RESEARCH COMMUNICATION

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

Glycogen synthase kinase-3 (GSK-3), a protein-serine kinase implicated in cell-fate determination and differentiation, phosphorylates several regulatory proteins that are activated by dephosphorylation in response to hormones or growth factors. GSK-3β is phosphorylated *in vitro* at serine 9 by p70 S6 kinase and p90*rsk*-1, resulting in its inhibition [Sutherland, Leighton, and Cohen (1993) Biochem. J. **296**, 15–19]. Using HeLa cells expressing GSK-3β or a mutant containing alanine at residue 9, we demonstrate that serine 9 is modified in intact cells and is targeted specifically by p90*rsk*-1, and that phosphorylation leads to loss of activity. Since p90*rsk*-1 is directly activated by mitogen-activated protein kinases, agonists of this pathway, such as insulin, repress GSK-3 function.

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, YPYDVPDYASLGGPN, recognized by monoclonal antibody 12CA5 [13] and subcloned into pTM1 [14]. N-terminally HA-tagged p90*rsk*-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). 10^6 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 1ml 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% CO_2. A 1ml 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 [^{32}P]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 Na_3VO_4, 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 Na_3VO_4, 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-tosylamido-2-phenethyl chloromethyl ketone (TPCK)–trypsin (Worthington Biochemical Corp., Bedford, 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 YRRAAVPPSPSLSRHSSPHQSEDEE, 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-3βA9 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-^{32}P]ATP (NEN-Dupont) in 25 mM Tris, pH 7.5/1 mM DTT/10 mM $MgCl_2$, 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 [^{32}P]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 [^{32}P]phosphate (Figure 1b). The mutant GSK-3βA9 polypeptide reproducibly contained half of the ^{32}P radioactivity compared with wild-type GSK-3β. Coexpression of p90*rsk*-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 p90*rsk*-1 in this event. Although p70 S6 kinase targeted serine 9 *in vitro*, this interaction was not observed in intact cells.

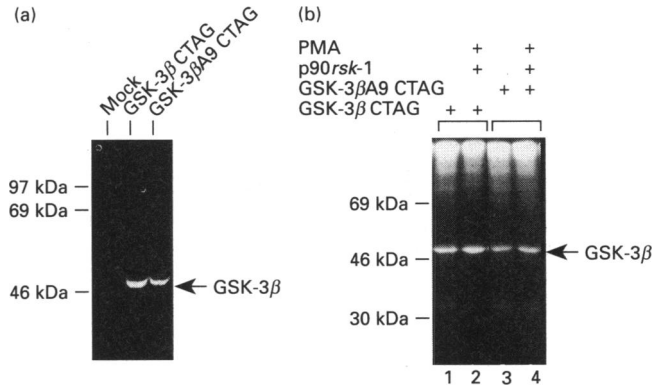

Figure 1 Analysis of GSK-3β proteins expressed in HeLa S3 cells

(**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, [^{32}P]phosphate-labelled HeLa S3 cells. Cells were treated with 160nM PMA where indicated.

GSK-3β is phosphorylated at serine 9 *in vivo*

GSK-3β proteins from radiolabelled cells coexpressing either GSK-3β or GSK-3βA9 with p90*rsk*-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 (f)]. Two of these six phosphopeptide spots were missing in the map of GSK-3βA9 (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 p90*rsk*-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 p90*rsk*-1 [5]. The *in vitro* phosphopeptide map contains three spots (Figure 2e, peptides 1a, 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-

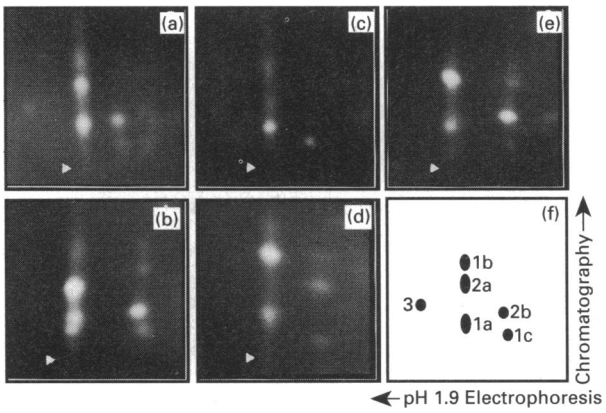

Figure 2 Tryptic phosphopeptide maps of [^{32}P]phosphate labelled GSK-3β proteins

(**a**) Immunoprecipitated GSK-3β; (**b**) immunoprecipitated GSK-3β + GSK-3β phosphorylated by p90*rsk*-1 *in vitro*; (**c**) immunoprecipitated GSK-3βA9; (**d**) immunoprecipitated GSK-3βA9 + GSK-3β phosphorylated by p90*rsk*-1 *in vitro*; (**e**) GSK-3β phosphorylated by p90*rsk*-1 *in vitro*; (**f**) schematic diagram of major phosphopeptide spots appearing on presented maps (see text for details). Origins are indicated by arrows.

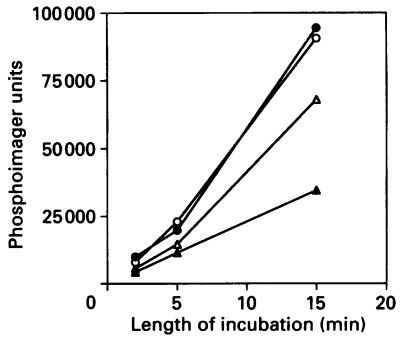

Figure 3 Protein kinase activity of immunoprecipitated proteins

Phospho-GS1 peptide was incubated with the immunoprecipitates for the indicated times and the amount of incorporated [^{32}P]phosphate was determined following Phosphorimager analysis; △, ▲, GSK-3β; ○, ●, GSK-3βA9; ▲, ● indicate cotransfection of p90*rsk*-1 and stimulation with 160 nM PMA.

peptide pattern (Figure 2, compare a and d). Furthermore, the mixture of GSK-3β phosphorylated *in vitro* and immunoprecipitated GSK-3β did not reveal any additional spots (Figure 2b). Phosphopeptides 2a and 2b are most likely a result of partial trypsinolysis of the same region of GSK-3β containing serine 9. In support of this, both 2a and 2b are absent from GSK-bA9. Peptides 1a, 1b and 1c are probably all derived from the region encompassing phosphotyrosine 216 of GSK-3β, since the equivalent peptides are absent from a tryptic map of a tyrosine phosphorylation-site mutant of the fission yeast GSK-3 homologue (S. Plyte, J. R. Woodgett, J. D. Burke and K. Gould, unpublished work). Together, these results indicate that GSK-3β is phosphorylated at serine 9 in cells.

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 p90*rsk*-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-3β expressed alone (Figure 3). In contrast, the activity of the mutant GSK-3βA9 was unchanged upon coexpression with p90*rsk*-1 (Figure 3). These results confirm that p90*rsk*-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 macrolide, 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 underlies 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 p90*rsk*-1 *in vivo* 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

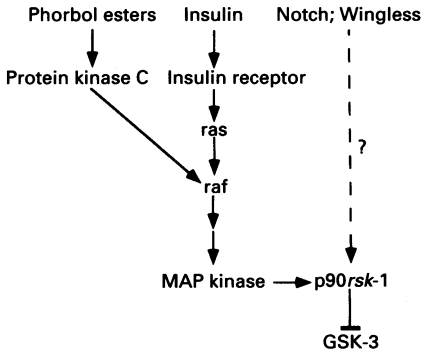

Figure 4 Scheme for negative regulation of GSK-3 in cells by insulin, phorbol esters and other agonists of the ras pathway

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 p90*rsk*-1 is capable of inhibiting GSK-3 in cells implicates regulation via the ras/mitogen-activated protein (MAP) kinase signalling-pathway since p90*rsk*-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 unpublished observation). It will be of interest to determine whether these signalling pathways utilize a similar mechanism in regulating GSK-3.

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REFERENCES

1 Plyte, S., Hughes, K., Nikolakaki, E., Pulverer, B. and Woodgett, J. R. (1992) Biochim. Biophys. Acta **1114**, 147–162
2 Woodgett, J. R. (1991) Trends Biochem. Sci. **16**, 177–181
3 Parker, P. J., Caudwell, F. B. and Cohen, P. (1983) Eur. J. Biochem. **130**, 227–234
4 Woodgett, J. R. (1990) EMBO J. **9**, 2431–2438
5 Siegfried, E., Perkins, L. A., Capaci, T. M. and Perrimon, N. (1990) Nature (London) **345**, 825–829
6 Bourouis, M., Moore, P., Ruel, L., Grau, Y., Heitzler, P. and Simpson, P. (1990) EMBO J. **9**, 2877–2884
7 Boyle, W. B., Smeal, T., Defize, L. H. K., Angel, P., Woodgett, J. R., Karin, M., and Hunter, T. (1991) Cell **64**, 573–584
8 Nikolakaki, E., Coffer, P., Hemelsoet, R., Woodgett, J. R. and Defize, L. H. K. (1993) Oncogene **8**, 833–840
9 de Groot, R. P., Auwerx, J., Bourouis, M. and Sassone-Corsi, P. (1993) Oncogene **8**, 841–847
10 Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F. and Woodgett, J. R. (1993) EMBO J. **12**, 803–808
11 Sutherland, C., Leighton, I. A. and Cohen, P. (1993) Biochem J. **296**, 15–19
12 Sutherland, C. and Cohen P. (1994) FEBS Lett. **338**, 37–42
13 Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A. and Wigler, M. (1988) Mol. Cell. Biol. **8**, 2159–2165
14 Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. and Fuerst, T. R. (1990) Nature (London) **348**, 91–92
15 Elroy-Stein, O., Fuerst, T. and Moss, B. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6126–6130
16 Rose, J. K., Buonocore, L. and Whitt, M. A. (1991) BioTechniques **10**, 520–525
17 Woodgett, J. R. (1992) in Cell Lines in Neurobiology: A Practical Approach (Wood, J., ed.), pp. 133–159. IRL Press, Oxford
18 Boyle, W. J., van der Geer, P. and Hunter, T. (1991) Methods Enzymol. **201**, 110–149
19 Fiol, C. J., Wang, A., Roeske, R. W. and Roach, P. J. (1990) J. Biol. Chem. **265**, 6061–6065
20 Schägger, H. and von Jagow, G. (1987) Anal. Biochem. **166**, 368–379
21 Price, D. J., Grove, J. R., Calvo, V., Avruch, J. and Bierer, B. E. (1992) Science **257**, 973–977
22 Woodgett, J. R. (1994) Seminars Cancer Biol. **5**, 1–7
23 Welsh, G. I. and Proud, C. G. (1993) Biochem J. **294**, 625–629
24 Sturgill, T. W., Ray, L. B., Erikson, E. and Maller, J. L. (1988) Nature (London) **334**, 715–718
25 Ruel, L., Bourouis, L., Heitzler, P., Pantesco, V. and Simpson, P. (1993) Nature (London) **362**, 557–560
26 Siegfried, E., Wilder, E. L. and Perrimon, N. (1994) Nature (London) **367**, 76–80

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