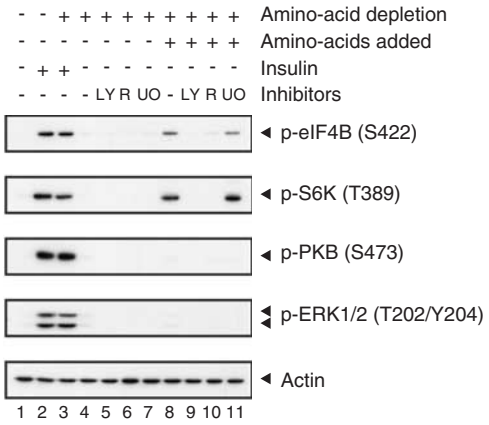

Figure 4 eIF4B Ser422 phosphorylation after amino-acid refeeding is mTOR dependent. A14 cells were serum starved overnight, and then for additional 4 h starved in medium with or without amino acids. Cells were then stimulated with either 1 μg/ml insulin or amino acids for 30 min, after pretreatment for 30 min with or without LY294002 (50 μM), rapamycin (20 ng/ml) or U0126 (15 μM). Cells were subsequently lysed and equal amounts of protein were analysed for levels of phospho-eIF4B (S422), phospho-p70S6K (T389), phospho-PKB (S473), phospho-ERK1/2 (T202/Y204) and actin. eIF4B, eukaryotic translation initiation factor 4B; mTOR, mammalian target of rapamycin.

stimulation, we starved A14 cells in medium without amino acids followed by stimulation for 30 min with amino acids after pretreatment with or without LY294002, rapamycin or UO126. As a control, cells were also stimulated with insulin. As shown in Figure 4, amino-acid stimulation resulted in strong phosphorylation of eIF4B (Ser422) and p70S6K, whereas PKB phosphorylation was undetectable (Figure 4, lane 8). Preincubation with either LY294002, which inhibits both classes I and III PI3K, or rapamycin inhibited the phosphorylation of eIF4B completely (Figure 4, lanes 9 and 10). These results indicate that in contrast to growth factor signaling, amino-acid refeeding leads to eIF4B phosphorylation in an mTOR/p70S6K-dependent and PKB-independent manner.

Regulation of eIF4B Ser406 phosphorylation in response to insulin

To determine whether eIF4B could additionally be phosphorylated by PKB on Ser406, we generated a FLAG-tagged eIF4B in which this residue had been mutated to alanine. A14 cells were transfected with wild-type or mutant eIF4B, serum starved overnight and subsequently stimulated with insulin before immunoprecipitating the FLAG-tagged protein. Insulin stimulation resulted in phosphorylation of eIF4B as detected by both the phospho-eIF4B Ser422 antibody as well as the phospho-PKB substrate antibody (Figure 5a, lanes 1 and 2). However, when Ser406 was mutated to an alanine, this abolished reactivity with the phospho-PKB substrate antibody (Figure 5a, lanes 3 and 4), whereas there was no effect on Ser422 phosphorylation. This demonstrates that the phospho-PKB antibody specifically recognizes eIF4B Ser406, allowing us to make use of this to analyse Ser406 phosphorylation *in vivo*.

To determine whether PKB activity is also required for insulin-induced eIF4B Ser406 phosphorylation, A14 cells were transfected with FLAG-tagged eIF4B, serum starved overnight and subsequently treated with a specific PKB inhibitor before stimulation with insulin. Insulin-stimulated, robust phosphorylation of Ser422 and inhibition of PKB abrogated this, whereas the phosphorylation of Ser406 was not affected by PKB inhibition (Figure 5b, lane 4). This clearly indicates that while PKB activity is required for insulin-induced Ser422 phosphorylation, other signaling pathways mediate the phosphorylation of Ser406. To define which signal transduction pathways regulate the eIF4B Ser406 phosphorylation *in vivo*, A14 cells were transfected with FLAG-tagged eIF4B, serum starved overnight and treated with or without inhibitors, LY294002, rapamycin, UO126, or a combination of rapamycin and U0126 before stimulation with insulin. Pretreatment with LY294002, rapamycin or UO126 had little effect on the phosphorylation of eIF4B on Ser406 (Figure 5c), whereas a combination of rapamycin and U0126 abrogated the phosphorylation of this residue (Figure 5c, lane 10). In contrast to eIF4B Ser422, inhibition of the PI3K/PKB signaling module by LY294002 has no effect on Ser406 phosphorylation. Our data suggest that Ser406 phosphorylation is an mTOR- and MEK-dependent event.

Phosphorylation of eIF4B regulates translation initiation in vivo

Taken together, our data suggest that the phosphorylation of eIF4B Ser422 by PKB, and Ser406 through an MEK/mTOR-dependent pathway regulate translation initiation. To investigate this, we developed a novel translation control luciferase assay based on a system previously described by Wiesenthal *et al*. (2006). This translation control luciferase assay makes use of an evolutionary conserved upstream open reading frame (uORF) of the transcription factor C/EBPα. This uORF controls the ratio of two proteins expressed from a single mRNA by regulated reinitiation (Calkhoven *et al*., 2000): a full-length protein expressed from a proximal initiation site and an amino-terminally truncated protein expressed from a different reading frame at a distal site. At high translational activity, the uORF is recognized and the truncated protein is translated as a result of efficient reinitiation. The uORF of C/EBPα was used for the generation of a reporter assay, which is able to measure translation and translation reinitiation. Two plasmids were generated: one vector encodes the uORF from C/EBPα with the first startcodon followed by the firefly luciferase (Fl-Cα-Gl3), which measures translation, and a second vector which contains the uORF and the second AUG followed by the luciferase gene (Tr-Cα-Gl3), which measures translation reinitiation (Figure 6a). The levels of the luciferase signal from the Tr-Cα-Gl3 are therefore directly proportional to the

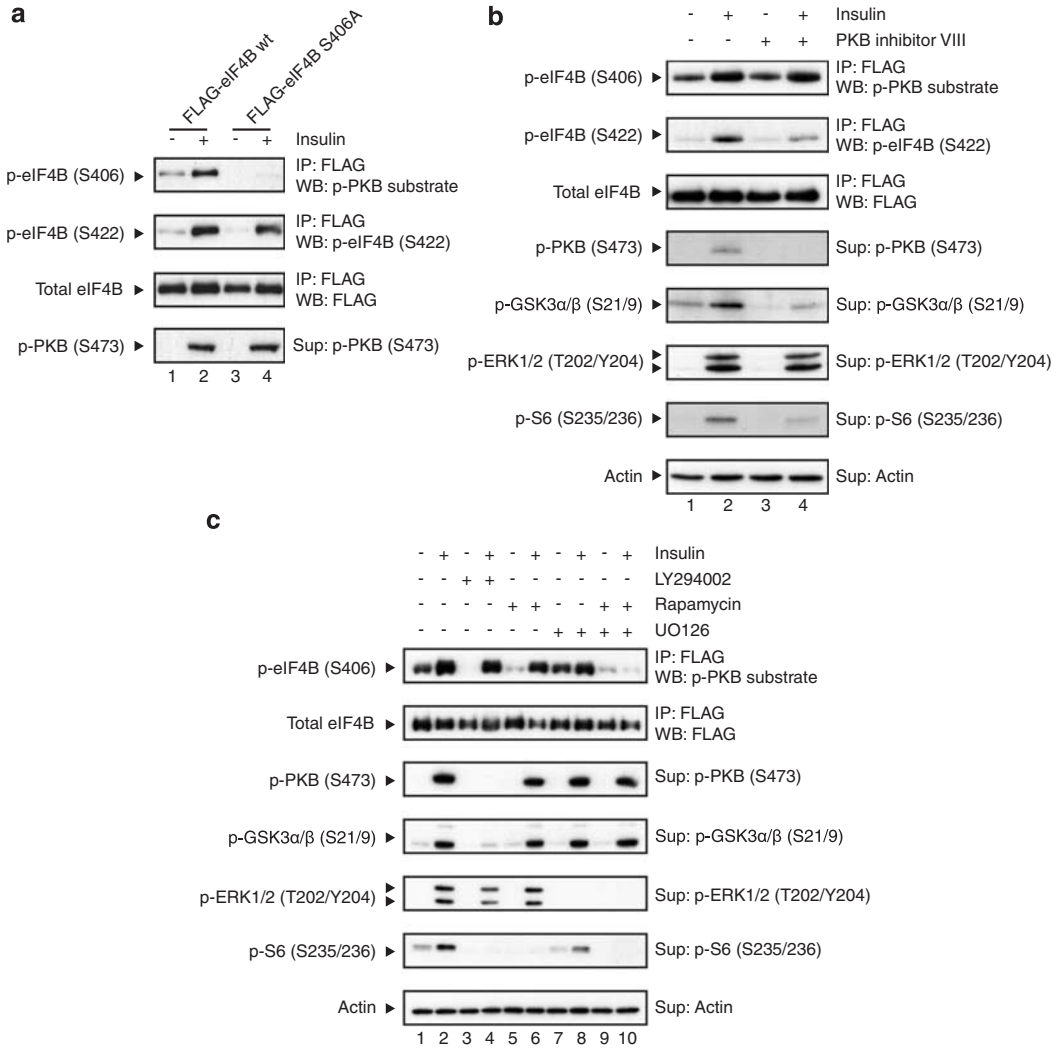

Figure 5 eIF4B Ser406 phosphorylation after insulin stimulation is MEK and mTOR dependent. (**a**) A14 cells were transfected with FLAG-tagged wild-type eIF4B or mutant eIF4B in which Ser406 had been mutated to alanine. Cells were serum starved overnight and subsequently stimulated for 15 min with insulin (1 μg/ml) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analysed for levels of phospho-PKB substrate, phospho-eIF4B (S422) and FLAG as a loading control. The whole-cell lysate was analysed for phospho-PKB (S473) as a control for insulin stimulation. (**b**) A14 cells were transfected with FLAG-tagged eIF4B. Cells were serum starved overnight and subsequently stimulated for 15 min with insulin after pretreatment for 30 min with or without PKB inhibitor VIII (10 μM) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analysed for levels of phospho-eIF4B (S422), phospho-PKB substrate and FLAG as a loading control. The whole-cell lysate was analysed for phospho-PKB (Ser473), phospho-GSK3α/β (S21/9), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/S236) and actin. (**c**) A14 cells were transfected with FLAG-tagged eIF4B. Cells were serum starved overnight and subsequently stimulated for 15 min with insulin (1 μg/ml) after or without pretreatment for 30 min with either LY294002 (50 μM), rapamycin (20 ng/ml), U0126 (15 μM) or a combination of rapamycin (20 ng/ml) and U0126 (15 μM) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analysed for levels of phospho-PKB substrate and FLAG as a loading control. The whole-cell lysate was analysed for phospho-PKB (Ser473), phospho-GSK3α/β (S21/9), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/S236) and actin. eIF4B, eukaryotic translation initiation factor 4B; mTOR, mammalian target of rapamycin; PKB, protein kinase B.

translational activity of the cell and, an increase in Tr-Cα-Gl3/ Fl-Cα-Gl3 ratio correlates with an increase in this activity.

To determine whether Ser406 and Ser422 phosphorylation affects the translational activity of cells, cells were transiently transfected to express various mutant forms of Flag-tagged eIF4B together with Fl-Cα-Gl3 or Tr-Cα-Gl3. As shown in Figure 6b, expression of wt eIF4B resulted in an increased ratio of the two luciferase signals, reflecting a higher translational activity, whereas mutation of Ser406, Ser422 or both phosphorylation sites abrogated this effect. Taken together, these data indicate that the phosphorylation of eIF4B on both Ser406 and Ser422 is an important mechanism by which AGC kinase family members can positively regulate translational activity.

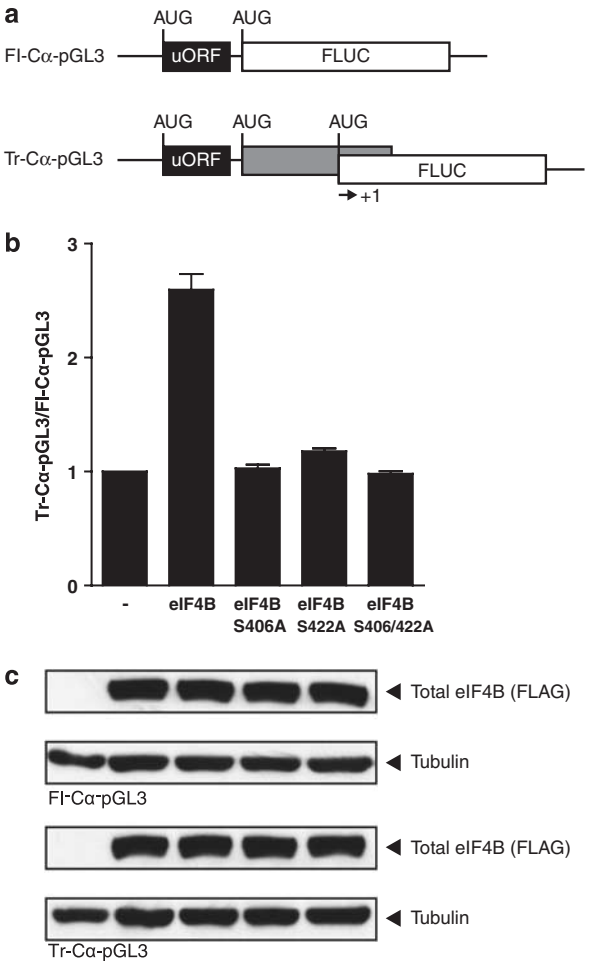

Figure 6 eIF4B Ser406 and Ser422 phosphorylation is required for optimal translational activity. (**a**) Schematic representation of reporter constructs. Fl-Cα-Gl3 encodes the upstream open reading frame (uORF) from C/EBPα with the first start codon followed by the firefly luciferase, whereas Tr-Cα-Gl3 contains the uORF and the second AUG followed by the firefly luciferase gene. AUG1 lies out of frame with the luciferase AUG2. Fl-Cα-Gl3 measures translation initiation and Tr-Cα-Gl3 measures translation reinitiation. (**b**) Cells were transiently transfected with the various mutant forms of Flag-tagged eIF4B together with Fl-Cα-pGL3 or Tr-Cα-pGL3 constructs and pGL4.74 renilla luciferase-expressing vector for normalization. After 24 h, firefly and renilla luciferase activity in whole-cell lysates was determined by luminescence. After normalization, the ratio of normalized Tr-Cα-pGL3 to Fl-Cα-pGL3 luciferase activity was calculated. Three independent transfection studies were performed. (**c**) Samples from luciferase assay were analysed for expression levels of eIF4B with a FLAG antibody and tubulin as a loading control. eIF4B, eukaryotic translation initiation factor 4B; uORF, upstream open reading frame.

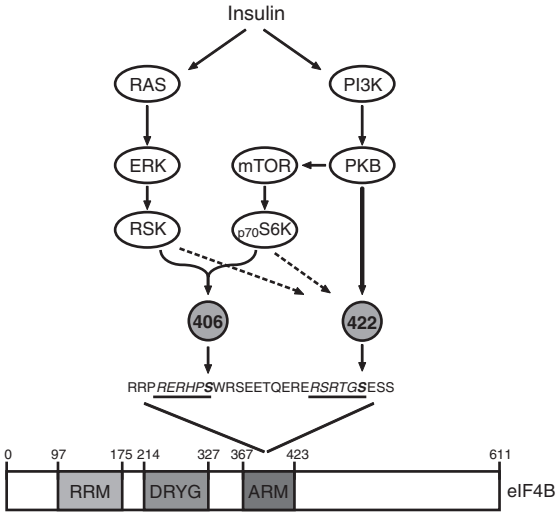

Figure 7 Signaling pathways regulating eIF4B Ser406 and Ser422 phosphorylation. Insulin activates the PI3K/PKB/mTOR pathway as well as the RAS/MEK/ERK pathway. Upon activation by insulin, the RAS/MEK/ERK and mTOR pathways are required for regulating eIF4B Ser406 phosphorylation, whereas PKB phosphorylates Ser422. Both residues are important in the regulation of translation activation. eIF4B, eukaryotic translation initiation factor 4B; mTOR, mammalian target of rapamycin; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase.

Discussion

In this study, we have demonstrated that PKB can phosphorylate eIF4B on Ser422 *in vitro*. Upon mitogen-stimulation, phosphorylation on Ser442 is also regulated by PKB *in vivo*, as blocking PKB activity either by the addition of a specific PKB inhibitor or the PI3K inhibitor LY294002 was sufficient to abolish phosphorylation on this residue. In contrast, mTOR inhibitor rapamycin and MEK inhibitor U0126 had no effect on Ser422 phosphorylation *in vivo*. Furthermore, we have identified a novel eIF4B phosphorylation site, Ser406, which is also phosphorylated upon mitogen stimulation. As Ser406 represents a consensus phosphorylation motif for AGC kinase family members and its phosphorylation can be abrogated by the inhibition of both MEK and mTOR, we propose that the phosphorylation of this residue is regulated by RSK and p70S6K (Figure 7). Importantly, using a novel translational control luciferase assay, phosphorylation of both residues was found to modulate the translational activity of eIF4B.

Previously, Shahbazian *et al*. (2006) reported that the phosphorylation of eIF4B on Ser422 is synergistically regulated by p70S6K and RSK upon serum-stimulation. Phosphorylation of this residue upon insulin stimulation was reported to be solely dependent on p70S6K. However, in this study, cells were pretreated with rapamycin for an extended period of time (up to 18 h). Recently, it has become clear that prolonged treatment with rapamycin can inhibit PKB activity in a cell-type-specific manner (Sarbassov *et al*., 2006). Sarbassov *et al*. (2006) provided compelling evidence that mTOR in combination with rictor (mTORC2) was sensitive to long-term rapamycin treatment, and this complex was previously reported by the same group as the long sought after PDK2 kinase, which phosphorylates PKB at Ser473 leading to its activation (Sarbassov *et al*., 2005, 2006). In our study, cells were pretreated with rapamycin for only a short period of time (less than 2 h) to ensure that only mTOR in combination with raptor (mTORC1), the upstream activator of p70S6K, and not mTORC2, was inhibited. This had no effect on PKB

activation as shown in Figure 3a allowing us to specifically analyse the effect of mTOR/p70S6K inhibition.

Members of the AGC protein kinase family have highly homologous kinase domains and similar substrate specificities, and can therefore be considered as potentially 'promiscuous' when it comes to phosphorylation of target proteins. Therefore, care must be taken in drawing conclusions from *in vitro* assays where, it is likely that various members of the AGC kinase family may phosphorylate substrates at the same site. However, *In vivo* phosphorylation of substrates is likely to be a highly regulated process. In the case of eIF4B, three AGC kinase family members have now been shown to phosphorylate Ser422 *in vitro*, p70S6K, RSK and PKB, respectively. Whereas insulin specifically utilizes PKB to phosphorylate this residue, serum may also utilize RSK to regulate Ser422 phosphorylation. Shahbazian *et al*. (2006) show a temporal effect of MEK inhibitor U0126 and mTOR inhibitor rapamycin after serum stimulation. U0126 effects the early phase of eIF4B phosphorylation, whereas rapamycin effects the late phase. In this study, we have shown that for insulin stimulation, RSK does not play a role in Ser422 phosphorylation, but this kinase is crucial in Ser406 phosphorylation. Therefore, it is safe to conclude that eIF4B phosphorylation and activation are regulated in a stimulus- and cell-type-dependent manner, and this could be the reason why the Ser406 site was not identified by Raught *et al*. (2004) in their phosphomapping experiment after serum stimulation.

Although we have shown that PKB is the dominant kinase regulating Ser422 phosphorylation after insulin stimulation, this does not discount a role for other AGC family members in eIF4B phosphorylation. Indeed, although our data demonstrate that mTOR activity is not required for insulin-mediated Ser422 phosphorylation, it is required for phosphorylation of Ser422 after amino-acid refeeding. Regulation of eIF4B phosphorylation may be a fundamental process in the regulation of protein translation in response to diverse extracellular stimuli. Our data suggest that the utilization of various AGC kinase family members allows this mechanism of translational control to be regulated through distinct stimulus-specific intracellular signaling pathways.

The effects of eIF4B phosphorylation on translation have, to a limited degree, been studied previously and eIF4B phosphorylation has been reported to correlate with high translational activity. In accordance with this, phosphorylation of eIF4B on Ser422 has been shown to stimulate its interaction with eIF3, an important player in translation initiation (Holz *et al*., 2005; Shahbazian *et al*., 2006). In addition, Holz *et al*. (2005) found that expression of an eIF4B S422D mutant increased cap-dependent translation. Here, we have developed a novel readout for translation initiation using the translation control luciferase assay. Our data reveal that mutation of the 422 or 406 residues abrogates the eIF4B-mediated increase in translational activity. These results demonstrate that the phosphorylation of eIF4B by AGC protein kinase family members in the arginine-rich motif region indeed activates eIF4B, resulting in a positive effect on translation initiation.

In this study, we provide evidence that the phosphorylation of eIF4B can be regulated by both RAS and PKB signaling. Both pathways have been shown to be deregulated in a plethora of neoplasias. What role could eIF4B phosphorylation play in the process of transformation? eIF4B has been shown to play a critical role in stimulating the helicase activity of eIF4A to unwind the inhibitory secondary structures in the 5′ untranslated region of mRNAs. These highly structured mRNAs are poorly translated, when the translation initiation activity is decreased (Lodish, 1976). Highly structured mRNAs often encode those proteins, which are the components of pathways critical to cell growth, such as growth factors, transcription factors, tyrosine kinases and receptors (Ruggero and Pandolfi, 2003). Rajasekhar *et al*. (2003) recently demonstrated that upon PKB and RAS signaling, the profile of mRNA associated to polysomes was drastically altered; these mRNAs mainly encoded for the proteins mentioned. Therefore, the activation of eIF4B by dysregulated RAS and PKB signaling may be critical in the induction of cellular transformation.

Until recently, the effects PKB has on regulating translation were thought to be through increased mTOR activity. The inhibitory effects of rapamycin on PKB-induced transformation appeared to reveal the importance of mTOR as a downstream mediator of PKB signaling. However, the recent evidence for the fact that the prolonged rapamycin treatment can itself inhibit PKB activation re-emphasizes the importance of PKB itself as an oncogenic factor in regulating growth and proliferation. We suggest that oncogenic transformation as a result of uncontrolled PKB activity could be directly mediated by enhanced eIF4B activity, providing a novel rationale for the design of therapeutic strategies to inhibit tumor cell growth.

Materials and methods

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% HyClone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells (Dijkers *et al*., 2002a). For the generation of clonal Ba/F3 cells, stably expressing myrPKB:ER*, the SRα-myrPKB:ER* construct was electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1 mg/ml G418 (Gibco) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with phosphate-buffered saline and resuspended in AimV medium (Gibco). A14 cells and COS cells were cultured in Dulbecco's modified Eagles Medium (Gibco) with 8% fetal calf serum (Gibco). A14 cells are NIH 3T3-derived cells that overexpress the insulin receptor (Burgering *et al*., 1991). A14 cells were serum starved in Dulbecco's modified Eagle's medium supplemented with 0.1% fetal calf serum. For amino-acid refeeding, A14 cells were serum starved overnight, and then for additional 4 h starved in medium without amino acids, followed by stimulation with modified Eagle's mediumamino-acids solution (Gibco).

Constructs
Pc-DNA3-FLAG-eIF4B and pcDNA3-FLAG-eIF4BS422A have been previously published (Raught *et al*., 2004). pcDNA3-FLAG-eIF4BS406A was generated from pcDNA3-FLAG-eIF4B by side-directed mutagenesis. For the construction of Fl-Cα-pGL3, the 135-nt rat C/EBPα 5′UTR was cut (EcoRI blunted/NcoI) from rC/EBPαwt-pcDNA3 (Calkhoven *et al*., 2000), and cloned into pGL3-promoter firefly luciferase vector (HindIII blunted/NcoI) (Promega, Madison, WI, USA). This construct was used to emulate full-length C/EBPα translation.

For Tr-Cα-pGL3, a plasmid was constructed containing 485 nt of rat C/EBPα-cDNA ranging from the Cap site to the AUG start codon normally used for Tr-C/EBPα expression (Calkhoven *et al*., 2000)—an NcoI fragment was produced by PCR, covering the sequence between Fl-C/EBPα start codon to the Tr-C/EBPα start codon. At the Tr-C/EBPα initiation site, the start codon for luciferase expression was shifted (+1) out of frame with the Fl-C/EBPα frame. The PCR fragment was cloned into (NcoI) Fl-Cα-pGL3. PCR primers: 5′-gtggatagcggtttgactcacg-3′ (binding to cytomegalovirus promoter of C/EBPα-pcDNA3) and 5′-ttccatggggcaccgccggggcc-3′ (+1 mutation and NcoI-site). This construct was used to emulate truncated C/EBPα translation.

Antibodies and reagents
Monoclonal antibodies against phospho-PKB (Ser473) and the polyclonal antibodies against phospho-eIF4B (Ser422), phospho-PKB substrate, phospho-GSK3α/β (S21/9) and phospho-p70S6K (T389) were from Cell Signaling Technologies (Hitchin, Hertfordshire, UK). Actin antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-FOXO3 (Thr32) and phospho-FOXO3 (Ser253) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Phospho-MAPK42/44(Thr202/Tyr204) and phosphor-S6 (S235/S236) were from New England Biolabs (Hitchin). The Anti-FLAG M2 monoclonal antibody peroxidase conjugate, 4-hydroxytamoxifen (4-OHT) and insulin were purchased from Sigma (Seelze, Germany). LY294002, U0126 and rapamycin were obtained from Biomol International LP (Hamburg, Germany) and PKB inhibitors IV, V and VIII were from Calbiochem (San Diego, CA, USA).

Western blotting
A14 cells were lysed in 1 × sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 min. BaF3 cells were lysed in lemmli buffer (0.12 M Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 0.05 μg/μl bromophenol blue and 35 mM β-mercaptoethanol), boiled for 5 min and the protein concentration was determined. Equal amounts of sample were analysed by SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA) and probed with respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Immunoprecipitation
For immunoprecipitation assays, either COS or A14 cells (9-cm dishes) were transfected with a total of 10 μg of plasmid DNA by the calcium phosphate or polyethyleneimine precipitation method. In the following morning, the cells were washed with phosphate-buffered saline and fresh medium was added to the cells. For serum starvation, cells were again washed with phosphate-buffered saline at the end of the day and Dulbecco's modified Eagle's medium containing 0.1% fetal calf serum was added to the cells. After another 24 h of growth, cells were stimulated as indicated and lysed in RIPA lysis buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodiumdeoxycholin, 5 mM EDTA (ethylenediaminetetraacetic acid), 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF (phenylmethanesulphonylfluoride or phenylmethylsulphonyl fluoride)). Lysates were centrifuged at maximum speed for 10 min to remove DNA and cellular debris. A part of the lysate was taken as a control for stimulations, 5 × sample buffer was added to a final concentration of 1 × (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 min. The rest of the lysate was incubated for at least 2 h with FLAG M2 agarose beads from Sigma at 4 °C and subsequently beads were washed four times with RIPA lysis buffer and boiled in 1 × sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue).

Kinase assay
After immunoprecipitation and washing, kinase buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP) and 200 ng of active PKBα (Upstate Biotechnology Inc) was added to the FLAG M2 agarose beads and incubated at 37 °C for 30 min. After incubation, 5 × sample buffer was added to a final concentration of 1 × (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 min.

Tandem mass spectrometry
Immunoprecipitated eIF4B was digested with Trypsin (Roche, Basel, Switzerland) and enriched for phosphorylated peptides using a ~5-mm length TiO_2 microcolumn, packed in GELoader tip with a 3 M Empore C8 plug from an extraction disc, essentially as described by Larsen *et al*. (2005). Peptides were loaded onto this column in buffer A (80% acetonitrile and 0.1% trifluoric acid)/200 g/l DHB (2,5-dihydroxibenzoic acid). Columns were washed once in buffer A/DHB followed by a wash in buffer A. The bound peptides were eluted with 20 μl 1% ammonia in 5 μl 10% formic acid. Samples were directly subjected to nanoflow liquid chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100 μm ID, 2 cm). Peptides were separated on an aqua C18 reversed phase column (kind gift from Professor A Heck, dimensions; 75 μM ID, 20 cm) at a flow rate of 200 nl/min with a 60-min linear acetonitrile gradient from 0 to 90%. The liquid chromatography system was directly coupled to a QTOF micro tandem mass spectrometer (Micromass Waters, Manchester, UK). A survey scan was performed from 400–1200 AMU/s and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches using MASCOT software (Matrixscience) against SWISSPROT and the NCBI nonredundant database, allowing for the detection of phosphorylation residues, with a 0.25 kDa mass tolerance for both precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

Translational control luciferase assay
Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections were performed using FUGENE (Roche) according to the manufacture's instructions. Briefly, cells were seeded at a density of 4×10^4 cells per well of a 96-well plate and grown to 80–90% confluency. The cells were cotransfected with 0.1 μg DNA of Fl-Cα-pGL3 or Tr-Cα-pGL3 constructs/well, 0.2 μg eIF4B-pcDNA3 vector and 0.1 μg pGL4.74 renilla luciferase-expressing vector for normalization (Promega). Fresh media was added after 12 h, and the cells were grown for another

24 h. Firefly and renilla luciferase activity in whole-cell lysates was determined by luminescence (Mithras, Berthold, Bad Wildbad, Germany). After normalization, the ratio of normalized Fl-Cα-pGL3 to Tr-Cα-pGL3 luciferase activity was calculated.

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References

Burgering BM, Medema RH, Maassen JA, van de Wetering ML, van der Eb AJ, McCormick F *et al*. (1991). Insulin stimulation of gene expression mediated by p21ras activation. *EMBO J* **10**: 1103–1109.

Calkhoven CF, Muller C, Leutz A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev* **14**: 1920–1932.

Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L *et al*. (2002b). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* **156**: 531–542.

Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L *et al*. (2002a). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* **156**: 531–542.

Duncan R, Hershey JW. (1984). Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. *J Biol Chem* **259**: 11882–11889.

Duncan R, Hershey JW. (1985). Regulation of initiation factors during translational repression caused by serum depletion. Covalent modification. *J Biol Chem* **260**: 5493–5497.

Gani R. (1976). The nucleoli of cultured human lymphocytes. I nucleolar morphology in relation to transformation and the DNA cycle. *Exp Cell Res* **97**: 249–258.

Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL *et al*. (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J Clin Invest* **112**: 197–208.

Gingras AC, Raught B, Sonenberg N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* **68**: 913–963.

Guertin DA, Sabatini DM. (2005). An expanding role for mTOR in cancer. *Trends Mol Med* **11**: 353–361.

Hershey JW, Merrick WC. (2000). Pathway and mechanism of initiation of protein synthesis. In: Sonenberg N, Hershey JWB, Merrick WC (eds). *Translational Control of Gene Expression*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp 33–88.

Holland EC, Sonenberg N, Pandolfi PP, Thomas G. (2004b). Signaling control of mRNA translation in cancer pathogenesis. *Oncogene* **23**: 3138–3144.

Holland EC, Sonenberg N, Pandolfi PP, Thomas G. (2004a). Signaling control of mRNA translation in cancer pathogenesis. *Oncogene* **23**: 3138–3144.

Holz MK, Ballif BA, Gygi SP, Blenis J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123**: 569–580.

Inoki K, Li Y, Zhu T, Wu J, Guan KL. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* **4**: 648–657.

Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* **4**: 873–886.

Lawson TG, Lee KA, Maimone MM, Abramson RD, Dever TE, Merrick WC *et al*. (1989). Dissociation of double-stranded polynucleotide helical structures by eukaryotic initiation factors, as revealed by a novel assay. *Biochemistry* **28**: 4729–4734.

Lodish HF. (1976). Translational control of protein synthesis. *Annu Rev Biochem* **45**: 39–72.

Luo J, Manning BD, Cantley LC. (2003). Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* **4**: 257–262.

Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. (2006). mTOR, translation initiation and cancer. *Oncogene* **25**: 6416–6422.

Methot N, Pause A, Hershey JW, Sonenberg N. (1994). The translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. *Mol Cell Biol* **14**: 2307–2316.

Methot N, Pickett G, Keene JD, Sonenberg N. (1996a). *In vitro* RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. *RNA* **2**: 38–50.

Methot N, Song MS, Sonenberg N. (1996b). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. *Mol Cell Biol* **16**: 5328–5334.

Naranda T, Strong WB, Menaya J, Fabbri BJ, Hershey JW. (1994). Two structural domains of initiation factor eIF-4B are involved in binding to RNA. *J Biol Chem* **269**: 14465–14472.

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* **344**(Pt 2): 427–431.

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P *et al*. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci USA* **102**: 14238–14243.

Obata T, Yaffe MB, Leparc GG, Piro ET, Maegawa H, Kashiwagi A *et al*. (2000). Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J Biol Chem* **275**: 36108–36115.

Obenauer JC, Cantley LC, Yaffe MB. (2003). Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res* **31**: 3635–3641.

Pandolfi PP. (2004). Aberrant mRNA translation in cancer pathogenesis: an old concept revisited comes finally of age. *Oncogene* **23**: 3134–3137.

Parker PJ, Parkinson SJ. (2001). AGC protein kinase phosphorylation and protein kinase C. *Biochem Soc Trans* **29**: 860–863.

Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. (2003). Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol Cell* **12**: 889–901.

Raught B, Gingras AC, Sonenberg N. (2000). Regulation of ribosomal recruitment in eukaryotes. In: Sonenberg N, Hershey JWB, Merrick WC (eds). *Translational Control of Gene Expression*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, pp 245–295.

Raught B, Peiretti F, Gingras AC, Livingstone M, Shahbazian D, Mayeur GL *et al*. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *EMBO J* **23**: 1761–1769.

Richter JD, Sonenberg N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433**: 477–480.

Rogers Jr GW, Richter NJ, Lima WF, Merrick WC. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4 H, and eIF4F. *J Biol Chem* **276**: 30914–30922.

Rozen F, Edery I, Meerovitch K, Dever TE, Merrick WC, Sonenberg N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol Cell Biol* **10**: 1134–1144.

Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C *et al*. (2004). The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat Med* **10**: 484–486.

Ruggero D, Pandolfi PP. (2003). Does the ribosome translate cancer? *Nat Rev Cancer* **3**: 179–192.

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF *et al*. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* **22**: 159–168.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098–1101.

Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J *et al*. (2006). The mTOR/PI3 K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J* **25**: 2781–2791.

van Gorp AG, Pomeranz KM, Birkenkamp KU, Hui RC, Lam EW, Coffer PJ. (2006). Chronic Protein Kinase B (PKB/c-akt) Activation Leads to Apoptosis Induced by Oxidative Stress-Mediated Foxo3a Transcriptional Up-regulation. *Cancer Res* **66**: 10760–10769.

Wiesenthal V, Leutz A, Calkhoven CF. (2006). A translation control reporter system (TCRS) for the analysis of translationally controlled processes in the vertebrate cell. *Nucleic Acids Res* **34**: e23.

Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR *et al*. (2002). Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J Biol Chem* **277**: 39379–39387.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)