Mitogen inactivation of glycogen synthase kinase-3β in intact cells via serine 9 phosphorylation

Vuk STAMBOLIC and James R. WOODGETT†
Department of Medical Biophysics, University of Toronto and Division of Cell and Molecular Biology, Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Canada M4X 1K9

INTRODUCTION
The glycogen synthase kinase-3 (GSK-3) family of protein-serine kinases was initially characterized as a regulator of glycogen metabolism [1,2]. The protein kinase phosphorylates residues on glycogen synthase, the rate-limiting enzyme of glycogen synthesis, that are specifically dephosphorylated in response to insulin [3]. In mammals, two genes encode closely related proteins termed GSK-3α and β [4]. The kinase is highly conserved and homologues have been cloned from plants, yeasts and mould [1]. The Drosophila homologue is a homeotic gene termed zeste-white3 or shaggy, suggesting the kinase has roles in a number of signal transduction pathways regulating cell growth, differentiation and development [5,6]. GSK-3 phosphorylates the product of the mammalian protooncogene c-jun, a component of the activator protein-1 (AP-1) transcription factor, at sites proximal to its DNA-binding domain, reducing DNA-binding affinity [7]. Furthermore, GSK-3 inhibits transactivation of AP-1-responsive reporter genes implying that interaction between GSK-3 and c-jun occurs in vivo [8,9].

Previous work has demonstrated GSK-3 to be active in resting cells. The enzyme is phosphorylated on tyrosine in vivo and this phosphorylation is required for activity [10]. Recent studies have demonstrated that GSK-3α and β are phosphorylated and inhibited by two growth factor-stimulated protein kinases in vitro [11,12]. In this report, we present evidence for the existence of such regulation of GSK-3β in intact cells.

MATERIALS AND METHODS
All reagents were purchased from Sigma Chemical Co. (Mississauga, Ontario, Canada) unless indicated otherwise.

DNA constructs
Serine 9 of human GSK-3β was mutated to alanine using the pAlter-1 method (Promega, Madison, WI, U.S.A.). This wild type and mutant GSK-3β were C-terminally tagged with the haemagglutinin (HA) epitope, YPYDVPDYASLGPPN, recognized by monoclonal antibody 12CA5 [13] and subcloned into pTM1 [14]. N-terminally HA-tagged p90rsk-1 kinase and human p70 S6 kinase were similarly engineered into pTM1.

Cell culture and transfections
HeLa S3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) (both GIBCO-BRL, Burlington, Ontario, Canada). 106 HeLa S3 cells to be transfected were plated on 35 mm dishes. On the following day they were infected with vTF7-3, a recombinant vaccinia virus encoding T7 RNA polymerase [14,15] at a multiplicity of infection (M.O.I.) of 10 by incubation for 30 min at 37°C in serum-free DMEM. DNA (2.5 mg) to be transfected was incubated with the mixture of 1 ml of serum-free DMEM and 30 ml of liposomes [16] for 5–10 min at room temperature to allow binding of DNA to liposomes. The viral solution was replaced with the DNA-liposome solution and incubated with the cells for 3 h at 37°C in 5% CO2. A 1 ml volume of DMEM + 10% FCS was then added to cells without replacing the transfection mixture. For cell labelling, 1 ml of phosphate-free DMEM + 2% dialysed FCS was added instead and cells were incubated with 1 mCi/ml of [32P]phosphate (NEN-Dupont, Mississauga, Ontario, Canada) for 16 h before lysis.

Western-blot analysis
Proteins were resolved by 12.5% SDS/PAGE, electroblotted to poly(vinylidene difluoride) (PVDF) membrane (Millipore, Bedford, MA, U.S.A.), blocked in 4% dried milk/1 × PBS/0.05% Tween-20 and probed with the polyclonal rabbit antisera against GSK-3. Bound immunoglobulins were detected using enhanced chemiluminescence (NEN-Dupont).

Abbreviations used: GSK-3, glycogen synthase kinase-3; MAP, mitogen-activated protein; AP-1, activator protein-1; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; M.O.I., multiplicity of infection; PVDF, poly(vinylidene difluoride); PMA, phorbol 12-myristate 13-acetate; hA, haemagglutinin.

The nucleotide sequence reported for GSK-3β appears in GenBank under accession number L33801.
† To whom correspondence should be addressed.
Immunoprecipitation of expressed proteins

For immunoprecipitation of radioactively labelled proteins, cells were lysed 24 h post-transfection in RIPA buffer [17] containing 5 mM EDTA, 50 mM NaF, 100 mM Na3VO4, 1 mM benzamidine and 5 mg/ml aprotinin. Proteins were immunoprecipitated by incubation with the monoclonal antibody 12CA5 (a gift from Joe Avruch, Harvard Medical School), which is reactive with the epitope tag, for 1 h at 4 °C with subsequent precipitation with protein A-Sepharose and extensive washing in lysis buffer. Immunoprecipitation of proteins for the kinase assay was performed following the same protocol except that instead of RIPA, Gentle-Soft buffer [17] containing 5 mM EDTA, 50 mM NaF, 100 mM Na3VO4, 1 mM benzamidine and 5 mg/ml aprotinin was used.

Tryptic phosphopeptide analysis

Tryptic phosphopeptide analysis was performed according to Boyle et al. [18]. Radioactively labelled proteins were immunoprecipitated, resolved by 12.5 % SDS/PAGE, transferred to PVDF membrane and visualized by autoradiography (Kodak XAR). The bands were excised, soaked in 0.5 % PVP-360 in 100 mM acetic acid for 30 min at 37 °C, washed, and incubated overnight with 10 mg of 1-lysylamido-2-phenethyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemical Corp., Bed- ford, MA, U.S.A.) in 50 mM ammonium bicarbonate. The liquid phase was lyophilized, oxidized with performic acid and subsequently lyophilized three times with water. The samples were solubilized in pH 1.9 buffer, applied to thin-layer cellulose plates (BDH, Toronto, Ontario, Canada), separated by electrophoresis in pH 1.9 buffer for 30 min at 1000 V in the first dimension, followed by ascending chromatography in the second dimension. Phosphopeptides were visualized by autoradiography with intensifying screens for 3–7 days.

Kinase assay

The kinase activity of immunoprecipitated GSK-3β proteins was tested by their ability to phosphorylate phospho-GS1 peptide [19]. Phospho-GS1 is a synthetic 25 amino acid peptide YRRAPVPPSP SSLRHSPPHSEQEDEE, derived from glycogen synthase and prephosphorylated by casein kinase II at serine 20 (underlined) and purified by reverse-phase h.p.l.c., creating three consensus sites for GSK-3-specific phosphorylation (bold). GSK-3β and GSK-3β/A9 immunoprecipitates from transfected HeLa S3 cells were incubated at 30 °C with 30 μM of phospho-GS1 peptide in the presence of 50 μM [γ-32P]ATP (NEN-Dupont) in 25 mM Tris, pH 7.5/1 mM DTT/10 mM MgCl2, reactions stopped at different time-points and peptides resolved by tricine/SDS gel-electrophoresis [20]. Phosphorylated peptides were visualized by PhosphorImager (Molecular Dynamics Inc.) and incorporation of [32P]phosphate quantified using ImageQuant (MDI) software.

RESULTS

A point mutation of GSK-3β induces significant changes in its phosphorylation in intact cells

To determine whether serine phosphorylation and corresponding inhibition of GSK-3β occurs in vivo we expressed wild-type and mutant GSK-3β in HeLa S3 cells using recombinant vaccinia virus/T7 expression [14,15]. Epitope-tagged human, wild-type GSK-3β and a mutant in which serine 9 was replaced by alanine (GSK-3βA9) were lipofected into HeLa S3 cells previously infected with vaccinia virus/T7 polymerase (Figure 1a). To investigate the phosphorylation status of GSK-3β and the phosphorylation-site mutant in intact cells, the proteins were immunoprecipitated from transfected cells metabolically labelled with [32P]phosphate (Figure 1b). The mutant GSK-3βA9 polypeptide reproducibly contained half of the [32P]radioactivity compared with wild-type GSK-3β. Coexpression of p90rsk-1, one of the protein kinases capable of phosphorylating GSK-3β at serine 9 in vitro [11], enhanced this effect, particularly following stimulation with phorbol 12-myristate 13-acetate (PMA) (Figure 1b). In contrast, cotransfection of p70 S6 kinase had no detectable effect on GSK-3β phosphorylation (data not shown, see below) but, when purified, was able to phosphorylate GSK-3β in vitro in agreement with Sutherland et al. [11]. These observations, albeit using overexpressed proteins, indicated that the mutation of a single residue (serine 9 to alanine) induces significant changes in GSK-3β phosphorylation within cells and implied a possible role for p90rsk-1 in this event. Although p70 S6 kinase targeted serine 9 in vitro, this interaction was not observed in intact cells.

Figure 1  Analysis of GSK-3β proteins expressed in HeLa S3 cells

(a) immunoblot analysis of the expression of GSK-3β and GSK-3βA9 in transfected HeLa S3 cells; (b) immunoprecipitation of GSK-3β proteins from transfected, [32P]phosphate-labelled HeLa S3 cells. Cells were treated with 160 mM PMA where indicated.

GSK-3β is phosphorylated at serine 9 in vitro

GSK-3β proteins from radiolabelled cells coexpressing either GSK-3β or GSK-3β/A9 with p90rsk-1 (Figure 1b, lanes 2 and 4) and treated with PMA were subjected to tryptic phosphopeptide mapping (Figure 2) [17]. At least six phosphopeptides are present in the GSK-3β/peptide map (Figure 2a; see also scheme (i)). Two of these six phosphopeptide spots were missing in the map of GSK-3β/A9 (Figure 2c, peptides 2a and 2b), confirming an altered pattern of GSK-3β phosphorylation as a result of the mutation.

To determine whether these phosphopeptide changes were due to the absence of serine 9 phosphorylation, a tryptic phosphopeptide map was prepared from purified baculovirus-expressed GSK-3β phosphorylated by p90rsk-1 in vitro (Figure 2e). Phosphoamino-acid analysis of this preparation revealed phosphoserine (not shown), consistent with the reported exclusive serine 9 phosphorylation of GSK-3β by p90rsk-1 [5]. The in vitro phosphopeptide map contains three spots (Figure 2c, peptides 2a, 2a and 2b), two of which correspond to the spots missing in the map of the immunoprecipitated mutant (Figure 2c). Mixing the peptides phosphorylated in vitro with those from immunoprecipitated GSK-3βA9 reconstituted the wild-type phospho-
Figure 2  Tryptic phosphopeptide maps of [32P]phosphate labelled GSK-3β proteins
(a) Immunoprecipitated GSK-3β; (b) immunoprecipitated GSK-3β + GSK-3β phosphorylated 
by p90rsk-1 in vitro; (c) immunoprecipitated GSK-3βA9; (d) immunoprecipitated GSK-3βA9 + 
GSK-3β phosphorylated by p90rsk-1 in vitro; (e) GSK-3β phosphorylated by p90rsk-1 in vitro; 
(f) schematic diagram of major phosphopeptide spots appearing on presented maps (see text 
for details). Origins are indicated by arrows.

Figure 3  Protein kinase activity of immunoprecipitated proteins
Phospho-GS1 peptide was incubated with the immunoprecipitates for the indicated times 
and the amount of incorporated [32P]phosphate was determined following Phosphorimager analysis; 
△, ▲, GSK-3β; ○, ●, GSK-3βA9; △, ● indicate cotransfection of p90rsk-1 and 
stimulation with 160 nM PMA.

The effect of serine 9 phosphorylation of GSK-3 in vivo is inhibitory
To assess the effect of serine 9 phosphorylation on GSK-3β activity, immunoprecipitated GSK-3β and GSK-3βA9 were 
assayed using a GSK-3-specific peptide substrate [19]. Immunoprecipitated GSK-3β consistently exhibited lower activity 
towards the phospho-GS1 peptide substrate than immunoprecipitated 
GSK-3βA9 (Figure 3). Furthermore, when coexpressed with p90rsk-1 in cells stimulated with PMA, the immunoprecipitated 
GSK-3β exhibited a further 2–3-fold decrease in activity towards the phospho-GS1 peptide compared with the 
GSK-3βA9 expressed alone (Figure 3). Contrast, the activity of the mutant GSK-3βA9 was unchanged upon coexpression with 
p90rsk-1 (Figure 3). These results confirm that p90rsk-1 plays an active role in serine 9 phosphorylation and is a likely candidate 
for direct regulation of GSK-3.

Coexpression of p70 S6 kinase with GSK-3β and GSK-3βA9 had no detectable effect on activity (data not shown), implying 
that even though p70 S6 kinase is able to phosphorylate GSK-3β on serine 9 in vitro, such interaction does not occur in vivo. 
In addition, treatment of cells with the immunosuppressant macrodide, rapamycin, which specifically inhibits activation of p70 S6 
kinase [21], had no effect on the phosphorylation of peptides 2a and 2b (not shown).

DISCUSSION
Unlike most protein kinases, GSK-3 displays significant activity 
in unstimulated cells and many candidate GSK-3 substrates (e.g. c-jun, glycogen synthase, ATP citrate lyase, tau, armadillo, [22]) 
are phosphorylated under such ‘resting’ conditions. Upon agonist-challenge several of these substrates become dephosphorylated. 
For example, in skeletal muscle insulin treatment leads to dephosphorylation of glycogen synthase at GSK-3 target sites, which 
derides the anabolic effects of the hormone on glycogen [3]. Addition of insulin to CHO cells expressing 
insulin receptors causes rapid inactivation of GSK-3, an effect reversible by incubation with protein phosphatase 2A [23]. 
Likewise, phorbol esters cause dephosphorylation of c-jun at sites which inhibit DNA binding and which are targeted by 
GSK-3 in vitro [7]. Phorbol esters also cause inhibition of GSK-3 in U937 cells (K. Hughes and J. R. Woodgett, unpublished 
observation). These observations suggested that GSK-3 may be subject to negative regulation (see Fig. 4).

In the present work we have presented evidence in support of 
such an inhibitory mechanism. Phosphorylation of GSK-3β by 
p90rsk-1 in vitro on serine 9 causes inactivation, and mutation of 
that residue to alanine renders the kinase insensitive to inhibitory 
phosphorylation. Phosphorylation in vitro of the homologous

Phorbol esters  Insulin  Notch; Wingless
Protein kinase C  Insulin receptor  ras
MAP kinase  p90rsk-1  GSK-3

Figure 4  Scheme for negative regulation of GSK-3 in cells by insulin, 
phorbol esters and other agonists of the ras pathway
See text for details.
serine in GSK-3α is also inhibitory [12], suggesting a common mechanism of inactivation of GSK-3 family members, including the Drosophila Shaggy proteins.

The finding that p9Orsk-1 is capable of inhibiting GSK-3 in cells implicates regulation via the ras/mitogen-activated protein (MAP) kinase signalling-pathway since p9Orsk-1 is itself regulated by phosphorylation by p42/p44 MAP kinases [24]. Rsk-1 inhibition of GSK-3 may be the major mechanism via which insulin stimulates glycogen deposition in skeletal muscle. If so, abnormal control of GSK-3 may contribute to the pathology of diabetes.

Genetic epistasis experiments in Drosophila have placed the GSK-3 homologue, Shaggy, downstream of the Wingless and Notch signalling pathways [25,26]. Preliminary results suggest that the GSK-3 activity is inhibited in cells expressing mammalian Wnt proteins (K. Hughes, T. Dale and J. R. Woodgett un-published observation). It will be of interest to determine whether these signalling pathways utilize a similar mechanism in regulating GSK-3.

We thank Ken Hughes for purified GSK-3β, Joe Avruch for the p90rsk-1 cDNA, Dennis Templeton for the p70 S6 kinase 17 expression plasmid and much advice, and Bernie Moss for the vaccinia virus plasmids. This study was supported by operating and career grants from the Canadian MRC to J.R.W.

REFERENCES

4 Woodgett, J. R. (1990) EMBO J. 9, 2431–2438

Received 18 July 1994/8 August 1994; accepted 19 August 1994