

Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin

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Received 8 December 1997

Abstract Eukaryotic initiation factor eIF2B catalyses a key regulatory step in mRNA translation. eIF2B and total protein synthesis are acutely activated by insulin, and this requires phosphatidylinositol 3-kinase (PI 3-kinase). The ϵ -subunit of eIF2B is phosphorylated by glycogen synthase kinase-3 (GSK-3), which is inactivated by insulin in a PI 3-kinase-dependent manner. Here we identify the phosphorylation site in eIF2B as Ser⁵⁴⁰ and show that treatment of eIF2B with GSK-3 inhibits its activity. Ser⁵⁴⁰ is phosphorylated in intact cells and undergoes dephosphorylation in response to insulin. This is blocked by PI 3-kinase inhibitors. Insulin-induced dephosphorylation of this inhibitory site in eIF2B seems likely to be important in the overall activation of translation by this hormone.

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Key words: Eukaryotic initiation factor; Phosphorylation; Insulin; Glycogen synthase kinase-3

1. Introduction

mRNA translation is an important control point in gene expression in eukaryotic cells [1,2] and acutely regulated by a variety of agents, including hormones, mitogens, growth factors and stress conditions. Insulin acutely activates protein synthesis in a range of mammalian cell types, including tissues of major importance for whole body protein metabolism such as skeletal muscle and fat cells. Considerable efforts have therefore been made to identify the regulatory mechanisms by which this occurs [3]. Recent data indicate that the activation of protein synthesis by insulin requires signalling pathways involving phosphatidylinositol 3-kinase (PI 3-kinase), based on the use of inhibitors of PI 3-kinase (such as wortmannin) and of mutants of the insulin receptor substrate, IRS-1 [4,5].

The primary effect of insulin on protein synthesis is at the level of translation initiation [1,2] although effects on elongation have also been reported [6]. Translation initiation requires a set of (eukaryotic) initiation factors (eIFs) which are involved in the process of binding the ribosome to the

mRNA and locating the correct start codon (reviewed in [7]). The initiator methionyl-tRNA (Met-tRNA_i) is brought to the ribosome by initiation factor eIF2 in a complex also containing GTP, which is subsequently hydrolysed during the initiation process. After each initiation event, the active GTP-bound form of eIF2 must be regenerated from [eIF2.GDP] by a nucleotide-exchange reaction mediated by eIF2B, a heteropentameric protein which plays an important role in regulating mRNA translation [8]. Since eIF2 is required for every initiation event, regulation of the activities of eIF2 and eIF2B can modulate overall rates of translation initiation. Previous studies have shown that insulin activates this step of translation initiation in skeletal muscle and other types of cells [9–13]. Activation of eIF2B by insulin requires PI 3-kinase [13].

Here we show that glycogen synthase kinase-3 (GSK-3) phosphorylates eIF2B at a conserved serine residue in its ϵ -subunit (Ser⁵⁴⁰) and provide evidence that this results in the inactivation of eIF2B. GSK-3 itself is inactivated in response to insulin, and other agents which stimulate protein synthesis, in a PI 3-kinase-dependent manner [13–19]. We show that Ser⁵⁴⁰ is phosphorylated in intact cells and that insulin brings about its dephosphorylation through a PI 3-kinase-dependent mechanism. These findings point to the operation of a novel signalling pathway by which insulin can activate overall translation initiation.

2. Materials and methods

2.1. Chemicals and biochemicals

Chemicals and biochemicals were obtained respectively from BDH (Poole, Dorset, UK) and Sigma Chemical Co. (Poole, Dorset, UK), unless otherwise stated. eIF2 and eIF2B were prepared from rabbit reticulocyte lysates [20]. GSK-3 α and - β were generously provided by Professor Philip Cohen (University of Dundee). The *Drosophila melanogaster* homologue of GSK-3 was kindly provided by Drs Ken Hughes and Jim Woodgett (Toronto, Canada). Casein kinases-1 and -2 were from Promega (Madison, USA). Synthetic peptides were prepared by Dr G. Bloomberg (University of Bristol, UK).

2.2. Cells

CHO.T cells were maintained as described previously [21]. Prior to insulin treatment, cells were stepped down into medium lacking serum for 24 h. Insulin (20 nM) was added for 10 min before extraction of the cells. Where used, wortmannin (100 nM) or LY294002 (20 μ M) were added 30 min before the treatment with insulin.

2.3. Analysis of translation factor activity and phosphorylation

Measurement of eIF2B activity, the *in vitro* phosphorylation of eIF2B and the phosphorylation of the eIF2B-based peptides were performed as outlined earlier [20,22]. CNBr and proteinase digestions, phosphoamino acid analysis, peptide mapping and tricine gel electrophoresis were carried out as described [23]. Amino acid se-

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quence analysis and identification of the site in eIF2B phosphorylated by GSK-3 was performed by Dr D.C. Pappin (Imperial Cancer Research Fund Laboratories, London) [24]. CHO.T cells were extracted and fractionated by MonoQ chromatography as described [12].

2.4. PCR amplification of partial cDNAs for eIF2Be

Total RNA was isolated using Trizol reagent (Gibco BRL) following the manufacturer's directions. cDNA was prepared from each RNA sample using AMV reverse transcriptase (Promega cDNA synthesis kit). Oligonucleotides were designed using conserved regions of the nucleotide sequences of rat, rabbit and human eIF2Be. These primers (not shown) flank the region encoding the site in rabbit eIF2Be which is phosphorylated by GSK-3. The corresponding regions of Chinese hamster and mouse eIF2Be were amplified by PCR using these primers. In each case a single product of the predicted size was obtained. The amplified DNA was purified and sequenced directly using an ABI 373A DNA sequencer.

2.5. Antisera

An anti-phosphopeptide antibody was raised in rabbit against the peptide AELDS(p)RAGSPLC (where S(p) denotes phosphoserine), which corresponds to the site in eIF2B phosphorylated in vitro by GSK-3, as described previously [25,26]. For immunisation, the peptide was conjugated to keyhole limpet haemocyanin using the heterobifunctional crosslinker sulpho-MBS (*m*-maleimidobenzoyl-*N*-hydroxy-sulphosuccinimide ester).

3. Results and discussion

3.1. GSK-3 phosphorylates eIF2B at a conserved serine residue in its ϵ -subunit

As reported previously, purified eIF2B was readily phosphorylated by GSK-3 β [27] (Fig. 1A), and this occurred exclusively on the largest (ϵ)-subunit. Fig. 1A shows that eIF2Be is also phosphorylated by the other isoform of GSK-3 found in mammalian cells (α) and by its homologue from *D. melanogaster*, the product of the *zeste-white* or *shaggy* gene (reviews [19,28]). The only radiolabelled phosphoamino acid detected following treatment of purified eIF2B with highly purified GSK-3 was phosphoserine (Fig. 1B); in a previous study using less highly purified GSK-3 we also detected some phosphothreonine [27], but this has not been observed in any of our subsequent studies. The initial rate of phosphorylation of eIF2Be by GSK-3 was rapid and comparable to that of glycogen synthase (GS). However, the final stoichiometry of phosphorylation was often low, typically being about 0.1 mol P/mol eIF2Be, although it varied slightly between preparations of eIF2B (0.08–0.14). Singh et al. [29] recently reported a very similar stoichiometry for the phosphorylation of eIF2B

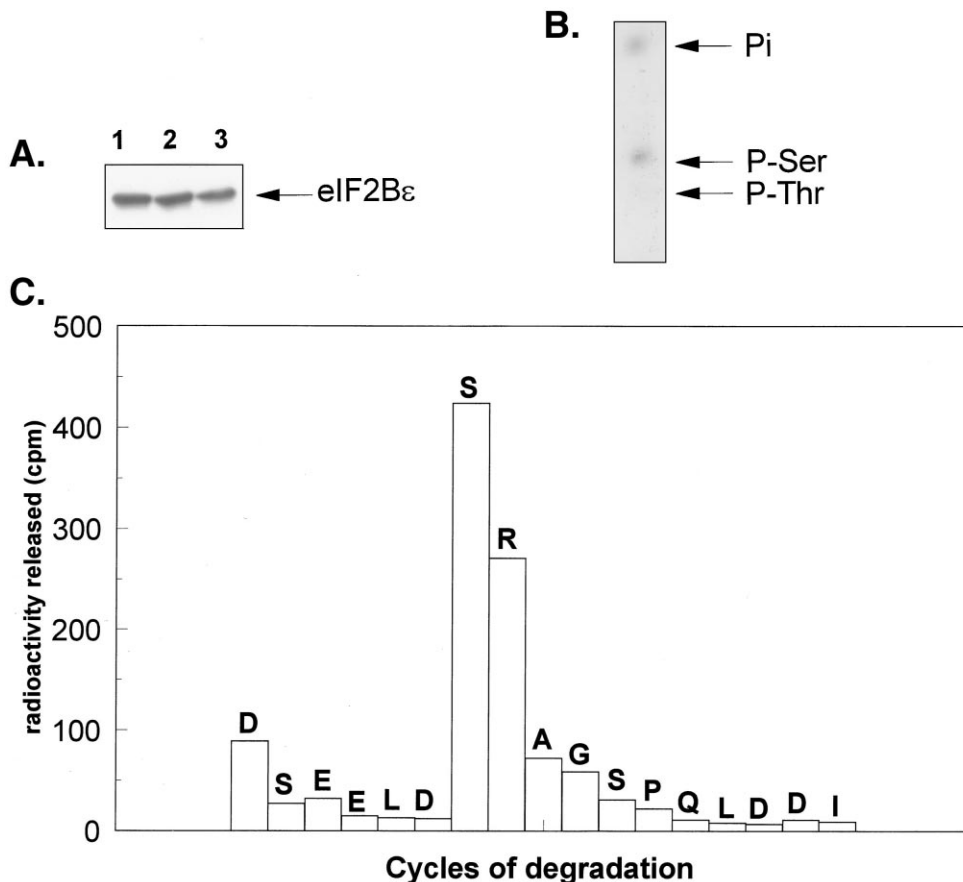


Fig. 1. GSK-3 phosphorylates Ser⁵⁴⁰ in eIF2Be. A: Phosphorylation of eIF2B by GSK-3 α (lane 1), GSK-3 β (lane 2) and Shaggy (lane 3). Purified eIF2B was phosphorylated in vitro using either the α or β isoenzyme of mammalian GSK-3 or the *Drosophila* homologue Shaggy. The arrow shows the position of eIF2Be. B: Phosphoamino acid analysis of eIF2B radiolabelled using purified GSK-3. The positions of inorganic phosphate (Pi), P-Ser, P-Thr and the origin are indicated. C: Location of the site in eIF2B phosphorylated by GSK-3 using solid phase sequencing. CNBr fragments from purified rabbit eIF2B phosphorylated by GSK-3 were resolved by tricine gel electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF, Problott, Applied Biosystems). The site phosphorylated by GSK-3 was identified using a Milligen L600 solid-phase sequencer. The stained protein band was covalently immobilised on the surface of the Problott membrane by treatment with poly(allylamine) and 1,4-phenylenediisothiocyanate. Cycle fractions were split to deliver 50% to the HPLC for identification of phenylthiohydantoin derivatives, the remainder being collected for Cerenkov counting. The results from this sequence analysis were compared with the published amino acid sequence to identify the site as Ser⁵⁴⁰ of the ϵ subunit.

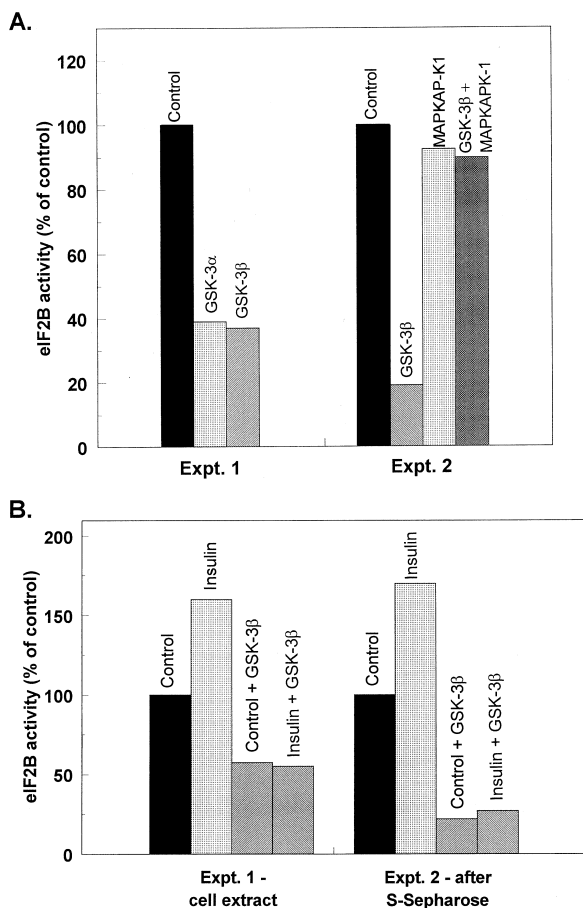


Fig. 2. Effect of GSK-3 on eIF2B activity in cell extracts. The effect of GSK-3 on the activity of eIF2B was determined in reticulocyte lysates (A) and in extracts from control and insulin-treated CHO.T cells (B). Cell lysates or extracts were incubated with ATP (0.2 mM) and MgCl₂ (2 mM) at 30°C for 20 min, with or without GSK-3. A: Experiment 1: Samples of reticulocyte lysate were treated with GSK-3 (α or β isoform) for 20 min prior to measurement of eIF2B activity. Experiment 2: Samples of reticulocyte lysate were treated with GSK-3 β , MAPKAP-K1 or GSK-3 β preincubated with MAPKAP-K1 and then assayed for eIF2B activity. Control are samples incubated without GSK-3. Similar results were obtained in six separate experiments. B: Experiment 1: Samples of CHO.T cell extracts from control or insulin-treated cells were incubated without or with GSK-3 prior to assay of eIF2B activity. In experiment 2, eIF2B was partially purified from extracts of control or insulin-treated CHO.T cells using fast flow S-Sepharose as described previously [12]. eIF2B was eluted with buffer containing 0.4 M NaCl. The partially purified eIF2B was then treated with or without GSK-3 and assayed for activity.

by GSK-3 (0.12 mol P/mol eIF2B). This combination of high initial rate but low plateau stoichiometry is likely to reflect a particular feature of the substrate specificity of GSK-3. Phosphorylation of a target residue by GSK-3 often depends on

the prior phosphorylation of the protein at a nearby serine, e.g. in GS [28,30]. Thus the maximal level of phosphorylation by GSK-3 of a site for which it requires a priming phosphoserine is limited by the level of phosphorylation of this 'priming site'. Most proteins that we isolate from reticulocyte lysates are almost entirely in their dephosphorylated states [31–33]. Thus the low stoichiometry of phosphorylation of eIF2B by GSK-3 may reflect a similarly low level of phosphorylation of a priming site in the purified factor.

To identify the residue(s) phosphorylated by GSK-3, eIF2Be was radiolabelled by GSK-3, subjected to CNBr digestion and the resulting fragments were analysed by polyacrylamide gel electrophoresis using the tricine gel system [34]. Autoradiography revealed a single labelled CNBr fragment of apparent M_r about 6500 which was then subjected to automated Edman degradation and the release of radiolabel was monitored at each cycle (Fig. 1C). Apart from a small amount appearing in the first cycle (Asp), no radioactivity was released before cycle 7, the first cycle where a Ser was encountered. Release of radioactivity trailed into the next cycle (Arg), but thereafter no further radioactivity was released and none remained associated with the peptide after the end of the sequencing run (which did not proceed as far as the C-terminus of the peptide). The sequence data obtained here (DSEELDSRAGSPQLDDI) correspond to a region towards the C-terminus of eIF2Be [35], the Ser labelled by GSK-3 being Ser⁵⁴⁰.

This sequence contains a Ser at +4 relative to the residue phosphorylated by GSK-3 which could act as a priming phosphorylation site. Our recent experiments employing synthetic peptides based on the sequence around Ser⁵⁴⁰ in eIF2Be [36] demonstrated that prior phosphorylation of the serine at +4 is essential for the phosphorylation of the residue corresponding to Ser⁵⁴⁰ by GSK-3. These data offer strong support to the idea that phosphorylation of this site in eIF2Be itself also requires such a priming phosphorylation event. Consistent with this, prior treatment of purified eIF2B with protein phosphatase-1 reproducibly abolished its subsequent phosphorylation by GSK-3 (data not shown).

When these experiments were performed, sequence data for eIF2Be were available for only one species (rabbit [37]), which we used to design primers for PCR to amplify partial cDNAs corresponding to this region of eIF2Be from four other species (Table 1). In each case, serines are present at the positions corresponding to both the phosphorylation site for GSK-3 (Ser⁵⁴⁰) and Ser⁵⁴⁴ (the potential priming site) of the rabbit sequence. We have sought to identify which protein kinase might phosphorylate Ser⁵⁴⁴ and thus act as a 'priming kinase', and have tested a number of enzymes, including casein kinases-1 and -2, which can phosphorylate eIF2Be [20,29] but failed to enhance the phosphorylation of eIF2B by GSK-3, and several proline-directed kinases (since Ser⁵⁴⁴ is followed

Table 1
Sequence around the GSK-3 phosphorylation site in eIF2Be from various mammalian species

DSEEPD S RGSGSPQMDDIKVF	Human	U23028
DPEELD S RAGSPQLDDIRVF	Rat	U19516
DSEELD S RAGSPQLDDIKVF	Rabbit	U23037
DSEELD S RAVSPQSDDIKVF	Hamster	This work
DPEELD S RAGSPQLDDIRVF	Mouse	This work

The site phosphorylated by GSK-3 in the rabbit factor and the corresponding residue in the polypeptide from other species is shown bold. The putative priming serine is underlined.

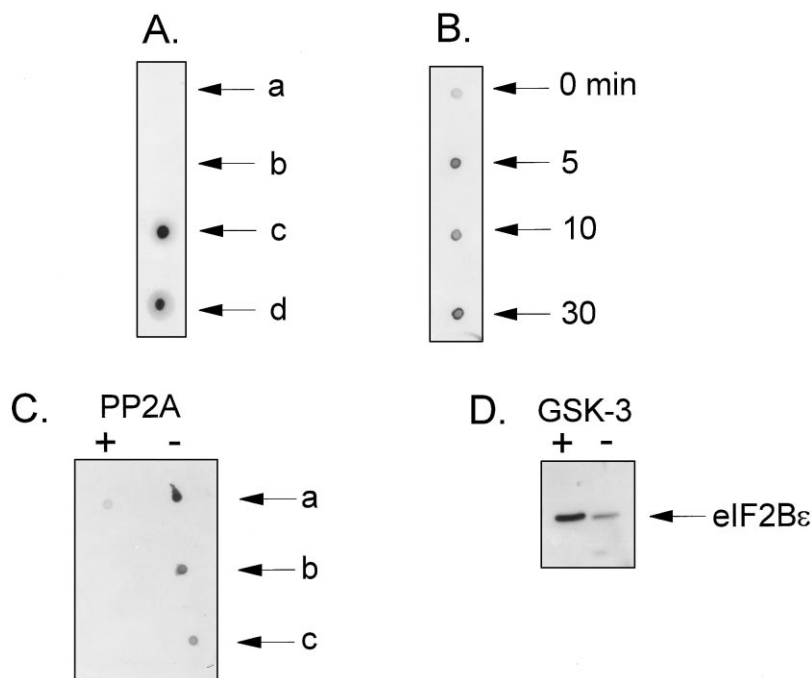


Fig. 3. Development of an anti-phosphopeptide antibody recognising Ser⁵⁴⁰(P) in eIF2Bε. A: Dot blot in which the antibody raised against the peptide AELDS⁵⁴⁰(p)RAGSPC was tested for reactivity against a panel of peptides whose sequences are based on that around Ser⁵⁴⁰ in eIF2B: (a) RRAAEELDSRAGSPQL; (b) SRAGS⁵⁴⁴(p)PQSDC; (c) AELDS⁵⁴⁰(p)RAGS⁵⁴⁴(p)PQSDC; (d) AELDS⁵⁴⁰(p)RAGSPC. B: Dot blot showing reactivity of the anti-phosphopeptide antibody against purified rabbit reticulocyte eIF2B pretreated with GSK-3 for the times indicated. C: Three different preparations of purified rabbit eIF2B (a–c) were analysed by dot blot using the anti-phosphopeptide antibody with (+) or without (–) prior incubation with protein phosphatase-2A (PP-2A). D: Western blot (using the anti-phosphopeptide antibody) of purified reticulocyte eIF2B either untreated (–) or preincubated with GSK-3β and ATP for 30 min (+). The position of eIF2Bε is indicated.

by a proline) but none of them phosphorylated eIF2Bε. Further work is therefore needed to identify the putative priming kinase.

3.2. GSK-3-mediated phosphorylation inhibits the activity of eIF2B

Since GSK-3 only phosphorylated purified eIF2B to a low stoichiometry, we considered it unlikely that phosphorylation by GSK-3 would affect its activity significantly, and this was indeed the case (data not shown). We therefore examined the effect of GSK-3 on the activity of eIF2B in cell extracts, based on the arguments that in cell extracts either Ser⁵⁴⁴ in eIF2Bε might already be more fully phosphorylated or the putative 'priming kinase' might be present, permitting a higher level of GSK-3-mediated phosphorylation and thus giving rise to a larger change in eIF2B activity. Preincubation of reticulocyte lysates with GSK-3 (α or β) resulted in a marked fall in eIF2B activity (to about 35–40% of the control value, Fig. 2A) relative to the activity in controls which were not incubated or incubated without GSK-3. This effect was abolished by preincubation of the GSK-3 with MAPKAPK-1 (which inactivates GSK-3 [38,39]), indicating that it is due to the activity of GSK-3 (Fig. 2A).

We also examined the effect of GSK-3 on the activity of eIF2B in extracts from CHO.T cells and NIH.3T3 HIR cells. Both cell types express the human insulin receptor, and insulin activates eIF2B in both ([13] and our unpublished data). Preincubation of CHO.T cell extracts with GSK-3β reduced eIF2B activity substantially, and to a greater extent in extracts from insulin-treated cells than controls. Indeed, after pretreatment with GSK-3β, the activities of eIF2B in the control and

insulin-treated cell extracts were almost equal (Fig. 2B). The change in activity was retained after partial purification of the eIF2B by ion exchange (Fig. 2B), indicating that it reflects reduced intrinsic activity of the factor rather than altering its binding to a loosely associated regulator. Similar data were obtained using extracts of control and insulin-treated NIH.3T3 HIR cells (data not shown).

In these experiments, the maximal extents of inactivation observed were in the range 80–90%. The residual activity might either represent failure to achieve 100% occupancy of Ser⁵⁴⁰ or genuine basal activity of the phosphorylated factor. Although we have an anti-phosphopeptide antiserum with which we can assess the relative level of phosphorylation of Ser⁵⁴⁰ (see below), this approach does NOT give *absolute* levels of phosphorylation and direct correlations of % phosphorylation and % activity are not therefore possible.

3.3. Ser⁵⁴⁰ in eIF2Bε is phosphorylated in vivo and undergoes dephosphorylation in response to insulin

To study the phosphorylation of Ser⁵⁴⁰ in intact cells, we raised an antibody against a phosphopeptide corresponding to the sequence surrounding this serine (AELDS(p)RAGSPC, the cysteine being added to facilitate coupling of the peptide to the carrier). When used against a panel of peptides related to this sequence, it reacted with peptides containing a phosphate at this site whether or not there was also a phosphate group at Ser⁵⁴⁴ (Fig. 3A). Thus the antibody can react with Ser⁵⁴⁰(P) irrespective of the phosphorylation state of Ser⁵⁴⁴. It did not react with their non-phosphorylated counterparts (Fig. 3A). The antibody also reacted with eIF2B purified from rabbit reticulocytes (Fig. 3B–D) showing that Ser⁵⁴⁰ is

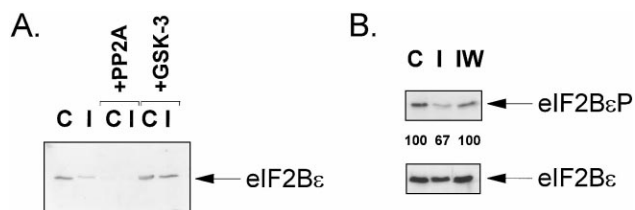


Fig. 4. Insulin brings about dephosphorylation of Ser⁵⁴⁰ in eIF2B. eIF2B was partially purified from CHO.T cells by MonoQ chromatography. The elution position of eIF2B was determined by measuring eIF2B activity in each of the fractions and by Western blotting with appropriate antisera. A: Fractions containing eIF2B activity were subjected to SDS-PAGE and Western blotting using the anti-phosphopeptide antibody. The arrows show the position of eIF2Bε. Samples of fractions from control (C) or insulin-treated (I) cells were also treated either with PP2A or GSK-3, as indicated, for 30 min at 30°C prior to analysis. Similar data were obtained in three separate experiments. B: Fractions from control cells (C) or cells treated with insulin in the absence (I) or presence (IW) of wortmannin were subjected to SDS-PAGE and Western blotting using the anti-phosphopeptide antiserum (upper blot) and the numbers below each lane indicate the densitometric readings expressed as % control (corrected for the amount of eIF2B loaded, determined using an anti-eIF2Bε monoclonal antibody, lower blot).

already partially phosphorylated in these preparations. The strength of the signal increased markedly when the eIF2B was pretreated with GSK-3 (Fig. 3B,D), demonstrating that it reflects the level of phosphorylation of Ser⁵⁴⁰. Conversely treatment of the eIF2B with phosphatase 2A resulted in a total loss of reactivity (Fig. 3C).

The anti-phospho-Ser⁵⁴⁰ antibody was then used to analyse the level of phosphorylation of this site in eIF2B partially purified from control or insulin-treated CHO.T cells. A clear signal in the position of the ε-subunit of eIF2B was obtained showing that this site is indeed phosphorylated in intact cells (Fig. 4A). Furthermore, the signal was substantially reduced for eIF2B partially purified from insulin-treated cells as compared to control cells. As expected, the signal was totally abolished by treatment of these fractions with phosphatase 2A and increased by pretreatment with GSK-3 (Fig. 4A). In particular, treatment with GSK-3 eliminated the difference in the strength of the signal for eIF2B from control and insulin-treated cells. A selective inhibitor of PI 3-kinase, wortmannin, blocked the ability of insulin to induce dephosphorylation of Ser⁵⁴⁰ in eIF2B (Fig. 4B), consistent with the requirement for PI 3-kinase for the inhibition of GSK-3. The strengths of the signals were quantitated by densitometric analysis of the ECL image. Insulin caused a drop in the signal seen with the anti-phosphopeptide antiserum, and wortmannin completely prevented this effect (intensity of signal: control cells, 100%; insulin-treated cells, 67 ± 4%; cells treated with insulin in the presence of wortmannin, 110 ± 15%). Similar data were obtained using another inhibitor of PI 3-kinase, LY294002. The relatively small effect of insulin on the phosphorylation of this site is reflected in the modest activation of eIF2B caused by insulin in CHO.T cells (Fig. 2B; [13]: larger effects on eIF2B activity are seen in other cell types [12,22,40]).

4. Conclusions

These data demonstrate that eIF2B is phosphorylated in vivo at the site which is labelled in vitro by GSK-3 (Ser⁵⁴⁰)

and that this inhibitory site undergoes dephosphorylation in intact cells in response to insulin. The activation of protein synthesis and of eIF2B by insulin, and the dephosphorylation of Ser⁵⁴⁰, all appear to be dependent on PI 3-kinase. Taken together, the data suggest that the activation of protein synthesis by insulin may be mediated to a large extent through the inactivation of GSK-3, and the consequent dephosphorylation and activation of eIF2B, which is required for overall translation initiation.

Acknowledgements: This work was supported by Project and Programme Grants from the Wellcome Trust. We are grateful to Dr Andrew Czernik (Rockefeller University, New York, USA) for helpful advice on the design of phosphopeptides for antiserum production. Some of the eIF2B used was prepared by Dr Susan Oldfield.

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