A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of Axin and β-catenin

Gareth M. Thomas[a], Sheelagh Frame[a], Michel Goedert[c], Inke Nathke[b], Paul Polakis[d], Philip Cohen[a,*]

[a] *MRC Protein Phosphorylation Unit, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, UK*
[b] *Department of Anatomy and Physiology, MSI/WTB Complex, University of Dundee, Dow Street, Dundee DD1 5EH, UK*
[c] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*
[d] *Onyx Pharmaceuticals, 3031 Research Drive, Richmond, CA 94806, USA*

Received 12 August 1999

Abstract The Axin-dependent phosphorylation of β-catenin catalysed by glycogen synthase kinase-3 (GSK3) is inhibited during embryogenesis. This protects β-catenin against ubiquitin-dependent proteolysis, leading to its accumulation in the nucleus, where it controls the expression of genes important for development. Frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) is a mammalian homologue of a GSK3-binding protein (GBP), which appears to play a key role in the correct establishment of the dorsal-ventral axis in *Xenopus laevis*. Here, we demonstrate that FRATtide (a peptide corresponding to residues 188–226 of FRAT1) binds to GSK3 and prevents GSK3 from interacting with Axin. FRATtide also blocks the GSK3-catalysed phosphorylation of Axin and β-catenin, suggesting a potential mechanism by which GBP could trigger axis formation. In contrast, FRATtide does not suppress GSK3 activity towards other substrates, such as glycogen synthase and eIF2B, whose phosphorylation is independent of Axin but dependent on a 'priming' phosphorylation. This may explain how the essential cellular functions of GSK3 can continue, despite the suppression of β-catenin phosphorylation.

Key words: Axin; Glycogen synthase kinase-3; β-Catenin; FRAT1

1. Introduction

Glycogen synthase kinase-3 (GSK3) is a serine/threonine-specific protein kinase that is thought to phosphorylate many proteins in vivo and therefore to play key roles in the regulation of a variety of cellular processes [1]. For example, GSK3 lies in an insulin-stimulated, phosphatidylinositide (PI) 3-kinase-dependent pathway that leads to the stimulation of glycogen and protein synthesis in mammalian cells. In this pathway, GSK3 is inhibited by phosphorylation at a specific serine residue catalysed by protein kinase B (PKB). This prevents GSK3 from phosphorylating and inhibiting glycogen synthase [2,3] and protein synthesis initiation factor eIF2B [4], contributing to the insulin-induced stimulation of glycogen and protein synthesis. The insulin-induced PI 3-kinase-dependent inhibition of GSK3 also underlies the dephosphorylation of the microtubule-associated protein Tau in neurons [5].

The inhibition of GSK3 is also critical for the specification of cell fates during embryogenesis. For example, inhibition of GSK3 is essential for the patterning of segments during early embryogenesis in *Drosophila* and occurs in response to a signalling pathway initiated by the glycoprotein Wingless [6]. The inhibition of GSK3 in this situation is not mediated by PKB and appears to involve the phosphorylation of a distinct serine residue(s) [7]. A homologous pathway operates in *Xenopus laevis* ventral mesoderm development and anterior-posterior neural patterning in which Wingless homologues (termed Wnts) mediate the inhibition of GSK3 [8].

The inhibition of GSK3 during embryogenesis suppresses its ability to phosphorylate β-catenin, protecting β-catenin against ubiquitin-dependent proteolysis. This leads to the accumulation of β-catenin in the nucleus where it controls the expression of genes important for development [9]. The GSK3-catalysed phosphorylation of β-catenin depends on the presence of Axin, a protein that forms a complex with GSK3 and β-catenin [9–11]. Axin is also a substrate for GSK3 and the Wnt-induced dephosphorylation of Axin causes it to bind β-catenin with a reduced efficiency [12]. Thus, Wnt signaling lowers Axin's affinity for β-catenin, disengaging β-catenin from the degradation machinery.

In early *X. laevis* development, localised inhibition of GSK3 is required for correct establishment of the dorsal-ventral axis. However, no Wnts that might mediate this process have yet been identified, raising the possibility that a different mechanism is involved. Recently, a novel GSK3-binding protein (GBP) has been identified in *Xenopus* embryos [13] and zebrafish [14], which may function in vivo to stabilise β-catenin. Injection of GBP antisense RNA prevents correct axis formation, while injection of GBP mRNA into the ventral region of the embryo leads to the formation of a second axis and therefore produces a tadpole with two heads [13]. These observations are consistent with GBP functioning as an inhibitor of GSK3 in vivo, but whether GBP can act in the absence of Wnt signalling or whether it cooperates with as yet unidentified Wnts to achieve stabilisation of β-catenin is unclear. Moreover, the effects of GBP on GSK3 activity in vitro have not yet been reported.

GBP exhibits limited homology with a previously identified mammalian protein termed frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) [15], while another homologue, FRAT2 (Fig. 1), mimics the effect of GBP on secondary axis formation [13]. *FRAT1* is an oncogene upregulated during infection with Moloney murine leukaemia virus and FRAT1 expression confers a selective advantage to tumour cells that overexpress Myc and Pim1 [15]. The C-terminal half of GBP

*Corresponding author. Fax: (44) (1382) 223 778.
E-mail: pcohen@bad.dundee.ac.uk

PII: S0014-5793(99)01161-8

is sufficient to bind GSK3 and to mediate its effects on axis formation [13]. Here, we show that FRATtide, a 39 residue peptide from the C-terminus of FRAT1, binds to GSK3 and prevents the binding of Axin. FRATtide also blocks the GSK3-catalysed phosphorylation of Axin and β-catenin, suggesting a potential way in which GBP could control axis formation. Interestingly, FRATtide does not inhibit GSK3 activity towards substrates whose phosphorylation is independent of Axin and which possess a 'priming' phosphorylation. This selectivity may allow for many essential cellular functions of GSK3 to continue, despite the suppression of β-catenin phosphorylation.

2. Materials and methods

2.1. Materials

Glutathione-Sepharose and CNBr-activated Sepharose were purchased from Pharmacia and nickel-nitrilotriacetate-agarose from Qiagen (West Sussex, UK). All other reagents were from Sigma (Poole, UK) and E. Merck (Poole, UK).

2.2. Proteins

The peptide SQPETRTGDDDPHRLLQQLVLSGNLIKEAVRRLHSRRLQ (FRATtide, residues 188–226 of human FRAT1), the phosphopeptide YRRAAVPPSPSLSRHSSPHQSpEDEEE (GS-1, closely related to a sequence towards the C-terminus of glycogen synthase and phosphorylated at the most C-terminal serine) and the phosphopeptide RRAAEELDSRAGSpPQL (corresponding to two arginines followed by residues 534–547 of the ε subunit of eIF2B and phosphorylated at the most C-terminal serine) were synthesised by Dr. S. Bloomberg, University of Bristol, UK. The underlined residues indicate sites phosphorylated by GSK3. The α and β isoforms of GSK3 [16,17] and glycogen synthase [18,19] were purified from rabbit skeletal muscle. Affinity-purified His-tagged human GSK3β, obtained from Sf9 cells, was used in the Axin and β-catenin phosphorylation experiments. Glutathione *S*-transferase (GST) fused in frame to residues 281–500 of human Axin in the vector pGEX-3X (Pharmacia) was expressed in *Escherichia coli*. Bacteria were grown to the mid-log phase, induced at 30°C with isopropylthiogalactoside, lysed by sonication in Triton X-100-containing buffer and purified by chromatography on glutathione-Sepharose. After washing, the GST-Axin[281–500] was eluted with buffer containing 10 mM glutathione. Murine GST-β-catenin (a gift from Dr. A. Huber, Stanford University, CA, USA) and the 412 residue form of human Tau [20] were expressed in *E. coli* and purified. A polyclonal anti-peptide antibody that recognises GSK3α specifically was raised in sheep [21] and a monoclonal mouse antibody against the β isoform of GSK3 was purchased from Transduction Laboratories (Lexington, KY, USA). Horseradish peroxidase-linked secondary antibodies (sheep anti-mouse IgG and rabbit anti-sheep IgG) were from the Scottish Antibody Production Unit (Carluke, UK) and Pierce (Chester, UK), respectively.

2.3. Cell culture and transfection

Human embryonic kidney 293 cells were transfected as in [22] with 10 μg of pEBG-2T vector DNA expressing GST or GST-FRATtide. 24 h later, the cells were lysed in 1 ml of 20 mM Tris-acetate, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) β-mercaptoethanol, 1 mM benzamidine, 4 μg/ml leupeptin. Lysates were centrifuged at 4°C for 5 min at $13\,000\times g$ and the supernatant used for analysis.

2.4. Coupling of FRATtide to Sepharose

CNBr-activated Sepharose (1 ml settled volume) was coupled to 1 ml of 0.5 mM FRATtide in 0.1 M $NaHCO_3$, pH 8.0, 0.5 M NaCl according to the manufacturer's protocol, except that 0.5 M NaCl was included during coupling and initial washing steps. Finally, the beads were washed four times with 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.03% (m/v) Brij 35 (buffer A) and stored as a 50% slurry in buffer A until use.

2.5. Phosphorylation of Tau with MAP kinase

Tau (4 μM) was phosphorylated to 2.5 mol/mol by incubation for 24 h at 30°C in buffer A with or without 0.5 U/ml p42 MAP kinase, 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP. One unit of MAP kinase was that amount which catalysed the phosphorylation of 1 nmol myelin basic protein in 1 min [23].

2.6. Assay of GSK3

The assay (0.02 ml) was carried out in buffer A, containing GSK3 (2 mU), protein (2 μM) or peptide (20 μM) substrate, 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP. FRATtide was also present where indicated. After 10 min at 30°C, assays with protein substrates were stopped by addition of 5 μl of 10% (w/v) sodium dodecyl sulfate (SDS), heated for 5 min at 95°C and then electrophoresed on a 12% (w/v) SDS-polyacrylamide gel. Gels were autoradiographed to detect phosphorylated proteins and stained with Coomassie blue. Assays with peptides were terminated by spotting 16 μl of the reaction mix on to P81 phosphocellulose paper followed by immersion in 75 mM phosphoric acid. The P81 papers were washed and analysed by Cerenkov counting as described [24]. One unit of GSK3 was that amount which catalysed the incorporation of 1 nmol of phosphate into peptide GS-1 in 1 min. All assays were carried out at dilutions where substrate phosphorylation was linear with respect to time.

2.7. Disruption of the GSK3-Axin complex by FRATtide

Glutathione-Sepharose was washed three times with 10 volumes of ice-cold buffer A. 2 μg of GST-Axin[281-500] in 50 μl of buffer A was then added to 10 μl (settled volume) of the washed glutathione-Sepharose and the mixture was incubated for 30 min at 4°C on a shaking platform. The beads were briefly centrifuged, the supernatant removed and the beads then washed four times with 1 ml of buffer A containing 150 mM NaCl to remove any unbound GST-Axin[281-500]. GSK3 (50 ng) in 10 μl of buffer A plus 1 mg/ml bovine serum albumin (BSA) was incubated for 10 min at room temperature with 10 μl of water or 0.2 mM FRATtide in water and then added to the GST-Axin beads. After incubation for 1 h at 4°C on a shaking platform, the beads were centrifuged and the supernatant was removed and denatured in SDS. The beads were washed twice in buffer A containing 150 mM NaCl and then also denatured in SDS. The SDS-denatured proteins were electrophoresed on 10% (w/v) SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblotted using the anti-GSK3α antibody (1 μg/ml) or anti-GSK3β antibody (500 ng/ml) using the ECL system (Amersham International, UK).

2.8. GSK3-catalysed phosphorylation of Axin and β-catenin

His-tagged human GSK3β was incubated with various concentrations of FRATtide or water for 10 min at an ambient temperature in 0.01 ml of buffer A plus 1 mg/ml BSA. Assays (0.02 ml) were carried out as described above in buffer A containing 0.1 mg/ml BSA, 25 mU of GSK3β and 92 nM Axin and/or 1 μg of GST-β-catenin as indicated in the figure legends.

3. Results and discussion

The C-terminal half of GBP, which is sufficient to bind GSK3 and to mediate the effects of GBP on axis formation [13], contains only one region of about 30 residues that is strikingly similar to its mammalian homologues FRAT1 [15] and FRAT2 (Fig. 1). The importance of this region for the function of GBP is indicated by the finding that mutation of two residues that lie within it (Lys-139 and Glu-140) abolishes binding to GSK3 and axis-inducing activity [13]. We therefore expressed a 39 residue peptide corresponding to residues 188–226 of FRAT1 (FRATtide) as a GST fusion in 293 cells and, after affinity purification from the cell lysates on glutathione-Sepharose, tested for the presence of GSK3 by immunoblotting. These experiments demonstrated that GSK3α (Fig. 2A) and GSK3β (data not shown) were complexed to GST-FRATtide, but not to GST, and that the complex was stable to washing in buffer containing 0.5 M NaCl (Fig. 2A). Immunoblotting of the supernatants showed that $>90\%$ of the

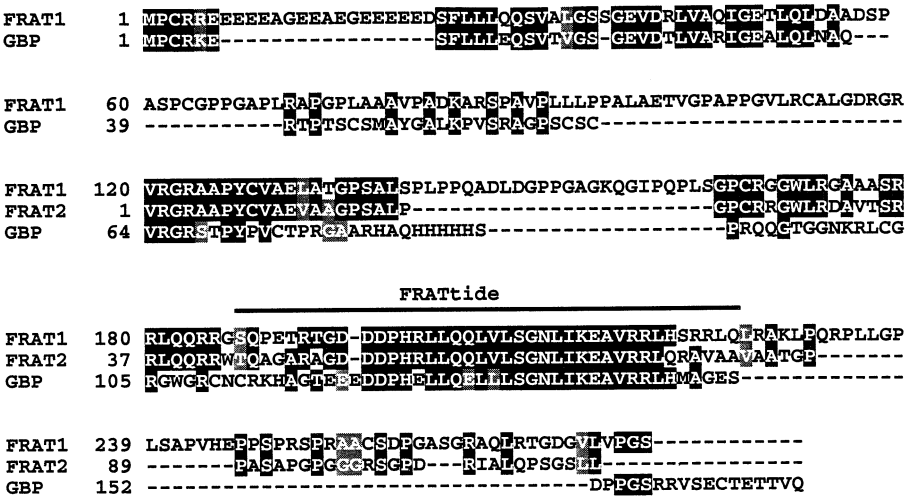

Fig. 1. Amino acid sequences of *Xenopus* GBP, human FRAT1 and human FRAT2. Identities are shaded in black and similarities in grey. The region near the C-terminus of FRAT1 that is strikingly conserved in GBP and FRAT2, termed FRATtide, is indicated.

GSK3α and 80% of the GSK3β had been depleted from the lysate by GST-FRATtide (data not shown). The affinity of FRATtide for GSK3 was also demonstrated by quantitative binding of purified GSK3α (Fig. 2B) or GSK3β (Fig. 2C) to FRATtide coupled to Sepharose. Minimal GSK3 was found associated with Sepharose beads lacking bound FRATtide (Fig. 2B,C).

FRATtide blocked the interaction of GSK3 with GST-Axin[281-500] (Fig. 3), suggesting that the binding sites on GSK3 for FRAT/GBP and Axin may overlap (despite the lack of sequence similarity between them). FRATtide also prevented the GSK3-catalysed phosphorylation of Axin[281-500] (Fig. 4A). Since the GSK3-catalysed phosphorylation of β-catenin requires the presence of Axin ([9–11] and see Fig. 4B), these findings implied that Axin-dependent phosphorylation of β-catenin should also be blocked by FRATtide. This was indeed found to be the case (Fig. 4B).

These observations may be relevant to GBP's ability to promote axis formation. Thus, displacement of Axin from GSK3 by GBP may mimic or facilitate the action of Wnts by helping to suppress the GSK3-catalysed phosphorylation of β-catenin. This would be expected to lead to stabilisation of β-catenin [9–11] and hence to regulation of the expression of genes essential for axis formation. In turn, the suppression of Axin phosphorylation should promote its degradation [25] and reduce its binding to β-catenin [12], thereby amplifying the effect of GBP to stabilise β-catenin.

In addition to its role in regulating embryogenesis, GSK3 participates in insulin signal transduction where its inhibition by PKB underlies some of the important metabolic effects of this hormone, such as the activation of glycogen synthase and eIF2B and dephosphorylation of Tau (Section 1). Indeed, the peptide substrate used to assay GSK3 routinely (GS-1) is closely related to the sequence surrounding the serine residues on glycogen synthase that are phosphorylated by GSK3. Interestingly, FRATtide did not affect the activity of GSK3 towards the peptide GS-1 (Fig. 5A, see also Fig. 2B,C) or the glycogen synthase protein itself (Fig. 5C). Consistent with these observations, the overexpression of GST-FRATtide in 293 cells did not cause any activation (dephosphorylation) of glycogen synthase, nor did it affect the ability of insulin-like

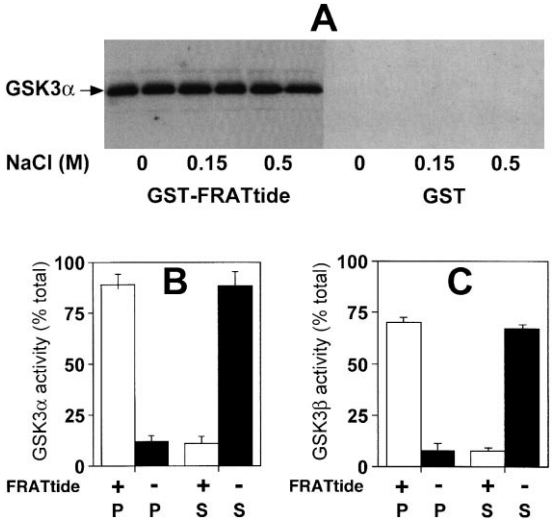

Fig. 2. FRATtide forms a complex with GSK3. (A) Human embryonic kidney 293 cells expressing either GST or GST-FRATtide were lysed and 0.5 mg of lysate protein was added to 10 μl of glutathione-Sepharose equilibrated in lysis buffer. After mixing for 90 min at 4°C, the beads were washed four times with lysis buffer plus the indicated concentrations of NaCl. Proteins attached to the beads were denatured in SDS, electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblotted using anti-GSK3α antibody (1 μg/ml). GST and GST-FRATtide were expressed to similar levels as judged by immunoblotting with anti-GST antibodies (not shown). (B and C) GSK3α (B) or GSK3β (C), both at 1 U/ml in buffer A plus 1 mg/ml BSA, were added to 10 μl (settled volume) of Sepharose-FRATtide (open bars) or Sepharose alone (closed bars). After incubation with shaking for 1 h at 4°C, the suspension was centrifuged briefly to pellet the beads and the supernatant removed and placed on ice. The beads were washed twice with buffer A plus 0.15 M NaCl and excess liquid was removed. The pellets (P) and supernatants (S) were then assayed for GSK3 activity using the GS-1 peptide. The data show the average of duplicate assays for a single experiment and are plotted relative to the activity of control incubations in which Sepharose and Sepharose-FRATtide were omitted and stored on ice. Similar results were obtained in three (B) or two (C) further analyses.

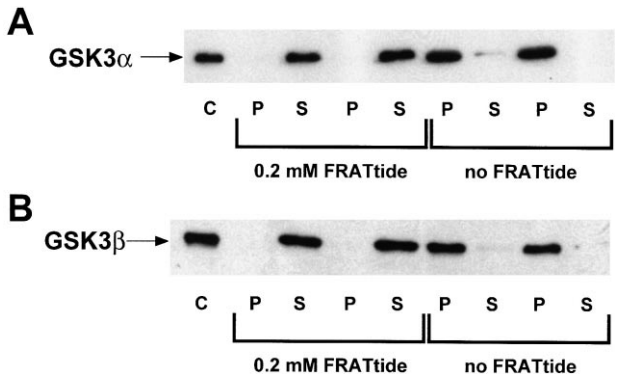

Fig. 3. FRATtide prevents binding of GSK3 to Axin. GSK3α or GSK3β (duplicate samples of each) were pre-incubated with or without 0.2 mM FRATtide, added to GST-Axin[281-500] that had been pre-coupled to glutathione-Sepharose and incubated for 60 min. GSK3 bound to the pelleted beads (P) and unbound material remaining in the supernatant (S) were analysed by immunoblotting. The control lane (C) shows a GSK3 standard.

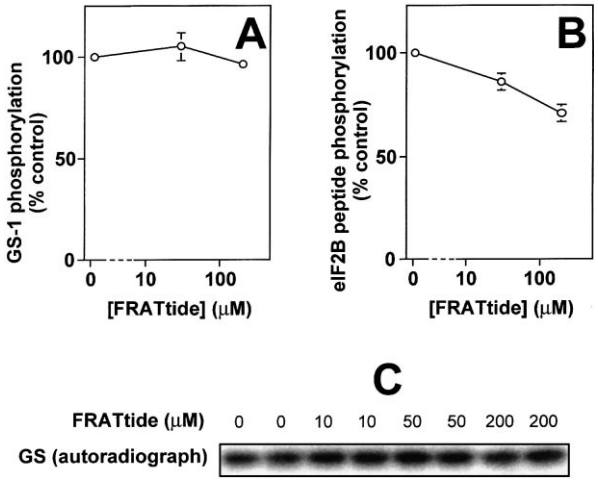

Fig. 5. Lack of an effect of FRATtide on GSK3 kinase activity towards three substrates. The phosphorylation of GS-1 (A), the eIF2B-derived peptide (B) and glycogen synthase (C) by GSK3 (0.1 U/ml) was assayed in the presence of the indicated concentrations of FRATtide. GSK3 was incubated with FRATtide for 10 min before the addition of substrate and Mg[γ-^{32}P]ATP. In C, the reaction was terminated by addition of 0.2 volumes of 10% (w/v) SDS and, after electrophoresis on a 12% SDS-polyacrylamide gel, the gel was autoradiographed.

growth factor-1 to activate glycogen synthase in these cells (data not shown). FRATtide also had little effect on GSK3 activity towards a synthetic peptide corresponding to the sequence surrounding the phosphorylation site on eIF2B (Fig. 5B).

In contrast to the GS-1 peptide, glycogen synthase and the eIF2B peptide, the GSK3-catalysed phosphorylation of Tau was completely inhibited by FRATtide (closed circles in Fig. 6). Axin was not present in these assays, implying that the inhibition of Tau phosphorylation by FRATtide was not occurring by the mechanism invoked for inhibition of β-catenin phosphorylation (i.e. displacement of Axin from GSK3). A second/alternative mechanism of inhibition was also suggested by the observation that β-catenin phosphorylation is blocked by slightly lower concentrations of FRATtide than Axin phosphorylation and that FRATtide reduced the level of β-catenin phosphorylation below that observed in the absence of Axin (Fig. 4B). This should not be the case if dissociation of Axin from GSK3 was the sole mechanism by which FRATtide inhibits the phosphorylation of Axin and β-catenin.

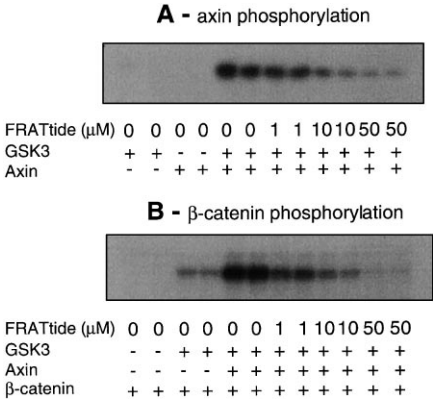

Fig. 4. FRATtide prevents the GSK3-catalysed phosphorylation of Axin and β-catenin. The phosphorylation of Axin[281-500] (92 nM) (A) and β-catenin (430 nM) (B) by His-tagged human GSK3β (1.0 U/ml, 94 nM) was assayed in the presence of the indicated concentrations of FRATtide. GSK3 was incubated with FRATtide for 10 min before the addition of substrate and Mg[γ-^{32}P]ATP. The reactions were terminated after 20 min at 30°C by addition of 0.2 volumes of 8% (w/v) SDS and then incubated with nickel-nitrilotriacetate-agarose to remove the GSK3β. The supernatant was removed and electrophoresed on an 8% SDS-polyacrylamide gel, followed by autoradiography.

GSK3 preferentially phosphorylates proteins and peptides at serine or threonine residues that are followed by another phosphoserine, frequently located four residues C-terminal to the GSK3 site [26,27]. Indeed, glycogen synthase [27], the GS-1 peptide [28] and the eIF2B-derived peptide [29] are not phosphorylated by GSK3, unless such a 'priming' phosphorylation is present (see Section 2). The proteins whose phosphorylation by GSK3 was unaffected by FRATtide (Fig. 5) all contained a 'priming phosphorylation' and were phosphorylated at high rates in vitro, while those proteins whose phosphorylation was prevented by FRATtide (Axin, β-catenin and Tau) were expressed in *E. coli*, did not possess a priming phosphorylation and were phosphorylated by GSK3 in vitro at rates 100–1000-fold slower than the peptide GS-1. We therefore wondered whether resistance to inhibition by FRATtide was conferred by a priming phosphorylation. Indeed, prior phosphorylation of Tau with p42 MAP kinase, which phosphorylates Ser-235 (and other residues) and allows GSK3 to phosphorylate Thr-231 [30], increased the rate of Tau phosphorylation about 50-fold and abolished inhibition by FRATtide (Fig. 6).

There is currently no evidence to support the idea that a 'priming' phosphorylation is a prerequisite for the GSK3-catalysed phosphorylation of Axin or β-catenin in vivo. High levels of Axin and β-catenin phosphorylation may be attained in vivo because their rates of dephosphorylation in the GSK3-Axin-β-catenin ternary complex are very slow. This is consistent with the observation that maximal dephosphorylation of Axin and accumulation of β-catenin only occurs after stimulation with Wnt3A for several hours [12]. In contrast, the insulin-induced dephosphorylation of glycogen synthase oc-

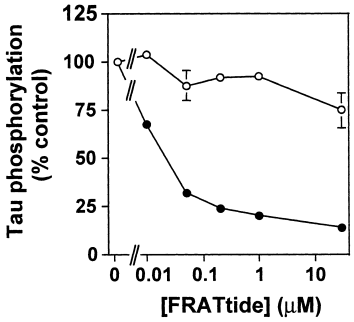

Fig. 6. The ability of FRATtide to prevent the GSK3-catalysed phosphorylation of Tau depends on phosphorylation at a 'priming' site. Tau that had been pre-phosphorylated with p42 MAP kinase (open circles) or not pre-phosphorylated (closed circles) was incubated for 10 min using GSK3 (0.1 U/ml) and Mg[γ-^{32}P]ATP in the presence of the indicated concentrations of FRATtide. Results are plotted relative to the rate of phosphorylation in the absence of FRATtide. No phosphorylation of Tau occurred in the absence of added GSK3.

curs with a half-time of less than 5 min and is maximal after 15 min [31].

The peptide GS-1 did not prevent FRATtide from forming a complex with GSK3 (data not shown), indicating that the failure of FRATtide to inhibit phosphorylation of this substrate was not explained by the inability of FRATtide to bind to GSK3 in the presence of this or other 'primed' substrates. However, FRATtide might prevent 'unprimed' substrates from interacting with GSK3 and 'primed' substrates might be resistant to inhibition by FRATtide because they interact with GSK3 much more strongly. Whatever the explanation, the inability of FRATtide to prevent the GSK3-catalysed phosphorylation of several of its physiological substrates is likely to be significant, allowing many of the essential functions of GSK3 to proceed normally despite the suppression of β-catenin and Axin phosphorylation.

Acknowledgements: We thank Chris Proud for eIF2B peptides, Andrew Paterson and Giselle Wiggin for GSK3 from Sfg cells and Nick Helps and Stefan Hoppler for valuable discussions. This work was supported by the UK Medical Research Council, British Diabetic Association, Royal Society and Louis Jeantet Foundation (to P.C.).

References

[1] Welsh, G.I., Wilson, C. and Proud, C.G. (1996) Trends Cell. Biol. 6, 274–279.
[2] Cross, D.E.A., Alessi, D.R., Cohen, P., Andjelkovich, M. and Hemmings, B.A. (1995) Nature 378, 785–789.
[3] Cohen, P. (1999) Phil. Trans. R. Soc. Lond. B354, 485–495.
[4] Welsh, G.I., Miller, C.M., Loughlin, A.J., Price, N.T. and Proud, C.G. (1998) FEBS Lett. 421, 125–130.
[5] Hong, M. and Lee, V.M. (1997) J. Biol. Chem. 272, 19547–19553.
[6] Perrimon, N. (1994) Cell 76, 781–784.
[7] Ruel, L., Stambolic, V., Ali, A., Manoukian, A.S. and Woodgett, J.R. (1999) J. Biol. Chem. 274, 21790–21796.
[8] Moon, R.T., Brown, J.D. and Torres, M. (1997) Trends Genet. 13, 157–162.
[9] Ben-Ze'ev, A. and Geiger, B., Curr. Opin. Cell Biol. 10, pp. 629–639.
[10] Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998) EMBO J. 17, 1371–1384.
[11] Hart, M.J., de los Santos, R., Albert, I.N., Rubinfeld, B. and Polakis, P. (1998) Curr. Biol. 8, 573–581.
[12] Willert, K., Shibamoto, S. and Nusse, R. (1999) Genes Dev. 13, 1768–1773.
[13] Yost, C., Farr, G.H., Pierce, S.B., Ferkey, D.M., Chen, M.M. and Kimelman, D. (1998) Cell 93, 1031–1041.
[14] Sumoy, L., Kiefer, J. and Kimelman, D. (1999) Dev. Genes Evol. 209, 48–58.
[15] Jonkers, J., Korswagen, H.C., Acton, D., Breuer, M. and Berns, A., EMBO J. 16, pp. 441–450.
[16] Sutherland, C., Leighton, I.A. and Cohen, P. (1996) Biochem. J. 296, 15–19.
[17] Sutherland, C. and Cohen, P. (1994) FEBS Lett. 338, 37–42.
[18] Nimmo, H.G., Proud, C.G. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[19] Pitcher, J., Smythe, C., Campbell, D.G. and Cohen, P. (1987) Eur. J. Biochem. 169, 497–502.
[20] Goedert, M. and Jakes, R. (1990) EMBO J. 9, 4225–4230.
[21] Cross, D.A.E., Alessi, D.R., Vandenheede, J.R., McDowell, H.E., Hundal, H.S. and Cohen, P. (1994) Biochem. J. 303, 21–30.
[22] Webster, G. and Perkins, N.D. (1999) Mol. Cell. Biol. 19, 3485–3495.
[23] Alessi, D.R., Cohen, P., Ashworth, A., Cowley, S., Leevers, S.J. and Marshall, C.J. (1995) Methods Enzymol. 255, 279–290.
[24] Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C. and Cohen, P. (1992) EMBO J. 11, 3985–3994.
[25] Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S. and Kikuchi, A. (1999) J. Biol. Chem. 274, 10681–10684.
[26] Picton, C., Woodgett, J., Hemmings, B. and Cohen, P. (1982) FEBS Lett. 150, 191–196.
[27] Wang, Y. and Roach, P.J. (1993) J. Biol. Chem. 268, 23876–23880.
[28] Fiol, C.J., Wang, A., Roeske, R.W. and Roach, P.J. (1990) J. Biol. Chem. 265, 6061–6065.
[29] Welsh, G.I., Patel, J.C. and Proud, C.G. (1997) Anal. Biochem. 244, 16–21.
[30] Goedert, M., Jakes, R., Crowther, R.A., Cohen, P., Vanmechelen, E., Vandermeeren, M. and Cras, P. (1994) Biochem. J. 301, 871–877.
[31] Cross, D.A.E., Watt, P.W., Shaw, M., van der kaay, J., Downes, C.P., Holder, J.C. and Cohen, P. (1997) FEBS Lett. 406, 211–215.