Evidence that the dephosphorylation of Ser⁵³⁵ in the ϵ -subunit of eukaryotic initiation factor (eIF) 2B is insufficient for the activation of eIF2B by insulin

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Eukaryotic initiation factor (eIF) 2B is a guanine-nucleotide exchange factor that plays a key role in the regulation of protein synthesis. It is activated by insulin, serum and other agents that stimulate general protein synthesis. The largest (ϵ) subunit of eIF2B is a substrate for glycogen synthase kinase (GSK)-3 *in vitro*, and phosphorylation by GSK3 inhibits the activity of eIF2B. The site of phosphorylation has previously been identified as Ser⁵³⁵. GSK3 is inactivated by phosphorylation in response to insulin or serum. In Chinese-hamster ovary cells, insulin and serum bring about the dephosphorylation of Ser⁵³⁵ *in vivo*, concomitantly with the phosphorylation of GSK3, and these effects are mediated through signalling via phosphoinositide 3-kinase. We have made use of inhibitors of GSK3 to determine whether GSK3 is responsible for phosphorylation of Ser⁵³⁵ *in vivo* and to explore the role of phosphorylation of Ser⁵³⁵ in the regulation of eIF2B. Treatment of cells with LiCl or with either

of two recently developed GSK3 inhibitors, SB-415286 and SB-216763, brought about the dephosphorylation of Ser⁵³⁵, which strongly indicates that this site is indeed a target for GSK3 *in vivo*. However, these compounds did not elicit significant activation of eIF2B, indicating, consistent with conclusions from one of our previous studies, that additional inputs are required for the activation of eIF2B. Our results also show that each of the inhibitors used affects overall protein synthesis and have additional effects on translation factors or signalling pathways apparently unrelated to their effects on GSK3, indicating that caution must be exercised when interpreting data obtained using these compounds.

Key words: glycogen synthase kinase 3, mRNA translation, mammalian target of rapamycin (mTOR).

INTRODUCTION

Eukaryotic initiation factor (eIF) 2B plays an important role in the process of mRNA translation and in its control [1]. It facilitates the exchange of guanine nucleotides by eIF2, the protein that mediates the attachment of the initiator methionyl (Met)-tRNA_i to the 40 S ribosomal subunit [2]. eIF2 can only bind Met-tRNA_i when it is complexed with GTP. Binding of the eIF2·GTP·Met-tRNA_i ternary complex to the 40 S ribosomal subunit is a key step in translation initiation, since it is the anticodon on this tRNA that recognizes the start codon in the mRNA. eIF2 activity is regulated under a wide variety of conditions: stressful conditions inhibit it by bringing about the phosphorylation of the smallest subunit (α) of eIF2, which converts eIF2 into a potent competitive inhibitor of eIF2B [2]; conversely, stimuli that activate protein synthesis elicit an increase in the activity of eIF2B [1]. Such stimuli include insulin [3–6], phorbol esters [4], growth factors [7–9], mitogenic activation of T-lymphocytes [10], amino acids [11] and, in pancreatic β -cells, glucose [12].

eIF2B comprises five subunits, α – ϵ [2], of which the largest (ϵ) appears to be the catalytic subunit that promotes nucleotide exchange on eIF2 [13–16]. eIF2B ϵ is a phosphoprotein that contains sites for phosphorylation by at least four different protein kinases [17]. These include protein kinases CK1 and CK2 (formerly known as casein kinases 1 and 2) whose activities are

not thought to be regulated by stimuli such as insulin [18] and glycogen synthase kinase 3 (GSK3), which is inactivated by insulin and certain other stimuli that stimulate eIF2B and protein synthesis [19,20]. Phosphorylation of the eIF2B complex from mammalian or insect cells by GSK3 *in vitro* causes inhibition of its GDP/GTP exchange activity [5,16]. The site phosphorylated by GSK3 is Ser⁵³⁵, which is conserved in all mammalian eIF2B ϵ sequences and in the insect factor [5,16]. Inactivation of GSK3 by insulin is thought to proceed via a signalling pathway involving phosphoinositide (PI) 3-kinase and protein kinase B (PKB), which directly phosphorylates and inactivates GSK3 [20,21].

We have previously shown that the activation of eIF2B by insulin is dependent upon PI 3-kinase, but independent of other signalling pathways, such as the mitogen-activated protein kinase and mammalian target of rapamycin (mTOR) pathways [7,9]. Stimulation of cells with insulin causes dephosphorylation of the Ser⁵³⁵ site in eIF2B ϵ [5], and this effect is blocked by wortmannin, an inhibitor of PI 3-kinase, consistent with the idea that inactivation of GSK3 by insulin plays an important role in the activation of eIF2B *in vivo*. However, there are clearly other inputs into the control of eIF2B. For example, activation of eIF2B by certain growth factors involves mitogen-activated protein kinase/ERK (extracellular-signal-regulated protein kinase) kinase (MEK) signalling rather than dephosphorylation of Ser⁵³⁵ in eIF2B ϵ [7,9], and the activation of eIF2B by insulin is dependent upon the presence of amino acids and glucose in the

Abbreviations used: CHO, Chinese-hamster ovary; DYRK, dual specificity tyrosine phosphorylated and regulated kinase; eIF, eukaryotic initiation factor; 4E-BP1, eIF4E-binding protein 1; Erk, extracellular signal-regulated protein kinase; GSK, glycogen synthase kinase; MEK, mitogen-activated protein kinase/ERK kinase; mTOR, mammalian target of rapamycin; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; rp, ribosomal protein; S6K1, 70-kDa rp S6 kinase 1.

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cellular medium [22], even though insulin, under this condition, still promotes the dephosphorylation of the GSK3 site as it does in control cells given amino acids. Amino acids may also modulate the regulation of eIF2B in other ways [11]. The control of eIF2B therefore appears to involve multiple inputs.

To study further the role of GSK3 in the phosphorylation and control of eIF2B, we have made use of a recently developed phospho-specific antiserum that recognizes eIF2B ϵ when phosphorylated at Ser⁵³⁵ [5], inhibitors of potential upstream signalling pathways and newly-available cell-permeant inhibitors of GSK3 [23]. These compounds inhibit GSK3 in a competitive manner with respect to its substrate, ATP [23]. The results indicate that Ser⁵³⁵ is indeed phosphorylated by GSK3 *in vivo*, but that dephosphorylation of this residue is in itself insufficient to bring about activation of this factor. Thus additional inputs are required for the stimulation of this key regulator of translation initiation.

EXPERIMENTAL

Chemicals and biochemicals

Chemicals and biochemicals were obtained from BDH–Merck or Sigma, unless indicated otherwise. SB-415286 and SB-216763 were kindly provided by GlaxoSmithKline [23]. The antibody against eIF2B ϵ phosphorylated at Ser⁵³⁵ (residue 540 in the numbering system for the rabbit protein) was prepared as described previously [5]. The antibody used to detect eIF2B ϵ irrespective of its state of phosphorylation was raised in rabbit against recombinant rat eIF2B ϵ expressed in *Spodoptera* cells using a baculovirus expression system. The antibody for ribosomal protein (rp) S6 (rpS6) phosphorylated at Ser²³⁵ has been described previously [24], and was kindly given by Dr Dario Alessi (MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.). Other phosphospecific antisera were from commercial sources. Enhanced chemiluminescence (ECL[®]) reagents were from Amersham Nycomed. Membranes for Western blotting were from Millipore.

Culture and treatment of cells

Chinese-hamster ovary (CHO).K1 cells were maintained in Ham's F12 medium with 10% (v/v) foetal bovine serum. Cells were starved of serum for 16–18 h before use. After treatment, cells were immediately lysed using our standard extraction buffer containing 20 mM Tris/HCl, 50 mM β -glycerophosphate, pH 7.4, 1.5 mM EGTA, 1 mM dithiothreitol and inhibitors of proteases and protein phosphatases [19,25]. Protein concentrations were determined using the Bradford method [26].

Gel electrophoresis and Western blotting

These techniques were performed using SDS/PAGE containing 12.5% acrylamide and 0.1% methylene bis-acrylamide. Similar amounts of cell extract protein (usually 20 μ g) were applied to each lane of the gel. Equal loading was confirmed by Western-blot analysis with antisera that recognize the protein of interest irrespective of its state of phosphorylation. After electrophoresis, proteins were transferred electrophoretically to membranes (PVDF) for immunoblotting. Blots were developed with the appropriate secondary antibodies coupled to horseradish peroxidase and visualized with enhanced chemiluminescence.

Assays of eIF2B and protein synthesis

The guanine-nucleotide exchange activity of eIF2B in cell extracts was determined as described previously [8], using substrate complexes formed between purified eIF2 (from rabbit reticulocytes) and [³H]GDP. The same quantity of cellular protein (usually 20 μ g) was used in each assay within a set and assays were performed in triplicate. All assays were performed so that release of [³H]GDP lay within the linear range of the test. Protein synthesis was assayed by the incorporation of [³⁵S]methionine into trichloroacetic acid-insoluble material as described previously [8].

RESULTS AND DISCUSSION

Serum or insulin induce the dephosphorylation on Ser⁵³⁵ in eIF2B ϵ

eIF2B is activated by variety of stimuli including insulin, serum and phorbol esters [1]. To examine whether the effects of these stimuli on the phosphorylation of the GSK3 site in eIF2B ϵ (Ser⁵³⁵), we employed an antibody that detects eIF2B ϵ only when this site is phosphorylated [5].

To study the control of the phosphorylation of Ser⁵³⁵, serum-starved CHO.K1 cells were treated with the stimulus under study for the indicated time and then lysed. Equal amounts of cell protein from each sample were subjected to SDS/PAGE and then immunoblotting. As shown in Figures 1(A) and 1(B), treatment of CHO.K1 cells with serum or insulin caused a marked decrease in the signal observed with this antibody, indicative of a decrease in the phosphorylation of this site in eIF2B ϵ . In the case of serum, the dephosphorylation of Ser⁵³⁵ was almost complete by 30 min. For insulin, marked dephosphorylation was seen by 10 min, after which there was a small reversal of this effect (presumably due to termination or decay of the insulin signal). Insulin and serum have both previously been shown to activate eIF2B in several cell types [3–6,10]. The phorbol ester PMA also elicited a decrease in the phosphorylation

Figure 1 Changes in the phosphorylation of eIF2B and GSK3 α and β in response to serum, insulin or PMA in CHO.K1 cells

Cells were stimulated with 10% (v/v) foetal calf serum, 100 nM insulin or 1 μ M PMA (TPA) for the times indicated (min) (A) or for 30, 10 or 30 min, respectively (B). Identical amounts of cell protein were analysed by SDS/PAGE followed by Western blotting. The phosphorylation of the ϵ -subunit of eIF2B was examined using an antibody that only recognizes eIF2B ϵ when it is phosphorylated at Ser⁵³⁵, whereas the phosphorylation of GSK3 was examined using an antibody against GSK3 that recognizes both the α - and β -isoforms only when they are phosphorylated at Ser²¹ or Ser⁹ respectively. C, control; (P), phosphorylated. (C) Con, control; Ser: serum; Ins: insulin. Results shown are typical of similar results obtained in the 3–5 experiments performed.

Figure 2 Regulation of signalling pathways and eIF2B phosphorylation in CHO.K1 cells

Cells were stimulated for 30 min with 10% (v/v) foetal calf serum (**A**) or for 10 min with 100 nM insulin (**B**). Prior to stimulation, the cells were pretreated with or without U0126 and/or LY294002 (for 30 min). Identical amounts of cell protein were applied to each lane, and equal protein loading was confirmed using an antibody that recognizes eIF2Bε irrespective of its state of phosphorylation (results not shown). Immunoblots were probed with antibodies that specifically recognize the p42 (lower band) and p44 (upper band) isoforms of Erk phosphorylated at Tyr²⁰⁴ and Thr²⁰², α-γ isoforms of PKB phosphorylated at Ser⁴⁷³, GSK3α and -β phosphorylated at Ser²¹ or Ser⁹ respectively, and eIF2Bε phosphorylated at Ser⁵³⁵ (as indicated by the labelled arrows). Essentially identical results were obtained in three separate experiments.

of eIF2Bε (Figure 1A), although its effect was much smaller than those of insulin or serum.

GSK3 has been shown to phosphorylate Ser⁵³⁵ *in vitro* [5]. To examine whether its dephosphorylation was associated with inactivation of GSK3, we made use of an antiserum that recognizes GSK3 only when it is phosphorylated at the inhibitory site (Ser⁹ in GSK3β and Ser²¹ in GSK3α). Phosphorylation of these residues was undetectable in control cells. Insulin and serum each caused a marked increase in the phosphorylation of these sites (Figure 1B), consistent with our previous observation that these stimuli inactivate GSK3 in CHO cells [27]. The effect of PMA was much smaller, consistent with the small effect of this agent on the phosphorylation of Ser⁵³⁵ and with our previous study [27] showing that PMA has a rather modest effect on the activity of GSK3 in CHO cells.

Effects of insulin or serum on phosphorylation of Ser⁵³⁵ are mediated via PI 3-kinase

The phosphorylation and inactivation of GSK3 is thought to be mediated through PKB, which is activated by signalling through PI 3-kinase [20,21], and this is consistent with our previous observation that the PI 3-kinase inhibitor wortmannin prevents inactivation of GSK3 by insulin [27]. However, p90^{RSK}, a downstream kinase in the MEK/Erk pathway, can also phosphorylate and inactivate GSK3 *in vitro* [28,29] and has been implicated in the regulation of GSK3 *in vivo* [30]. To examine which signalling events are involved in modulating the phos-

Figure 3 Effect of LiCl and KCl on the phosphorylation of eIF2Bε (**A**) and GSK3 (**B**) in CHO.K1 cells

Cells were treated for 2 h with LiCl or KCl with concentrations as indicated. Immunoblots were probed with antibodies that specifically recognize (**A**) eIF2Bε phosphorylated at Ser⁵³⁵ or (**B**) GSK3α and -β phosphorylated at Ser²¹ or Ser⁹ respectively. Results are representative of findings from three (**B**) or four (**A**) separate experiments.

phorylation of Ser⁵³⁵ in eIF2Bε in CHO.K1 cells, we first studied which pathways are activated by serum or insulin, using antisera designed to recognize only the phosphorylated and activated forms of PKB and Erk. Serum and insulin each brought about the phosphorylation of PKB and Erk (Figures 2A and 2B). As expected, the phosphorylation of Erk was blocked by the MEK inhibitor U0126 [31], whereas the phosphorylation of PKB was eliminated by the PI 3-kinase inhibitor LY294002. LY294002 also noticeably decreased the phosphorylation of Erk: this is similar to our previous report that inhibition of PI 3-kinase by wortmannin blunted the phosphorylation of Erk by serum or insulin in CHO cells [27].

The phosphorylation of GSK3 was not reduced by blocking Erk activation using U0126 (Figures 2A and 2B), but was abolished by LY294002. This is consistent with the idea that inactivation of GSK3 is mediated by PI 3-kinase/PKB rather than through MEK/Erk/p90^{RSK}. Similarly, U0126 had no effect on the phosphorylation of Ser⁵³⁵ in eIF2Bε, whereas the dephosphorylation of Ser⁵³⁵ induced by serum or insulin was completely prevented by LY294002. These results are entirely consistent with our previous finding that (in CHO cells) the activation of eIF2B by insulin is prevented by a dominant-interfering mutant of PI 3-kinase, but not by a dominant negative mutant of Sos, the exchange factor for Ras, which blocks Erk activation [6].

The above results support the idea that Ser⁵³⁵ in eIF2Bε is phosphorylated by GSK3 *in vivo*, and the notion that signalling through PI 3-kinase, PKB and GSK3, and the dephosphorylation of Ser⁵³⁵ are involved in the activation of eIF2B by insulin. To test the role of GSK3 itself in the phosphorylation of Ser⁵³⁵, we made use of the fact that low millimolar concentrations of Li⁺ ions inhibit GSK3 [32,33]. Several studies have shown that treatment of cells with Li⁺ mimics the effect of inactivation of GSK3 (see, for example, [32–35]). More recently, other cell-permeant inhibitors of GSK3 have also become available [23].

LiCl and other inhibitors of GSK3 elicit dephosphorylation of Ser⁵³⁵ in eIF2Bε

Treatment of CHO cells with LiCl caused the almost complete dephosphorylation of Ser⁵³⁵ in eIF2Bε (Figure 3A), whereas treatment with similar concentrations of KCl (as a negative control) had no effect. LiCl also caused an increase in the phosphorylation of the regulatory N-terminal phosphorylation sites in GSK3 (Figure 3B). This effect does not appear to have been reported before, and suggests that LiCl may inhibit GSK3 both by a direct effect on its activity and by promoting its phosphorylation.

Figure 4 Effects of GSK3 inhibitors on the phosphorylation of eIF2 β and GSK3

(A) CHO.K1 cells were treated with insulin (Ins; 100 nM, 15 min), KCl (K; 10 mM, 1 h), LiCl (Li; 10 mM, 1 h), DMSO (DM; the vehicle for the SB compounds, 0.1% by volume, 1 h), SB-216763 (SB2; 30 μ M, 1 h), SB-415286 (SB4; 100 μ M, 1 h), or SB4 for 1 h followed by insulin for 15 min (Ins/SB4; concentrations as above). Cells were then lysed and equal amounts of extracted protein were analysed by SDS/PAGE and Western blotting using an antibody that detects eIF2 β only when phosphorylated at Ser⁵³⁵ {upper panel; eIF2 β (S535[P])} or, as a loading control, one that detects eIF2 β irrespective of its state of phosphorylation (lower panel, eIF2 β). Essentially identical results were obtained in four separate experiments. C, control. (B) CHO.K1 cells were treated with insulin (Ins; 100 nM, 15 min), PMA (TPA; 1 μ M, 30 min), DMSO (DM; the vehicle for the SB compounds, 0.1% by volume, 60 min), SB-216763 (SB2; 30 μ M, 30 or 60 min as indicated) or SB-415286 (SB4; 100 μ M, 30 or 60 min as indicated). Cells were then lysed and equal amounts of extracted protein (20 μ g) were analysed by SDS/PAGE and Western blotting using an antibody that detects GSK3 only when phosphorylated at its N-terminal regulatory site.

Table 1 Effects of inhibitors of GSK3 on the activity of eIF2B

CHO cells were starved of serum overnight and then treated with SB-216763 (30 μ M, 1 h), SB-415286 (100 μ M, 1 h), KCl (10 mM, 1 h) or LiCl (10 mM, 1 h). Cells were then extracted and samples (equal amounts of protein, usually 20 μ g) were assayed in triplicate for eIF2B activity. Alternatively, protein synthesis was measured by incorporation of [³⁵S]methionine into trichloroacetic-acid insoluble material as described previously [8]. For each experiment, results from triplicate determinations were averaged and expressed as the means $\pm$ S.E.M. Results are expressed as a percentage of the control: for KCl and LiCl, this was untreated cells, whereas for SB-216763 and SB-415286, results from cells treated with the vehicle (DMSO, 0.1% by volume) were taken as the control.

Condition	eIF2B activity (% of control)	Number of experiments performed	Protein synthesis (% of control)	Number of experiments performed
SB-216763	0.27 $\pm$ 0.04	5	69.8 $\pm$ 3.8	7
SB-415286	1.02 $\pm$ 0.08	6	42.0 $\pm$ 3.6	8
KCl	1.05 $\pm$ 0.07	5	141.1 $\pm$ 4.8	7
LiCl	1.10 $\pm$ 0.10	5	124.0 $\pm$ 2.5	6

Low molecular mass cell-permeant inhibitors of GSK3 have recently been developed [23]. We tested their effects on the phosphorylation of eIF2 β . As shown in Figure 4(A), treatment of CHO cells with either of these inhibitors caused the complete dephosphorylation of Ser⁵³⁵ in eIF2 β . This finding offers further strong support to the idea that GSK3 is, indeed, responsible for the phosphorylation of Ser⁵³⁵ in eIF2 β *in vivo*. Interestingly, SB-216763 caused a small, but reproducible, increase in the phosphorylation of GSK3 α/β (Figure 4B). This effect was much smaller than that of insulin or PMA on the phosphorylation of GSK3 (Figure 4B). Since the much larger change in the phosphorylation of GSK3 in response to these agents corresponds to a fall in GSK3 activity of about 30–70% [19,27], it was unlikely

that SB-216763 elicited a significant change in the intrinsic activity of GSK3 and we have not studied this. The major effect of SB-216763 on GSK3 activity is due to its ability to act as a competitive inhibitor of GSK3 with respect to its substrate ATP [23].

Dephosphorylation of Ser⁵³⁵ is insufficient to activate eIF2B

Since treatment of cells with LiCl, SB-216763 and SB-415286 led to dephosphorylation of Ser⁵³⁵, a site at which phosphorylation has been shown to inactivate mammalian or insect eIF2B [5,16], it was important to test whether these agents caused the activation of eIF2B. In fact, none of these three agents elicited activation of eIF2B (Table 1). Indeed, treatment of cells with SB-216763 actually caused a marked inhibition of eIF2B (Table 1). This effect, in particular, was puzzling. To study this further, we examined the state of phosphorylation of the α -subunit of eIF2, since this regulates eIF2B activity. eIF2(α P) acts as a potent competitive inhibitor of eIF2B and increased phosphorylation of eIF2 α thus inhibits the nucleotide exchange activity of eIF2B [36]. SB-216763 treatment caused a marked increase in the phosphorylation of eIF2 α (detected using a phosphospecific antibody, Figure 5A), whereas none of the other treatments perceptibly affected the level of phosphorylation of eIF2 α (Figure 5A). These results from experiments in CHO cells are similar to our previous finding using Swiss 3T3 cells [4] that the activation of eIF2B by insulin does not involve the dephosphorylation of eIF2 α .

Treatment of CHO.K1 cells with SB-216763 at concentrations as low as 10 μ M elicited an increase in the phosphorylation of eIF2 α (Figure 5B). SB-216763 may activate one of the four protein kinases that are known to phosphorylate eIF2 α in mammalian cells [37–41]. It is a lipophilic compound and one possibility is that it interferes with the function of the endoplasmic reticulum and activates the RNA-dependent protein kinase-like endoplasmic-reticulum kinase ('PERK') [39]. Phosphorylation of eIF2 α clearly has pronounced effects on mRNA translation and recent results indicate that it may also affect transcription in mammalian cells, for example, by inducing translation of the mRNA for activating transcription factor-4 [42]. Thus great caution must be exerted when interpreting any results relating to the control of mammalian cell function obtained using this compound.

To assess the effects of the GSK3 inhibitors and LiCl on protein synthesis, we pretreated CHO cells for 45 min with the reagent under study, and then added [³⁵S]methionine for a further 30 min, before incorporation of label into trichloroacetic-acid insoluble material was assessed. As shown in Table 1, treatment of cells with either SB-216763 or SB-415286 led to inhibition of protein synthesis. In the case of SB-216763 this may reflect the increased phosphorylation of eIF2 α and inhibition of eIF2B reported above. The effect of this compound on protein synthesis was smaller than its effect on eIF2B activity, although the latter is of course measured *in vitro*. Since SB-415286 did not affect eIF2 α phosphorylation, the inhibition of protein synthesis that it causes must be due to other effects of this compound (see below). In the case of LiCl, activation of protein synthesis was observed, but this effect is not specific to LiCl, as KCl elicited a similar degree of activation (Table 1).

Agents used as GSK3 inhibitors can also exert other effects on signalling pathways

Given the above results, we were prompted to test whether the inhibitors tested here, LiCl and SB-415286, also affected cellular

Figure 5 Effects of GSK3 inhibitors on the state of phosphorylation of eIF2 α , 4E-BP1, S6K1 and rpS6

CHO, K1 cells were treated with the indicated agents [see Figure 4 for details; PMA (TPA) treatment here was for 30 min at 1 μ M; in some cases different doses of SB-216763 or SB-415286 were used, as stated]. Samples of cell extract (equalized for protein content) were then analysed by SDS/PAGE and Western blotting. (A) Western blots in which an antibody was used that detects eIF2 α irrespective of its state of phosphorylation (upper panel, eIF2 α) or only when phosphorylated at Ser⁵¹ [lower panel, eIF2 α (S51[P])]. Control cells (C) were not treated. (B) Dose-response for effects of SB-216763 on the phosphorylation of eIF2 α . Control cells (0) received 0.1% (v/v) DMSO (the vehicle), whereas other cells were treated with the indicated final concentrations of SB-216763. Western blotting was as for (A). (C) Western blots in which an antiserum that detects 4E-BP1 irrespective of its state of phosphorylation was used. On the gel systems used here, 4E-BP1 migrates as multiple bands depending on its state of phosphorylation, more highly phosphorylated species migrating more slowly. For 4E-BP1, two main bands are evident, indicated as β and γ (in some cases, a trace of the fastest migrating α -species is visible). Control cells (C) were not treated. (D) CHO, K1 cells were starved of serum overnight and then treated with DMSO (0.1% v/v) or the concentration indicated in parentheses of SB-415286 (μ M). In some cases (+), insulin (100 nM, 15 min) was added for 15 min prior to lysis of the cells; (–) indicates cells that did not receive insulin. Western blotting was as in (C), except that here the α -, β - and γ -forms of 4E-BP1 are visible, as indicated by labelled arrows (β is the middle one). (E) As in (C), but here lysates of CHO, K1 cells were subjected to SDS/PAGE and Western-blot analysis for S6K1. Several bands are seen, corresponding to species of S6K1 differing in their states of phosphorylation (indicated by arrows, the most highly phosphorylated species migrated most slowly). Note the very faint band running below the main group is not S6K1, but is an unrelated protein that cross-reacts with this antibody. (F) CHO, K1 cells were treated as in (D), but Western blots were developed with anti-S6K1 antibody. (G and H) Cells were treated as described in (C) and (D), but the Western blots were developed using an antibody that recognized rpS6 when phosphorylated at Ser²³⁵. The results shown are typical of findings from at least three separate experiments in each case. Ins, insulin; DM, DMSO; SB2, SB-216763; SB4, SB-415286; TP, TPA (PMA).

signalling pathways. A signalling pathway of particular importance for the control of mRNA translation is the mTOR pathway [43,44]. We tested the effects of the agents used here on two targets of the mTOR pathway: these are the eIF4E-binding protein (4E-BP1) [45], and the 70-kDa rpS6 [46]. We also checked the phosphorylation state of a cellular substrate for S6 kinase 1 (S6K1), rpS6, as a readout of cellular S6K1 activity.

As we have shown previously for CHO cells [47], 4E-BP1 is mainly found in its partially phosphorylated β -species, with a trace of the most highly phosphorylated γ -species. Upon treatment of cells with insulin, all the protein migrated as the γ -species (Figure 5C). DMSO, SB-216763 and KCl had no discernible effect on the basal level of phosphorylation of 4E-BP1. However, LiCl treatment led to a marked increase in the phosphorylation of 4E-BP1, as manifested by a shift of most of the protein into the γ -species (Figure 5C). These findings suggested that LiCl may activate mTOR signalling. In addition, there was a hint that SB-415286 treatment caused the disappearance of the small amount of the γ -isoform seen in control cells suggesting that it may interfere with mTOR signalling (Figure 5C). To study this further, we tested the effect of a range of concentrations of SB-415286 on the phosphorylation of 4E-BP1 (Figure 5D). In this experiment, some of the 4E-BP1 was present as the fastest migrating α -form in the serum-starved cells. Insulin elicited a shift of the protein into the most highly phosphorylated γ -form, and this was partially blocked by SB-415286 at 100 μ M, as manifested by the fact that more than half the protein remained in the β -species at the highest concentration of SB-415286 tested (Figure 5D).

To study this further, we also looked at S6K1: this protein undergoes mTOR-dependent phosphorylation at multiple sites *in vivo* and this is associated with its activation [46]. The more highly phosphorylated forms of S6K1 migrated more slowly than the less phosphorylated species of the protein. In control cells, S6K1 existed mostly in its most mobile form, with a small amount of a phosphorylated slower-moving species (Figure 5E). Insulin or PMA caused the protein to shift into more highly phosphorylated species. As observed for 4E-BP1, DMSO, KCl and SB-216763 had no visible effect on the migration of S6K1 in control cells. In contrast, LiCl again caused a (partial) shift to more phosphorylated forms and SB-415286 appeared to cause a shift into the fastest-moving dephosphorylated species. When tested across a range of concentrations, SB-415286 was found to inhibit the insulin-induced phosphorylation of S6K1 especially at 50 or 100 μ M, with a smaller effect being evident at 20 μ M (Figure 5F). The band-shift analyses are indicative of changes in the activity of S6K1, but to obtain a more direct assessment of the *in vivo* activity of S6K1, we looked at the phosphorylation of its best-characterized substrate, rpS6, using a phospho-specific antibody. As shown in Figure 5(G), insulin increased the phosphorylation of rpS6. In line with their lack of effect on the migration of S6K1, KCl and DMSO had no obvious effect on rpS6 phosphorylation, whereas SB-216763 caused a slight increase. LiCl elicited a rather more pronounced increase in rpS6 phosphorylation, which matches its ability to elicit the mobility shift of S6K1 shown in Figure 5(E). In agreement with the apparent reduction in S6K1 phosphorylation, SB-415286 markedly inhibited the increase in rpS6 phosphorylation elicited by insulin. When tested across a range of concentrations, it was clear that even at 20 μ M, SB-415286 inhibited the insulin-induced increase in rpS6 phosphorylation (Figure 5H). At 50 or 100 μ M, the effect of insulin was completely inhibited.

It thus appears that SB-415286 interferes with mTOR signalling, whereas LiCl may (albeit rather weakly) stimulate it. This could be due to an effect on mTOR itself or on the poorly

understood components that lie downstream of mTOR and link it to the phosphorylation of 4E-BP1 and S6K1. Thus, as for SB-216763, but for different reasons, results obtained in experiments in which SB-415286 is used to study the role of GSK3 *in vivo* must be treated with caution, as they might reflect its ability to interfere with mTOR signalling. For example, an effect of SB-415286 on a biological endpoint may reflect a role for GSK3 or an involvement of mTOR signalling.

The inhibition of mTOR signalling by SB-415286 could contribute to the inhibition of protein synthesis caused by this compound (Table 1). However, another much more potent inhibitor of mTOR signalling, rapamycin, only has a slight effect on protein synthesis in CHO cells over short time periods, such as those studied here (typically inhibition by 5–10%; A. Flynn, J. Vyas and C. G. Proud, unpublished work). It is therefore most unlikely that the inhibition of protein synthesis caused by SB-415286 is entirely due to its effect on mTOR signalling and it must presumably have further unidentified effects. Although the activation of mTOR signalling caused by LiCl may contribute to its ability to activate [³⁵S]methionine incorporation, the observation that KCl had a similar effect suggests that the picture is more complicated, for example, that treatment of cells with these salts has other effects which influence protein synthesis. As discussed above, caution must be exercised when interpreting the effects of any of these treatments on cell physiology.

Concluding remarks

The aim of the experiments described in Table 1 and Figure 4 was to establish whether inhibition of GSK3 and dephosphorylation of eIF2B ϵ was sufficient to elicit activation of eIF2B. None of the three GSK3 inhibitors tested caused the activation of eIF2B. However, the results for SB-216763 were not helpful here, as it caused phosphorylation of eIF2 α and hence inhibition of eIF2B. LiCl and SB-415286 have effects on signalling through mTOR, which detracts in general from their utility as specific signalling inhibitors. However, since mTOR signalling appears not to be involved in the control of eIF2B [6], and since the effects on mTOR signalling operate in opposite directions, these additional effects do not detract from the conclusion that inhibition of GSK3 and dephosphorylation of Ser⁵³⁵ in eIF2B ϵ is insufficient to activate eIF2B. Thus these results are in striking agreement with the conclusions from our previous study [22], examining the interplay between nutrients and insulin in the control of eIF2B. In CHO cells deprived of nutrients, Ser⁵³⁵ undergoes dephosphorylation in response to insulin, but eIF2B is not activated. This again argued that dephosphorylation of this site is not enough to activate eIF2B. The implication of this finding is that additional inputs are required, and that these are dependent upon the nutrient status of the cells, as summarized in Scheme 1. The cells used in the present study were maintained in medium containing nutrients; it therefore seems likely that the additional input for activation of eIF2B requires both insulin and nutrients. The nature of this additional input(s) remains to be established. Our recent study of the phosphorylation of eIF2B *in vivo* [17] demonstrated that only the ϵ -subunit was phosphorylated under the serum-starved conditions we employed. In that study [17], we identified five sites of phosphorylation in eIF2B ϵ . These are substrates (*in vitro*) for CK1, CK2, GSK3 and members of the dual specificity tyrosine phosphorylated and regulated kinase (DYRK) group. Of these enzymes, only GSK3 is thought to be regulated by insulin and none of them is known to be affected by nutrients. However, the homologues of DYRK in lower eukaryotes are involved in nutrient signalling (for example, yeast Yak1 [48,49] and *Dictyostelium* YakA [50]), and it is possible that

Scheme 1 Interplay between inputs to the control of eIF2B activity in CHO.K1 cells

The insulin receptor signals via PI 3-kinase (PI 3-k) to inactivate GSK3, leading to the dephosphorylation of Ser⁵³⁵ in eIF2B ϵ . This input is insufficient to activate eIF2B, however, and additional input(s) are also required. The simplest view of this is that one additional input is required, which is activated by insulin and dependent upon the availability of nutrients (amino acids, glucose). However, more complex situations are possible. See the text for further discussion of this point.

mammalian DYRKs are also modulated by nutritional conditions. It is also possible that insulin elicits the phosphorylation of additional sites in eIF2B ϵ or even another subunit of the complex. Recent studies on the activation of eIF2B by growth factors imply a role for signalling pathways other than the PKB/GSK3 pathway [7,9]. Given the key role that eIF2B plays in regulating mRNA translation, a process that requires both amino acids and energy, it is not unexpected that its control involves multiple inputs to coordinate its activity with hormonal, mitogenic and nutritional stimuli. The present study gives further impetus to studies designed to establish a full understanding of the control of eIF2B in animal cells.

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REFERENCES

- Proud, C. G. (2001) Regulation of eukaryotic initiation factor eIF2B. *Prog. Mol. Subcell. Biol.* **26**, 95–114.
- Hinnebusch, A. G. (2000) Mechanism and regulation of methionyl-tRNA binding to ribosomes. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 185–243, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Kimball, S. R. and Jefferson, L. S. (1988) Effect of diabetes on guanine nucleotide exchange factor activity in skeletal muscle and heart. *Biochem. Biophys. Res. Commun.* **156**, 706–711.
- Welsh, G. I. and Proud, C. G. (1992) Regulation of protein synthesis in Swiss 3T3 fibroblasts. Rapid activation of the guanine-nucleotide-exchange factor by insulin and growth factors. *Biochem. J.* **284**, 19–23.
- Welsh, G. I., Miller, C. M., Loughlin, A. J., Price, N. T. and Proud, C. G. (1998) Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett.* **421**, 125–130.
- Welsh, G. I., Stokes, C. M., Wang, X., Sakaue, H., Ogawa, W., Kasuga, M. and Proud, C. G. (1997) Activation of translation initiation factor eIF2B by insulin requires phosphatidylinositol 3-kinase. *FEBS Lett.* **410**, 418–422.

- 7 Kleijn, M. and Proud, C. G. (2000) The activation of eukaryotic initiation factor (eIF)2B by growth factors in PC12 cells requires MEK/Erk signalling. *FEBS Lett.* **476**, 262–265
- 8 Kleijn, M., Welsh, G. I., Scheper, G. C., Voorma, H. O., Proud, C. G. and Thomas, A. A. M. (1998) Nerve and epidermal growth factors induce protein synthesis and eIF2B activation in PC12 cells. *J. Biol. Chem.* **273**, 5536–5541
- 9 Quevedo, C., Alcazar, A. and Salinas, M. (2000) Two different signal transduction pathways are implicated in the regulation of initiation factor 2B activity in insulin-like growth factor-1-stimulated neuronal cells. *J. Biol. Chem.* **275**, 19192–19197
- 10 Welsh, G. I., Miyamoto, S., Proud, C. G. and Safer, B. (1996) T-cell activation leads to rapid stimulation of translation initiation factor eIF-2B and inactivation of glycogen synthase kinase-3. *J. Biol. Chem.* **271**, 11410–11413
- 11 Kimball, S. R., Horetsky, R. L. and Jefferson, L. S. (1998) Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. *J. Biol. Chem.* **273**, 30945–30953
- 12 Gilligan, M., Welsh, G. I., Flynn, A., Bujalska, I., Proud, C. G. and Docherty, K. (1996) Glucose stimulates the activity of the guanine nucleotide-exchange factor eIF-2B in isolated rat islets of Langerhans. *J. Biol. Chem.* **271**, 2121–2125
- 13 Pavitt, G. D., Ramaiah, K. V. A., Kimball, S. R. and Hinnebusch, A. G. (1998) eIF2 independently binds two distinct eIF2B subcomplexes that catalyse and regulate guanine-nucleotide exchange. *Genes Dev.* **12**, 514–526
- 14 Gomez, E. and Pavitt, G. D. (2000) Identification of domains and residues within translation initiation factor eIF2B ϵ required for guanine nucleotide-exchange reveals a novel activation function promoted by eIF2B complex formation. *Mol. Cell. Biol.* **20**, 3965–3976
- 15 Fabian, J. R., Kimball, S. R., Heininger, N. K. and Jefferson, L. S. (1997) Subunit assembly and guanine nucleotide exchange factor activity of eukaryotic initiation factor eIF2B subunits expressed in Sf9 cells. *J. Biol. Chem.* **272**, 12359–12365
- 16 Williams, D. D., Pavitt, G. D. and Proud, C. G. (2001) Characterisation of the initiation factor eIF2B and its regulation in *Drosophila melanogaster*. *J. Biol. Chem.* **276**, 3733–3742
- 17 Wang, X., Paulin, F. E. M., Campbell, L. E., Gomez, E., O'Brien, K., Morrice, N. and Proud, C. G. (2001) Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon subunit and their roles *in vivo*. *EMBO J.* **20**, 4349–4359
- 18 Litchfield, D. W., Dobrowolska, G. and Krebs, E. G. (1994) Regulation of casein kinase-II by growth factors: a re-evaluation. *Cell Mol. Biol. Res.* **40**, 373–381
- 19 Welsh, G. I. and Proud, C. G. (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem. J.* **294**, 625–629
- 20 Cohen, P., Alessi, D. R. and Cross, D. A. E. (1997) PDK1, one of the missing links in insulin signal transduction? *FEBS Lett.* **410**, 3–10
- 21 Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature (London)* **378**, 785–789
- 22 Campbell, L. E., Wang, X. and Proud, C. G. (1999) Nutrients differentially modulate multiple translation factors and their control by insulin. *Biochem. J.* **344**, 433–441
- 23 Coghlan, M. P., Culbert, A. A., Cross, D. A. E., Corcoran, S. L., Yates, J. W., Pearce, N. J., Rausch, O. L., Murphy, G. J., Carter, P. S., Cox, L. R. et al. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. Biol.* **7**, 793–803
- 24 Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R. and Proud, C. G. (2001) Regulation of elongation factor 2 kinase by p90^{RSK1} and p70 S6 kinase. *EMBO J.* **20**, 4370–4379
- 25 Dickens, M., Chin, J. E., Roth, R. A., Ellis, L., Denton, R. M. and Tavaré, J. M. (1992) Characterization of insulin-stimulated protein serine/threonine kinases in CHO cells expressing human insulin receptors with point and deletion mutations. *Biochem. J.* **287**, 201–209
- 26 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **77**, 248–254
- 27 Welsh, G. I., Foulstone, E. J., Young, S. W., Tavaré, J. M. and Proud, C. G. (1994) Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochem. J.* **303**, 15–20
- 28 Sutherland, C. and Cohen, P. (1994) The α -isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70 S6 kinase or MAP kinase-activated protein kinase-1 *in vitro*. *FEBS Lett.* **338**, 37–42
- 29 Sutherland, C., Leighton, I. A. and Cohen, P. (1993) Inactivation of glycogen synthase kinase-3 β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.* **296**, 15–19
- 30 Eldar-Finkelman, H., Segar, R., Vandenheede, J. R. and Krebs, E. G. (1995) Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein kinase signaling pathway in NIH/3T3 cells. *J. Biol. Chem.* **270**, 987–990
- 31 Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.* **273**, 18623–18632
- 32 Stambolic, V., Ruel, L. and Woodgett, J. R. (1996) Lithium inhibits glycogen synthase kinase 3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664–1668
- 33 Klein, P. S. and Melton, D. A. (1996) A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8455–8459
- 34 Summers, S. A., Kao, A. W., Kohn, A. D., Backus, G. S., Roth, R. A., Pessin, J. E. and Birnbaum, M. J. (1999) The role of glycogen synthase kinase 3 β in insulin-stimulated glucose metabolism. *J. Biol. Chem.* **274**, 17934–17940
- 35 Lucas, F. R., Gool, R. G., Gordon-Weekes, P. R. and Salinas, P. C. (1998) Inhibition of GSK-3 β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J. Cell. Sci.* **111**, 1351–1361
- 36 Rowlands, A. G., Panniers, R. and Henshaw, E. C. (1988) The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. *J. Biol. Chem.* **263**, 5526–5533
- 37 Kaufman, R. J. (2000) Double-stranded RNA-activated protein kinase PKR. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 503–528, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 38 Chen, J.-J. (2000) Heme-regulated eIF2 α kinase. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 529–546, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 39 Ron, D. and Harding, H. P. (2000) PERK and translational control by stress in the endoplasmic reticulum. In *Translational Control of Gene Expression* (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds.), pp. 547–560, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 40 Berlanga, J. J., Santoyo, J. and de Haro, C. (1999) Characterization of a mammalian homolog of the GCN2 eukaryotic initiation factor 2 α kinase. *Eur. J. Biochem.* **265**, 754–762
- 41 Sood, R., Porter, A. C., Ma, K., Quilliam, L. A. and Wek, R. C. (2000) Pancreatic eukaryotic initiation factor-2 α kinase (PEK) homologues in humans, *Drosophila melanogaster* and *Caenorhabditis elegans* that mediate translational control in response to endoplasmic reticulum stress. *Biochem. J.* **346**, 281–93
- 42 Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonners-Weir, S. and Kaufman, R. J. (2001) Translational control is required for the unfolded protein response and *in vivo* glucose homeostasis. *Mol. Cell* **7**, 1165–1176
- 43 Dennis, P. B., Fumagalli, S. and Thomas, G. (1999) Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Opin. Genet. Devel.* **9**, 49–54
- 44 Gingras, A.-C., Raught, B. and Sonenberg, N. (2001) Control of translation by the target of rapamycin proteins. *Prog. Mol. Subcell. Biol.* **27**, 143–174
- 45 Gingras, A.-C., Raught, B. and Sonenberg, N. (1999) eIF4 translation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68**, 913–963
- 46 Avruch, J., Belham, C., Weng, Q., Hara, K. and Yonezawa, K. (2001) The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog. Mol. Subcell. Biol.* **26**, 115–154
- 47 Wang, X., Campbell, L. E., Miller, C. M. and Proud, C. G. (1998) Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* **334**, 261–267
- 48 Thevelien, J. M. and de Winder, J. H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **33**, 904–918
- 49 Garrett, S., Menold, M. M. and Broach, J. R. (1991) The *Saccharomyces cerevisiae* YAK1 gene encodes a protein kinase that is induced by arrest early in the cell cycle. *Mol. Cell. Biol.* **11**, 4045–4052
- 50 Souza, G. M., Lu, S. and Kuspa, A. (1998) YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*. *Development* **125**, 2291–2302