The kinase DYRK phosphorylates protein-synthesis initiation factor eIF2Bε at Ser^{539} and the microtubule-associated protein tau at Thr^{212}: potential role for DYRK as a glycogen synthase kinase 3-priming kinase

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The substrate specificity of glycogen synthase kinase 3 (GSK3) is unusual in that efficient phosphorylation only occurs if another phosphoserine or phosphothreonine residue is already present four residues C-terminal to the site of GSK3 phosphorylation. One such substrate is the ϵ-subunit of rat eukaryotic protein-synthesis initiation factor 2B (eIF2Bϵ), which is inhibited by the GSK3-catalysed phosphorylation of Ser^{535}. There is evidence that GSK3 is only able to phosphorylate eIF2Bϵ at Ser^{535} if Ser^{539} is already phosphorylated by another protein kinase. However, no protein kinases capable of phosphorylating Ser^{539} have so far been identified. Here we show that Ser^{539} of eIF2Bϵ, which is followed by proline, is phosphorylated specifically by two isoforms of **d**ual-specificity t**y**rosine phosphorylated and **r**egulated **k**inase (DYRK2 and DYRK1A), but only weakly or not at all by other 'proline-directed' protein kinases tested. We also establish that phosphorylation of Ser^{539} permits GSK3 to phosphorylate Ser^{535} *in vitro* and that eIF2Bϵ is highly phosphorylated at Ser^{539} *in vivo*. The DYRK isoforms also phosphorylate human microtubule-associated protein tau at Thr^{212} *in vitro*, a residue that is phosphorylated in foetal tau and hyperphosphorylated in filamentous tau from Alzheimer's-disease brain. Phosphorylation of Thr^{212} primes tau for phosphorylation by GSK3 at Ser^{208} *in vitro*, suggesting a more general role for DYRK isoforms in priming phosphorylation of GSK3 substrates.

Key words: Alzheimer's disease, Down's syndrome, insulin action, protein phosphorylation, signal transduction.

INTRODUCTION

Eukaryotic initiation factor 2B (eIF2B) catalyses an important regulatory step in the initiation of mRNA translation, i.e. the exchange of guanine nucleotides on eIF2. When bound to GTP, eIF2 can interact with the initiator methionyl-tRNA (Met-tRNA$_i$) and recruit it to the 40 S ribosomal subunit, where it participates in recognition of the AUG start codon [1]. Following hydrolysis of the GTP to GDP and P_i, eIF2 is released from the ribosome as an inactive [eIF2·GDP] complex. The rate of release of GDP is very slow under physiological conditions, and eIF2B accelerates this, allowing eIF2 to reassociate with GTP and to participate in another round of translation initiation. This is a key regulatory step in translation initiation in eukaryotes from yeast to mammals [2].

The activity of eIF2B is regulated in response to a wide variety of conditions. Stressful conditions inhibit it, while stimuli that activate protein synthesis increase the activity of eIF2B. A number of stresses activate protein kinases that phosphorylate the α-subunit of eIF2, converting it from a substrate for eIF2B into a potent competitive inhibitor [3]. Under many conditions where protein synthesis is activated, there is, however, no change in the phosphorylation of eIF2α. Such conditions include treatment of cells with insulin, phorbol esters and growth factors [4–6], the exposure of pancreatic β-cells to high levels of glucose [7] and mitogenic activation of primary T-lymphocytes [8]. The activation of eIF2B in such situations appears to be due to direct inputs into eIF2B itself. One such input is the phosphorylation of eIF2B by glycogen synthase kinase-3 (GSK3). This occurs at a single conserved site (Ser^{535} in the rat enzyme) in the catalytic ϵ-subunit of eIF2B and leads to inhibition of its guanine-nucleotide-exchange activity [9]. GSK3 is inactivated in response to insulin, phorbol esters and growth factors and this is associated with dephosphorylation of the GSK3 site on eIF2Bϵ and the activation of this exchange factor [9]. These events therefore provide a mechanism by which such stimuli enhance the regeneration of active [eIF2·GTP] and thus stimulate the attachment of Met-tRNA$_i$ to the 40 S subunit.

For many substrates, phosphorylation by GSK3 requires prior phosphorylation at a 'priming' site at position +4 relative to the GSK3 site [10]. All known mammalian eIF2Bϵ sequences do indeed contain such a serine residue (Ser^{539} in rat eIF2Bϵ), and studies using synthetic peptides based on this sequence have shown that phosphorylation of Ser^{539} is essential for phosphorylation of Ser^{535} by GSK3 [9]. However, it was previously unclear (i) whether this was also true of eIF2Bϵ itself, (ii) whether Ser^{539}

Abbreviations used: DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; eIF, eukaryotic protein-synthesis initiation factor; ERK, extracellular-signal-regulated protein kinase; FKHR, forkhead in rhabdosarcoma; GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; His-tagged, hexahistidine-tagged; JNK, c-Jun N-terminal kinase; Met-tRNA$_i$, initiator methionyl-tRNA; Ni-NTA, Ni^{2+}-nitrilotriacetate; SAPK, stress-activated protein kinase; *Sf*9 cells, *Spodoptera frugiperda* cells; Woodtide, the synthetic peptide KKISGRLSPIMTEQ.

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was phosphorylated *in vivo* and (iii) which kinase(s) could act at this site. Here we address these issues and, in particular, show that members of the DYRK (**d**ual-specificity t**y**rosine phosphorylated and **r**egulated **k**inase) group of protein kinases can phosphorylate Ser^{539} of eIF2Bϵ specifically and thus prime phosphorylation of Ser^{535} by GSK3 *in vitro*. DYRK isoforms may play a more general role in priming phosphorylation of GSK3 substrates. Thus we show here that they phosphorylate the microtubule-associated protein tau at Thr^{212} *in vitro*, thereby priming it for phosphorylation by GSK3 at Ser^{208}.

MATERIALS AND METHODS

Materials

Peptides were synthesized by Dr G. Bloomberg (Department of Biochemistry, University of Bristol, Bristol, U.K.) and sequencing-grade trypsin, Asp-N and Lys-C proteinases were purchased from Roche Diagnostics (Mannheim, Germany). Sequelon arylamine membranes were from Milligen (Bedford, MA, U.S.A).

Proteins

Vectors encoding GST–DYRK1A[1–500] and GST–DYRK2 were expressed in *Escherichia coli* [11] and purified on GSH–Sepharose by Dr Zsuzsanna Gupa [Division of Signal Transduction Therapy (DSTT), University of Dundee, Dundee, Scotland U.K.]. The enzymes were dialysed against 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1 % 2-mercaptoethanol/50 % (v/v) glycerol and stored at 1.5 mg/ml at −20 °C. A hexahistidine (His)-tagged GSK3β expressed in insect (*Spodoptera frugiperda*) *Sf*21 cells, purified on Ni^{2+}-nitrilotriacetate (Ni-NTA)–agarose (Qiagen) and chromatographed on S-Sepharose to remove associated DYKR1A activity (which also binds to Ni-NTA-agarose by virtue of a natural His-tag in the sequence) ([12] and the accompanying paper [13]), was provided by Dr A. Paterson (DSTT). A cDNA encoding rat GST–eIF2Bϵ was provided by Dr Fiona Paulin (School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.), expressed in *E. coli* and purified on GSH–Sepharose, as for the DYRK isoforms. The GST tag was then cleaved by incubation with 20 μg/ml thrombin (Sigma) for 20 min at 30 °C, and the thrombin removed using benzamidine–agarose (Sigma). The 412-amino-acid isoform of human brain tau and its $Thr^{212} \rightarrow$ Ala mutant were expressed in *E. coli* from cDNA clone htau 46 and purified as described in [14]. The amino acid numbering corresponds to that of the 441-residue isoform of human brain tau [15].

Protein kinase assays

Phosphorylation reactions were carried out at 30 °C in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA, using 30 μM YRRAAVPPSPSLSRHSSPHQSpEDEEE for GSK3, 50 μM KKISGRLSPIMTEQ (Woodtide) for DYRK, 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (10^6 c.p.m./nmol). The standard substrate peptides were replaced by eIF2Bϵ or tau where indicated. One unit of protein kinase activity was that amount which catalysed the phosphorylation of 1 nmol of the standard synthetic peptide substrate in 1 min.

Digestion of ^{32}P-labelled proteins

^{32}P-labelled eIF2Bϵ or tau were denatured in 62.5 mM Tris (pH 6.8)/2 % (w/v) SDS/10 % (v/v) glycerol/0.01 % Bromophenol Blue/1 % (v/v) 2-mercaptoethanol and subjected to SDS/PAGE. The radioactive bands were excised and washed extensively with 50 % (v/v) acetonitrile/0.1 mM NH_4HCO_3, dried, and then digested for 18 h at 30 °C with 1 μg/ml of the appropriate proteinase in 50 mM ammonium bicarbonate containing 0.1 % (v/v) n-octyl glucoside. The peptides obtained were analysed on a Vydac (Hesperia, CA, U.S.A.) C_{18} column.

MS

Phosphopeptides were analysed on a Perseptive Biosystems (Framingham, MA, U.S.A.) Elite STR matrix-assisted laser-desorption–ionization time-of-flight ('MALDI-TOF') mass spectrometer with saturated α-cyanocinnamic acid as the matrix. The mass spectrum was acquired in the reflector mode and was internally mass-calibrated. The peptide ions were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector to identify their position in the eIF2Bϵ or tau sequence.

Phospho-specific antibodies and immunoblotting

Incubations were carried out for 1 h at ambient temperature in 5 % (w/v) non-fat dried milk in 50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 % Tween 20, unless otherwise indicated. The antibody that recognizes eIF2Bϵ phosphorylated at Ser^{535} (equivalent to Ser^{540} in rabbit eIF2Bϵ) was raised against a synthetic phosphopeptide as described previously [9]. An antibody specific for eIF2Bϵ phosphorylated at Ser^{539} of the rat protein was similarly raised against the peptide SRAG^{p}SPQSDC (where ^{p}S indicates phosphoserine). The specificity of the antibody was tested against the panel of peptides described in [9] and it was found to react only with peptides containing phosphate on the position corresponding to Ser^{539} (results not shown). An antibody specific for pThr^{212} in tau was purchased from Biosource (Calmarillo, CA, U.S.A.) and used at a 1:5000 dilution. The phosphorylation-independent anti-tau antibody BR134 was used at 1:2500 dilution. Tau protein was extracted from newborn- and adult-rat brain and immunoblotted as described in [16].

RESULTS

Phosphorylation of Ser^{539} in eIF2Bϵ is required for phosphorylation of Ser^{535} by GSK3

We have shown previously that GSK3 phosphorylates the residue equivalent to Ser^{535} in rat eIF2Bϵ [9]. On the basis of results obtained for other substrates for GSK3 [10] and on the use of synthetic peptides corresponding to the region including Ser^{535} of eIF2Bϵ, we anticipated that phosphorylation of the serine residue at position 539 would be important for the phosphorylation of Ser^{535} by GSK3. Since Ser^{539} of eIF2Bϵ is followed by a proline residue, it must be phosphorylated *in vivo* by a 'proline-directed' protein kinase. However, none of the proline-directed protein kinases we tested initially, such as cyclin B-cdk, mitogen-activated protein kinase family members [extracellular-signal-regulated protein kinase 2 (ERK2), stress-activated protein kinase 1 (SAPK1)/c-Jun N-terminal kinase (JNK), SAPK2a/p38, SAPK2b/p38β2, SAPK3/p38γ, SAPK4/p38δ] or kinase interacting with stathmin ('KIS') [17], phosphorylated bacterially expressed eIF2Bϵ at significant rates *in vitro*. In order to be able to phosphorylate Ser^{539} *in vitro* to test its possible role in priming the phosphorylation of Ser^{535} by GSK3, we mutated Ala^{537} to proline in the expectation that the creation of a Pro-Xaa-Ser-Pro sequence would render Ser^{539} a better substrate for ERK2. Indeed, whereas wild-type eIF2Bϵ was only phosphorylated very weakly at Ser^{539} by ERK2, eIF2Bϵ[A537P] was phosphorylated

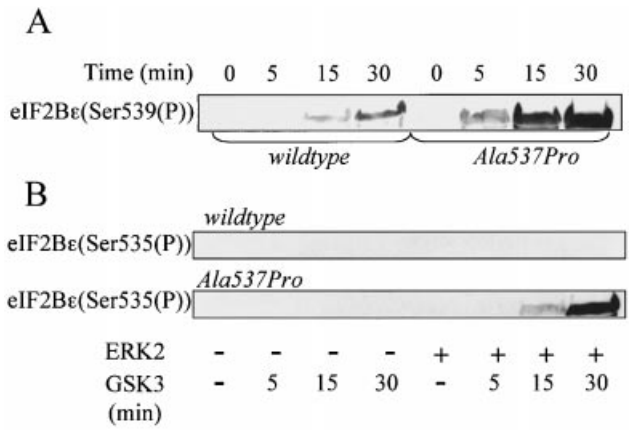

Figure 1 Phosphorylation of the mutant eIF2Bε[A537P] by ERK2 primes phosphorylation of Ser^{535} by GSK3

(**A**) Wild-type eIF2Bε or eIF2Bε[A537P] were expressed as GST-fusion proteins in *E. coli* and purified. Each protein (1 μg) was incubated *in vitro* with unlabelled MgATP and ERK2 (1 unit/ml) in a total volume of 0.05 ml. At the times indicated, samples (15 μl) were removed, subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with an antibody that recognizes eIF2Bε only when it is phosphorylated at Ser^{539}. Immunoblots were revealed by enhanced chemiluminescence (ECL®, Amersham). (**B**) Wild-type eIF2Bε or eIF2Bε[A537P] were incubated with or without MgATP and ERK2 as in (**A**). After 30 min, GSK3 was added to a final activity of 1 unit/ml. Samples were withdrawn at the times indicated and analysed as in (**A**), except that immunoblotting was performed with an antibody that recognizes eIF2Bε phosphorylated at Ser^{535}.

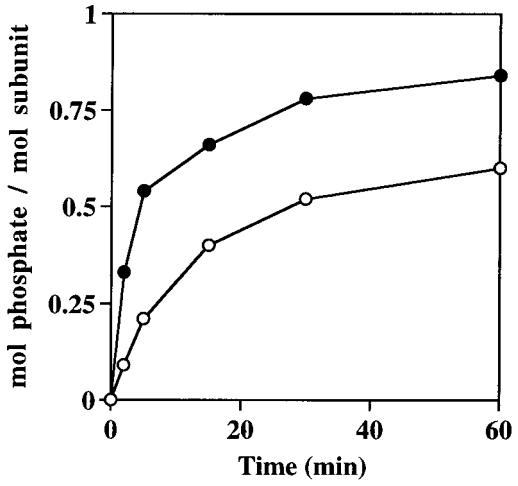

Figure 2 Phosphorylation of eIF2B by DYRK isoforms

eIF2Bε (1 μM) was phosphorylated for the times indicated by incubation at 30 °C with GST–DYRK1A[1-500] (○) or GST-DYRK2 (●), each at 1 unit/ml. The reactions were stopped by precipitation in 20% (w/v) trichloroacetic acid. The samples were centrifuged at 13000 ***g*** for 10 min at 4 °C and the supernatants discarded. The pellets were washed four times with ice-cold 10% trichloroacetic acid, before being subjected to Cerenkov counting.

more rapidly at this site (Figure 1A). GSK3 barely phosphorylated either wild-type eIF2Bε or eIF2Bε[A537P] but, following phosphorylation by ERK2, eIF2Bε[A537P] was readily phosphorylated by GSK3 at Ser^{535} (Figure 1B). These data demonstrate that the phosphorylation of Ser^{539} greatly facilitates the phosphorylation of Ser^{535} by GSK3. Given that none of the proline-directed kinases tested could phosphorylate wild-type eIF2Bε on Ser^{539} at significant rates, the results suggested that another 'proline-directed' protein kinase must be responsible for the phosphorylation of this site.

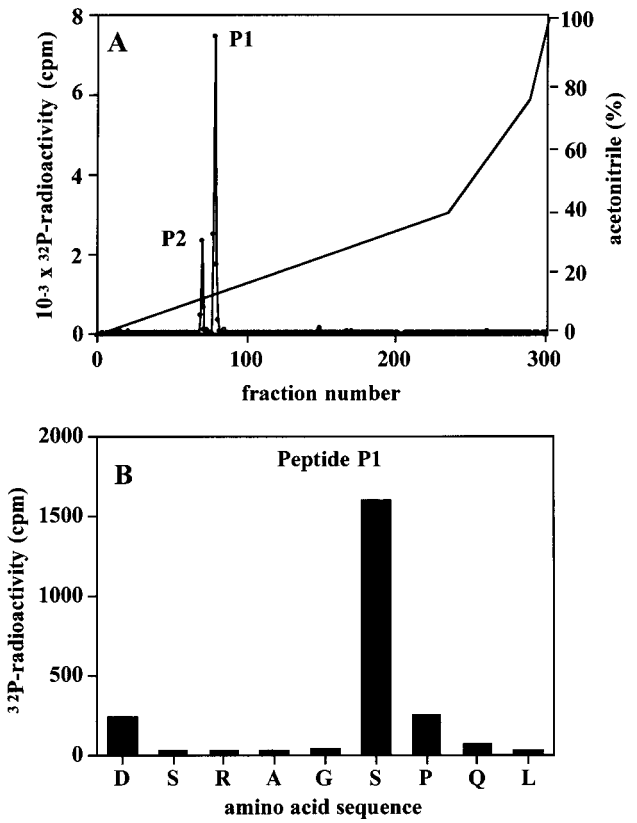

Figure 3 Identification of the residue on eIF2Bε phosphorylated by DYRK2

(**A**) eIF2Bε (1 μM) was maximally phosphorylated by incubation for 30 min and 30 °C with GST–DYRK2 (1 unit/ml) and digested with Asp-N proteinase. The digest was applied to a Vydac C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with an acetonitrile gradient (diagonal line) at a flow rate of 1 ml/min. Fractions (0.5 ml each) were collected and analysed for ^{32}P radioactivity by Cerenkov counting. Two major ^{32}P-labelled peptides, P1 and P2, accounted for 85% of the ^{32}P radioactivity applied to the column. (**B**) Phosphopeptide P1 from (**A**) was sequenced by Edman degradation using an Applied Biosystems 429A protein sequencer. ^{32}P radioactivity released after each cycle was measured in a separate experiment by solid-phase Edman degradation of the peptide coupled to a Sequelon arylamine membrane [30].

Identification of DYRK isoforms as Ser^{539} kinases

Recently, we identified Ser^{329} as an *in vivo* phosphorylation site of the transcription factor FKHR (forkhead in rhabdosarcoma) and found that DYRK1A was able to phosphorylate this site specifically *in vitro* (see the accompanying paper [13]). As Ser^{329} is followed by a proline residue, and DYRK1A shows preference for serine or threonine residues that lie in Ser-Pro or Thr-Pro sequences [18], we tested whether DYRK isoforms were capable of phosphorylating eIF2Bε at Ser^{539}.

DYRK2, a cytoplasmic DYRK isoform showing 45% identity (61% similarity) to the catalytic domain of DYRK1A, phosphorylated bacterially expressed eIF2Bε *in vitro*, the phosphorylation approaching a plateau near 1 mol/mol (Figure 2). DYRK1A, the nuclear isoform, also phosphorylated eIF2Bε, although a little less efficiently (Figure 2). Following phosphorylation by DYRK2, digestion with Asp-N proteinase followed by chromatography on a Vydac C_{18} column showed one major (P1) and one minor (P2) phosphopeptide (Figure 3A),

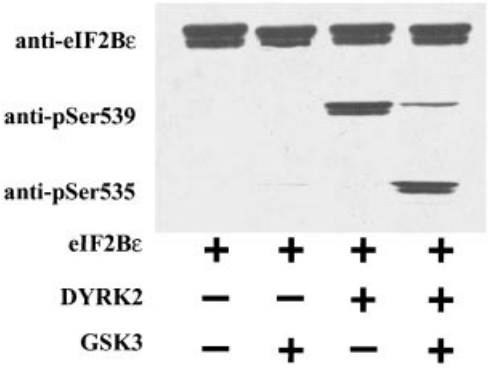

Figure 4 Phosphorylation of eIF2Bε at Ser^{539} by DYRK2 'primes' the subsequent phosphorylation of Ser^{535} by GSK3

Bacterially expressed eIF2Bϵ (1 μM) was either left unphosphorylated or phosphorylated for 30 min with GST–DYRK2 as described in Figure 1, but using unlabelled ATP. The solutions were then made 1 unit/ml in GSK3 and phosphorylation continued for a further 30 min. Aliquots of each reaction mixture (50 μl) were subjected to SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted with two different phospho-specific antibodies that recognize eIF2Bϵ when it is phosphorylated at Ser^{539} or Ser^{535} (anti-pSer539, anti-pSer535) respectively or with an antibody that recognizes the unphosphorylated and phosphorylated forms of eIF2Bϵ equally well (anti-eIF2Bϵ).

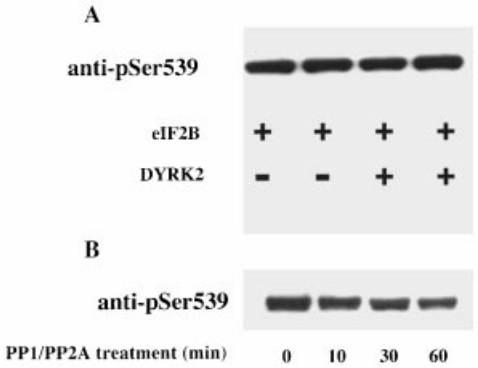

Figure 5 Phosphorylation of Ser^{539} in the eIF2B complex

(**A**) The heteropentameric eIF2B complex (2 μg) purified from HeLa cells was incubated for 30 min with MgATP in the absence or presence of 1 unit/ml of DYRK2. At this point, the incubations were terminated with SDS, electrophoresed on a 4–12%-polyacrylamide-gradient gel (Novex), transferred to a nitrocellulose membrane and immunoblotted with the anti-PSer^{539} antibody. (**B**) The purified eIF2B complex (10 μg) was incubated at 30 °C in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1% (v/v)/2-mercaptoethanol/10 units/ml protein phosphatase 2A/10 units/ml protein phosphatase 1γ/2 mM $MnCl_2$ in a total reaction volume of 50 μl. Aliquots of the reaction mixture (10 μl) were withdrawn at the times indicated and terminated by the addition of microcystin to a final concentration of 1 μM. The samples were denatured in SDS, subjected to SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with the anti-pSer^{539} antibody.

which were identified by Edman sequencing and MS. Peptide P1 comprised residues 534–542 and Peptide P2 comprised residues 534–543, the latter being generated by incomplete cleavage of the Asp–Asp bond between residues 542 and 543. Peptides P1 (Figure 3B) and P2 (results not shown) were both phosphorylated at Ser^{539}. DYRK1A also phosphorylated eIF2Bϵ specifically at Ser^{539} (results not shown).

Phosphorylation of Ser^{539} by DYRK primes phosphorylation of Ser^{535} by GSK3

The identification of DYRK isoforms as specific Ser^{539} kinases *in vitro* allowed us to investigate whether the phosphorylation of this residue is required for the phosphorylation of wild-type eIF2Bϵ at Ser^{535} by GSK3 *in vitro*. This experiment was performed using two phospho-specific antibodies that recognize eIF2Bϵ only when it is phosphorylated at either Ser^{535} [9] or Ser^{539}. These studies confirmed that DYRK2 phosphorylates eIF2Bϵ at Ser^{539} and showed that GSK3 only phosphorylates Ser^{535} significantly if Ser^{539} has already been phosphorylated by DYRK2 (Figure 4). Interestingly, the phosphorylation of Ser^{535} inhibits immunoreactivity towards phosphorylated Ser^{539} (Figure 4), presumably because binding of the antibody is suppressed by the presence of phosphoserine at Ser^{535} and/or because dephosphorylated Ser^{535} is part of the epitope recognized by the phospho-specific Ser^{539} antibody. Essentially the same results were obtained when DYRK1A replaced DYRK2 (results not shown).

eIF2Bε is phosphorylated at Ser^{539} in the purified eIF2B complex

The eIF2B heteropentameric complex isolated from HeLa cells was phosphorylated at Ser^{539}, as judged by immunoblotting with the Ser^{539} phospho-specific antibody. Phosphorylation could not be increased significantly by incubation with MgATP and DYRK2 (Figure 5A), implying that this residue is likely to be almost maximally phosphorylated *in vivo*. The eIF2B complex was also rather resistant to dephosphorylation by protein phosphatases 1γ and 2A, with only a small degree of dephosphorylation occurring even after prolonged incubation with high concentrations of these phosphatases (Figure 5B).

Phosphorylation of tau by DYRK isoforms

In order to examine whether DYRK isoforms were capable of phosphorylating other proteins, we studied the phosphorylation of tau, a protein known to be phosphorylated at multiple Ser-Pro and Thr-Pro sequences *in vivo*. We found that tau was phosphorylated by DYRK2 (1 unit/ml) *in vitro*, phosphorylation reaching about 1 mol/mol of protein after 60 min and approaching 2 mol/mol after 240 min (results not shown). After phosphorylation by DYRK2 to 1 mol/mol, followed by digestion with trypsin and chromatography on a Vydac C_{18} column, one major phosphopeptide and a number of minor phosphopeptides were obtained (Figure 6A). The major peptide was identified by Edman sequencing and MS. It comprised residues 210–224 and was phosphorylated at Thr^{212} (Figure 6B). DYRK1A also phosphorylated tau preferentially at Thr^{212}, albeit slightly less efficiently (results not shown). These findings were confirmed by immunoblotting with an antibody specific for Thr^{212} after phosphorylation of tau by DYRK1A and DYRK2 (Figure 7A). Phosphorylation of Thr^{212} is not accompanied by a change in the mobility of tau. Using the same antibody, tau from newborn-, but not adult-rat, brain was shown to be phosphorylated at Thr^{212} (Figure 7B).

Phosphorylation of tau at Thr^{212} primes phosphorylation by GSK3 at Ser^{208}

Residue 208 in tau is a serine residue, raising the possibility that phosphorylation of Thr^{212} by DYRK might prime phosphorylation by GSK3 at Ser^{208}, by analogy with eIF2Bϵ. We therefore prephosphorylated tau with DYRK2 by incubation for 4 h at 30 °C with magnesium acetate and unlabelled ATP to maximally phosphorylate Thr^{212}. Phosphorylation by GSK3 (5 units/ml) was then carried out for 10 min after the addition of

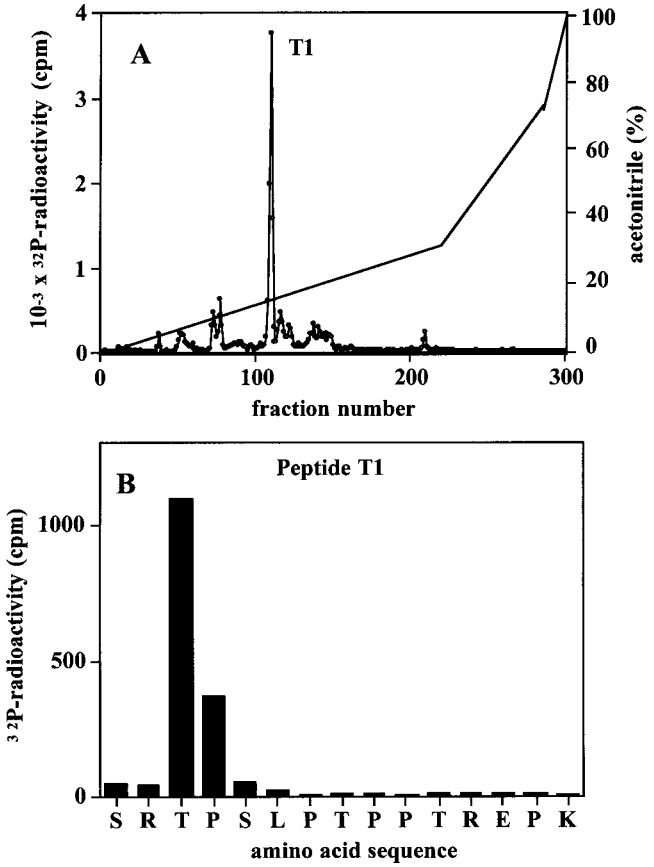

Figure 6 Identification of the major residue on tau phosphorylated by DYRK2

(**A**) Tau (1 μM) was maximally phosphorylated by incubation for 30 min at 30 °C with GST–DYRK2 (1 unit/ml) and Mg[γ-^{32}P]ATP and digested with trypsin as described in the Materials and methods section. The digest was chromatographed on a Vydac C_{18} column as described in the legend to Figure 2(A). One major ^{32}P-labelled peptide T1 accounted for 50% of the radioactivity applied to the column. (**B**) The phosphopeptide T1 from (**A**) was analysed by gas-phase and solid-phase sequencing as described in the legend to Figure 3(B) and ^{32}P radioactivity released after each cycle of Edman degradation was analysed.

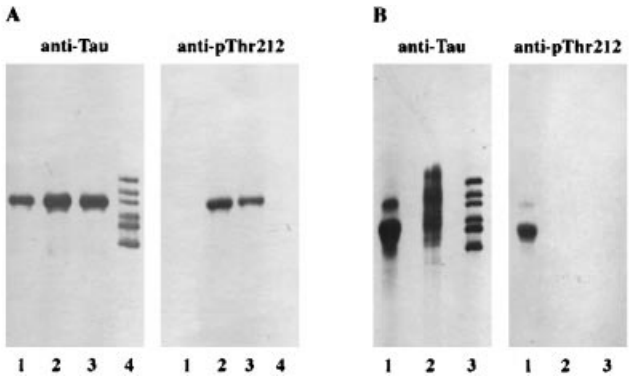

Figure 7 Tau is phosphorylated at Thr^{212} in newborn-rat brain

Immunoblotting of recombinant human tau phosphorylated with DYRK (**A**) and rat brain tau (**B**) using a phosphorylation-independent anti-tau antibody ('anti-Tau') and an antibody specific for pThr^{212} ('anti-pThr212'). (**A**) lanes: 1, tau; 2, tau phosphorylated with DYRK1A; 3, tau phosphorylated with DYRK2; 4, mixture of recombinant human brain tau isoforms. (**B**) Lanes: 1, tau from newborn-rat brain; 2, tau from adult-rat brain; 3, mixture of bacterially expressed human brain tau isoforms (ranging from 46 to 68 kDa).

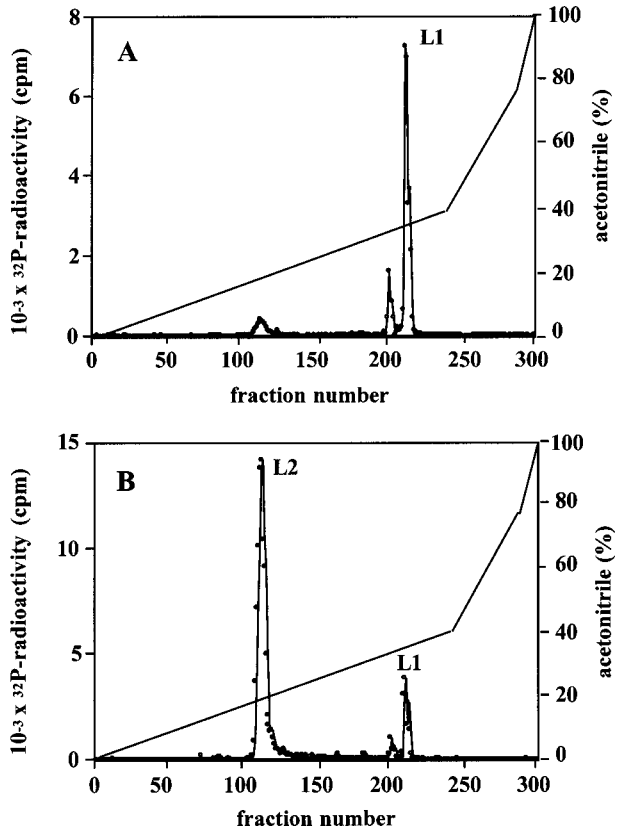

Figure 8 Separation of phosphopeptides from tau obtained by digestion with Lys-C proteinase

(**A**) Tau (2 μM) was phosphorylated for 10 min with GSK3 (5 units/ml), as described in the Materials and methods section, using [γ-^{32}P]ATP. The reaction was stopped by denaturation in SDS and the mixture subjected to PAGE. The ^{32}P-labelled band corresponding to the tau protein was excised and digested with Lys-C, as described in the Materials and methods section. The digest was applied to a Vydac C_{18} column and analysed as described in Figure 2. One major ^{32}P-labelled peptide, L1, was obtained. (**B**) tau (2 μM) was phosphorylated for 4 h with DYRK2 (1 unit/ml), as described in the Materials and methods section, using unlabelled ATP. GSK3 was then added to 5 units/ml and phosphorylation continued for a further 10 min using [γ-^{32}P]ATP. The reaction was stopped by denaturation in SDS, and the mixture analysed as in (**A**). A major phosphopeptide, L2, and a minor phosphopeptide, L1, were obtained.

Mg[γ-^{32}P]ATP. Under these conditions, GSK3 phosphorylated tau to 0.8 mol/mol of protein. In contrast, tau preparations that had not been pre-phosphorylated with DYRK were only phosphorylated to 0.1 mol/mol (results not shown). Samples of the GSK3-phosphorylated tau were subjected to SDS/PAGE and the ^{32}P-labelled bands excised, digested with Lys-C proteinase and chromatographed on the Vydac C_{18} column (see the legend to Figure 8). Tau phosphorylated by GSK3 (without DYRK pre-phosphorylation) showed one major ^{32}P-labelled peptide L1 (Figure 8A) which, by Edman sequencing and MS (results not shown), was found to correspond to the peptide comprising residues 396–438 phosphorylated predominantly at Ser^{404}. Tau phosphorylated by GSK3 after pre-phosphorylation with DYRK also phosphorylated peptide L1 (Figure 8B) at Ser^{404} (results not shown), but there was much greater labelling of another peptide L2 (Figure 8B). L2 was shown by MS to correspond to the peptide comprising residues 190–224, and Edman sequencing revealed that it was phosphorylated predominantly at Ser^{208} (results not shown). These results were

confirmed by experiments using a mutant form of tau in which Thr^{212} was changed to alanine. This mutant was poorly phosphorylated by GSK3, even after pre-phosphorylation with DYRK2 (results not shown).

DISCUSSION

A requirement for Ser^{539} phosphorylation to permit the GSK3-catalysed phosphorylation of eIF2Bϵ at Ser^{535} had been inferred previously from studies with synthetic peptides [9], but the absence of any protein kinase capable of phosphorylating this site significantly *in vitro* had precluded the demonstration that these hierarchical phosphorylations occur in the full-length protein. Here we show that DYRK isoforms phosphorylate Ser^{539} specifically (Figure 3) and that this is indeed required to prime phosphorylation at Ser^{535} by GSK3 (Figure 4). We also show that the ϵ-subunit of the eIF2B complex isolated from HeLa cells is almost fully phosphorylated at Ser^{539}, because little or no increase in the phosphorylation of this site occurs upon incubation with DYRK and MgATP (Figure 5A). The high level of phosphorylation of Ser^{539} may be explained by its very low rate of dephosphorylation (Figure 5B). The resistance of other pSer-Pro sequences to dephosphorylation has been noted previously [19].

The finding that DYRK isoforms phosphorylate eIF2Bϵ as well as FKHR [13] raised the possibility that these protein kinases have broad substrate specificities *in vivo*. It was therefore of interest to study the phosphorylation of tau by DYRK, because this microtubule-associated protein is known to be phosphorylated at numerous Ser-Pro and Thr-Pro sequences *in vivo*, many of which become hyperphosphorylated in the brains of patients with Alzheimer's disease and several other neurodegenerative diseases. We found that DYRK phosphorylates tau predominantly at one site, namely Thr^{212} (Figure 6), a residue known to be phosphorylated in Alzheimer's-disease brain [20] and, as shown here, in tau from the brain of newborn rats (Figure 7). Moreover, the phosphorylation of Thr^{212} 'primed' the phosphorylation of tau at Ser^{208} by GSK3. Ser^{208} has also been reported to be phosphorylated in tau from Alzheimer's-disease brain [20]. Since Ser^{208} does not lie in an obvious consensus sequence for any other known kinase, GSK3 may be responsible for the phosphorylation of this residue *in vivo*.

In the present study we confirmed previous work showing that bacterially expressed 'unprimed' tau is preferentially phosphorylated at Ser^{404} [21]. However, bacterially expressed tau is a poor substrate for GSK3 *in vitro* unless it is first phosphorylated by other protein kinases to prime the phosphorylation of other sites. For example, the phosphorylation of tau at Ser^{235} by the mitogen-activated protein kinase ERK2 increases the rate of phosphorylation by GSK3 50-fold, by 'priming' the phosphorylation of Thr^{231} [16,22]. Similarly, we find that the phosphorylation of Thr^{212} by DYRK stimulates the rate of phosphorylation of tau about 8-fold by 'priming' the phosphorylation of Ser^{208} (results not shown).

DYRK isoforms are phosphorylated at one or both adjacent tyrosine residues in the activation loops of their catalytic domains [12]. However, the name 'dual-specificity tyrosine-phosphorylated and regulated kinase' is misleading, because tyrosine phosphorylation probably arises from autophosphorylation catalysed by DYRK itself [12,23], and DYRK1A activity in mammalian cells is similar under all conditions we have tested so far (see the accompanying paper [13]). This is consistent with the high level of phosphorylation of Ser^{539} of eIF2Bϵ in the purified eIF2B complex and its proposed role as a 'GSK3-priming' kinase. The finding that DYRK isoforms 'prime' both eIF2Bϵ and tau for phosphorylation by GSK3 suggests that these enzymes may play more general roles as 'GSK3-priming' kinases *in vivo*.

DYRK1A is reported to have a predominantly nuclear location, while DYRK2 is mainly cytosolic [11]. It is therefore likely that DYRK1A phosphorylates nuclear substrates, such as the transcription factor FKHR, while DYRK2 probably phosphorylates cytosolic substrates, such as eIF2B and tau. However, genetic evidence, or the availability of specific inhibitors, will be needed to establish whether FKHR, eIF2B and tau are true physiological substrates of DYRK.

DYRK is remarkably specific for particular Ser/Thr-Pro sequences. It phosphorylates just one of eight such sequences in FKHR, one of five such sequences in eIF2Bϵ and one of 17 such sequences in tau. A feature of the sites phosphorylated by DYRK isoforms in FKHR (see the accompanying paper [13]), eIF2Bϵ and tau (the present paper) is an arginine residue located two or three residues N-terminal to the site of phosphorylation. In order to investigate whether this arginine residue is critical, we mutated it to alanine or lysine in Woodtide, KKISGRLSPIMTEQ, the standard synthetic peptide substrate. The mutation to alanine or lysine virtually abolished phosphorylation by DYRK1A or DYRK2. We also found that a very similar peptide, KKISRALSPIMTEQ, in which the arginine residue is positioned three (and not two) residues N-terminal to the phosphorylation site, was phosphorylated with similar efficiency to Woodtide (Y. L. Woods, unpublished work). These experiments indicate that an N-terminal arginine residue is critical for substrate specificity, and this may facilitate the identification of additional substrates for DYRK isoforms. It will be particularly important to identify substrates in the brain because mutants in the *Drosophila* 'minibrain' gene, *mnb*, the product of which is a DYRK homologue, are defective in the generation of particular sets of neurons. Moreover, DYRK1A is one of the proteins encoded by the 'Down's-syndrome critical region' on chromosome 21 [12,24–27], and its overexpression may contribute to this disorder. Furthermore, transgenic mice with a three-fold overexpression of a smaller (180 kb) DNA sequence within this region (of which the gene encoding DYRK1A accounts for over 100 kb) have learning difficulties [28]. Down's syndrome is also associated with the pathology of Alzheimer's disease [29], including filamentous deposits made of hyperphosphorylated tau protein.

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