## RESEARCH COMMUNICATION

# Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling

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The β-isoform of glycogen synthase kinase-3 (GSK3β) isolated from rabbit skeletal muscle was inactivated 90–95% following incubation with MgATP and either MAP kinase-activated protein kinase-1 (MAPKAP kinase-1, also termed RSK-2) or p70 S6 kinase ($p70^{S6K}$), and re-activated with protein phosphatase 2A. MAPKAP kinase-1 and $p70^{S6K}$ phosphorylated the same tryptic peptide on GSK3β, and the site of phosphorylation was identified as the serine located nine residues from the N-terminus of the protein. The inhibitory effect of Ser-9 phosphorylation on GSK3β activity was observed with three substrates, (inhibitor-2, c-jun and a synthetic peptide), and also with glycogen synthase provided that 0.15 M KCl was added to the assays. The results suggest that Ser-9 phosphorylation underlies the reported inhibition of GSK3β by insulin and that GSK3 may represent a point of convergence of two major growth-factor-stimulated protein kinase cascades.

## INTRODUCTION

Glycogen synthase kinase-3 (GSK3) was originally identified as an inactivator of glycogen synthase in skeletal muscle [1] and later shown to phosphorylate other proteins such as inhibitor-2 (a regulatory subunit of protein phosphatase-1) [2] and the type-II regulatory subunit of cyclic AMP-dependent protein kinase [3]. Subsequently, it was implicated in the phosphorylation of transcription factors, such as c-jun and c-myc [4–7], and in the regulation of several developmental processes in *Drosophila* [8,9].

Until recently, there was no evidence that the activity of GSK3 was under hormonal control. However, a protein kinase originally identified in the liver as an ATP-citrate lyase kinase [10], and reported to be inhibited in insulin-stimulated adipocytes [11], was recently identified as the α-isoform of GSK3 (GSK3α) [12]. Furthermore, a protein kinase which phosphorylates eIF2B (a nucleotide exchange factor required for the initiation of protein synthesis) and is inhibited in insulin-stimulated Chinese-hamster ovary cells was identified as GSK3β [13]. These observations led us to explore potential mechanisms by which insulin might regulate GSK3. Here, we demonstrate that two protein kinases, which are activated by insulin and other growth factors, inactivate GSK3β by phosphorylating a serine located nine residues from its N-terminus. Our results suggest that Ser-9 phosphorylation may underlie the inhibition of GSK3β by insulin, and that GSK3 could represent a point of convergence of two major growth-factor-stimulated protein kinase cascades.

## MATERIALS AND METHODS

### Materials

Glycogen synthase peptide-1 (GS peptide-1) (KKPLNRTLSVASLPGLamide) was synthesized by Mr. F. B. Caudwell in this Unit, while GS peptide-2 (YRRAAVPPSPSLSRHSSPHQSEDEEE) was provided by Dr. M. Goedert, MRC Laboratory for Molecular Biology, Cambridge, U.K. The C-terminal serine residue of GS peptide-2 was phosphorylated by incubation with unlabelled ATP and casein kinase-2 from rat liver (a gift from Professor L. A. Pinna, University of Padova, Italy) and repurified by chromatography on a Vydac $C_{18}$ column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 10 mM ammonium acetate (pH 6.5) from which it eluted at 22% (v/v) acetonitrile. MAP kinase-activated protein (MAPKAP) kinase-1 [14] and MAPKAP kinase-2 [15] were purified from rabbit skeletal muscle. GSK3 co-purified with MAPKAP kinase-2 through the first five steps of purification, but was resolved during subsequent chromatography on a Mono S column (see Figure 1a). GSK3 (28 μg), with an estimated purity of about 25% (see Figure 1b), was isolated from 1500 g of muscle. p70 S6 kinase ($p70^{S6K}$) was partially purified from the livers of rats injected with cycloheximide [16] by chromatography on QAE-Sephadex, Q-Sepharose and Mono S columns and gel-filtration on a Superose 12 column. These $p70^{S6K}$ preparations, which had specific activities of about 30 units/mg and were about 10% pure, were free of MAPKAP kinase-1, as they did not phosphorylate the peptide LRRASLG, which is a good substrate for MAPKAP kinase-1 [17]. A highly purified preparation of $p70^{S6K}$ expressed in insect cells was provided by Dr. G. Thomas (Friedrich Miescher Institute, Basel, Switzerland). The p42 isoform of MAP kinase was expressed in *Escherichia coli* as a glutathione transferase fusion protein and activated with purified MAP kinase kinase as described [15]. The trimeric form of protein phosphatase 2A, termed $PP2A_1$, was isolated from rabbit skeletal muscle [18] by Dr. G. Moorhead in this Unit. Bacterially expressed c-jun was provided by Dr. E. Black and Dr. D. Gillespie (Beatson Cancer Institute, Glasgow, U.K.).

### Enzyme assays

MAPKAP kinase-1 and $p70^{S6K}$ were assayed with a peptide related to the C-terminus of ribosomal protein S6 (Gly-245,Gly-246)S6(218–249) (30 μM) [16]. MAPKAP kinase-2 was assayed with GS peptide-1 as described [15]. GSK3 was assayed in an identical manner to MAPKAP kinase-2, except that 20 μM GS

Abbreviations used: GSK3, glycogen synthase kinase-3; GS peptide-1, glycogen synthase peptide-1; MAP kinase, mitogen-activated protein kinase; MAPKAP, MAP kinase-activated protein; PP2A, protein phosphatase 2A; $PP2A_1$, trimeric holoenzyme form of PP2A; PP1G, glycogen-associated form of protein phosphatase-1; $p70^{S6K}$, p70 S6 kinase.

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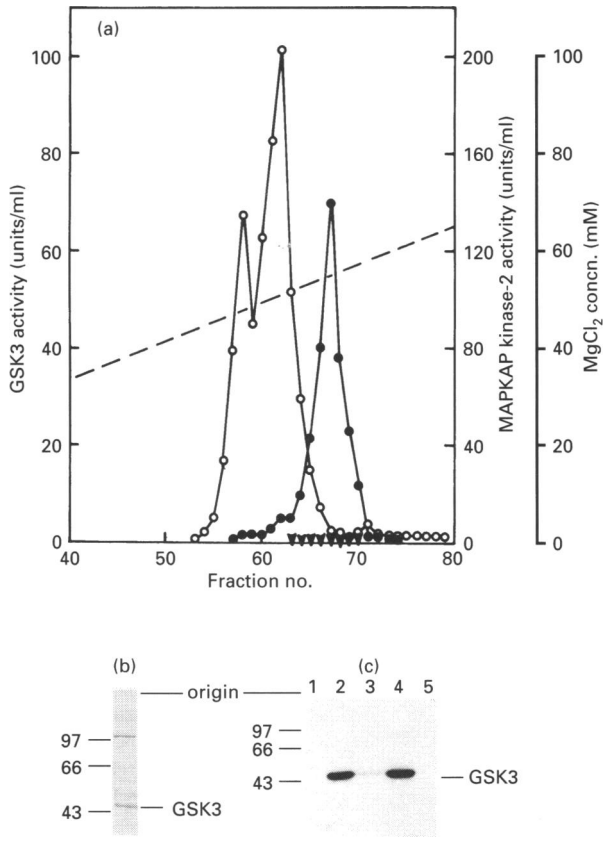


**Figure 1 Purification of GSK3 and its phosphorylation by MAPKAP kinase-1 and p70$^{S6K}$**

(**a**) GSK3 is resolved from MAPKAP kinase-2 by chromatography on a Mono S column, the sixth step of purification of the latter enzyme [15]. Fractions were assayed for MAPKAP kinase-2 using GS peptide-1 (○) and for GSK3 using GS peptide-2 in its dephosphorylated form (▼) or after prior phosphorylation with casein kinase-2 (●). The broken line indicates the $MgCl_2$ gradient. (**b**) SDS/PAGE of GSK3 from (**a**). The gel was stained with Coomassie Blue and migration is from top to bottom. The positions of GSK3 and the marker proteins glycogen phosphorylase (97 kDa), BSA (66 kDa) and ovalbumin (43 kDa) are indicated. (**c**) Phosphorylation of GSK3 by MAPKAP kinase-1 and p70$^{S6K}$. GSK3 (15 units/ml) was incubated with Mg[$\gamma$-$^{32}$P]ATP ($10^6$ c.p.m./nmol) and either 0.5 unit/ml p70$^{S6K}$ for 5 min or 2 units/ml MAPKAP kinase-1 for 15 min. The samples were subjected to SDS/PAGE as in (**b**), dried and autoradiographed. Lanes 1 and 2, p70$^{S6K}$ incubated with MgATP in the absence and presence of GSK3 respectively; lane 3, GSK3 incubated for 15 min with MgATP in the absence of either p70$^{S6K}$ or MAPKAP kinase-1; lanes 4 and 5, MAPKAP kinase-1 incubated with MgATP in the presence and absence of GSK3 respectively. The 47 kDa $^{32}$P-labelled band in lanes 2–4 is GSK3 and the incorporated $^{32}$P radioactivity in lanes 2 and 4 was 0.7 mol of phosphate/mol of GSK3, based on an estimated purity of 25% (see **b**) and protein concentrations measured by the method of Bradford [40].

peptide-2 was used as the substrate instead of 30 $\mu$M GS peptide-1. One unit of protein kinase activity was that amount which catalysed the phosphorylation of 1 nmol of peptide in 1 min. Protein phosphatase 2A (PP2A) was assayed as described [18] and one unit of activity was that amount which catalysed the dephosphorylation of 1 nmol of glycogen phosphorylase in 1 min.

### Phosphorylation of GSK3

GSK3 (15 units/ml) was incubated with MAPKAP kinase-1 or p70$^{S6K}$ in 40-$\mu$l incubations containing 10 mM Mops (pH 7.0), 0.5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM ATP. At various times, aliquots were diluted 10-fold into ice-cold 10 mM Mops (pH 7.0), containing 1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.01% Brij 35 and 1.0 mg/ml BSA and assayed for GSK3 activity at a 5-fold further dilution.

### Isolation of the major tryptic phosphopeptide from GSK3

GSK3 (4 ml, 50 units/ml) was phosphorylated for 5 min as described above, except that [$\gamma$-$^{32}$P]ATP ($10^6$ c.p.m./nmol) replaced unlabelled ATP. The reactions were stopped by addition of Na-EDTA and NaF at final concentrations of 20 mM and 50 mM respectively, and the samples concentrated by centrifugation through a Centricon 30 membrane (Amicon). The preparation was then diluted and reconcentrated and this procedure repeated until free ATP comprised < 10% of the $^{32}$P radioactivity. The native $^{32}$P-labelled GSK3 (0.25 ml) was then incubated for 20 min at 30 °C with 0.25 ml of 0.2 mg/ml trypsin and the reactions terminated by addition of 0.11 ml of 30% (w/v) trichloroacetic acid. After standing on ice for 2 min, the suspensions were centrifuged for 5 min at 13000 *g* and the supernatants, containing 92% of the $^{32}$P radioactivity (300 pmol), were chromatographed on a Vydac $C_{18}$ column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear 0–40% acetonitrile gradient at a flow rate of 0.8 ml/min. Fractions (0.4 ml) were collected and $^{32}$P radioactivity was recorded with an on-line monitor. The major phosphopeptide (120 pmol) was dried, incubated at −10 °C for 2 h with 0.02 ml of performic acid to convert cysteine residues into cysteic acid and methionine into methionine sulphone, and the reactions terminated by addition of 0.4 ml of water and freeze-drying. The peptide was then rechromatographed on the $C_{18}$ column as before, from which it eluted as a single major peak (46 pmol) at 18% acetonitrile, as compared with 23% acetonitrile before oxidation.

## RESULTS AND DISCUSSION

Two protein kinase 'cascades' have been identified whose activation is triggered by insulin and other growth factors that signal through receptor protein tyrosine kinases. One leads to the activation of MAP kinase (reviewed in [19]) and the other to the activation of p70 ribosomal protein S6 kinase (p70$^{S6K}$) [20,21]. Two physiological targets for MAP kinase are themselves protein kinases, which we have termed MAPKAP kinase-1 and MAPKAP kinase-2 [14,15]. MAPKAP kinase-1 is an isoform of p90 ribosomal protein S6 kinase (RSK-2) [14,17], and distinct from p70$^{S6K}$, which is not activated by MAP kinase. One physiological substrate for MAPKAP kinase-2 is heat-shock protein 25 [22], which is not a substrate for MAPKAP kinase-1 or p70$^{S6K}$. Conversely, MAPKAP kinase-2 does not phosphorylate ribosomal protein S6 [15]. We therefore examined the effects of these four growth-factor-stimulated protein kinases on GSK3.

The specificity of GSK3 is unusual in that it phosphorylates serine and threonine residues positioned N-terminal to another phosphoserine [23,24], the preferred consensus sequence being S/TXXXS(P) [24]. Accordingly, we assayed GSK3 using a synthetic peptide similar to the region in glycogen synthase phosphorylated by GSK3. This peptide (termed here GS peptide-2) is a substrate for GSK3 provided that the C-terminal serine residue is first phosphorylated by casein kinase-2 [24,25] (Figure 1). Using this substrate, a form of GSK3 was purified 15000-fold from rabbit skeletal muscle extracts by exploiting its co-purification with MAPKAP kinase-2 [15]. This form of GSK3 is only resolved from MAPKAP kinase-2 at the sixth step of purification

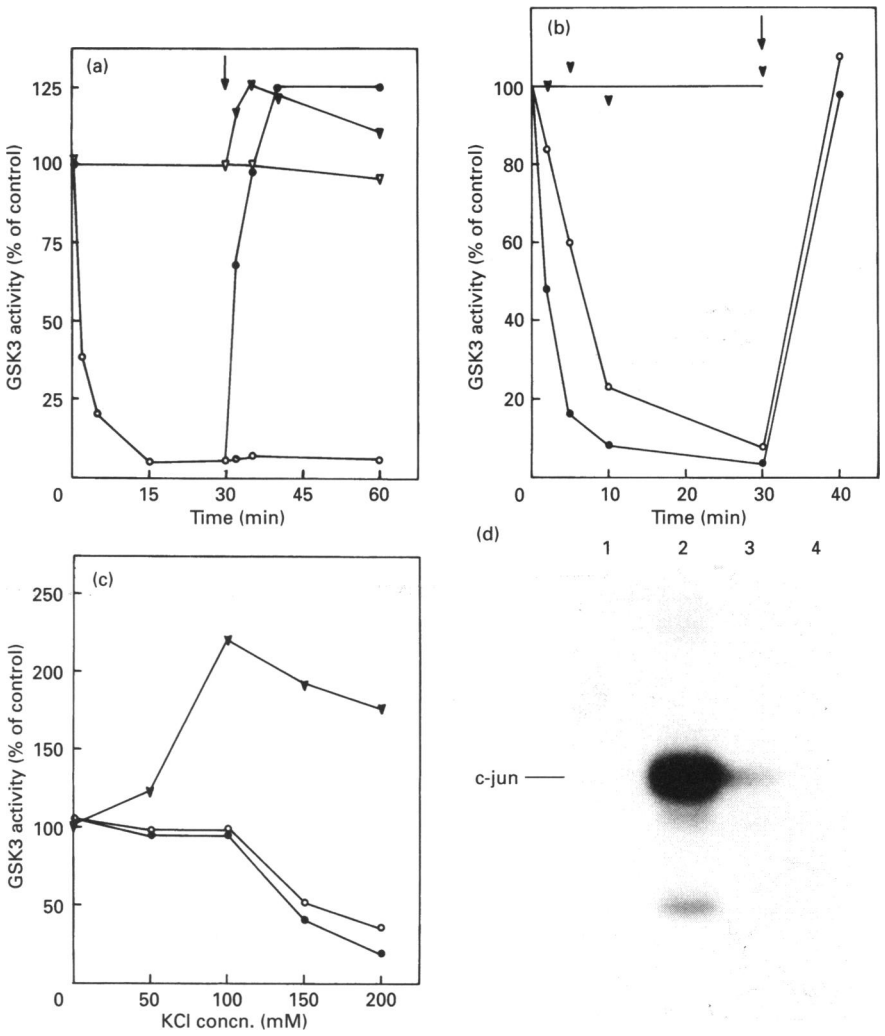


**Figure 2 GSK3 is inactivated by MAPKAP kinase-1 and p70$^{S6K}$ and re-activated by PP2A**

(**a**) Inactivation and re-activation of GSK3 by MAPKAP kinase-1 and PP2A. GSK3 (15 units/ml) was incubated with MgATP in the presence (○) and absence (▽) of MAPKAP kinase-1 (3 units/ml). After 30 min, phosphorylation was stopped with excess Na-EDTA, and $PP2A_1$ included at a concentration of 0.5 unit/ml in the presence (open symbols) and absence (closed symbols) of okadaic acid (2 $\mu$M). GSK3 was assayed with GS peptide-2 at the times indicated. Similar results were obtained in several experiments with two GSK3 and two MAPKAP kinase-1 preparations. (**b**) Inactivation of GSK3 by MAPKAP kinase-1 and P70$^{S6K}$. GSK3 (15 units/ml) was incubated with MgATP and 0.5 unit/ml MAPKAP kinase-1 (○), MgATP and 0.1 unit/ml p70$^{S6K}$ (●), or with MgATP alone (▼). After 30 min, the phosphorylations were stopped with Na-EDTA and dephosphorylation initiated with $PP2A_1$ (0.6 unit/ml). GSK3 activity was assayed with GS peptide-2 at the times indicated. Similar results were obtained in several experiments with two GSK3, two MAPKAP kinase-1 and three p70$^{S6K}$ preparations. (**c**) Phosphorylation by MAPKAP kinase-1 and p70$^{S6K}$ inhibits the phosphorylation of glycogen synthase by GSK3 in the presence, but not the absence, of KCl. GSK3 (15 units/ml) was incubated for 30 min with MgATP in the presence of 0.1 unit/ml p70$^{S6K}$ (●), 0.5 unit/ml MAPKAP kinase-1 (○) or in the absence of either protein kinase (▼). GSK3 was then assayed at the concentrations of KCl indicated using glycogen synthase (3 $\mu$M) as substrate instead of GS peptide-2. (**d**) Phosphorylation of GSK3 by MAPKAP kinase-1 inhibits the phosphorylation of c-jun. GSK3 was incubated for 30 min with MgATP and MAPKAP kinase-1 (0.5 unit/ml) and assayed for GSK3 activity in the absence of KCl using c-jun (0.14 mg/ml) as substrate. The samples were subjected to SDS/PAGE and autoradiography. Lanes 2 and 3, GSK3 was preincubated with MgATP in the absence and presence of MAPKAP kinase-1 respectively; lane 1, GSK3 and MAPKAP kinase-1 were omitted from the incubations; lane 4, GSK3, but not MAPKAP kinase-1, was omitted.

of the latter enzyme (Figure 1), after which step it is a major protein staining band, although only approx. 25% pure (Figure 1b).

The GSK3 preparation was rapidly inactivated by incubation with MgATP and either MAPKAP kinase-1 (Figure 2a) or p70$^{S6K}$ (Figure 2b). No inhibition occurred if either MgATP (results not shown) or protein kinases (Figure 2a) were omitted. MAPKAP kinase-1 is inactivated by incubation with PP2A and re-activated by MAP kinase [17] and these treatments abolished and restored, respectively, the ability of MAPKAP kinase-1 to inhibit GSK3 (results not shown). Similarly, preincubation of the partially purified p70$^{S6K}$ preparation with PP2A or inclusion of the standard S6 peptide substrate in the phosphorylation reactions prevented the inactivation of GSK3 (results not shown). A highly purified preparation of p70$^{S6K}$ expressed in insect cells (generously provided by Dr. G. Thomas, Friedrich Miescher Institute, Basel, Switzerland) inhibited GSK3 in an identical manner to the partially purified p70$^{S6K}$. These experiments demonstrated that the inactivation of GSK3 was catalysed by MAPKAP kinase-1 and p70$^{S6K}$ and not by other protein kinases present as trace contaminants. The concentrations of MAPKAP kinase-1 (0.5 unit/ml) and p70$^{S6K}$ (0.1 unit/ml) required to inactivate GSK3 in 15 min at 30 °C (Figure 2b) are about 10-fold and 15-fold lower than their reported concentrations in insulin-

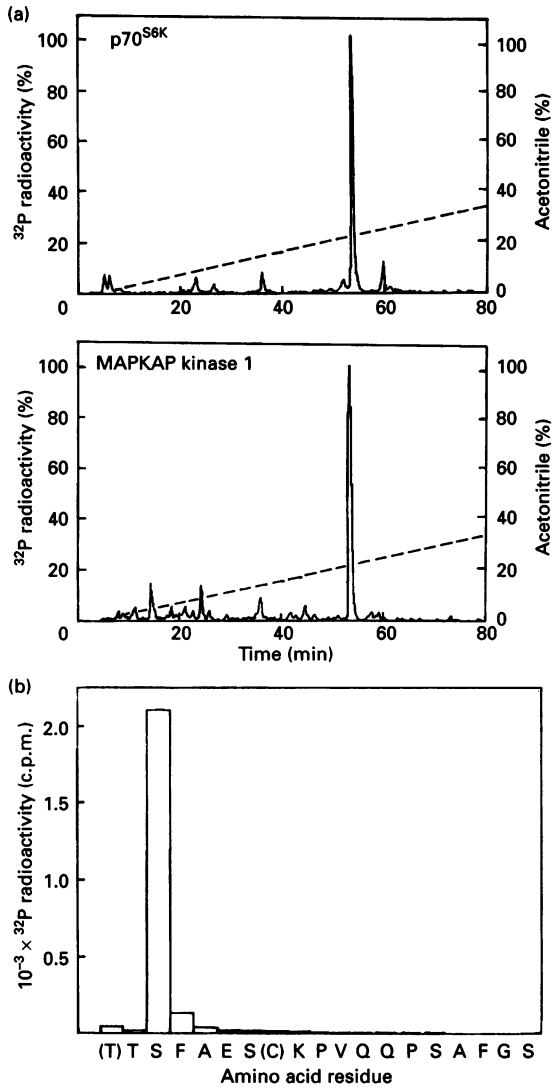


**Figure 3 Purification and sequence analysis of the tryptic phosphopeptide from GSK3 labelled by p70$^{S6K}$ and MAPKAP kinase-1**

(**a**) Chromatography of tryptic phosphopeptides from GSK3. GSK3 phosphorylated with Mg[$\gamma$-$^{32}$P]ATP and either p70$^{S6K}$ or MAPKAP kinase-1 was digested with trypsin and chromatographed on a $C_{18}$ column equilibrated in 0.1% trifluoroacetic acid as described in the Materials and methods section. The full line shows the $^{32}$P radioactivity and the broken line the acetonitrile gradient. (**b**) Identification of the residue in GSK3 phosphorylated by p70$^{S6K}$. The major tryptic phosphopeptide from (**a**) was further purified as described in the Materials and methods section, dissolved in 10% (v/v) trifluoroacetic acid and 10 pmol of peptide (3300 c.p.m.) sequenced twice, first by conventional gas-phase sequencing to identify amino acid residues, and secondly by solid-phase sequencing after coupling the peptide covalently to an arylamine membrane [15]. The Figure shows $^{32}$P radioactivity released, and phenylthiohydantoin-derived amino acid (single letter code) identified after each cycle of Edman degradation. The sequence corresponds to residues 7 to 24 of rat GSK3$\beta$ and residues in parentheses were not identified by peptide sequencing. The sequence of the tryptic peptide isolated after phosphorylation by MAPKAP kinase-1 was identical. The corresponding sequence in GSK3$\alpha$ (residues 19–36) is TSSFAEPGGGGGGGGGGG [27].

stimulated skeletal muscle [14] and cycloheximide-treated rat liver [16] respectively. Inactivated GSK3 was rapidly re-activated by incubation with PP2A, and re-activation was prevented if okadaic acid, a specific PP2A inhibitor [26], was added together with the phosphatase (Figure 2). GSK3 that had not been inactivated by MAPKAP kinase-1 or p70$^{S6K}$ was activated 20–30% by PP2A (Figure 2a), indicating that the GSK3 preparation was partially phosphorylated. GSK3 was not inactivated by the 42 kDa isoform of MAP kinase or MAPKAP kinase-2 (results not shown).

Inactivation of GSK-3 by either MAPKAP kinase-1 or p70$^{S6K}$ was accompanied by the incorporation of near stoichiometric amounts of phosphate into the enzyme (about 0.7 mol per mol, Figure 1c) and no further phosphorylation occurred when both kinases were combined, suggesting that they phosphorylate the same residue. This was confirmed by tryptic digestion and chromatography on a $C_{18}$ column, which revealed that both protein kinases phosphorylated an identical tryptic peptide (Figure 3a). After further purification, the phosphopeptide was sequenced and found to correspond to residues 7–28 of GSK3$\beta$ [27]. The site of phosphorylation was identified as Ser-9 (Figure 3b). The identification of the enzyme preparation as GSK3$\beta$ is consistent with its apparent molecular mass of 47 kDa (Figure 1b) [27] and with the presence of GSK3$\beta$ mRNA in skeletal muscle, although only the GSK3$\alpha$ isoform has previously been detected in skeletal muscle by immunoblotting [27]. The sequence surrounding Ser-9 is consistent with the reported consensus sequences for phosphorylation by p70$^{S6K}$ [28] and MAPKAP kinase-1 [29].

The potent inactivation of GSK3$\beta$ by MAPKAP kinase-1 or p70$^{S6K}$ was not only observed using GS peptide-2 (Figures 2a and 2b) but also with three other substrates, namely the transcription factor c-jun (Figure 2d), inhibitor-2 ([2], results not shown) and glycogen synthase (Figure 2c). However, with glycogen synthase, the inhibitory effect of phosphorylation on activity was only observed if physiological concentrations of KCl were present in the assays (Figure 2c). With glycogen synthase as substrate, dephosphorylated GSK3$\beta$ was activated 2-fold by 0.1–0.2 M KCl, whereas phosphorylated GSK3$\beta$ was unaffected by 0.1 M KCl and strongly inhibited at higher concentrations (Figure 2c). In contrast, when GS peptide-2 or inhibitor-2 were the substrates, 0.15 M KCl suppressed the activity of either the phosphorylated or dephosphorylated forms of GSK3$\beta$ by $80 \pm 10$%. Phosphorylation of GSK3$\beta$ therefore inhibited its activity towards GS peptide-2, inhibitor-2 or c-jun by $> 90$% in the absence (Figure 2) or presence (results not shown) of 0.15 M KCl.

Our findings may explain how GSK3$\beta$ is inhibited acutely by insulin in Chinese-hamster ovary cells, as the inhibition of GSK3$\beta$ by this hormone is reversed by treatment with PP2A [13]. The sequence surrounding Ser-23 of GSK3$\alpha$ (RARTSSFAE) [27] is similar to that surrounding Ser-9 of GSK3$\beta$, suggesting that the reported inhibition of GSK3$\alpha$ by insulin in adipocytes [11] may be mediated by the same mechanism. Recently, we have identified GSK3$\alpha$ as a minor peak of activity (with GS peptide-2 as substrate) which elutes from S-Sepharose (step 5 of the purification [15]) just before GSK3$\beta$, and have also shown that (after further purification by chromatography on Mono S and Mono Q columns) it is inactivated by MAPKAP kinase-1 or p70$^{S6K}$ and re-activated by PP2A in a similar manner to GSK3$\beta$ (C. Sutherland and P. Cohen, unpublished work).

In skeletal muscle, the activation of glycogen synthase by insulin involves the dephosphorylation of serine residues that are phosphorylated by GSK3 [30] and dephosphorylated by the glycogen-associated form of protein phosphatase-1 (PP1G) [31]. We have reported previously that MAPKAP kinase-1 activates PP1G by phosphorylating a specific serine residue on its glycogen-binding subunit, and demonstrated that the phosphorylation of this serine residue increases *in vivo* in response to insulin [32]. The present data now suggest that the inhibition of GSK3 may also contribute to the stimulation of glycogen synthesis by insulin. The requirement for isotonic KCl in the assays to detect the inhibitory effects of phosphorylation on GSK3 activity when

glycogen synthase is the substrate (Figure 2c) may explain why regulation of GSK3 by insulin has not been detected previously in muscle.

One mechanism by which phorbol esters enhance the binding of DNA by the activation protein-1(AP1) transcription-factor complex is by stimulating the dephosphorylation of c-jun at serine residues proximal to the DNA-binding region that are phosphorylated by GSK3 *in vitro* [4]. Since phorbol esters (acting through protein kinase C) increase both MAPKAP kinase-1 and $p70^{S6K}$ activity [33], the inhibition of GSK3 by either MAPKAP kinase-1 or $p70^{S6K}$ may explain how these tumour promoters stimulate the dephosphorylation of the inhibitory phosphoserines in c-jun [4]. MAPKAP kinase-1 homologues have been reported to undergo translocation from the cytosol to the nucleus following cell stimulation by growth factors [34], and an alternatively spliced form of $p70^{S6K}$ appears to be located in the nucleus. The association of GSK3 with the nucleus has also been reported (data of Defize cited in [7]). However, protein kinase C could be involved more directly, because it has been reported to phosphorylate and partially inhibit GSK3$\beta$ (but not GSK3$\alpha$) *in vitro* [35]. The association of protein kinase C with nuclei has also been reported [36].

The sequence surrounding Ser-9 of rabbit GSK3$\beta$ (RPRTTSFAE) is almost perfectly conserved in the 'shaggy' gene product of *Drosophila* (RPRTSSFAE), the sequence of which shows 88% overall identity with mammalian GSK3$\beta$ [37]. 'Shaggy' is essential for the development of the syncitial blastoderm, the specification of cell fates in embryonic development [38,39] and for normal larval neurogenesis (where it is implicated in a signal-transduction pathway lying downstream of the putative receptor 'notch') [8,9]. The striking conservation of the sequence surrounding Ser-9, even in *Drosophila*, suggests that inhibition of GSK3 by phosphorylation is likely to regulate many cellular processes, and that it may represent a point of convergence of two major growth-factor-stimulated protein kinase cascades.

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