Biochem. J. (1993) 294, 625–629 (Printed in Great Britain)

RESEARCH COMMUNICATION

Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B

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We have studied the control of insulin-regulated protein kinases in Chinese hamster ovary cells transfected with the human insulin receptor (CHO.T cells). Among these enzymes is one that is obtained after chromatography of cell extracts on Mono-S, whose activity is decreased (7.3 ± 1.9-fold) within 10 min of insulin treatment. This enzyme phosphorylates glycogen synthase and the largest subunit of protein synthesis eukaryotic initiation factor (eIF)-2B (the guanine nucleotide exchange factor). The kinase appears to be glycogen synthase kinase-3 (GSK-3), on the basis of: (1) its ability to phosphorylate a peptide based on the phosphorylation sites for GSK-3 in glycogen synthase, and (2) the finding that the fractions possessing this activity contain immunoreactive GSK-3, whose peak is coincident with that of kinase activity, as judged by immunoblotting using antibodies specific for the α- and β-isoforms of GSK-3. The decrease in kinase activity induced by insulin was reversed by treatment of the column fractions with protein phosphatase-2A. These data indicate that insulin rapidly causes inactivation of GSK-3 and that this is due to phosphorylation of GSK-3. The implications of these findings for the control of glycogen and protein metabolism are discussed.

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) was originally identified as phosphorylating the regulatory enzyme of glycogen synthesis (glycogen synthase) at a cluster of serine residues now termed sites 3a–3d [1–4]. Phosphorylation of these sites inhibits the activity of GS, and the level of phosphorylation is decreased by insulin in skeletal muscle. Insulin acts to increase glycogen synthase activity and glycogen synthesis, indicating that the phosphorylation of the sites modified by GSK-3 may play an important role in the insulin-mediated regulation of glycogen synthesis [3,4]. Previous studies have indicated either that insulin activates GSK-3 (measured by an indirect assay [5–7]) or that insulin causes inactivation of the kinase (measured against ATP citrate lyase [8,9]). The former data are clearly inconsistent with the known effects of insulin on the phosphorylation of glycogen synthase.

GSK-3 has been cloned and shown to exist (in mammalian cells) as two isoenzymes (termed α and β) [10]. It has a close homologue in *Drosophila*, the product of the *zeste-white3* or *shaggy* homeotic gene [11,12]. GSK-3 phosphorylates a number of other regulatory proteins, including c-*jun*, protein phosphatase inhibitor-2, the regulatory subunit of cyclic AMP-dependent protein kinase and ATP citrate lyase [2,8,9,13–15]. Recently we have found that GSK-3 also phosphorylates, at least *in vitro*, protein synthesis initiation factor eIF-2B, which mediates the recycling of eukaryotic initiation factor-2 (eIF-2), the protein responsible for the attachment of initiator Met-tRNA to the ribosome during the initiation of translation in eukaryotic cells (reviewed in [16]). eIF-2B is an important regulatory component of translation: changes in its activity play an important role in controlling peptide-chain initiation under a variety of conditions [16,17]. A well-characterized mechanism for controlling eIF-2B activity is mediated by alterations in the level of phosphorylation of its substrate eIF-2 (specifically the α-subunit of eIF-2). Insulin (and other stimuli such as epidermal growth factor and phorbol esters) acutely activates eIF-2B in fibroblasts [18], by a mechanism that is independent of alterations in the phosphorylation of eIF-2α, suggesting direct regulation of eIF-2B, for example by phosphorylation. In the course of seeking kinases which phosphorylate eIF-2B and exploring their regulation by, in particular, insulin, we have found that the activity of GSK-3 is acutely decreased by insulin in Chinese hamster ovary cells which over-express the insulin receptor. This observation may have important implications for the control of protein synthesis, glycogen metabolism and other cellular processes.

MATERIALS AND METHODS

Chemicals and biochemicals were obtained respectively from BDH (Poole, Dorset, U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.), unless otherwise indicated. Glycogen synthase, protein phosphatase-2A (PP-2A), GSK-3β (from rabbit muscle) and the GSK-3 peptide substrate (YRRAAVPPSPSLSRHS-SPHQSEDEEE) were generously provided by Professor P. Cohen (University of Dundee, Dundee, U.K.). The 'priming' site phosphorylated by casein kinase-2 is underlined and the residues corresponding to phosphorylation sites for GSK-3 in glycogen synthase are shown bold. Antibodies to the α and β isoforms of GSK-3 and GSK-3β (expressed in baculovirus) were gifts from Dr. J. Woodgett and Dr. K. Hughes (Ontario Cancer Research Centre, Toronto, Canada). eIF-2B was prepared from rabbit reticulocyte lysates by Dr. S. Oldfield in this laboratory as described previously [19]. Casein kinase-2 was prepared from rat liver as described previously [19].

CHO.T cells (Chinese hamster ovary cells transfected with DNA encoding the human insulin receptor) were grown and maintained as described previously [20]. They were extracted as

Abbreviations used: CHO.T cells, Chinese hamster ovary cells transfected with cDNA encoding the human insulin receptor; eIF, eukaryotic initiation factor; GSK-3, glycogen synthase kinase-3; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A.

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previously described [20] into a buffer containing 50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.5 mM Na_3VO_4, 0.1 mM phenylmethanesulphonyl fluoride, 1 μM microcystin-LR and 1 μg/ml each of pepstatin, antipain and leupeptin. The extracts were then fractionated as described [20], except that in this case a Mono-S HR 5/5 column (Pharmacia) was used.

The ability of the Mono-S fractions to phosphorylate purified rabbit reticulocyte eIF-2B was assayed by incubating 10 μl of each fraction with eIF-2B for 15 min at 30 °C in a final volume of 25 μl containing 20 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM microcystin-LR, 1.5 mM EGTA, 0.1 mM Na_3VO_4 and 0.2 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). Reactions were stopped by the addition of SDS/PAGE sample buffer followed by boiling. Samples were then applied to standard SDS/polyacrylamide gels followed by radioautography of the gel [19].

Assays for the ability of the Mono-S fractions to phosphorylate glycogen synthase or the GSK-3 peptide substrate were carried out in the above buffer, except that the eIF-2B was replaced by either glycogen synthase (2 μg) or GSK-3 peptide substrate (25 μM). The glycogen synthase was pretreated as described previously to remove any traces of glycogen synthase kinase or phosphatase activity [21]. The GSK-3 peptide was prephosphorylated by incubation with casein kinase 2 (approx. 0.5 unit/ml, prepared from rat liver as described [19]) for 30 min before use. Appropriate controls using peptide which was not prephosphorylated were also carried out. In the case of glycogen synthase the reactions were stopped by spotting on to 3MM paper squares (Whatman). These papers were then immediately immersed in ice-cold 15% (v/v) trichloroacetic acid. In the case of the GSK-3 peptide the reactions were stopped by spotting on P81 phosphocellulose paper squares (Whatman) followed by immersion in 3% H_3PO_4. All papers were then washed four times in fresh acid as appropriate and radioactivity was then determined by the Čerenkov method.

To determine the role of phosphorylation in the kinase activity, the fractions were pretreated for 30 min with PP-2A under standard assay conditions but without ATP. Okadaic acid (2 μM) was then added prior to the measurement of kinase activity against eIF-2B or the GSK-3 peptide, which was carried out as above and initiated by addition of radiolabelled ATP.

Immunoblotting was carried out as described previously [20].

RESULTS AND DISCUSSION

To search for insulin-regulated kinases capable of phosphorylating eIF-2B, and which might be involved in controlling its activity, we have used CHO.T cells, which over-express the human insulin receptor and have been used successfully to study other insulin-regulated kinases [20].

CHO.T cell extracts were fractionated by ion-exchange chromatography and the resulting fractions were assayed for their ability to phosphorylate eIF-2B purified from rabbit reticulocytes. As shown in Figures 1(a) and 1(b), when CHO.T cell extracts were fractionated on Mono-S, a substantial peak of activity against the largest (84 kDa, p84 or ε) subunit of eIF-2B was observed. The activity in this region of the profile was greatly diminished in fractions derived from extracts of insulin-treated cells. Figures 1(a) and 1(b) show a typical experiment; this decrease in kinase activity was reproducibly observed in ten separate experiments, although the magnitude of the effect was

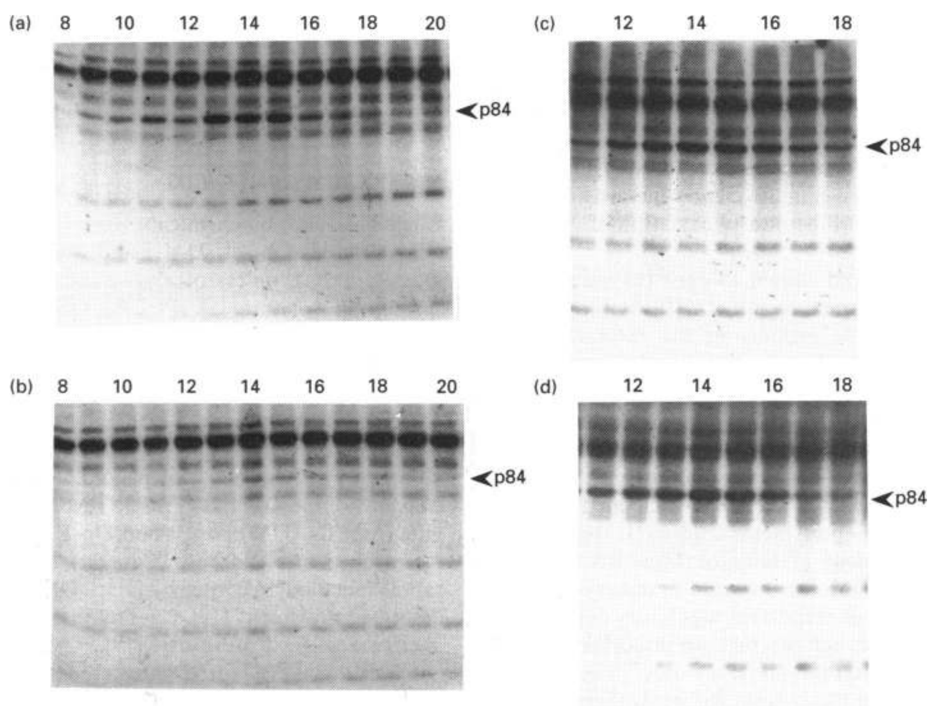

Figure 1 Assay of Mono-S fractions from CHO.T cells for protein kinase activity against initiation factor eIF-2B

Panel (**a**) and (**b**) show the kinase activities, using purified eIF-2B as substrate, of fractions from Mono-S columns from control and insulin-treated cells respectively. Panels (**c**) and (**d**) show the activities of the same fractions following treatment with PP-2A. Alternate fractions are numbered and the labelled arrowhead indicates the position of the 84 kDa (ε) subunit of eIF-2B. These fractions span the range of the gradient from 100 to 150 mM NaCl. The radiolabelled proteins seen in all lanes are proteins present in the eIF-2B used that undergo phosphorylation when incubated with [γ-^{32}P]ATP in the absence of column fractions. When column fractions were incubated in the absence of added eIF-2B, no radiolabelled polypeptides were apparent.

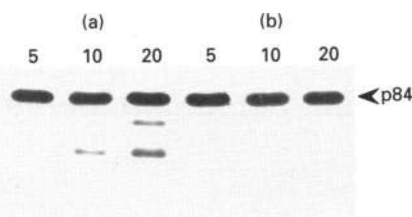

Figure 2 Time course of phosphorylation of eIF-2B by purified GSK-3

(**a**) Phosphorylation by GSK-3β from rabbit skeletal muscle; (**b**) phosphorylation by recombinant GSK-3β expressed in baculovirus. Times (in min) are indicated and the arrowhead shows the migration position of the 84 kDa subunit eIF-2B. The radiolabelled bands in Figure 1 that are due to the eIF-2B preparation are not apparent here, since highly purified and concentrated GSK-3 was used, rather than the relatively crude Mono-S fractions. Thus it was only necessary to expose the autoradiograph for a relatively short time, and weakly labelled bands are therefore not visible.

rather variable. In eight experiments where the activity was determined by densitometry of autoradiographs, the decrease was 7.3±1.9-fold. Pretreatment with insulin was for 10 min, so the inactivation of the kinase in response to insulin is rapid. We have not attempted shorter times.

This observation clearly raised the question of the identity of this insulin-inactivated kinase. We have found that, *in vitro*, highly purified GSK-3 can phosphorylate purified eIF-2B on its 84 kDa subunit. Figure 2 shows time courses of the phosphorylation of eIF-2Bε by the β isoform of GSK-3. Phosphorylation was on serine and threonine residues (results not shown). Since GSK-3 elutes from cation-exchange resins such as Mono-S at roughly similar salt concentrations to those eluting the eIF-2Bε kinase seen here [22], it was possible that the eIF-2Bε kinase was GSK-3, and a number of approaches were used to address this possibility.

As discussed above, GSK-3 phosphorylates a number of substrates including, clearly, glycogen synthase (at four sites termed sites 3a–3d [1,2]). The fractions from Mono-S containing eIF-2Bε kinase activity were therefore assayed for their ability to phosphorylate glycogen synthase. Kinase activity against glycogen synthase was evident in many fractions from the column (Figure 3a), consistent with the fact that glycogen synthase possesses phosphorylation sites for many protein kinases (see, e.g., [4]). However, in the fractions containing the activity against eIF-2Bε, the glycogen synthase kinase activity was decreased markedly by insulin (Figure 3a). Fractions from the profile shown from insulin-treated cells displayed 3-fold less glycogen synthase kinase activity than those from control cells, similar to the fall in the eIF-2Bε kinase activity. This is consistent with the notion that the insulin-inactivated eIF-2Bε kinase is GSK-3, but since glycogen synthase is a substrate for many kinases, it does not prove it. Additional, more specific, data were therefore sought.

Phosphorylation of sites 3a–3d in glycogen synthase by GSK-3 requires a priming phosphorylation at a serine at position +4 relative to site 3d, since the substrate specificity of GSK-3 (at least in this case) is based on a phosphoserine at +4 relative to the target serine [23,24]. The kinase thought to be responsible for the phosphorylation of the 'priming site' (termed site-5) is casein kinase-2 [24]. We have therefore used a synthetic peptide whose sequence is based on that around sites 3a–3d and site-5 in glycogen synthase, which only becomes a substrate for GSK-3 after phosphorylation at the most C-terminal serine by casein kinase-2. The fractions containing eIF-2Bε kinase activity also showed activity against the synthetic peptide substrate, but this

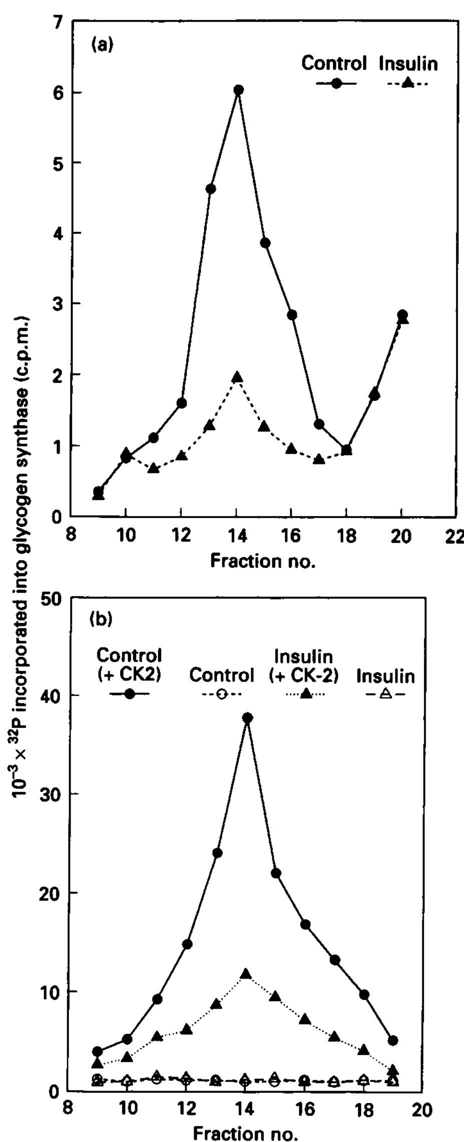

Figure 3 Kinase activity of Mono-S fractions from CHO.T cells against glycogen synthase (a) and the peptide substrate for GSK-3 (b)

Fractions were from control or insulin-treated cells as indicated, and in the case of the GSK-3 peptide, assays were performed with peptide pretreated with casein kinase 2 (' +CK-2') or not. Experimental details are given in the Materials and methods section.

was completely dependent on its prior prephosphorylation (using unlabelled ATP) by casein kinase-2 (Figure 3b). Fractions derived from insulin-treated cells showed decreased activity against the peptide, as they did against glycogen synthase or eIF-2Bε. Since GSK-3 is the only protein kinase known to phosphorylate this peptide in a manner dependent on prior casein kinase-2 treatment, this evidence strongly supports the idea that the kinase is indeed GSK-3.

To study the role of phosphorylation in the regulation of the activity of the insulin-inactivated kinase, appropriate fractions from Mono-S runs of control and insulin-treated cells were treated with PP-2A prior to assay against the GSK-3 peptide (Table 1) or eIF-2B (Figures 1c and 1d). When activity was measured against the peptide, addition of PP-2A to fractions from insulin-treated cells resulted in a marked increase in activity. In contrast, PP-2A treatment of fractions from control cells led to only a small increase in kinase activity (Table 1). PP-2A

Table 1 Effect of PP-2A on the activity of fractions from Mono-S against the peptide substrate for GSK-3

Assays were performed as described in Figure 2, and the phosphatase pretreatment was as in Figure 1. Treatments are defined as follows: CK-2, peptide pretreated with casein kinase-2; PP-2A, fractions pretreated with PP-2A.

		^{32}P radioactivity incorporated (c.p.m.)					
		Control cells			Insulin-treated cells		
Fraction no.	Treatment...	–	CK-2	CK-2/PP-2A	–	CK-2	CK-2/PP-2A
13		1943	37751	48782	1843	16243	46642
14		112	43248	51393	1412	24014	49898
15		1841	38168	49730	1639	21144	43126

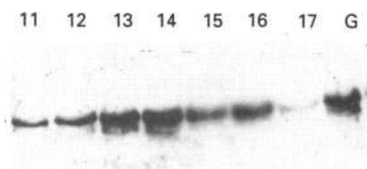

Figure 4 Immunoblot analysis of fractions from the Mono-S profile of control CHO.T cells treated with anti-GSK-3 antibodies

Numbers correspond to those of the fractions: lane G contained purified GSK-3β as a marker.

treatment virtually eliminated the difference in activity between the fractions from the two columns. PP-2A also abolished the difference in activity of fractions from the two columns as measured against eIF-2B (Figures 1c and 1d). This strongly indicates that the inactivation of GSK-3 by insulin is a consequence of its phosphorylation on serine or threonine, presumably by a protein kinase that is activated by insulin. In control cells there is presumably a low basal level of phosphorylation of GSK-3, accounting for the modest increase in activity seen after PP-2A treatment of the fractions from these cells.

To confirm that the column fractions contained GSK-3, antibodies to this protein were used. GSK-3 exists as two isoenzymes (α and β) and a mixture of antibodies against these two species was employed. Immunoblot analysis revealed a peak of immunoreactive GSK-3 in the fractions displaying kinase activity; the two peaks were coincident (Figure 4). No difference in the apparent amount of GSK-3 was evident when blots from insulin-treated and control cells were compared, indicating that the effect of insulin was not due to a decrease in the amount of GSK-3 protein. This is consistent with the data obtained using PP-2A, which suggested that the inactivation of GSK-3 by insulin was a consequence of its phosphorylation.

Earlier findings on the effects of insulin on GSK-3 activity are conflicting. Yang and co-workers, using an indirect assay involving the activation of protein phosphatase-1 (PP-1), have provided data indicating that insulin activates GSK-3 [5,6]. On the other hand, Benjamin's group have shown that insulin inactivates a protein kinase which phosphorylates ATP citrate lyase [8], subsequently identified as GSK-3 [9]. Inactivation of GSK-3 by insulin, as reported here and by Benjamin's group, is consistent with the decreased phosphorylation of the GSK-3 sites in ATP citrate lyase and glycogen synthase which occurs in response to insulin [3,4,25,26]. In the case of ATP citrate lyase, it is not clear what role, if any, phosphorylation by GSK-3 plays in the control of its activity or of flux on the pathway in which it participates, i.e. fatty acid biosynthesis. For glycogen synthase, inactivation of GSK-3 would provide a second mechanism by which insulin could decrease the phosphorylation of this enzyme at the regulatory GSK-3 sites, in addition to the activation of the glycogen-bound form of PP-1 [27].

The phosphorylation of c-*jun* by GSK-3 also inhibits its function, namely its binding to its target promoter, the phorbol ester response element [13]. Inactivation of GSK-3 by insulin could therefore serve to activate c-*jun*, switching on genes under its control. In the case of the initiation factor eIF-2B, GSK-3 could be involved in controlling peptide-chain initiation, which is activated by insulin in many cell types [18]. However, many questions remain to be answered here, including the effect of GSK-3 phosphorylation on the activity of eIF-2B, whether eIF-2B is phosphorylated at the GSK-3 site(s) *in vivo*, and whether the level of phosphorylation actually changes in response to insulin. This could potentially provide a mechanism for the coordinated regulation of transcription and translation, and of other biosynthetic pathways such as glycogen synthesis. Since many stimuli (including, for example, growth factors) also increase protein synthesis and other biosynthetic pathways, it will be important to study the effects of these agents on the activity of GSK-3.

Hughes et al. [28] have recently reported that GSK-3 activity is modulated by tyrosine phosphorylation. However, our data in Figure 1 and Table 1 suggest that the inactivation of GSK-3 by insulin is mediated by phosphorylation on serine and/or threonine. This is presumably mediated by an insulin-activated protein kinase. Goode et al. [29] have reported that GSK-3β is inactivated by protein kinase C. Several studies have concluded that protein kinase C may play a role in insulin action [30–32], although others have questioned this and the matter remains highly controversial [33,34]. Our own recent data (obtained, it should be pointed out, using Swiss 3T3 cells rather than CHO.T cells) have indicated that, while protein kinase C does appear to play a role in the overall activation of protein synthesis by insulin, it is not required for the activation of eIF-2B by this hormone [35]. Further investigation is clearly required to elucidate the mechanism by which insulin inactivates GSK-3, and the role (if any) of protein kinase C in this.

We are very grateful to Philip Cohen (Dundee, U.K.) for glycogen synthase, GSK-3β (from rabbit muscle), PP-2A and the GSK-3 substrate peptide, to Jim Woodgett and Kenneth Hughes (Toronto, Canada) for anti-GSK-3 antibodies and for baculovirus-expressed GSK-3β, and to Leland Ellis (Houston, U.S.A.) for CHO.T cells. eIF-2B was generously provided by Susan Oldfield in this laboratory.

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Received 8 June 1993/9 July 1993; accepted 13 July 1993