Further evidence that the inhibition of glycogen synthase kinase-3β by IGF-1 is mediated by PDK1/PKB-induced phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216

Morag Shaw*, Philip Cohen, Dario R. Alessi

MRC Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, UK

Received 19 September 1997

Abstract 293 cells were transfected with wild-type GSK3β (WT-GSK3β) or a mutant in which the PKB phosphorylation site (Ser-9) was altered to Ala (A9-GSK3β). Upon stimulation with IGF-1 or insulin, WT-GSK3β was inhibited 75% or 60%, respectively, whereas the activity of the A9-GSK3β mutant was unaffected. Incubation of WT-GSK3β with $PP2A_1$ (a Ser/Thr-specific phosphatase) completely reversed the IGF-1- or insulin-induced inhibition. IGF-1 stimulation did not induce any tyrosine dephosphorylation of WT-GSK3β or A9-GSK3β. Coexpression of WT-GSK3β in 293 cells with either PKBα (also known as AKT) or PDK1 (the 'upstream' activator of PKB) mimicked the IGF-1- or insulin-induced phosphorylation of Ser-9 and inactivation of GSK3β.

Key words: Insulin; Insulin-like growth factor-1; Protein kinase B; Glycogen synthase kinase-3; Glycogen synthase; 3-Phosphoinositide-dependent protein kinase

1. Introduction

Glycogen synthase kinase-3 (GSK3) was originally identified as a protein kinase that phosphorylated and inactivated glycogen synthase, the enzyme which catalyses the last step in glycogen synthesis [1]. Subsequently, GSK3 was found to phosphorylate other proteins, including eIF2B (a nucleotide exchange factor required for the initiation of protein synthesis) [2] and the microtubule-associated protein Tau [3]. Unusually for an extracellular signal-regulated protein kinase, GSK3 is fully active in unstimulated cells and is inhibited in response to insulin/IGF-1, growth factors and other signals (reviewed in [4,5]). The inactivation of GSK3 is likely to contribute to the activation of glycogen synthesis [4] and protein synthesis [2] and to the dephosphorylation of Tau [3] that are induced by insulin/IGF-1.

Stimulation of rat L6 myotubes with insulin/IGF-1 induces the phosphorylation of GSK3α and GSK3β at Ser-21 and Ser-9, respectively. Recent evidence suggests that these residues are phosphorylated by isoforms of protein kinase B (PKB) under these conditions [4]. The phosphorylation of these residues in vitro inactivates GSK3α/GSK3β and inactivation can be completely reversed by dephosphorylation catalysed by Ser/Thr-specific protein phosphatases, such as PP1 and PP2A [4].

In unstimulated cells GSK3 is also phosphorylated on a tyrosine residue (Tyr-279 in GSK3α and Tyr-216 in GSK3β) which lies in the 'activation loop' between subdomains VII and VIII of the catalytic domain. This phosphotyrosine residue is essential for activity because its dephosphorylation with a protein tyrosine phosphatase (PTPase) inactivates GSK3 [6]. These findings raised the possibility that the inhibition of GSK3 by insulin, IGF-1 and other signals might not only be mediated by PKB-catalysed phosphorylation of Ser-21 (GSK3α) and Ser-9 (GSK3β), but also by a PTPase-catalysed dephosphorylation of Tyr-279 (GSK3α) and Tyr-216 (GSK3β). Several years ago it was reported that incubation of A431 cells with okadaic acid (an inhibitor of PP1/PP2A) inactivated GSK3 by promoting tyrosine dephosphorylation [7]. However, subsequent work showed that this was incorrect, because the okadaic acid-induced inactivation could be completely reversed by incubation of the GSK3 with PP1 [8]. More recently, it was reported that in Chinese hamster ovary (CHO) cells overexpressing the insulin receptor, the insulin-induced inhibition of GSK3β was accompanied by partial dephosphorylation of Tyr-216 and that a deletion mutant lacking the N-terminal nine residues of GSK3β could still be inhibited partially by insulin [9].

In this paper, we have reinvestigated this question in human embryonic kidney 293 cells overexpressing wild-type GSK3β (WT-GSK3β) and a mutant in which Ser-9 has been changed to Ala (A9-GSK3β). Our data indicate that the insulin/IGF-1-induced inhibition of GSK3β is mediated solely by the phosphorylation of Ser-9 and not by dephosphorylation of Tyr-216. We also provide further evidence that it is PKB which phosphorylates and inactivates GSK3β in these cells, by demonstrating that the overexpression of either PKB or 3-phosphoinositide-dependent protein kinase (PDK1, an 'upstream activator' of PKB [10,11]) is sufficient to induce phosphorylation of Ser-9 and the inactivation of GSK3β.

2. Materials and methods

2.1. Materials

Monoclonal antibodies recognising the sequence EFMPME (EE) were a gift from G. Walter (University of San Diego, CA, USA), and those recognising the (EQKLISEEDL) c-Myc (9E10) sequence were purchased from Boehringer (Lewes, UK). The antiphosphotyrosine antibody (PY120) was purchased from Transduction Laboratories (Mamhead, UK). The secondary anti-mouse IgG conjugated to horseradish peroxidase was a gift of the Scottish Antibody Production Unit (Carluke, UK). Protein G-Sepharose was from Pharmacia (Milton Keynes, UK). For sources of other reagents see [5,12].

2.2. Construction of expression vectors

A DNA construct expressing human GSK3β with the EFMPME (EE) epitope tag at the N-terminus (WT-EE-GSK3β) was prepared as follows. A standard PCR reaction was carried out using, as a template, the human GSK3β cDNA clone in the pBluescript SK^+ vector (a gift of Dr. J. Woodgett, Ontario Cancer institute) and the oligonucleotides GCGGTCCGGAACATAGTCCAGCACCAG and GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAG-

*Corresponding author. Fax: (44) (1382) 223778.

PII S0014-5793(97)01235-0

GGCGGCCCAGAACC that incorporate a *Bsp*EI site and a *Bgl*II site. A triple ligation was then set up in which the resulting PCR product was subcloned into the *Bgl*II-*Cla*I sites of the pCMV5 vector [13] as a *Bgl*II-*Bsp*EI fragment, together with the C-terminal *Bsp*EI-*Cla*I fragment of GSK3β. The structure of the construct was verified by DNA sequencing and purified using the Qiagen plasmid Mega kit according to the manufacturer's protocol. A GSK3β construct in which Ser-9 was mutated to Ala and possessing a c-Myc epitope at the C-terminus (A9-Myc-GSK3β) was prepared as in [14] and kindly provided in the pRMBS vector by Dr M. Goedert (MRC Laboratory of Molecular Biology, Cambridge, UK). The A9-Myc-GSK3β gene was then subcloned into *Xba*I/*Eco*RI sites of the pCMV5 vector.

The constructs for human haemagglutinin-tagged wild-type PKBα (HA-WT-PKBα), the K179A 'kinase dead' PKBα (HA-KD-PKBα) and the constitutively active PKBα (308D/473D PKBα) [15] and Myc-PDK1 [11] have been described previously.

2.3. Transfection of 293 cells and assay of GSK3 activity

293 cells were cultured on 10 cm diameter dishes and transfected with 1 μg/ml pCMV5 DNA constructs encoding WT-EE-GSK3β or A9-Myc-GSK3β with or without HA-PKBα or Myc-PDK1 using a modified calcium phosphate method [15]. 24 h post transfection, the cells were deprived of serum for a further 16 h, then stimulated for the times indicated with 100 ng/ml IGF-1 or insulin (100 nM) or buffer. Each dish of cells was lysed in 1.0 ml of ice-cold buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM sodium *ortho*pervanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 μM microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, and 0.1% (v/v) 2-mercaptoethanol). The lysate was centrifuged at 4°C for 10 min at 13 000×*g* and the supernatants (10 μg protein) incubated for 30 min on a shaking platform with 5 μl of protein G-Sepharose coupled to 1 μg of either the EE antibody or the 9E10 Myc antibody in order to immunoprecipitate the WT-EE-GSK3β and the A9-Myc-GSK3β enzymes, respectively. The protein G-Sepharose-antibody-GSK3β complex was washed twice with 1.0 ml of buffer A containing 0.5 M NaCl, and three times with buffer B (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol). The immunoprecipitates were incubated with or without $PP2A_1$ (25 mU/ml), and assayed using phospho-GS peptide-1 [16]. One unit of activity was that amount of enzyme which phosphorylated 1 nmol of peptide in 1 min. One mU of $PP2A_1$ was the amount of enzyme that dephosphorylated 1 nmol of glycogen phosphorylase in 1 min.

2.4. Immunoprecipitation and assay PKB

293 cells transfected with a DNA construct expressing either Myc-PDK1 or mock pCMV5 vector were stimulated with 100 ng/ml IGF-1, and lysed with 1.0 ml of ice-cold buffer A. Each PKB isoform (PKBα, PKBβ and PKBγ) was then immunoprecipitated separately from 0.1 mg of cell lysate using isoform-specific antibodies [17] and assayed for activity using the peptide GRPRTSSFAEG [18].

2.5. Immunoblotting of GSK3β immunoprecipitates

Cell lysate (0.4 mg protein) was incubated for 30 min on a shaking platform at 4°C with 45 μl of protein G-Sepharose coupled covalently to either 90 μg of EE-antibody or 90 μg of Myc-antibody. The suspension was centrifuged at 13 000×*g*, the GSK3 immunoprecipitates washed as described above, then denatured in 1% (by mass) SDS. Aliquots were electrophoresed on 10% SDS/polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with appropriate antibodies. Detection was performed using the enhanced chemiluminescence reagent (Amersham).

3. Results

3.1. IGF-1 or insulin stimulation of cells inhibits GSK3β by promoting phosphorylation of Ser-9 and not dephosphorylation of Tyr-216

293 cells were transfected with DNA constructs expressing either WT-EE-GSK3β or A9-Myc-GSK3β. Both forms of GSK3 were expressed at similar levels in unstimulated 293 cells (data not shown) and possessed similar specific activities (Fig. 1). IGF-1 stimulation of 293 cells induced a 70% inhibition of WT-EE-GSK3β within 6 min. This increased to 75% inhibition after 20 min which was maintained for up to 1 h (Fig. 1). Incubation of WT-EE-GSK3β from IGF-1-stimulated cells with $PP2A_1$ completely reversed this inhibition returning WT-EE-GSK3β to the specific activity observed in

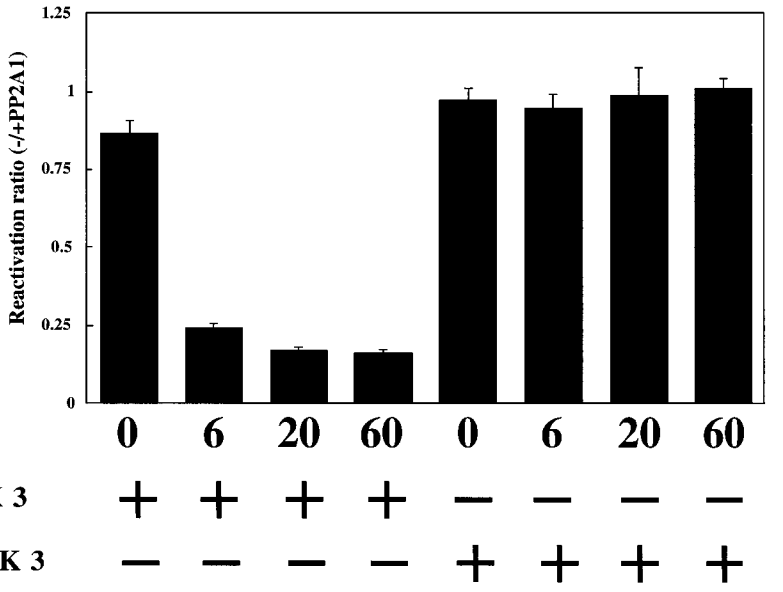


Fig. 1. Effect of IGF-1 on the activity of wild-type GSK3β and the A9 mutant. 293 cells were transiently transfected with DNA constructs expressing either WT-EE-GSK3β or A9-Myc-GSK3β. The cells were stimulated with 100 ng/ml IGF-1 for the times indicated and then lysed. The epitope-tagged GSK3β was immunoprecipitated, incubated without or with 25 mU/ml $PP2A_1$ and then assayed. GSK3β activity is expressed as a reactivation ratio, i.e. GSK3 activity measured without $PP2A_1$ treatment divided by GSK3 activity after $PP2A_1$ treatment. The data are presented as the mean ± S.E.M. for four separate experiments with each determination carried out in triplicate. No GSK3β activity was obtained in mock transfections in which 293 cells were transfected with empty pCMV5 vector alone (data not shown). The specific activity of both WT-EE-GSK3β and A9-Myc-GSK3β obtained after immunoprecipitation from unstimulated cells followed by treatment with $PP2A_1$ was 0.4 ± 0.06 U/mg for both forms of GSK3.

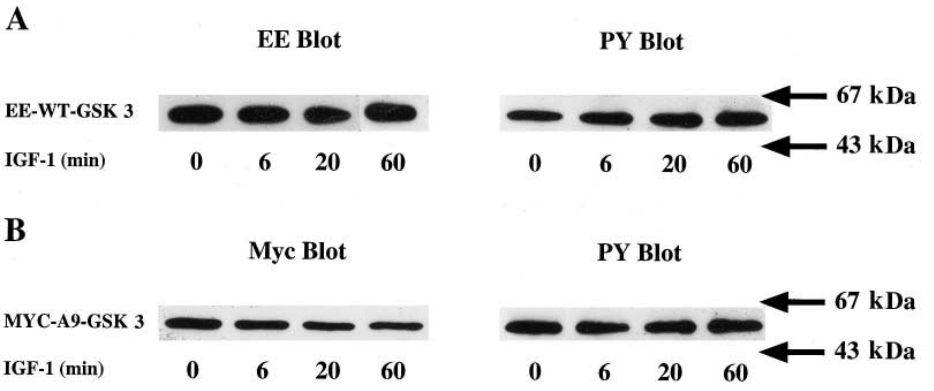


Fig. 2. IGF-1 does not affect the tyrosine phosphorylation of GSK3β. 293 cells were transiently transfected with DNA constructs expressing either WT-EE-GSK3β (A) or A9-Myc-GSK3β (B). The cells were stimulated with 100 ng/ml IGF-1 for the times indicated and then lysed. The epitope-tagged GSK3β was immunoprecipitated from 400 μg of lysate using either the Myc or EE antibodies coupled covalently to protein G-Sepharose. Aliquots of the immunoprecipitates corresponding to 150 μg lysate protein were electrophoresed on 10% SDS/polyacrylamide gels, transferred to nitrocellulose and immunoblotted with anti-EE, anti-Myc or anti-phosphotyrosine (pY) antibodies. No GSK3β immunoreactive band was observed in mock transfections using empty pCMV5 vector alone (data not shown). The results shown are representative of three independent experiments.

unstimulated 293 cells after $PP2A_1$ treatment (Fig. 1). Similar results were obtained when insulin (100 nM) was used instead of IGF-1. Insulin induced a 60% inhibition of WT-EE-GSK3β after 5 min which was also reversed by $PP2A_1$ (data not shown). IGF-1 stimulation also inhibited the endogenous GSK3 in 293 cells by 50%, and this inhibition was fully reversed by $PP2A_1$ (data not shown).

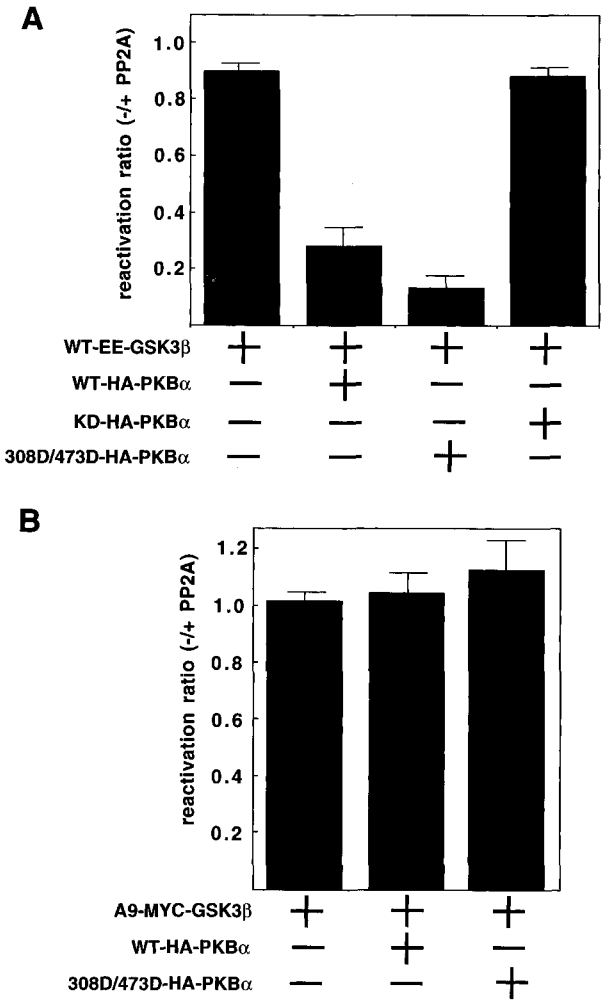
In contrast, IGF-1 stimulation of cells expressing A9-Myc-GSK3β did not result in any inhibition of the A9-Myc-GSK3β activity, nor was the A9-Myc-GSK3β activity in these immunoprecipitates further activated by incubation with $PP2A_1$ (Fig. 1).

Stimulation with IGF-1 did not cause any significant change in the tyrosine phosphorylation of WT-EE-GSK3β or A9-Myc-GSK3β, as judged by immunoblotting experiments using an anti-phosphotyrosine antibody (Fig. 2).

3.2. *GSK3β is phosphorylated at Ser-9 and becomes inhibited after cotransfection with either PKB or PDK1*

293 cells were transfected with DNA constructs expressing either WT-EE-GSK3β or A9-Myc-GSK3β in the presence or absence of DNA constructs expressing WT-HA-PKBα, or constitutively active PKBα (308D/473D-HA-PKBα) or catalytically inactive PKBα (KD-HA-PKBα). Coexpression of WT-EE-GSK3β with either WT-HA-PKBα or 308D/473D-HA-PKBα decreased activity by 68% and 85%, respectively, compared to cells transfected with WT-EE-GSK3β and KD-HA-PKBα or WT-EE-GSK3β alone (Fig. 3A). In contrast, A9-Myc-GSK3β was not inactivated when coexpressed with WT-HA-PKBα or 308D/473D-HA-PKBα (Fig. 3B).

WT-EE-GSK3β could also be inhibited up to 50% by cotransfection in 293 cells with Myc-PDK1, an 'upstream acti-



←

Fig. 3. WT-EE-GSK3β is inactivated by cotransfection with PKBα. 293 cells were transiently transfected with the PCMV5 vector expressing WT-EE-GSK3β (A) or A9-Myc-GSK3β (B) with or without constructs expressing wild-type WT-HA-PKBα, 'kinase dead' KD-HA-PKBα or a constitutively active 308D/473D PKBα. GSK3β was assayed after immunoprecipitation from the lysates and incubation with or without 25 mU/ml $PP2A_1$. GSK3β activity is expressed as a reactivation ratio, i.e. GSK3β activity measured without $PP2A_1$ treatment divided by GSK3 activity after $PP2A_1$ treatment. The data are presented as the mean ± S.E.M. for three separate experiments with each determination carried out in triplicate. No GSK3β activity was obtained in mock transfections in which 293 cells were transfected with empty pCMV5 vector alone (data not shown). The specific activities of WT-EE-GSK3β and A9-Myc-GSK3β after cotransfection with HA-PKBα and reactivation with $PP2A_1$ were both 0.25 ± 0.06 U/mg.

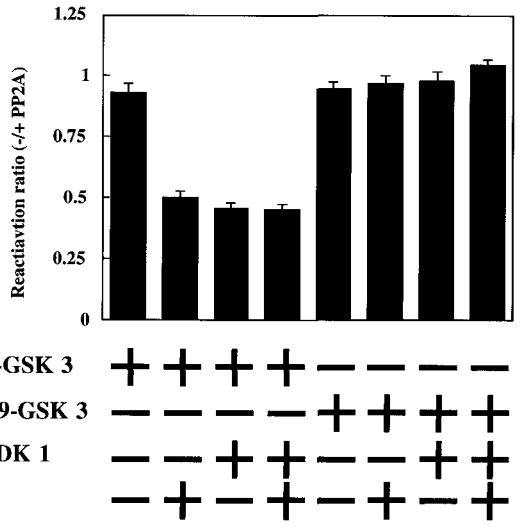


Fig. 4. GSK3β is inactivated by cotransfection with PDK1. 293 cells were transfected with DNA constructs expressing either WT-EE-GSK3β or A9-Myc-GSK3β with or without Myc-PDK1. The cells were then stimulated for 5 min with or without 100 ng/ml IGF-1 and lysed, and the tagged GSK3β immunoprecipitated and assayed as in Fig. 1. The data are presented ± S.E.M. for four experiments with each determination carried out in triplicate. The specific activities of WT-EE-GSK3β and A9-Myc-GSK3β after cotransfection with Myc-PDK1 and reactivation with $PP2A_1$ were both 1.8 ± 0.2 U/mg. The level of transfected PDK1 was similar in all transfections (data not shown).

vator' of PKB, and the inhibition was fully reversed by $PP2A_1$ (Fig. 4). In contrast A9-Myc-GSK3β was not inactivated by cotransfection with Myc-PDK1 and $PP2A_1$ (Fig. 4).

The inhibition of WT-EE-GSK3β by cotransfection with Myc-PDK1 suggested that the transfected PDK1 had activated the endogenous PKB in 293 cells which had then inactivated the transfected GSK3. This was confirmed by immunoprecipitating each PKB isoform from the lysates of 293 cells cotransfected with WT-EE-GSK3β and Myc-PDK1. These experiments revealed that Myc-PDK1 had activated PKBα and PKBγ, the latter being the dominant isoform present. There was hardly any PKBβ activity detectable in 293 cells transfected with PDK1 (Fig. 5).

4. Discussion

In this paper we have demonstrated that the inhibition of transfected or endogenous GSK3 by IGF-1 or insulin in 293 cells is mediated solely by the phosphorylation of Ser-9. Thus the inhibition could be fully reversed by incubation with $PP2A_1$, and did not occur if Ser-9 was mutated to Ala (Fig. 1). These results are supported by the finding that the inhibition of GSK3 isoforms by insulin can be fully reversed by treatment with Ser/Thr-specific protein phosphatases in rat L6 myotubes [18], rat skeletal muscle [12], rat adipocytes [12,19] or human myoblasts [20]. Murai et al. [9] reported that the insulin-induced inhibition of GSK3 in CHO cells overexpressing the insulin receptor could not be fully reversed by PP2A, but Welsh et al. [2,21] reported full reactivation of GSK3 in the same cell line. The inhibition of GSK3 induced by EGF or okadaic acid in A431 cells [8] can also be fully reversed by Ser/Thr-specific protein phosphatases [8]. The Ala-9 mutant of GSK3β has also been overexpressed in cells by others, and was also not inhibited by insulin/IGF-1 stimulation of neuronal NT2N cells [22] or after TPA stimulation of HeLa cells [23].

We were unable to detect any significant change in the phosphotyrosine content of either the wild-type or Ala-9 mutant of GSK3 after stimulation of transfected 293 cells with IGF-1 (Fig. 2), consistent with the inhibition of GSK3β being mediated solely by the phosphorylation of Ser-9. This conclusion is also consistent with experiments using insulin-stimulated L6 myotubes, in which the only tryptic peptide from GSK3 whose ^{32}P-content was altered was that containing Ser-21 (GSK3α) or Ser-9 (GSK3β) [18]. The ^{32}P-labelling of the phosphotyrosine-containing tryptic peptide from GSK3α or GSK3β was also unaffected by EGF stimulation of A431 cells [8]. We were unable to obtain any evidence in support of the work of Murai et al. [9] who reported that the tyrosine phosphorylation of GSK3 decreased in response to insulin in CHO cells and in response to EGF or phorbol esters in COS cells.

The protein kinase responsible for phosphorylating Tyr-216 (GSK3β) or Tyr-279 (GSK3α) in vivo is unclear. Some tyrosine phosphorylation was detected when the β-isoform was expressed in *E. coli* [24], suggesting that GSK3 is capable of autophosphorylating itself at Tyr-216, but this does not exclude the possibility that another protein kinase phosphorylates this residue in vivo. If the tyrosine phosphorylation of GSK3 occurs in vivo by autophosphorylation, then it might be predicted that the inhibition of GSK3 resulting from serine phosphorylation might be followed by a slower dephosphorylation of the tyrosine residue. However, we were unable to detect any decrease in tyrosine phosphorylation up to 1 h after stimulation with IGF-1 (Fig. 2), even though the inhibition of GSK3 was nearly maximal after 6 min (Fig. 1). This suggests that the dephosphorylation of Tyr-216 is very slow or

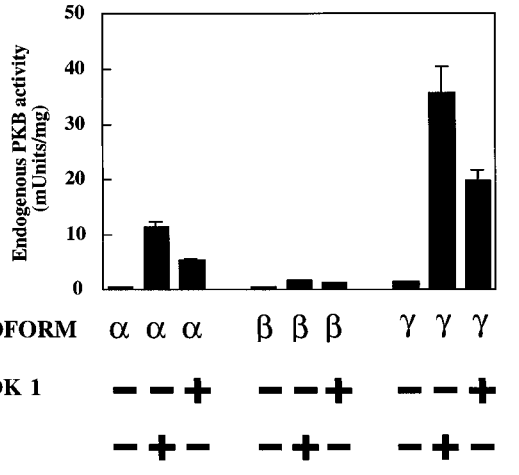


Fig. 5. PKBα and PKBγ are activated by transfection of PDK1 into 293 cells. 293 cells were transiently transfected with DNA constructs expressing Myc-PDK1. The cells were stimulated with or without 100 ng/ml IGF-1, lysed and the endogenous PKBα, PKBβ and PKBγ immunoprecipitated individually from the cell lysates (100 μg of protein) and assayed. The results are presented as PKB activity (mU/mg) ± S.E.M. for five separate experiments, with each determination carried out in triplicate. The efficiency of Myc-PDK-1 transfection into these cells was calculated as 60%. Therefore the data were corrected to take this into account (by multiplying the observed PKB activity in cells transfected with Myc-PDK-1 by a factor of 1.67).

that GSK3β is phosphorylated at Tyr-216 in vivo by another enzyme that is not inactivated by IGF-1.

Coexpression of GSK3β together with wild-type PKBα or a constitutively activated form of PKBα resulted in 70% and 85% inhibition of GSK3β activity, respectively, in unstimulated 293 cells (Fig. 3). This inhibition could be fully reversed by $PP2A_1$ and did not occur when Ser-9 was mutated to Ala. In contrast, a catalytically inactive mutant of PKBα did not inactivate GSK3β (Fig. 3). This shows for the first time that PKBα is capable of phosphorylating Ser-9 and inhibiting GSK3 in a cellular context. The inhibition of transfected GSK3β by wild-type PKB in unstimulated 293 cells can be explained by the very high level of expression of PKB in 293 cells (20-fold higher than GSK3β – data not shown) under these conditions. Hence a significant level of basal PKB activity is present in these cells.

The full activation of PKBα by insulin/IGF-1 requires its phosphorylation at Thr-308 and Ser-473 [15]. The phosphorylation of Thr-308 appears to be catalysed in vivo by PDK1, while Ser-473 is phosphorylated by a distinct enzyme that has yet to be identified and characterised [10,11]. We have previously demonstrated that cotransfection of PDK1 and PKBα in unstimulated 293 cells induced a partial activation of PKBα that resulted from the phosphorylation of Thr-308 [11]. In the present study we have demonstrated that transfected GSK3β becomes inhibited by Ser-9 phosphorylation following cotransfection with PDK1 (Fig. 4). This appears to be mediated by the activation of the endogenous PKB isoforms (principally PKBα and PKBγ) present in 293 cells (Fig. 5). The experiments presented in this paper support the contention that PDK1, PKB and GSK3 comprise a protein kinase cascade that is likely to be important in mediating many of the intracellular effects of insulin/IGF-1.

Acknowledgements: We thank Dr. M. Andjelkovic and Dr B.A. Hemmings for the PKB expression constructs, Dr J. Woodgett for the GSK3β clone, Dr M. Goedert for the A9 mutant of GSK3. The PKB isoform-specific antibodies were provided by Miss Kay Walker in this Unit. We thank the BBSRC and SmithKline Beecham for the award of a postgraduate studentship to M.S., and the MRC (D.R.A. and P.C.) and the Royal Society (P.C.) for financial support.

References

[1] Embi, N., Rylatt, D.B. and Cohen, P. (1980) Eur. J. Biochem. 107, 519–527.
[2] Welsh, G.I. and Proud, C.G. (1993) Biochem J. 294, 625–629.
[3] Lovestone, S., Reynolds, C.H., Latimer, D., Davis, D.R., Anderton, B.H., Gallo, J.M., Hanger, D., Mulot, S., Marquardt, B., Stabel, S., Woodgett, J.R. and Miller, C.C.J. (1994) Curr. Biol. 4, 1077–1086.
[4] Cohen, P., Alessi, D.R. and Cross, D.A.E. (1997) FEBS Lett. 410, 3–10.
[5] Welsh, G., Wilson, C. and Proud, C.G. (1996) Trends Cell Biol. 6, 274–279.
[6] Hughes, K., Nikolakai, E., Plyte, S.E., Totty, N.F. and Woodgett, J.R. (1993) EMBO J. 12, 803–808.
[7] Wu, J. and Yang, S.D. (1994) J. Biol. Chem. 269, 14341–14344.
[8] Saito, Y., Vandenheede, J.R. and Cohen, P. (1994) Biochem. J. 303, 27–31.
[9] Murai, H., Okazaki, M. and Kikuchi, A. (1996) FEBS Lett. 392, 153–160.
[10] Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B. and Cohen, P. (1997) Curr. Biol. 7, 261–269.
[11] Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reece, C., MacDougall, C.N., Harbison, D., Ashworth, A. and Bownes, M. (1997) Curr. Biol. 7, 776–789.
[12] Cross, D.A.E., Watt, P.W., Shaw, M., van der Kaay, J., Downes, C.P., Holder, J.C. and Cohen, P. (1997) FEBS Lett. 406, 211–215.
[13] Andersson, S., Davie, D.N., Dahlbäck, H., Jörnvall, H. and Russell, D.W. (1989) J. Biol. Chem. 264, 8222–8229.
[14] Sperber, B.R., Leight, S., Goedert, M. and Lee, V.M.Y. (1995) Neurosci. Lett. 197, 149–153.
[15] Alessi, D.R., Andjelkovic, M., Caudwell, F.B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. (1996) EMBO J. 15, 6541–6551.
[16] Sutherland, C., Leighton, I.A. and Cohen, P. (1993) Biochem. J. 296, 15–19.
[17] Walker, K., Deak, M., Alessi, D.R. and Cohen, P. (1997) submitted.
[18] Cross, D.A.E., Alessi, D.R., Cohen, P., Andjelkovic, M. and Hemmings, B.A. (1995) Nature 378, 785–789.
[19] Moule, S.K., Edgell, N.J., Welsh, G.I., Diggle, T.A., Foulstone, E.J., Heesom, K.J., Proud, C.G. and Denton, R.M. (1996) Biochem. J. 311, 595–601.
[20] Hurel, S.J., Rochford, J.J., Borthwick, A.C., Wells, A.M., Vandenheede, J.R., Turnbull, D.M. and Yeaman, S.J. (1996) Biochem. J. 320, 871–877.
[21] Welsh, G.I., Foulstone, E.J., Young, S.W., Tavare, J.M. and Proud, C.G. (1994) Biochem. J. 303, 15–20.
[22] Hong, M. and Lee, V.M.Y. (1997) J. Biol. Chem. 272, 19547–19553.
[23] Stambolic, V. and Woodgett, J.R. (1994) Biochem. J. 303, 701–704.
[24] Wang, Q.M., Fiol, C.J., DePaoli-Roach, A.A. and Roach, P.J. (1994) J. Biol. Chem. 269, 14566–14574.