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Phosphorylated seryl and threonyl, but not tyrosyl, residues are efficient specificity determinants for GSK-3β and Shaggy

Daniel D. Williams[1,a], Oriano Marin[1,b], Lorenzo A. Pinna[b], Christopher G. Proud[a,*]

[a] *Department of Anatomy and Physiology, Medical Sciences Institute/Wellcome Trust Building Complex, University of Dundee, Dundee, DD1 5EH, UK*

[b] *Dipartimento di Chimica Biologica, Università di Padova, CRIB and CNR (Centre for the study of Biomembranes), V.le G. Colombo, 3, I-35121 Padova, Italy*

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Abstract Glycogen synthase kinase-3 is involved in diverse functions including insulin signalling and development. In a number of substrates, phosphorylation by glycogen synthase kinase-3 is known to require prior phosphorylation at a Ser in the +4 position relative to its own phosphorylation site. Here we have used synthetic peptides derived from a putative glycogen synthase kinase-3 site in the *Drosophila* translation initiation factor eIF2Bε to investigate the efficacy of residues other than Ser(P) as priming residues for glycogen synthase kinase-3β and its *Drosophila* homologue Shaggy. glycogen synthase kinase-3β phosphorylated peptides with Ser(P) and Thr(P) in the priming position, but peptides with Tyr(P), Thr, Glu or Asp were not phosphorylated. The V_{max} for the Thr(P) peptide was three times higher than that of the Ser(P) peptide. These data suggest that glycogen synthase kinase-3 is unique among phosphate-directed kinases. The priming site specificity of Shaggy is similar to that of mammalian glycogen synthase kinase-3β. This unpredicted efficacy of Thr(P) in the priming position suggests that there may be other unidentified substrates for these kinases.

Key words: Glycogen synthase kinase-3; Specificity; Phosphate-directed; Shaggy; Protein kinase; *Drosophila*

1. Introduction

Glycogen synthase kinase-3 (GSK-3) was first identified by virtue of its ability to phosphorylate residues in skeletal muscle glycogen synthase (GS) which are important in the regulation of the activity of this enzyme by insulin [1]. Subsequent studies have revealed that it has other substrates which are implicated in the control of other cell functions, such as protein synthesis (the e-subunit of initiation factor eIF2B) and development (β-catenin) [1], and that GSK-3 is acutely regulated by insulin and other agents [1,2]. Insulin rapidly inactivates GSK-3 and leads to the phosphorylation of a conserved serine in the N-terminus of GSK-3. This is Ser^{9} in the β-isoform and Ser^{21} in the α-isoform of GSK-3 [3,4]. Since phosphorylation by GSK-3 inhibits both GS and eIF2B [5,6], the inactivation of GSK-3 in response to insulin provides a mechanism through which insulin may activate both glycogen and protein synthesis. A number of studies have shown that the signalling events which lie upstream of GSK-3 involve phosphatidylinositol 3-kinase [7–9] and that the protein kinase responsible for the phosphorylation and inactivation of GSK-3 is protein kinase B (PKB, [10–12]). Recent studies have shown that insulin brings about the dephosphorylation of the ε-subunit of mammalian eIF2B at Ser^{540}, the site phosphorylated by GSK-3 [6].

GSK-3 is also known to play key roles in the regulation of development in a wide range of species from insects to mammals [1]. Shaggy, the structural homologue of GSK-3 in *Drosophila*, plays multiple roles in development [1,13,14] and its mammalian homologue GSK-3β can complement a number of functions of the Shaggy gene in this organism [14]. Genetic evidence for paracrine signalling during the *Drosophila* epithelium development has indicated the involvement of Shaggy in the transduction of the Wingless signal [15]. Wingless acts through Dishevelled (whose function is currently unknown) to inactivate Shaggy, allowing Armadillo (the *Drosophila* homologue of β-catenin) to initiate the formation of sheet epithelium. Biochemical evidence from studies on Wingless in mouse $10T^{1}/_{2}$ fibroblasts has confirmed the role of Shaggy/GSK-3 in the Wingless signalling pathway and have also suggested that protein kinase C (PKC) lies upstream of Shaggy/GSK-3 [16].

Besides GS and eIF2Bε, a number of other substrates for GSK-3 have been identified including the type 1 phosphatase subunit R_{GI} [17], ATP-citrate lyase [18] and the transcription factor CREB (the cAMP response element binding protein [19]). Phosphorylation of this group of substrates requires prior phosphorylation of the C-terminal serine residue in the motif Ser-Xaa-Xaa-Xaa-Ser, where the more N-terminal serine residue is the target for GSK-3 [5,20]. However, it is not known whether phosphorylated residues other than Ser(P) (e.g. Thr(P), Tyr(P)) can serve this so called 'priming' function or whether other acidic residues (Asp, Glu) can do so. In the case of acidophilic, phosphate-directed protein kinases such as casein kinase-1 and -2 and Golgi apparatus casein kinase, at least some of these residues can substitute for Ser(P) and in some cases are actually better than Ser(P) [21–23].

Recent searching of *Drosophila* databases has revealed a genomic sequence that appears to be the homologue of eIF2Bε. This sequence (669 amino acids) shows similarity throughout its entire sequence to mammalian eIF2Bε (716 amino acids) and Gcd6p (712 amino acids), the homologue of eIF2Bε in *Saccharomyces cerevisiae* (identity at the amino acid level being 30 and 25% to these two proteins, respectively). The *Drosophila* sequence contains a serine at the position corresponding to Ser^{540} (the target for GSK-3) in the rabbit sequence. However, the possible priming site is occupied by a Thr rather than a Ser which is found in all the known mammalian eIF2Bε sequences (Table 1), raising the question

*Corresponding author. Fax: (44) (0) 1382 322424.
E-mail: cgproud@bad.dundee.ac.uk

[1] These authors contributed equally to this work.

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whether residues other than Ser(P), particularly Thr(P), can function in the C-terminal priming position to facilitate the phosphorylation by GSK-3. In this study, we have examined the efficacy of such residues in priming the phosphorylation by mammalian GSK-3β and Shaggy, using synthetic peptides.

2. Materials and methods

2.1. Chemicals and biochemicals

Chemicals and biochemicals were obtained from BDH (Poole, Dorset, UK) and Sigma-Aldrich (Gillingham, Dorset, UK), unless stated otherwise. [γ-^{32}P]ATP was obtained from Amersham and phosphocellulose (P81) and DEAE cellulose (DE81) paper were obtained from Whatman. Recombinant GSK-3β was kindly provided by Dr. A. Paterson (Dundee, MRC Protein Phosphorylation Unit). Dr. J.R. Woodgett (Ontario Cancer Institute) kindly provided a vector (pAC 5.1a) encoding *Drosophila* Shaggy with a hexa-His tag.

2.2. Synthetic peptides

The peptides T, Tp, Sp, E, D and Yp were synthesised by an automated peptide synthesiser ABI 431-A (Applied Biosystems) on 4-hydroxymethyl-phenoxymethylcopolystirene-1% divinylbenzene-resin (1.04 mmol/g). The synthesiser was equipped for using 9-fluorenylmethoxycarbonyl chemistry [24] and our own 2-(1H-benzotriazole-1-yl)-1,1,1,3,3 tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole protocol (see details in [25]) in a 0.05 mmol scale.

The incorporation of phospho-residues of Thr, Ser and Tyr was performed according to a building block approach [26] using the following Fmoc phospho-amino acid derivatives: Fmoc-Ser(PO(benzyl)OH, Fmoc-Thr(PO(benzyl)OH and Fmoc-Tyr(PO_3H_2)OH. Cleavage of peptides from the resin and side chain deprotection was carried out by treatment of the peptidyl resin with trifluoroacetic acid(TFA)/anisole/ethanedithiol/ethylmethylsulfide (95:3:1:1). The presence of the benzyl protecting group on phosphoresidues of serine and threonine, a mixture of TFA/triisopropylsilane/water (95:2.5:2.5), was preferred.

The crude peptides were purified by high performance liquid chromatography (HPLC) on a preparative reverse phase column Prep Nova-Pak HR C18, 6 m, 25×10 mm (Waters). The analytical HPLC and MALDI-TOF mass spectrometry analysis of the purified peptides showed a correct sequence and a purity of 95%.

2.3. Production of Shaggy protein

Drosophila melanogaster Schneider cells were transfected using the calcium phosphate procedure [27,28]. 3 days post-transfection, 3 ml of cells (at a density of 6×10^6 cells/ml) were harvested in lysis buffer containing 20 mM HEPES-KOH (pH 7.6), 0.27 M sucrose, 1 mM EDTA, 0.5% Triton X-100, 0.5 mM sodium orthovanadate, 0.1 mM DTT and protease inhibitors, leupeptin, aprotinin, pepstatin and benzamidine (all at 1 μg/ml) and 0.1 mM PMSF. The lysates were then incubated for 1.5 h at 4°C with Talon Metal Affinity Resin (Clontech, CA, USA) to bind the recombinant His-tagged Shaggy. Beads were then washed with lysis buffer and used as a source of Shaggy in assays using synthetic peptides.

2.4. Phosphorylation assays

Peptide phosphorylation assays were carried out essentially as described [14,29]. More specifically, phosphorylation was performed at 30°C in a 20 μl assay containing the various peptides (0.4 mM unless otherwise indicated), 16 mM HEPES-KOH (pH 7.5), 2 mM $MgCl_2$, 80 mM KCl, 0.1 mg/ml BSA, 25 μM ATP and 0.8 μCi [γ-^{32}P]ATP (specific activity ranged between 1500 and 4000 cpm/pmol). 32 mU of GSK-3β (0.028 μg) was used in each assay, where one unit is the amount of protein kinase transferring 1 nmol of phosphate/min to the standard substrate [30]. Reactions involving the phosphorylation of peptides by Shaggy were performed in a similar manner. To terminate reactions, samples were mixed with 5 μl of 0.5 M EDTA. Reaction mixtures containing basic peptides (SRAGS and SRAGSp) were spotted onto P81 paper and washed five times for 5 min with 1% (v/v) phosphoric acid. In the case of acidic peptides (all others), reaction mixtures were spotted onto DE81 paper and washed five times for 5 min in a solution containing 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl as described [31]. Incorporation of ^{32}P into the peptides was determined using the Cerenkov method. Kinetic analysis of the peptide phosphorylation was calculated from Eadie-Hofstee plots, using regression analysis of double reciprocal plots constructed from initial rate measurements.

3. Results

In a large proportion of the substrates for GSK-3, there is an almost absolute requirement for the prior phosphorylation of the more C-terminal serine residue in the motif Ser-Xaa-Xaa-Xaa-Ser in order for GSK-3 to phosphorylate its own target serine [5,20]. Our recent work on the role of GSK-3 in the regulation of the translation initiation factor eIF2B from mammals has indicated that this protein is no exception to this general rule [30]. Synthetic peptide substrates based on the mammalian eIF2Bε sequence (see Table 1 for details of the peptides) have previously been used to demonstrate the requirement for Ser(P) in the +4 position for phosphorylation by GSK-3 [32]. The Ser(P) at the +4 position is therefore said to be a 'priming' phosphorylation site [20,32,33]. In agreement with previous data [30], peptides containing Ser(P) were readily phosphorylated (Fig. 1A).

The discovery, through database searching, of a *Drosophila* homologue of eIF2Bε with a potential GSK-3 site (where the priming residue is a threonine) prompted us to investigate the efficiency of other residues in this position. To test whether Thr(P) could act to promote a GSK-3β-mediated phosphorylation, we synthesised the peptide EDDEDASRAVTPLPDD and two variants, 'Sp' and 'Tp', in which a Ser(P) or a Thr(P), respectively, was incorporated during synthesis (see Table 1). Phosphorylation of these peptides by GSK-3β was assessed over time (Fig. 1B). The peptide T, containing a non-phosphorylated threonine at position +4, was not phosphorylated significantly. In contrast, peptide TP was readily phosphorylated by recombinant GSK-3β (Fig. 1B), illustrating that Thr(P) can act as an efficient recognition determinant for GSK-3, at least in the case of this peptide. Three other peptides were also synthesised and tested for their ability to prime GSK-3β (shown in Table 1). These peptides, containing a Glu (potentially resembling Thr(P), peptide E in Table 1), an Asp (resembling Ser(P), peptide D in Table 1) or Tyr(P) (peptide Yp in Table 1) at the priming position, failed to undergo phosphorylation upon incubation with recombinant GSK-3β (Fig. 1B). Thus GSK-3β can recognise the motif Ser-Xaa-

Table 1
Synthetic peptides used in this study

Peptide name	Sequence
Peptides derived from *D. melanogaster* eIF2Bε (accession number AL021086)	
T	E D D E D A **S** R A V *T* P L P D D
Tp	E D D E D A **S** R A V T_P P L P D D
Sp	E D D E D A **S** R A V S_P P L P D D
E	E D D E D A **S** R A V *E* P L P D D
D	E D D E D A **S** R A V *D* P L P D D
Yp	E D D E D A **S** R A V Y_P P L P D D
Peptides derived from eIF2Bε (accession number U19516) ([6])	
SRAGS	R R A A E E L D **S** R A G *S* P Q L
SRAGS(P)	R R A A E E L D **S** R A G S_P P Q L

The GSK-3 phosphorylation site (rat) or putative GSK-3 (*D. melanogaster*) phosphorylation site is shown in bold and the priming site or variants introduced at that site are italicised. A $_P$ indicates a phosphorylated residue.

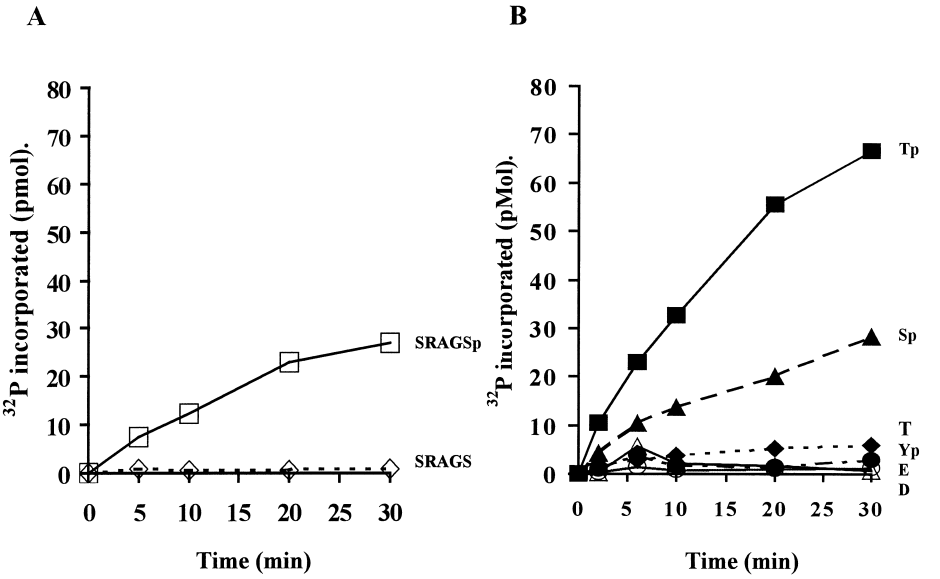

Fig. 1. Time courses of the phosphorylation of synthetic peptides based on (A) mammalian and (B) *Drosophila* eIF2Bε by mammalian GSK-3β. The peptides are designated as described in Table 1. Experimental conditions were as described in Section 2. The data are representative of at least three separate experiments.

Xaa-Xaa-Ser(P)/Thr(P), suggesting that there may be other substrates for this kinase containing Thr at the priming site.

The results in Fig. 1B suggest that GSK-3β phosphorylates a peptide with Thr(P) in the priming position at a higher rate than that seen with the corresponding peptide with Ser(P) in the same position. In order to compare directly the efficiencies of peptides Tp and Sp as substrates for GSK-3β, we carried out a detailed kinetic analysis using these two peptides. As shown in Table 2, the V_{max} of peptide Tp (768 pmol ^{32}P incorporated/min/μg) was higher than that of peptide Sp (249 pmol ^{32}P incorporated/min/μg), thus confirming that peptide substrate Tp is phosphorylated by GSK-3β at a higher rate. The 3-fold difference between peptides Tp and Sp is similar to that seen in Fig. 1B. The K_m values calculated for peptides Tp (40.0 μM) and Sp (39.2 μM) indicate that the affinity of GSK-3β for these substrates is essentially the same (Table 2). The kinetic values for peptide SRAGSp were also calculated as a control and were found to be similar to those reported previously [30]. Taken together, these results showed, in this case, that the peptide with a Thr(P) in the priming position is a more efficient substrate for GSK-3β than the peptide with a Ser(P) in the priming position. Further analysis will be required to determine whether or not this is true for other substrates.

These results clearly show that a Thr(P) in the critical position of the *Drosophila* eIF2Bε derived peptide, TP, is able to prime the substrate for the phosphorylation by recombinant mammalian GSK-3β. If *Drosophila* eIF2B is a physiological substrate for GSK-3 in fruit flies, Shaggy would have to be able to phosphorylate peptides containing Thr(P). However, the specificity determinants for Shaggy-mediated phosphorylation have not been tested before. To test this, His-tagged Shaggy was expressed in and purified from *Drosophila* Schneider cells. All five variants of the *Drosophila*-derived peptide were incubated with His-tagged Shaggy and their phosphorylation was assessed (Fig. 2). Here, we show for the first time, biochemically, that Shaggy requires a priming phosphorylation, four residues C-terminal of the target serine, and that this priming residue can be either a Ser(P) or Thr(P),

Table 2
Kinetic analysis for the phosphorylation of peptides Tp, Sp and SRAGSp by mammalian GSK-3β

Peptide	K_m (μM)	S.D. (+/−)	V_{max} (pmol/min/μg)	S.D. (+/−)	V_{max}/K_m
Tp	40.0	4.7	786	39.2	19.6
Sp	39.2	2.4	249	42.0	6.3
SRAGSp	95.3	40	206	5.6	2.1

Experimental conditions are described in Section 2 with the exception that the peptide concentrations were varied. Average values and S.D. from three or more experiments are shown.

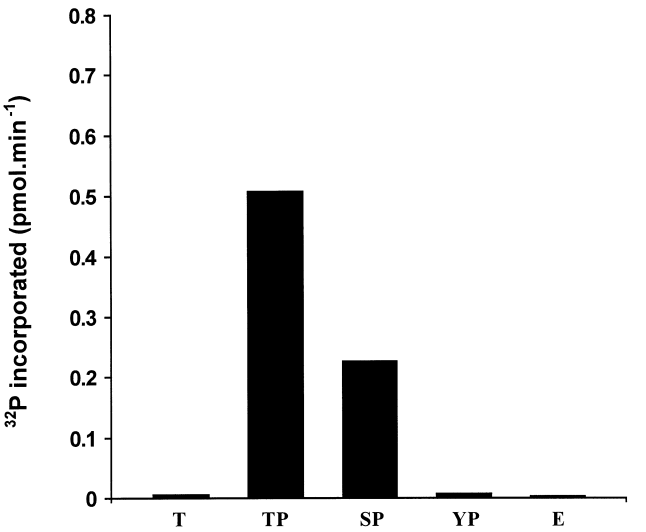

Fig. 2. Phosphorylation of synthetic peptides based upon *Drosophila* eIF2Bε by *Drosophila* His-tagged Shaggy. The peptides are designated as described in Table 1. The experimental conditions are described in Section 2. The data show an average of the results from two independent experiments.

Table 3
Specificity determinants for phosphate-directed kinases

Kinase	Priming position[a]	Specificity determinant at the priming position	Non-priming acidic residues	Reference
GEF-CK	n+2	Glu > Ser(P) > > Asp	Tyr(P), Thr(P)	[23]
CK2	n+3	Tyr(P) > Glu ≅ Asp ≅ Ser(P) > Thr(P)		[23]
CK1	n−3	Ser(P) > Thr(P) > > Asp > Glu	Tyr(P)	[23]
Syk	n−1	Thr(P) > Tyr(P) > Ser(P) > Glu		[34]
GSK-3β[b]	n+4	Thr(P) > Ser(P)	Tyr(P), Glu	This study
Shaggy[b]	n+4	Thr(P) > Ser(P)	Tyr(P), Glu	This study

[a]Relative to the phosphorylatable residue. In some cases (particularily Syk), this is not the only position where acidic/phosphorylated residues can act as specificity determinants.
[b]For this substrate.

as is the case with mammalian GSK-3β [6], indicating that *Drosophila* eIF2B is likely to be a physiological substrate for Shaggy. Interestingly, the same characteristics of phosphorylation observed for the recombinant GSK-3β with peptides Tp and Sp were seen with Shaggy. Again, the peptide with a Ser(P) in the priming position was phosphorylated less efficiently than that with a Thr(P) in the same position. As observed for mammalian GSK-3β, peptides T, E and Yp were not phosphorylated.

4. Discussion

Hierarchical phosphorylation, where phospho-acceptor sites are specified by the phosphorylation of other adjacent specific residues, has been described for a number of other protein kinases including the casein kinase from the Golgi apparatus of lactating mammary gland (GEF-CK), casein kinases-1 and -2 and the acidophilic protein tyrosine kinase Syk [23,34]. The order of effectiveness of different residues in the priming position for these kinases is shown in Table 3. As indicated, only the tyrosine-directed kinase Syk phosphorylates peptides containing a Thr(P) more efficiently than those with a Ser(P). Both GSK-3β and Shaggy also phosphorylate the peptide based on *D. melanogaster* eIF2Bε with a Thr(P) at a higher rate than when a Ser(P) is in the same position, however Tyr(P) and Glu are not recognised. This pattern differentiates GSK-3β and Shaggy from all the other phosphate-directed protein kinases studied to date.

An important unsolved question concerning the identity of the protein kinase responsible for priming both GSK-3β and Shaggy remains. The observation that the priming residue is followed by a proline suggests that members of the MAP kinase or cyclin-dependent kinase families may be responsible, since proline-directed kinases tend to phosphorylate Thr as readily as, if not better than, Ser [35].

In this study, we have shown phosphorylation of the probable *Drosophila* homologue of eIF2Bε by Shaggy in vitro, implying a possible role for Shaggy in the regulation of eIF2Bε in vivo. The involvement of Shaggy in at least two important developmental pathways, the Wingless [13] and Notch [14] signalling cascades, leads to speculation about the role of the translation initiation factor eIF2B in the *Drosophila* development through modulation of translation of specific transcripts. There is a well-established precedent for this in budding yeast (GCN4 [36,37]). In our future work, we aim to address whether the regulation of translation, by means of Shaggy-mediated phosphorylation of eIF2Be, is involved in development by studying the phosphorylation and control of *Drosophila* eIF2B.

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