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The α-isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70 S6 kinase or MAP kinase-activated protein kinase-1 in vitro

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Abstract

The α-isoform of glycogen synthase kinase-3 (GSK3α) was inactivated by 80% towards a synthetic peptide substrate upon incubation with Mg-ATP and either MAP kinase-activated protein (MAPKAP) kinase-1 or p70 S6 kinase. Inactivation by either kinase resulted from the phosphorylation of Ser-21 and was reversed by treatment with protein phosphatase $2A_1$. Phosphorylation also decreased GSK3α activity towards glycogen synthase, inhibitor-2 and c-jun. The specificity of GSK3α was similar to GSK3β, but with the synthetic peptide substrate heparin stimulated the dephosphorylated form of GSK3α (6-fold) more than GSK3β (1.8-fold). After phosphorylation, both isoforms were stimulated 15–20-fold by heparin.

Key words: MAP kinase; Ribosomal S6 kinase; Glycogen synthase kinase-3; Growth factor; Insulin

1. Introduction

Glycogen synthase kinase-3 (GSK3) was identified as an inactivator of glycogen synthase in skeletal muscle [1], and subsequently shown to phosphorylate many other proteins in vitro including inhibitor-2 (a regulatory subunit of protein phosphatase-1) [2], the type-II regulatory subunit of cyclic AMP-dependent protein kinase [3], ATP-citrate lyase [4,5], eIF2B [6] (a nucleotide exchange factor required for the initiation of protein synthesis), tau (a microtubule-associated protein) [7] and the transcription factors c-jun and c-myc [8–10]. *Drosophila* mutants lacking GSK3 are defective in several developmental processes, such as larval neurogenesis where it is implicated in a signal transduction pathway lying downstream of a putative receptor protein, termed 'notch' [11,12].

Recently, evidence has been accumulating that GSK3 is inhibited acutely by insulin. The activity of the α-isoform of GSK3 (GSK3α) decreased by about 50% upon stimulation of rat adipocytes with insulin [13], while the activity of the β-isoform (GSK3β) decreased by about 70% when Chinese hamster ovary cells that overexpress the insulin receptor were stimulated with insulin [6]. In the latter case, inhibition was reversed by protein phosphatase 2A (PP2A), suggesting that insulin may promote serine/threonine phosphorylation of GSK3β [6].

The first evidence that GSK3 activity is regulated by phosphorylation was obtained by Goode et al. [14], who reported that GSK3β was phosphorylated by protein kinase C (PK-C) in vitro decreasing its activity by about 50%. However, these investigators reported that GSK3α was not phosphorylated or inhibited by PK-C [14], indicating that this mechanism could not account for the inhibition of GSK3α by insulin in adipocytes [5]. Recently, we reported that MAP kinase-activated protein kinase-1 (MAPKAP kinase-1, also termed RSK2 [15]) and p70 S6 kinase ($p70^{S6K}$), which are both activated by insulin and other growth factors in vivo, phosphorylate GSK3β stoichiometrically at Ser-9 decreasing its activity by 95% [16]. These observations suggested that MAPKAP kinase-1 and/or $p70^{S6K}$, which are components of distinct protein kinase cascades [17–19], may be involved in the regulation of GSK3β by extracellular agonists. However, in view of the reported failure of PK-C to phosphorylate GSK3α [14], it was clearly essential to investigate whether the α-isoform was also regulated by MAPKAP kinase-1 and/or $p70^{S6K}$. In this paper we establish that these protein kinases do indeed inactivate GSK3α by phosphorylating Ser-21, the equivalent residue to Ser-9 of GSK3β.

2. Materials and methods

2.1. Materials

The trimeric form of protein phosphatase 2A, termed $PP2A_1$ (isolated by Dr. G. Moorhead as in [20]), glycogen synthase (isolated by Miss

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F. Douglas as in [21]). MAPKAP kinase-1 [15] and GSK3β [16] were purified from rabbit skeletal muscle. Highly purified p70^{S6K} expressed in Sf9 cells was provided by Dr G. Thomas (Friedrich Miescher Institute, Basel, Switzerland), bacterially expressed inhibitor-2 was provided by Dr A. DePaoli Roach, Indiana University School of Medicine, Indianapolis, USA) and bacterially expressed c-jun by Dr. E. Black and Dr. D. Gillespie (Beatson Cancer Institute, Glasgow, Scotland). Antibodies that recognise GSK3α and GSK3β were gifts from Dr. J. Woodgett, (Ontario Cancer Institute, Toronto, Canada) and the protein kinase inhibitor H7 was from Professor H. Hidaka (Nagoya University School of Medicine, Japan). Heparin from porcine intestinal mucosa (catalogue no. H3393) was purchased from Sigma Chemical Co. (Poole, Dorset, UK).

2.2. Enzyme assays

MAPKAP kinase-1 was assayed with a peptide related to the C-terminus of ribosomal protein S6 (30 μM) and p70^{S6K} with the peptide RRRLSSLA (30 μM) as in [22]. GSK3 was assayed in an identical manner using the peptide YRRAAVPPSPSLSRHSSPHQSEDEEE (20 μM, provided by Dr M. Goedart, MRC Laboratory for Molecular Biology, Cambridge, UK) phosphorylated at its C-terminal serine with casein kinase 2 from rat liver (a gift from Professor L. Pinna, University of Padova, Italy) [16] to convert it to a substrate for GSK3. One unit of protein kinase activity (U) was that amount which catalysed the phosphorylation of 1 nmol of peptide substrate in 1 min. GSK3 was also assayed with 3 μM glycogen synthase, 3 μM c-jun and 3 μM inhibitor-2. Before assay, inhibitor-2 was converted to a much more effective substrate for GSK3 by phosphorylation with casein kinase-2; then heated at 100°C for 5 min to inactivate casein kinase-2. PP2A$_1$ was assayed as described [20] and one unit of activity was that amount which catalysed the dephosphorylation of 1 nmole of glycogen phosphorylase in 1 min.

2.3. Inactivation and reactivation of GSK3

GSK3 (15 U/ml) was incubated in 20 mM MOPS (pH7.0), 0.01% (by vol.) Brij 35, 5% (by vol.) glycerol, 0.1% (by vol.) 2-mercaptoethanol (Buffer A), 10 mM Mg-acetate, 0.1 mM ATP, and either 3 U/ml MAPKAP kinase-1 or 0.7 U/ml p70^{S6K}. At various times, aliquots were removed, diluted appropriately into ice-cold buffer containing 20 mM MOPS, pH 7.0, 1.0 mg/ml bovine serum albumin, 1 mM EDTA and 0.1% (by vol.) β-mercaptoethanol and assayed for GSK3 activity at a further 5-fold dilution.

2.4. Isolation of the tryptic peptide from GSK3α

GSK3α (1.0 ml, 15 U/ml) was phosphorylated for 10 min with MAPKAP kinase-1 as described above, except that [γ^{32}P]ATP (10^6 cpm/nmol) replaced unlabelled ATP. The reaction was stopped by addition of Na-EDTA and NaF to final concentrations of 20 mM and 50 mM, respectively, and the sample concentrated to 0.1 ml by centrifugation through a centricon 30 membrane (Amicon). The preparation was rediluted to 1.0 ml with buffer A plus 50 mM NaF and re-concentrated, and this procedure repeated until free ATP comprised <10% of the ^{32}P-radioactivity. The native ^{32}P-labelled GSK3α (0.3 ml) was then incubated for 20 min at 30°C with 0.3 ml of 0.2 mg/ml trypsin and the reaction terminated by addition of 0.2 ml of 20% (by mass) trichloroacetic acid. After standing on ice for 2 min, the suspensions were centrifuged for 5 min at 13,000 × g and the supernatants, containing 95% of the ^{32}P-radioactivity, chromatographed on a Vydac C$_{18}$ column (Separations Group, Hesperia, CA, USA) equilibrated in 0.1% (by vol.) trifluoroacetic acid, pH 1.9. The column was developed with a linear acetonitrile gradient with an increase in acetonitrile concentration of 0.3% per min. Fractions of 0.4 ml were collected and ^{32}P-radioactivity recorded with an on-line monitor. The major phosphopeptide was dried, resuspended in 0.1 ml of 0.1% (by vol.) acetic acid and added to 0.1 ml of iron chelating Sepharose resin (Pharmacia) [23] equilibrated in 0.1% (by vol.) acetic acid. The resin was mixed end over end for 15 min at room temperature, then centrifuged for 0.5 min at 14 000 × g, and the supernatant discarded. The resin was washed 10 times with 0.4 ml portions of 0.1% (by vol.) acetic acid and ^{32}P-radioactivity eluted with three 0.4 ml portions of water:ethanol:triethylamine, pH 10.5 (9:9:2) followed by 0.4 ml of 0.1 M *N*-methylmorpholine acetate (pH 8.0). The pooled eluates, containing 70% of the applied ^{32}P-radioactivity, were dried and rechromatographed on the C$_{18}$ column as described above, except that trifluoroacetic acid was replaced by 10 mM ammonium acetate, pH 6.5.

3. Results

3.1. Identification of GSK3α and separation from GSK3β

The substrate specificity of GSK3 is unusual in that it phosphorylates Ser/Thr residues that lie N-terminal to another phosphoserine, the preferred consensus sequence being Ser/Thr-Xaa-Xaa-Xaa-Ser(P) [24,25]. The substrate used to assay GSK3 routinely is a synthetic peptide, termed GS peptide-2 [16], which only becomes a substrate after its most C-terminal serine residue is phosphorylated by casein kinase-2 ([25,26] and Fig. 1A). Using this substrate, we recently purified GSK3β 15,000-fold from rabbit skeletal muscle to a purity of 25% by a procedure involving successive chromatographies on CM-Sephadex, DEAE-cellulose, S-Sepharose and Mono-S. The major peak of GSK3 activity (GSK3β) from S-Sepharose was preceded by a minor peak of activity that was also active towards GS peptide-2 provided

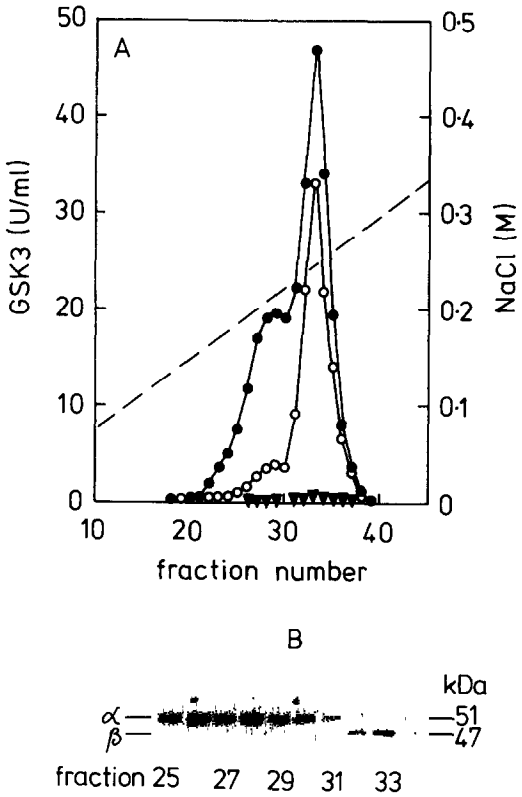


Fig. 1. Separation of GSK3α and GSK3β by chromatography on S-Sepharose. (A) GSK3 (450 U, 100 ml) from the DEAE-cellulose flow-through [16] was applied to the column equilibrated in 20 mM MOPS (pH 7.0), 1 mM EDTA, 0.01% (by vol.) Brij 35, 5% (by vol.) glycerol and 0.1% (by vol.) 2-mercaptoethanol. After washing with 50 ml of equilibration buffer, the column was developed with a 200 ml linear gradient to 0.4 M NaCl (broken line). Fractions (3 ml) were assayed for GSK3 activity in the absence (open circles) or presence (closed circles) of 0.1 mg/ml heparin using GS peptide-2 previously phosphorylated with casein kinase-2. The column was also assayed in the absence of heparin with GS peptide-2 not phosphorylated by casein kinase-2 (closed triangles). (B) Fractions from (A) were subjected to SDS/polyacrylamide gel electrophoresis and immunoblotted with antibodies that recognise GSK3α (α, 51 kDa) and GSK3β (β, 47 kDa) [27].

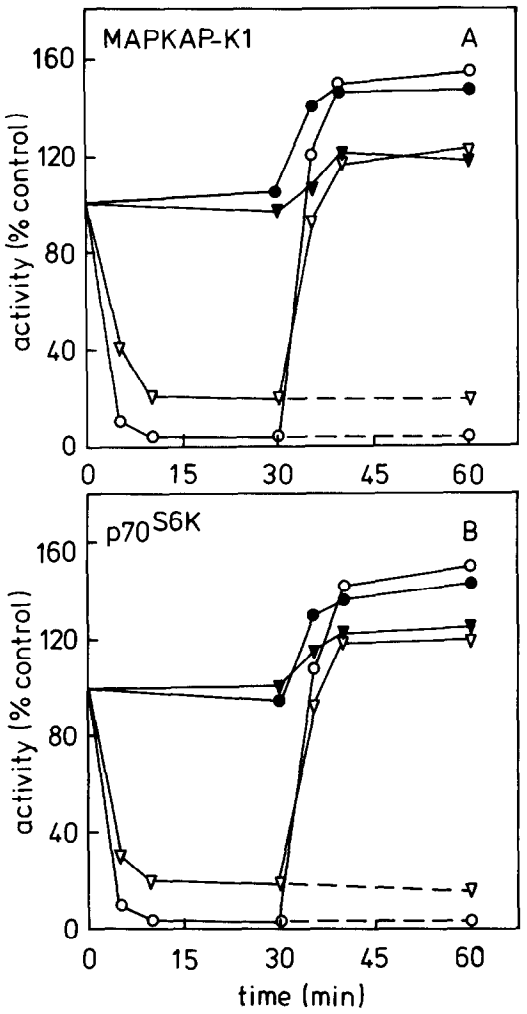
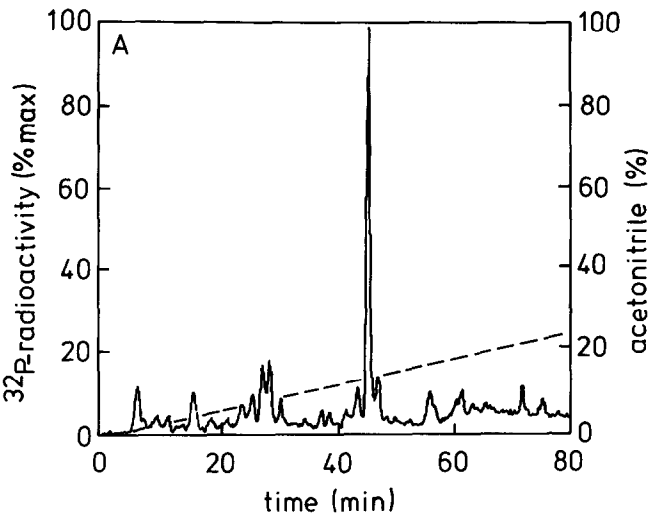


Fig. 2. Both isoforms of GSK3 are inactivated by MAPKAP kinase-1 (A) and $p70^{S6K}$ (B) and reactivated by protein phosphatase $2A_1$. (A) GSK3α (triangles) or GSK3β (circles) (15 U/ml) were incubated with Mg-ATP in the presence (open symbols) and absence (closed symbols) of MAPKAP kinase-1 (3 U/ml). After 30 min, phosphorylation was stopped with excess Na-EDTA, and $PP2A_1$ added (final concentration, 2 U/ml). The broken lines show experiments where okadaic acid (1 μM) was added together with $PP2A_1$. GSK3 activity was measured with GS peptide-2 at the times indicated. (B) The experiment and symbols are as described in (A), except that MAPKAP kinase-1 was replaced by $p70^{S6K}$ (0.7 U/ml).



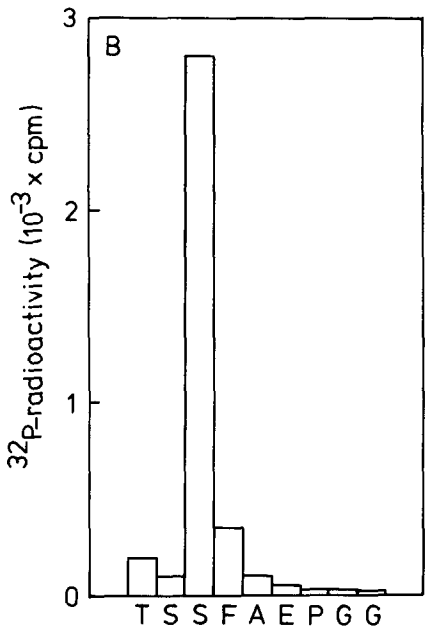


Fig. 3. Isolation and sequence analysis of the major tryptic phosphopeptide from GSK3α phosphorylated by MAPKAP kinase-1. (A) ^{32}P-labelled GSK3α maximally phosphorylated by MAPKAP kinase-1 (section 2.3) was digested with trypsin and chromatographed on a C_{18} column at pH 1.9 (section 2.4). The full line shows the ^{32}P-radioactivity and the broken line the acetonitrile gradient. (B) Identification of the residue in GSK3α phosphorylated by MAPKAP kinase-1. The major tryptic phosphopeptide from A was further purified (section 2.4) and 42 pmol (4,200 cpm) sequenced twice; firstly by conventional gas phase sequencing to identify amino acid residues, second by solid phase sequencing after coupling the peptide covalently to an arylamine membrane [22]. The figure shows ^{32}P-radioactivity released, and phenylthiohydantoin amino acid identified after each cycle of Edman degradation. The sequence corresponds to residues 19 to 27 of rat GSK3α although phenylthiohydantoin amino acids were identified unambiguously up to residue 36.

that it had been phosphorylated by casein kinase-2 (Fig. 1A). Furthermore, this minor peak was stimulated 5-fold to 6-fold by heparin (0.1 μg/ml), in contrast to GSK3β whose activity was only increased slightly by this glycosaminoglycan (Fig. 1A). Immunoblotting of the fractions with an antibody that recognises GSK3α and GSK3β revealed a cross-reacting species which co-eluted with the minor peak; its apparent molecular mass (51 kDa) was identical to GSK3α [27] (Fig. 1B). The major peak cross-reacted with a 47 kDa species, the same molecular mass as GSK3β (Fig. 1B).

3.2. Inactivation of GSK3α by MAPKAP kinase-1 and $p70^{S6K}$

The first half of the peak of GSK3α, which was free of GSK3β, was pooled and further purified by chromatography on Mono-S as in [16]. When this preparation, which was estimated to be 5–10% pure, was incubated with Mg-ATP and either MAPKAP kinase-1 or $p70^{S6K}$ its activity decreased and reached a plateau at about 80% inhibition (Fig. 2). No inhibition occurred if MAPKAP kinase-1 and $p70^{S6K}$ were omitted. Under the same conditions the activity of GSK3β was decreased by 95% (Fig. 2). GSK3α, like GSK3β, could be fully reactivated by incubation with $PP2A_1$ (Fig. 2) and reactivation was prevented by the inclusion of okadaic acid, a PP2A inhibitor (Fig. 2). GSK3α and GSK3β which had not been inactivated by phosphorylation were stimulated 20–30% and 40–45%, respectively, upon incubation with $PP2A_1$, indicating that the purified enzymes were partially phosphorylated.

3.3. Identification of the major residue on GSK3α phosphorylated by MAPKAP kinase-1 and $p70^{S6K}$

Incubation of GSK3α preparations with Mg-[γ-^{32}P]ATP and MAPKAP kinase-1 resulted in the phosphorylation of a single 51 kDa protein in the preparation (data not shown) and following tryptic digestion, chromatography on a C_{18} column at pH 1.9 revealed one major ^{32}P-peptide eluting at 14% acetonitrile (Fig. 3A). After further purification using an iron chelate column, which binds phosphopeptides relatively selectively [23], and chromatography on the C_{18} column at pH 6.5, the phosphopeptide was pure and its N-terminal sequence (Thr-Ser-Ser-Phe-Ala-Glu-Pro-$(Gly)_{10}$-Pro) corresponded to residues 19–36 of GSK3α. The site of phosphorylation was Ser-21 (Fig. 3B). Phosphorylation of GSK3α with $p70^{S6K}$ also labelled the 51 kDa band and tryptic digestion and chromatography on the C_{18} column yielded the same major phosphopeptide obtained after phosphorylation with MAPKAP kinase-1 (data not shown).

3.4. Effect of phosphorylation on GSK3α and GSK3β activity

When matched for activity towards GS peptide-2, GSK3α and GSK3β had similar relative activities toward glycogen synthase, inhibitor-2 and c-jun (Table 1). Phosphorylation of GSK3α decreased activity towards c-jun (70%) and inhibitor-2 (52%) somewhat less than activity towards GS peptide-2 (80%) (Table 1). When glycogen synthase was the substrate, the inhibitory effect of phosphorylation was more marked in the presence of 0.15M KCl (Fig. 4), as found previously for GSK3β [16]. The effect of phosphorylation on activity also varied with the glycogen synthase preparation. For example in the absence of KCl, phosphorylation of GSK3β had no effect on the rate of phosphorylation of one glycogen synthase preparation [16] (Fig. 4B), but inhibited by 50%, the rate of phosphorylation of another preparation prepared more recently. In the presence of KCl, the phosphorylated form of GSK3β (Fig. 4B) had a much lower activity than the dephosphorylated form with either glycogen synthase preparation. A similar result was obtained with GSK3α (data not shown). Glycogen synthase is known to aggregate on storage, which may affect its interaction with GSK3.

The phosphorylated forms of GSK3α and GSK3β were stimulated 15-fold to 20-fold by heparin when GS peptide-2 was the substrate and the effect of phosphorylation on activity was therefore far less pronounced when this glycosaminoglycan was included in the assays. The heparin concentration required for half maximal activation of phosphorylated or dephosphorylated GSK3α and GSK3β was 0.5–1 μg/ml. With other substrates heparin had little effect on the activity of either GSK3 isoform (Table 1).

Table 1
Effect of phosphorylation and heparin on the activities of GSK3α and GSK3β towards different substrates

	(a) Relative Activity		(b) Effect of phosphorylation (inhibition %)		(c) Effect of heparin (stimulation-fold)			
	GSK3α-D	GSK3β-D	GSK3α	GSK3β	GSK3α-D	GSK3α-P	GSK3β-D	GSK3β-P
GS peptide-2 (20 μM)	100	100	82 ± 2	95 ± 1	5.7 ± 0.8	18.9 ± 1	1.8 ± 0.3	14.2 ± 2
Glycogen synthase (3 μM)	45 ± 3	39 ± 2	63 ± 5*	90 ± 7*	1.1 ± 0.1*	1.1 ± 0.1*	1.1 ± 0.2*	0.8 ± 0.1*
Inhibitor-2 (3 μM)	6 ± 2	8.5 ± 2	52 ± 2	86 ± 6	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
c-jun (3 μM)	0.2 ± 0.1	0.4 ± 0.2	71 ± 6	96 ± 4	1.3 ± 0.2	1.7 ± 0.4	1.1 ± 0.2	2.5 ± 0.6

GSK3α and GSK3β were maximally phosphorylated with MAPKAP kinase-1 (section 2.3) and assayed with the substrates indicated. The table shows (a) the activity of dephosphorylated GSK3α (GSK3α-D) and GSK3β (GSK3β-D) towards glycogen synthase, inhibitor-2 and c-jun relative to GS peptide-2; (b) the effect of phosphorylation on activity measured as % inhibition, and (c) the effect of heparin (0.1 mg/ml) on the activities of the dephosphorylated (D) and phosphorylated (P) forms of each GSK3 isoform. The protein kinase inhibitor H7 was included in assays with glycogen synthase to inactivate MAPKAP kinase-1 which phosphorylates this substrate.
*0.15 M KCl was present in these assays for reasons discussed in the text. Activities are given as S.E.M. for three different experiments (GS-peptide-2 and c-jun) or the average of two experiments (inhibitor-2 and glycogen synthase).

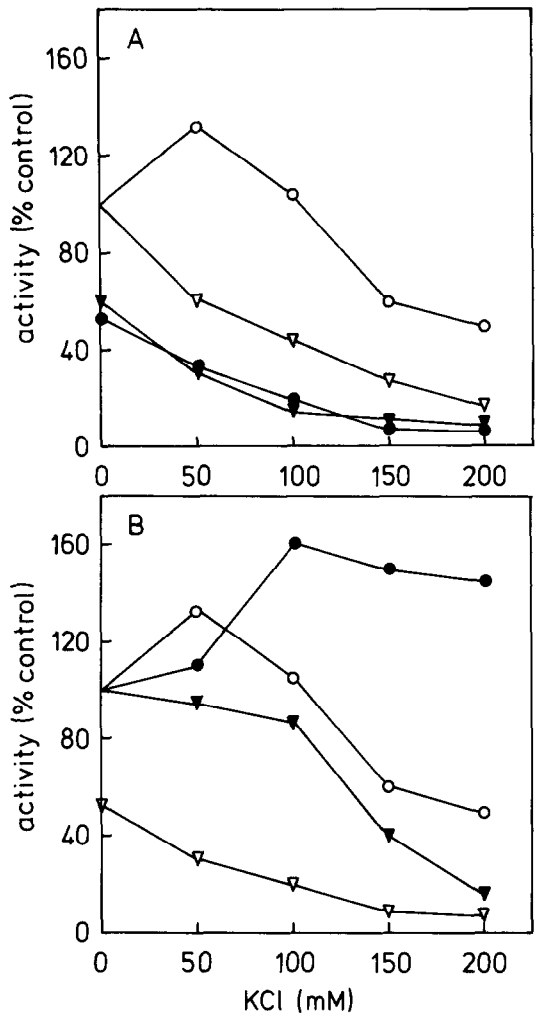


Fig. 4. Effect of phosphorylation by MAPKAP kinase-1 on the activity of GSK3 towards glycogen synthase at different concentrations of KCl. (A) The dephosphorylated forms of GSK3α (open triangles) and GSK3β (open circles) and the phosphorylated forms of GSK3α (closed triangles) and GSK3β (closed circles) were assayed with glycogen synthase (3 μM) at the KCl concentrations indicated. (B) Dephosphorylated GSK3β (circles) and phosphorylated GSK3β (triangles) were assayed with two different glycogen synthase preparations (open and closed symbols).

4. Discussion

In this paper we have demonstrated that GSK3α is phosphorylated at Ser-21 by MAPKAP kinase-1 and $p70^{S6K}$ in vitro and that phosphorylation is accompanied by the inhibition of activity. The sequence surrounding Ser-21 of GSK3α (RARTSSFAE) [27] is very similar to that surrounding Ser-9 of GSK3β (RPRTTSFAE) [27] and *Drosophila* GSK3 (RPRTSSFAE) [28], indicating a striking conservation of this regulatory phosphorylation site.

Although phosphorylation inhibited the GSK3α preparations somewhat less than the GSK3β preparations (Table 1), this difference may be an in vitro artefact. We have found that after chromatography on S-Sepharose, GSK3α is gradually converted to a 48 kDa species that is recognised by GSK3 antibodies, but cannot be phosphorylated by either MAPKAP kinase-1 or $p70^{S6K}$. The 48 kDa species, which has been observed previously [27] may be a form of GSK3α truncated at the N-terminus that cannot be inactivated by phosphorylation.

A heparin-stimulated protein kinase was detected several years ago, whose substrate specificity was similar to GSK3, but which lost sensitivity to heparin after tryptic digestion [29]. This could be the dephosphorylated form of GSK3α or the phosphorylated form of GSK3α and/or GSK3β, since these species are stimulated by heparin considerably with GS peptide-2 as substrate (Fig. 1, Table 1). However, the heparin-stimulated protein kinase identified previously was assayed with glycogen synthase and neither of the GSK3 isoforms we purified were stimulated significantly by heparin with this substrate. The identity of the heparin-stimulated protein kinase therefore remains uncertain.

It is clearly now critical to establish whether phosphorylation of Ser-21 (GSK3α) and Ser-9 (GSK3β) underlie the acute inhibition of these enzymes by insulin in rat adipocytes [5] and Chinese hamster ovary cells [6], and whether this regulatory mechanism operates in other cells following stimulation by growth factors and other agonists that activate $p70^{S6K}$ and the MAP kinase cascade. The identification of the inactivating phosphorylation sites and the elution positions of each tryptic phosphopeptide during C_{18} chromatography reported in this paper and in [16] will greatly facilitate these investigations.

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