The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser³²⁹ *in vitro*, a novel *in vivo* phosphorylation site

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Forkhead in rhabdomyosarcoma (FKHR) is a transcription factor that has been implicated in the control of gene expression by insulin, as well as the regulation of apoptosis by survival factors. These signals trigger the protein kinase B (PKB)-catalysed phosphorylation of FKHR at three residues (Thr²⁴, Ser²⁵⁶ and Ser³¹⁹) by a phosphoinositide 3-kinase-dependent pathway that results in the nuclear exit and inactivation of this transcription factor. Here, we have identified a conserved residue (Ser³²⁹) as a novel *in vivo* phosphorylation site on FKHR. Ser³²⁹ phosphorylation also decreases the ability of FKHR to stimulate gene transactivation and reduces the proportion of FKHR present in the nucleus. However, unlike the residues targeted by PKB, Ser³²⁹ is phosphorylated in unstimulated HEK-293 cells, and phosphorylation is not increased by stimulation with insulin-

like growth factor-1 or by transfection with 3-phosphoinositide-dependent protein kinase-1. We have also purified a protein kinase to near homogeneity from rabbit skeletal muscle that phosphorylates FKHR at Ser³²⁹ specifically and identified it as DYRK1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A). We find that FKHR and DYRK1A co-localize in discrete regions of the nucleus and can be co-immunoprecipitated from cell extracts. These experiments suggest that DYRK1A may phosphorylate FKHR at Ser³²⁹ *in vivo*.

Key words: Akt, apoptosis, insulin action, gene transcription, protein kinase B.

INTRODUCTION

Genetic studies in the nematode worm *Caenorhabditis elegans* have indicated that a specific member of the forkhead/winged-helix family of transcription factors (DAF16, where DAF means dauer arrest phenotype) lies 'downstream' of protein kinase B (PKB, also called Akt) in a phosphoinositide (PI) 3-kinase-dependent signalling pathway. Mutation of the insulin/insulin-like-growth-factor-1 (IGF-1) receptor homologue (DAF2), the catalytic subunit of PI 3-kinase (age-1), 3-phosphoinositide-dependent protein kinase-1 (PDK1) or the PKB isoforms (Akt-1 and Akt-2) results in increased longevity and constitutive dauer formation [1–3], a stage of developmental arrest, and reduced metabolic activity that enhances survival during periods of food deprivation and other environmental stresses. In each case, the mutation of DAF16 restores normal lifespan and prevents entry into the dauer stage [1–3]. These observations have suggested that DAF16 promotes entry into the dauer phase and enhances longevity and that signalling via the PI 3-kinase/PKB pathway acts to prevent these effects of DAF16 [1–3].

DAF16 possesses four consensus sequences for phosphorylation by PKB [2], three of which are conserved in the mammalian DAF16 homologues FKHR (forkhead in rhabdomyosarcoma), FKHL1 and AFX (acute-lymphocytic-leukaemia-1 fused gene

from chromosome X). Subsequent work has established that PKB phosphorylates FKHR, FKHL1 and AFX *in vitro* [4–10] and that these three sites become phosphorylated in a PI 3-kinase-dependent manner when a variety of mammalian cells are stimulated with insulin/IGF-1 [5–8,11]. Phosphorylation at these sites has been shown to suppress the ability of FKHR, FKHL1 and AFX to stimulate the transactivation of reporter genes [5,6,12] and to promote nuclear exit [4,5,10,11,13] and their interaction with 14-3-3 proteins [5]. These findings have established that FKHR and its close homologues are direct targets for PKB and that phosphorylation may contribute to repression of the transcription of some genes in response to insulin/IGF-1. The phosphorylation of FKHL1 may also mediate some of the anti-apoptotic effects of IGF-1 by inhibiting the transcription of proteins, such as the fibroblast-associated (FAS) ligand [5]. Similarly, it has been reported that the overexpression of AFX blocks cell-cycle progression, suggesting that PKB-mediated inactivation of AFX might promote oncogenesis [14].

Several reports in the literature have indicated that the phosphorylation of FKHR and its homologues may be more complex and involve the phosphorylation of other sites on these proteins. For example, it has been reported that a C-terminal fragment of FKHR, lacking all three sites of PKB phosphorylation, stimulates the transcription of reporter genes and that

Abbreviations used: AFX, acute-lymphocytic-leukaemia-1 fused gene from chromosome X; CH-, cyclohexyl-; DAF, dauer arrest phenotype; DTT, dithiothreitol; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; DYRKtide, the synthetic peptide RRRFRPASPLRGPPK; FAS, fibroblast-associated; FKHR, forkhead in rhabdomyosarcoma; GFP, green fluorescent protein; GST, glutathione S-transferase; IGF-1, insulin-like growth factor-1; IRS, insulin response sequence; Ni-NTA, Ni²⁺-nitrilotriacetate; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; PI, phosphoinositide; PKI, specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASGRTGRRNAIHD); MAPKAP-K2, mitogen-activated protein kinase-activated kinase-2; MALDI-TOF, matrix-assisted laser-desorption-ionization time-of-flight; S329A, mutation of Ser³²⁹ to Ala; Sf21, *Spodoptera frugiperda* 9 cells; TRITC, tetramethylrhodamine β -isothiocyanate; Woodtide, the synthetic peptide KKISGRSLPIMTEQ.

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transactivation can still be inhibited by insulin/IGF-1 [15]. It has also been reported that phosphorylation of another site(s) on AFX is triggered by activation of the small G-protein Ral [6]. Here we identify Ser³²⁹ as a new residue in FKHR that is phosphorylated in mammalian cells and identify dual-specificity tyrosine-phosphorylated and regulated protein kinase 1A (DYRK1A) as a protein kinase that phosphorylates this site specifically. Ser³²⁹ is conserved in FKHL1 and AFX, as well as in DAF16 of *C. elegans*.

MATERIALS AND METHODS

Materials

Tissue-culture reagents and IGF-1 were purchased from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). PKI, the specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASGRTGRRNAIHD), was synthesized by Mr F. B. Caudwell in the MRC Protein Phosphorylation Unit in Dundee, and other peptides by Dr G. Bloomberg (Department of Biochemistry, University of Bristol, Bristol, U.K.). A vector expressing glutathione S-transferase (GST)-FKHR has been described previously [8]. Mono S, heparin-Sepharose, GSH-Sepharose 4B, Protein G-Sepharose and cyclohexyl-(CH)-Sepharose were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Ni²⁺-nitrilotriacetic acid (Ni-NTA)-agarose was purchased from Qiagen (Crawley, West Sussex, U.K.). Phosphocellulose P81 paper was from Whatman International Ltd. (Maidstone, Kent, U.K.). Fluoromount-G was from Southern Biotechnology Associates, Inc. (Birmingham, AL, U.S.A.). Acetonitrile (HPLC grade) and trifluoroacetic acid were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.). Sequencing-grade trypsin and Fugene 6 transfection reagent were purchased from Roche Diagnostics (Mannheim, Germany).

Antibodies

A phosphopeptide was synthesized corresponding to residues 324–334 of FKHR (ISGRL^pSPIMTE, where ^pS indicates phosphoserine) and conjugated to both keyhole-limpet haemocyanin and BSA. The conjugates were mixed together and injected into a sheep at the Scottish Antibody Production Unit (Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.). After 6 weeks the antiserum was passed down a CH-Sepharose column to which the corresponding unphosphorylated peptide had been coupled, followed by affinity chromatography on CH-Sepharose to which the phosphopeptide antigen had been attached covalently. The phosphospecific antibody was eluted with 0.1 M glycine, pH 2.4, immediately adjusted to pH 8.0 with Tris/HCl, and stored at 4 °C. We also raised an antibody in sheep against the FKHR protein that was affinity-purified by chromatography on FKHR-Sepharose (anti-FKHR). This antibody recognizes phosphorylated and unphosphorylated FKHR equally well and immunoprecipitates FKHR efficiently from a number of cell lines. Another FKHR antibody that recognizes the Thr²⁴-phosphorylated protein (anti-pThr²⁴) has been described previously [8]. All FKHR antibodies described in the present paper are available from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Affinity columns were prepared and antibodies purified by Dr J. Leitch and Ms C. Clark of the Division of Signal Transduction Therapy at Dundee. An anti-DYRK1A, monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.), and immunoprecipitating antisera were raised in sheep against bacterially expressed GST-

DYRK1A and affinity-purified, first by passage down a column of GST-Sepharose (to remove the anti-GST antibodies), and then by chromatography on Sepharose to which DYRK1A had been attached covalently. Anti-(green fluorescent protein) (anti-GFP) monoclonal antibody was from Clontech (Palo Alto, CA, U.S.A.). FITC-conjugated anti-mouse IgG was from Dianova (Hamburg, Germany) and tetramethylrhodamine β -isothiocyanate (TRITC)-conjugated anti-rabbit IgG from Sigma (St Louis, MO, U.S.A.).

Cell culture, transient transfections and cell lysis

HEK-293 cells were cultured at 37 °C in an atmosphere of 5 % CO₂ in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal-calf serum. Transfection of the cells was carried out using the CaCl₂ precipitation method, employing 10 μ g of DNA/10 cm-diameter dish. Prior to lysis, cells were serum-starved for 12 h. Cells were harvested in 1 ml of ice-cold lysis buffer [50 mM Tris/acetate (pH 7.5)/1 mM EGTA/1 % (w/v) TritonX-100/1 mM EDTA/50 mM NaF/10 mM sodium β -glycerophosphate/5 mM sodium pyrophosphate/1 mM benzamide/0.2 mM PMSF/0.1 % 2-mercaptoethanol]. The lysates were centrifuged at 13 000 *g* and the supernatants removed, frozen immediately in liquid nitrogen and stored at –80 °C until use.

Luciferase reporter experiments

HEK-293 cells were transfected with 10 μ g of plasmid DNA/dish, including 3 μ g of a luciferase reporter gene construct containing an array of three insulin response sequences immediately upstream of the 81-bp thymidine kinase promoter (TK.IRS3) [12] plus 3 μ g of FKHR expression vector, 2 μ g of a cytomegalovirus-driven β -galactosidase vector and 2 μ g of empty vector. Cells were then stimulated as described in the Results section, harvested, and luciferase activity then assayed as described in [16].

Immunoprecipitation of FKHR from HEK-293-cell extracts

Cell lysate (500 μ g of protein) was added to 10 μ l of packed Protein G-Sepharose attached to 10 μ g of anti-FKHR antibody. The suspension was incubated for 2 h at 4 °C on a platform shaker, briefly centrifuged and the supernatant discarded. The resin was then washed twice with 1 ml of lysis buffer containing 0.5 M NaCl, to disrupt non-specific protein-antibody interactions, and twice with 1 ml of 50 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 mM EGTA/0.1 % 2-mercaptoethanol. The beads were then heated for 5 min at 100 °C in 0.1 ml of 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 the solubilized protein subjected to SDS/PAGE and immunoblotting.

Immunoblotting

All 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. Primary antibodies were used at 0.5 μ g/ml and, before use, phospho-specific antibodies were preincubated with the unphosphorylated form of the relevant phosphopeptide immunogen (10 μ g/ml).

Purification of Ser³²⁹ kinase from rabbit skeletal muscle

Three New Zealand White rabbits were administered a lethal dose of sodium pentobarbitone. Skeletal muscle from the hind

limbs and back was removed rapidly and placed on ice. Subsequent steps were carried out at 0–4 °C, unless stated otherwise. The muscle (2500 g) was minced, transferred to a Waring Blendor and homogenized in 8000 ml of 4 mM EDTA/1 mM benzamidine/0.1 mM PMSF/0.1 % 2-mercaptoethanol, pH 7.0. The homogenate was centrifuged for 45 min at 4000 *g* to pellet the myofibrils and the supernatant removed and filtered through glass wool. The extract was titrated to pH 6.1 with 1 M acetic acid to precipitate glycogen particles, centrifuged at 4000 *g* and the supernatant filtered through glass wool and adjusted to pH 7.0 with ammonium hydroxide. The solution was adjusted to 35 % (w/v) (NH₄)₂SO₄, and, after gently stirring for 60 min, the suspension was centrifuged for 10 min at 30000 *g* and the supernatant discarded. The pellet was resuspended in 50 ml of buffer B [50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.07 % 2-mercaptoethanol/1 mM benzamidine/0.1 mM PMSF] and dialysed overnight against buffer B. After centrifugation for 10 min at 100000 *g*, the supernatant was mixed for 1 h on a rotating platform with 3 ml of Ni-NTA-agarose equilibrated in Buffer B. The Ni-NTA-agarose was poured into a 10 ml Econopac column (Bio-Rad, Hercules, CA, U.S.A.), washed with buffer B containing 20 mM imidazole, pH 7.5, and 0.5 M NaCl until the absorbance of the eluate at 280 nm was below 0.03, and then in buffer B to remove NaCl. The Ser³²⁹ kinase activity was then eluted in buffer B containing 200 mM imidazole, pH 7.5, 150 mM NaCl and 0.03 % (w/v) Brij-35. Fractions (0.5 ml each) were collected and the most active fractions pooled and dialysed for 18 h against buffer C [50 mM Mops (pH 7.5)/50 % (v/v) glycerol/1 mM EGTA/1 mM EDTA/0.1 % 2-mercaptoethanol/0.03 % (w/v) Brij]. The solution was applied to a 1 ml Hitrap column of heparin-Sepharose equilibrated in buffer C. After washing with equilibration buffer until the *A*₂₈₀ was below 0.02, the column was developed with a 40 ml linear salt gradient to 1 M NaCl in the same buffer. The flow rate was 1 ml/min, and fractions (1 ml each) were collected. The most active fractions, eluted between 325 and 425 mM NaCl, were pooled, diluted with an equal volume of buffer C and applied to a 0.1 ml Mono S (PC 1.6/5) column equilibrated in buffer C. The column was developed with a 4 ml linear salt gradient to 1 M NaCl in the same buffer, and fractions (0.1 ml each) were collected and analysed as described in the Results section.

Protein kinases

Vectors encoding Myc-tagged PDK1 [17] and full-length GST-PKB α [18] (obtained from Dr M. Deak in the MRC Protein Phosphorylation Unit in Dundee) were expressed in HEK-293 cells and GST-PKB α was purified on GSH-Sepharose. The vector encoding GST-DYRK1A[1–500] [19] was expressed in *Escherichia coli*, and the protein purified on GSH-Sepharose, dialysed against 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/0.1 % 2-mercaptoethanol/50 % (v/v) glycerol and stored at 2 mg/ml at –20 °C. Human PKB α with an N-terminal hexahistidine tag was expressed in insect (*Spodoptera frugiperda*) Sf21 (CRL 1711) cells and purified by immobilized-metal-anion chromatography on Ni-NTA agarose by Dr A. Paterson of the Division of Signal Transduction Therapy, University of Dundee, Dundee, Scotland, U.K., who activated the protein by sequential phosphorylation with GST-mitogen-activated protein kinase-activated kinase-2 (MAPKAP-K2) (to phosphorylate Ser⁴⁷³) [20] and His₆-Myc-PDK1[51–566] [21] (to phosphorylate Thr³⁰⁸) [22]; the GST-MAPKAP-K2 was removed by chromatography on GSH-Sepharose.

Protein kinase assays

The activity of the protein kinase capable of phosphorylating FKHR at Ser³²⁹ and DYRK1A were measured using a synthetic peptide, termed Woodtide, corresponding to residues 324–334 of FKHR with two lysine residues added at the N-terminus to facilitate binding to phosphocellulose paper (KKISGRSLSPIMTEQ). Assays were carried out at 30 °C using 50 μ M Woodtide in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/2.5 μ M PKI/10 mM magnesium acetate/0.1 mM [γ -³²P]ATP (10⁶ c.p.m./nmol). One unit of enzyme activity was that amount which catalysed the phosphorylation of 1 nmol of Woodtide in 1 min.

Digestion of ³²P-labelled proteins

The ³²P-labelled band of the protein of interest was excised, washed extensively with 50 % (v/v) acetonitrile containing 0.1 mM ammonium bicarbonate, and dried. Following resuspension in 20 mM ammonium bicarbonate containing 0.1 % n-octyl glucoside, trypsin was added to a final concentration of 1 μ g/ml and digestion carried out for 18 h at 30 °C.

MS

Tryptic peptides were analysed on a Perseptive Biosystems (Framingham, MA, U.S.A.) Elite STR matrix-assisted laser-desorption–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 tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector (a search program algorithm supplied by the University of California, San Francisco, CA, U.S.A., but accessible via <http://prospector.ucsf.edu/ucshtml3.4/msfit.htm>).

Indirect immunofluorescence microscopy of FKHR and DYRK1A

COS-7 cells were seeded in six-wells on coverslips and transiently transfected with each 0.5 μ g of plasmid DNA encoding FKHR (in pcDNA3.1) and GFP-DYRK1A (in pEGFP-C1) [23], using the Eugene 6 transfection reagent according to the manufacturer's instructions. At 24 h after transfection, the cells were placed in serum-free medium for another 12 h and then washed twice with cold PBS (138 mM NaCl/2.6 mM KCl/10 mM Na₂HPO₄/1.8 mM KH₂PO₄, pH 7.4). The cells were fixed and permeabilized for 2 min with ice-cold methanol, blocked for 15 min at room temperature with 3 % (w/v) BSA in PBS and incubated for 2 h at 37 °C with primary antibodies (anti-FKHR polyclonal serum and anti-GFP monoclonal antibody, 1:100 each in 3 % BSA/PBS). Following incubation with secondary antibodies (1 h, 37 °C, FITC-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG, 1:300 in 3 % BSA/PBS), the cells were washed extensively with PBS, mounted with Fluoromount-G and revealed with a confocal laser-scanning microscope (Zeiss, Jena, Germany).

Co-immunoprecipitation of FKHR and DYRK1A from nuclear lysates

HEK-293T cells were seeded in 10-cm-diameter dishes and transfected with plasmids encoding either FKHR, GFP–

DYRK1A or GFP alone as a control using the Fugene 6 transfection reagent according to the manufacturer's instructions. At 36 h after transfection, the cells were harvested and nuclear extracts prepared as described in [24]. In brief, the plates were washed twice with ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. All subsequent steps were performed at 4 °C. After rinsing the plates with 2 ml of ice-cold hypotonic buffer [20 mM Hepes (pH 7.9)/1 mM EDTA/1 mM EGTA/20 mM NaF/1 mM Na_3VO_4 /1 mM $\text{Na}_3\text{P}_2\text{O}_7$ /1 mM dithiothreitol (DTT)/0.5 mM PMSF/1 mM benzamide], the cells were scraped into 500 ml of hypotonic buffer containing 0.2% (v/v) Nonidet P40 and centrifuged for 20 s at 16000 *g*. The supernatants were removed and the pellets extracted with 150 ml of high-salt buffer [20 mM Hepes (pH 7.9)/420 mM NaCl/1 mM EDTA/1 mM EGTA/20% (v/v) glycerol/20 mM NaF/1 mM Na_3VO_4 /1 mM $\text{Na}_3\text{P}_2\text{O}_7$ /1 mM DTT/0.5 mM PMSF/1 mM benzamide]. Following incubation for 30 min on an end-over-end rotator, the extracts were centrifuged for 30 min at 16000 *g* and the supernatants from this step collected (nuclear extracts). The salt concentration of the nuclear extracts was adjusted to 140 mM NaCl by adding 2 vol. of hypotonic buffer. Endogenous FKHR and GFP-DYRK1A were immunoprecipitated for 2 h with polyclonal antibodies, or a monoclonal anti-GFP antibody, bound to mixed Protein A/Protein G-Sepharose. The precipitates were washed twice with 1 ml of Tris-buffered saline containing 0.1% (v/v) Triton X-100. The beads were heated for 2 min at 100 °C in 30 μl of SDS sample buffer and then subjected to SDS/PAGE.

RESULTS

Identification of Ser³²⁹ as a novel phosphorylation site in FKHR

PKB α that had been expressed as a His-tagged fusion protein in *Sf*21 cells and affinity-purified by chromatography on Ni-NTA-agarose was used to phosphorylate GST-FKHR. After digestion with trypsin, the resulting phosphopeptides were chromatographed on a Vydac (Hesperia, CA, U.S.A.) C₁₈ column, which resolved three ³²P-labelled tryptic peptides, namely T1, T2a and T3 (Figure 1A), consistent with the known phosphorylation of FKHR at three residues (Thr²⁴, Ser²⁵⁶ and Thr³¹⁹, see the Introduction). The peptides were identified by Edman sequencing and MS. Peptide T1 (AASMDNNSKFAKSR), comprising residues 254–267, was phosphorylated at Ser²⁵⁶, while peptide T3 (comprising residues 22–150 and commencing SCTWPL ...) was phosphorylated at Thr²⁴. However, analysis of peptide T2a (comprising residues 317–354 and commencing ISSNASTISGR LSPIM ...), revealed that it was a diphosphorylated derivative phosphorylated at Ser³²⁹ as well as at the expected Ser³¹⁹ (Figure 2).

The sequence surrounding Ser³²⁹ does not conform to the consensus for phosphorylation by PKB, which is Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-, suggesting that Ser³²⁹ might not be phosphorylated by PKB, but by another protein kinase present as a contaminant. This was also indicated by experiments in which GST-PKB expressed in HEK-293 cells and purified by affinity chromatography on GSH-agarose was used to phosphorylate PKB. Tryptic digestion followed by Vydac C₁₈ chromatography also gave rise to three phosphopeptides, two of which were co-eluted with peptides T1 and T3. However, peptide T2a was absent and replaced by an earlier eluted phosphopeptide T2b (Figure 1B). This peptide (ISSNASTISGR) comprised residues 317–327 and was phosphorylated at Ser³¹⁹. This showed that Ser³²⁹ was phosphorylated by a protein kinase that contaminates preparations of His-tagged PKB, this phosphorylation preventing tryptic cleavage of the Arg–Leu bond between residues

Figure 1 Tryptic phosphopeptide maps obtained after phosphorylation with two different preparations of PKB

GST-FKHR was phosphorylated with His-tagged PKB (A) or GST-PKB (B) and digested with trypsin as described in the Materials and methods section. The digest was applied to a Vydac C₁₈ column equilibrated in 0.1% (v/v) trifluoroacetic acid and the column developed with an acetonitrile gradient (diagonal line). The flow rate was 1 ml/min, and fractions (0.5 ml each) were collected and analysed for ³²P radioactivity by Cerenkov counting. The major ³²P-labelled tryptic peptides T1, T2a, T2b and T3 were identified as described in the text.

Figure 2 Identification of the phosphorylation sites in peptide T2a

Phosphopeptide T2a from Figure 1(A) was sequenced by Edman degradation using an Applied Biosystems 429A protein sequencer. ³²P radioactivity released after each cycle was measured in a separate experiment by solid-phase Edman degradation of the peptides coupled to a Sequelon arylamine membrane (Milligen, Bedford, MA, U.S.A.) as described previously [34]. The amino acid sequence is shown using the single-letter code for amino acids.

(A) Cells were transfected with a vector expressing wild-type GST-FKHR or GST-FKHR[S329A]. In one experiment the cells were stimulated for 15 min with IGF-1 (100 ng/ml) and, in another experiment, GST-FKHR was co-expressed with Myc-tagged PDK1 as described in the Materials and methods section. Aliquots of the cell lysates (10 μ g of protein) were electrophoresed on SDS/10%-polyacrylamide gels and, after transfer to nitrocellulose, immunoblotted as described in the Materials and methods section with three of the FKHR antibodies in Figure 3. **(B)** HEK-293 cells were either left unstimulated, or stimulated for 15 min with IGF-1 (100 ng/ml). Endogenous FKHR was immunoprecipitated from the cell lysates using the anti-FKHR antibody, as described in the Materials and methods section. The immunoprecipitates were denatured in SDS, subjected to electrophoresis on 4–12% gradient polyacrylamide gels (Novex), transferred to nitrocellulose and immunoblotted with the same FKHR antibodies as in **(A)**.

(A) GST-FKHR ($2\ \mu\text{M}$) was left unphosphorylated or phosphorylated for 30 min with His-tagged PKB or GST-PKB (each at 1 unit/ml) as described in the Materials and methods section. Aliquots of each reaction mixture (40 pmol of GST-FKHR) were electrophoresed on SDS/10%-(w/v)-polyacrylamide gels and, after transfer to nitrocellulose membranes, immunoblotted as described in the Materials and methods section with antibodies that recognize the Ser³²⁹ (anti-pSer³²⁹) or Thr²⁴ (anti-pThr²⁴) phosphorylated forms of GST-FKHR, or an antibody that recognizes phosphorylated and dephosphorylated GST-FKHR equally well (anti-FKHR).

(B) Aliquots of unphosphorylated (U) GST-FKHR ($1\ \mu\text{g}$) or GST-FKHR phosphorylated (P) by His-tagged PKB as in **(A)** were spotted on to nitrocellulose membranes and blotted with either the anti-pSer³²⁹ or the anti-FKHR antibody. The experiment was performed after preincubating the anti-pSer³²⁹ antibody with the different competitor peptides indicated. The phosphopeptides comprised residues 19–29 of FKHR (phosphorylated at Thr²⁴), residues 251–261 (phosphorylated at Ser²⁵⁶), residues 314–324 (phosphorylated at Ser³¹⁹), residues 324–334 (phosphorylated at Ser³²⁹) or the unphosphorylated form of the peptide 324–334.

In order to investigate whether Ser³²⁹ was a site of phosphorylation *in vivo*, we raised an antibody against a peptide comprising residues 324–334 of FKHR, phosphorylated at Ser³²⁹. This antibody recognized FKHR phosphorylated by His-tagged PKB, but not unphosphorylated FKHR or FKHR phosphorylated by GST-PKB (Figure 3A). In contrast, an antibody that recognizes FKHR phosphorylated at Thr²⁴ detected FKHR whether it was phosphorylated by His-tagged PKB or by GST-PKB, while an antibody raised against recombinant FKHR protein recognized unphosphorylated and phosphorylated FKHR equally well. The specificity of the anti-pSer³²⁹ antibody was further established by competition studies, which showed that it was neutralized by the phosphopeptide antigen used to

We were also able to study the phosphorylation of the endogenous FKHR in HEK-293 cells after immunoprecipitating the protein from the lysates using an antibody raised against the whole FKHR protein (anti-FKHR). The anti-FKHR detected one major band in immunoblotting experiments (Figure 4B) that migrated in the expected position for the endogenous FKHR with an apparent molecular mass of 72 kDa. Further immunoblotting experiments using the same extracts and antibodies specific for the phosphorylation of particular residues showed that the endogenous FKHR was phosphorylated at Ser³²⁹ similarly in control and IGF-1-stimulated cells and phosphoryl-

Figure 5 Transactivation by wild-type GST-FKHR and GST-FKHR[S329A] in HEK-293 cells

Cells were transfected as described in the Materials and methods section, refed with serum-free medium for 24 h, then treated with/without 100 ng/ml IGF-1 for 12 h prior to harvesting. Basal TK.IRS3 promoter activity is negligible in HEK-293 cells, and luciferase activity largely reflects transactivation by FKHR. For each construct, luciferase activity in lysates is expressed relative to activity in cells treated with serum-free medium alone. Immunoblotting of the extracts showed similar levels of transfection in every dish of cells.

ated at Thr²⁴ only in response to IGF-1 (Figure 4B). Both phospho-specific antibodies also recognized a minor 76 kDa band that may represent the FKHL1 isoform, because the 76 kDa band was the major component recognized by a commercially available phospho-specific antibody raised against a synthetic phosphopeptide corresponding to the sequence surrounding Thr³² of FKHL1 (the residue equivalent to Thr²⁴ of FKHR) (G. Rena, unpublished work).

Mutation of Ser³²⁹ stimulates FKHR-dependent gene transactivation

The mutation of Ser³²⁹ to Ala (S329A), to prevent the phosphorylation of this residue, increased by about 4-fold the ability of co-transfected FKHR to stimulate transactivation of a luciferase reporter gene (Figure 5A). However, it had little or no effect on the ability of IGF-1 to inhibit FKHR-stimulated transactivation. Inhibition by IGF-1 was about 50 % with either wild-type FKHR or FKHR[S329A] (Figure 5). The wild-type and S329A mutant were expressed at similar levels in these experiments, as determined by immunoblotting (Figure 5)

The mutation of Ser³²⁹ increases the proportion of FKHR in the nucleus

We transfected wild-type GST-FKHR into HEK-293 cells and studied the subcellular localization of the protein by immunofluorescence (Figure 6A). In unstimulated cells, the wild-type protein was largely nuclear in 75 % of the cells and mainly cytosolic in 25 % of the cells. In contrast, GST-FKHR[S329A] was predominantly nuclear in 90 % of the cells (Figure 6B) and cytosolic in only 10 % of the cells. However, after stimulation for 30 min with IGF-1, the subcellular distribution of wild-type GST-FKHR and GST-FKHR[S329A] were indistinguishable, the proteins being equally divided between the nuclear and cytosolic compartments (Figure 6B). The level of phosphoryl-

Figure 6 The mutation of Ser³²⁹ to alanine increases the proportion of GST-FKHR in the nucleus of unstimulated cells

(A) HEK-293 cells were transfected with a vector encoding wild-type GST-FKHR or GST-FKHR[S329A]. The cells were incubated for 10 min with 100 nM wortmannin or stimulated for 30 min with 100 ng/ml IGF-1 and 10 % (v/v) serum. GST-FKHR was revealed by immunofluorescence with a GST antibody as described in [35]. (B) Quantification of the proportion of wild-type GST-FKHR and GST-FKHR[S329A] in the nucleus and cytosol of transfected HEK-293 cells incubated with wortmannin or stimulated with IGF-1 and serum. A total of 800 cells from four different experiments were analysed. The proportion of cells with a predominantly nuclear location (N) or predominantly cytoplasmic staining (C) are presented for wild-type and mutant FKHR in the wortmannin-treated (—) and IGF-1/serum-stimulated state (+).

ation of the three residues targeted by PKB (Thr²⁴, Ser²⁵⁶ and Ser³¹⁹) was similar in the wild-type and S329A mutant in the presence or absence of IGF-1 (results not shown).

Purification and identification of a protein kinase that phosphorylates FKHR at Ser³²⁹ specifically

We found that the Ser³²⁹ kinase contaminating His-tagged PKB α was present at similar levels in Ni-NTA-agarose eluates from control *Sf*21 cell lysates transfected with empty vectors. This indicated that the Ser³²⁹ kinase copurified with His-tagged PKB because it bound to Ni-NTA-agarose, implying the presence of a 'natural' His tag in this enzyme. We found a similar activity in several mammalian cell extracts when they were passed through Ni-NTA-agarose and analysed in the same manner. These included HEK-293 cells, rat brain, rat liver and rabbit skeletal muscle. In HEK-293 cells, the Ser³²⁹ kinase activity was not increased by stimulation for 10 min with IGF-1 (results not

Figure 7 Ser³²⁹ kinase phosphorylates the synthetic peptide Woodtide

(A) The peptide KKISGRSLSPIMTEQ (50 μ M) was phosphorylated for 10 min by a partially purified preparation of the Ser³²⁹ kinase from rabbit skeletal muscle, chromatographed on a Vydac C₁₈ column and ³²P radioactivity released after each cycle of Edman degradation was measured by solid-phase sequencing of the phosphopeptide as in Figure 2. (B,C) The Ser³²⁹ kinase from the final Mono S column of the purification from rabbit skeletal muscle (Table 1) was assayed by its ability to phosphorylate Woodtide (○ in B) or GST-FKHR at Ser³²⁹ (C). Phosphorylation of GST-FKHR at Ser³²⁹ was detected with the specific anti-pSer³²⁹ antibody as described in Figures 3(A) and 4. The diagonal line shows the NaCl gradient. A₂₈₀ is shown (●).

Table 1 Purification of the Ser³²⁹ kinase from rabbit skeletal muscle

A total of 2500 g of muscle was used in this preparation.

Step	Volume (ml)	[Protein] (mg/ml)	Total protein (mg)	Activity		Purification (fold)	Yield (%)
				(units per mg)	(Total units)		
1. Extract	8000	15	116800				
2. 35% (NH ₄) ₂ SO ₄ pellet	182	31	5642				
3. Ni-NTA-agarose	50	0.042	2.1	9	19	1	100
4. Heparin-Sepharose	5	0.06	0.3	40	12	4.4	63
5. Mono S	0.5	0.1	0.05	100	5	11.1	26

Figure 8 DYRK co-purifies with the Ser³²⁹ kinase

Fractions from the final chromatography on Mono S (Table 1) were assayed for Woodtide kinase activity (A) and analysed after electrophoresis on a SDS/10% polyacrylamide gel (B,C). In (B), the fractions were first incubated for 10 min with 10 mM magnesium acetate/0.1 mM [γ -³²P]ATP (10⁶ c.p.m./nmol) in the standard Ser³²⁹ kinase assay buffer and autoradiographed after electrophoresis. In (C), the gel was immunoblotted with anti-DYRK1A antibody after transfer to nitrocellulose membranes. The positions of the molecular-mass markers β -galactosidase (166 kDa) and transferrin (76 kDa) are indicated.

shown), a time at which activation of PKB is maximal [20]. This was consistent with the failure of IGF-1 to stimulate the phosphorylation of Ser³²⁹ (Figure 4).

The nickel-NTA-agarose eluate was capable of phosphorylating the synthetic peptide Woodtide, corresponding to the amino acid sequence surrounding Ser³²⁹ (see the Materials and methods section). Phosphorylation of this peptide occurred specifically at the position equivalent to Ser³²⁹ and not at the

Table 2 Identification of the major autophosphorylating band in Figure 8(B) as DYRK1A

Tryptic peptides from the major autophosphorylating band in Figure 8(B) were analysed on a Perspective Biosystems Elite STR MALDI-TOF mass spectrometer as described in the Materials and methods section. The tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the SF-FIT program of Protein Prospector. The three highest ranked protein entries that matched the submitted peptide ions were the mouse (MP86), rat (RP86) and human (HP86) DYRKs (Swiss-Prot accession numbers Q61214, Q63470 and Q13627 respectively). The Table summarizes the peptides from the rabbit Ser³²⁹ kinase that matched the murine DYRK1A protein kinase. The asterisk (*) indicates a methionine sulphone derivative; the double (**) indicates a pyrrolidone carboxylic acid derivative.

Mass		Peptide (residue nos.)	Peptide sequence
Measured	Theoretical		
881.46	881.40	459–465	MLDYDPK
904.51	904.56	452–458	FKDLILR
1101.55	1101.59	180–188	VEQEWAIVK
1288.73	1288.70	195–205	AFLNQAQIEVR
1514.77	1514.74	305–317	IVDFGSSCQLGQR
1567.85	1567.81	394–406	FFELPDGTWSLK
1604.92	1604.89	422–437	KLHNILGVETGGPGGR
1625.82	1625.80	155–167	WMDRYEIDSLIGK
1641.82	1641.79	155–167	WMDRYEIDSLIGK*
1641.82	1641.84	468–480	IQPYALQHSFFK
1676.92	1676.85	213–215	HDTEMKYIVHLK
1910.14	1910.11	373–390	IVEVLGIPPAHILDQAPK
3373.65	3373.55	710–740	QETGIAGHPTYQFSANTGPAHYMTEGHLAMR
3405.71	3405.54	710–740	QETGIAGHPTYQFSANTGPAHYMTEGHLAMR*
4107.12	4107.06	45–80	QPSISDQQVSALPYSDQIQQLTNQVMPDVMLQRR**

other serine or the threonine residue (Figure 7A). The Woodtide kinase activity co-purified through several chromatographic steps with the Ser³²⁹ kinase activity (Figure 7B), which was detected by phosphorylation of the FKHR protein followed by immunoblotting with the anti-pSer³²⁹ antibody (Figure 7C). The faster Woodtide kinase assay was therefore used to monitor the Ser³²⁹ kinase activity routinely during its purification.

The procedure for purifying the Ser³²⁹ kinase from rabbit skeletal-muscle extracts is described in the Materials and methods section, and a typical purification is summarized in Table 1. Activity could not be detected prior to Ni-NTA-agarose. However, pilot experiments in which different (NH₄)₂SO₄ fractions were passed through Ni-NTA-agarose established that the activity was largely precipitated at 35% (w/v) (NH₄)₂SO₄. About 50 µg of partially purified enzyme was isolated from 2.5 kg of muscle. Assuming a 50% recovery at each of the first two steps, the protein kinase was purified over 200 000-fold with an overall recovery of 10%.

After the final chromatography on Mono S, each fraction was incubated with Mg[γ-³²P]ATP and subjected to SDS/PAGE and autoradiography. This revealed a major autophosphorylating band of apparent molecular mass 85 kDa (Figure 8B) that co-eluted with the Woodtide kinase activity (Figure 8A). This band was excised, eluted and subjected to tryptic mass fingerprinting, revealing a close match to DYRK1A (Table 2). The binding of DYRK1A to Ni-NTA-agarose is consistent with the stretch of 13 consecutive histidine residues in the sequence [25]. The presence of DYRK1A was confirmed by immunoblotting with an antibody raised against recombinant DYRK1A, which recognized an 85 kDa protein that was co-eluted with the major autophosphorylated band (Figure 8C). Finally, we demonstrated that the majority of the Woodtide kinase activity in peak fractions from Mono S, was immunoprecipitated by an anti-DYRK1A antibody raised in sheep (results not shown).

The presence of DYRK1A in the final preparation suggested, but did not prove, that it was the Ser³²⁹ kinase. A GST-DYRK1A protein was therefore expressed in *E. coli* and purified by affinity

chromatography as described in the Materials and methods section. This preparation phosphorylated Woodtide specifically at the residue equivalent to Ser³²⁹ (results not shown). Moreover, the specific activity of the bacterially expressed kinase (150 units/mg at 50 µM Woodtide) was similar to that of the rabbit muscle preparation, which ranged from 90 to 100 units/mg for several different preparations. Conversely, the Ser³²⁹ kinase from rabbit skeletal muscle phosphorylated DYRKtide (RRFRPASPLRGPPK), a synthetic peptide shown previously to be phosphorylated by DYRK1A [19]. DYRKtide (50 µM) was phosphorylated 20% faster than Woodtide by either bacterially expressed DYRK1A or the Ser³²⁹ kinase from skeletal muscle.

The Ser³²⁹ kinase from rabbit skeletal muscle and bacterially expressed DYRK1A were matched for Woodtide kinase activity and used to phosphorylate the FKHR protein. After digestion with trypsin and chromatography on the Vydac C₁₈ column, the same major tryptic phosphopeptide was obtained after phosphorylation by the two preparations (Figures 9A and 9B). Edman sequencing and MS revealed that both peptides corresponded to the peptide comprising residues 317–354 phosphorylated specifically at Ser³²⁹ (Figures 9C and 9D). In conjunction with the results presented above, this establishes that the Ser³²⁹ kinase and DYRK1A are one and the same protein.

Co-localization and co-immunoprecipitation of FKHR and DYRK1A

If DYRK1A is the protein kinase responsible for phosphorylating Ser³²⁹ in cells, it should be present in the same subcellular compartment. As reported previously, DYRK1A is largely localized in the nucleus [23]. FKHR is also mainly present in the nucleus of unstimulated cells, although some FKHR is cytosolic. Both DYRK1A and FKHR showed a similar punctate staining pattern in unstimulated cells and a significant proportion of each protein co-localized when the images were merged (Figure 10). We were also able to demonstrate that antisera raised against FKHR were capable of immunoprecipitating transfected GFP–

Figure 9 Ser³²⁹ kinase from rabbit skeletal muscle and bacterially expressed DYRK1A both phosphorylate FKHR specifically at Ser³²⁹

GST-FKHR (2 μM) was phosphorylated for 30 min by the Ser³²⁹ kinase from rabbit skeletal muscle (A) and bacterially expressed DYRK1A (B) as described in the Materials and methods section, then digested with trypsin, chromatographed on a Vydac C₁₈ column and analysed exactly as described in the legend to Figure 1. In each case one major tryptic ${}^{32}\text{P}$ -labelled peptide, Ta or Tb, was obtained. The major ${}^{32}\text{P}$ peptides Ta and Tb, from (A) and (B), were analysed by Edman degradation and solid-phase sequencing in (C) and (D) respectively to determine the amino acid sequences and sites of phosphorylation, as described in the legend to Figure 2.

DYRK1A from the nuclear extracts of HEK 293T cells (Figure 11A). Conversely, when GFP-DYRK1A and FKHR were co-transfected into HEK-293 cells, FKHR could be immunoprecipitated with an anti-GFP antibody (Figure 11B).

DISCUSSION

In the present study we have identified Ser³²⁹ as a new site in FKHR that is phosphorylated in mammalian cells and have demonstrated that its mutation to alanine (to prevent phosphorylation) increases both the ability of FKHR to stimulate transactivation and the proportion of FKHR that is present in the nucleus (Figure 6B). Ser³²⁹ is conserved not only in all the mammalian FKHR homologues (FKHR, FKHL1 and AFX), but also in the *C. elegans* homologue DAF16 (Figure 12). The striking conservation of Ser³²⁹ suggests that the phosphorylation of this residue is likely to play an important role(s) in regulating FKHR homologues in diverse organisms.

We purified a protein kinase that phosphorylates FKHR at Ser³²⁹ specifically and identified it as DYRK1A. The key step in the purification was Ni-NTA-agarose and the presence of 13 consecutive histidines between residues 607 and 619 of DYRK1A [25] explains this unusual property. In the present study, DYRK1A-like activity (presumably a DYRK homologue) was detected as a contaminant in the preparation of His-tagged PKB α from insect *Sf21* cells. However, it should be noted that nearly all His-tagged proteins that are expressed in eukaryotic cells, and that have only been subjected to a one-step purification on Ni-NTA-agarose, are likely to be contaminated with a DYRK homologue.

DYRK1A and the closely related DYRK1B are localized in the nucleus [23,26], and in the present study we demonstrate that DYRK1A co-localizes with FKHR in discrete regions of the nucleus (Figure 10). We also found that DYRK1A and FKHR can be co-immunoprecipitated from nuclear extracts. These findings are consistent with the idea that DYRK1A phosphorylates FKHR at Ser³²⁹ *in vivo*. The genome of *C. elegans* also encodes a homologue of DYRK1A (accession number Z70308).

DYRK1A was identified while sequencing the Down's syndrome 'critical region' on chromosome 21 [25,27–30]. A three-fold overexpression in mice of a smaller (180 kb) DNA sequence within this region, of which the gene encoding DYRK1A accounts for over 100 kb, generates animals with learning defects [31]. The *Drosophila* (fruitfly) homologue of DYRK1A is termed 'minibrain', because mutant adults in which the expression of its encoding gene is disrupted lack neurons in certain regions of the brain [32]. These observations suggest an important role for DYRK1A in post-embryonic neurogenesis and in learning defects associated with Down's syndrome. In this connection, therefore, it is noteworthy that the FKHL1 isoform is implicated in the control of apoptosis in cerebellar granule neurons [5].

A closely related isoform of DYRK1A, termed DYRK1B, has been identified in mammalian cells. Four further isoforms, DYRK2, DYRK3, DYRK4A and DYRK4B [23], have also been identified that are less similar, sharing 40–50% identity with DYRK1A in the catalytic domain. All forms of DYRK, apart from DYRK1A, lack the stretch of consecutive histidine residues, explaining why they do not bind to Ni-NTA-agarose and are not co-purified with DYRK1A. It would be interesting to know whether the other DYRK isoforms phosphorylate FKHR specifically at Ser³²⁹. However, DYRK2, DYRK3 and DYRK4 lack the N-terminal nuclear localization sequence, explaining why DYRK2 is present in the cytosol [23]. In addition, DYRK2, DYRK3 and DYRK4 are expressed predominantly in

Figure 10 Intra-nuclear co-localization of FKHR and DYRK1A

Vectors expressing FKHR and GFP–DYRK1A were expressed and transiently transfected into COS7 cells. The expressed proteins were detected with a monoclonal anti-GFP antibody and a polyclonal anti-FKHR antiserum respectively and stained with FITC or TRITC-labelled secondary antibodies. Immunofluorescence was revealed by confocal microscopy. The merged image is shown in the middle panel. The scale bars shown represent a distance of 10 μm. Further details are given in the Materials and methods section.

Figure 11 Co-immunoprecipitation of endogenous FKHR and transfected DYRK1A in HEK-293T cells

(A) Cells were transiently transfected with a vector expressing GFP–DYRK1A. Nuclei were isolated by hypotonic lysis, and DYRK1A was immunoprecipitated (IP) from the nuclear extracts with a polyclonal anti-GFP (αGFP) or polyclonal anti-FKHR antiserum (αFKHR), but not with normal rabbit serum (NRS). Cell lysate (10 μg of protein) was transferred to nitrocellulose and detected by immunoblotting with the monoclonal anti-DYRK1A antibody. (B) The experiment was carried out as in (A), except that the cells were co-transfected with vectors expressing GFP–DYRK1A and FKHR, and FKHR was immunoprecipitated from the nuclear extracts with αFKHR or αGFP, but not with normal rabbit serum (NRS). Proteins were transferred to nitrocellulose and detected by immunoblotting with the polyclonal FKHR antibody. Similar results were obtained in another independent experiment.

the testis, while DYRK1B is found mainly in testis and muscle [23,26]

All DYRK homologues from mammals to yeast possess two tyrosine residues in a Tyr-Xaa-Tyr motif located in the ‘activation loop’ between kinase subdomains VII and VIII. DYRK1A expressed in *E. coli* is phosphorylated on tyrosine, but a catalytically inactive mutant is not. Moreover, mutation of both tyrosine residues of the Tyr-Xaa-Tyr motif to phenylalanine generates an inactive enzyme that is not phosphorylated on tyrosine [25], and the yeast homologue Yak1 can be inactivated by prior incubation with protein-tyrosine phosphatases [33]. These observations suggest that phosphorylation at one or both of the two tyrosine residues in the activation loop is essential for DYRK1A to phosphorylate exogenous substrates on serine/threonine residues. In the present study we have obtained no evidence that DYRK1A activity is regulated by reversible phosphorylation *in vivo*. The protein kinase was equally active in unstimulated and IGF-1-stimulated cells, consistent with the similar phosphorylation of FKHR at Ser³²⁹ under these conditions. However, it is possible that other signals may regulate DYRK1A by modulating the activity of a protein-tyrosine phosphatase that dephosphorylates the Tyr-Xaa-Tyr motif. It is also possible that these tyrosine residues are not phosphorylated *in vivo* by DYRK1A, but by a distinct protein kinase that has yet to be identified and whose activity is modulated by extracellular signals.

The phosphorylation of FKHR at Ser³²⁹ by DYRK1A (Figure 9) is consistent with the reported preference of this protein kinase for a proline residue located immediately C-terminal to the site of phosphorylation [19]. However, other important determinants of specificity must exist, because none of the other eight Ser-Pro and Thr-Pro sequences in FKHR were phosphorylated by DYRK1A. In contrast, we found that another ‘proline-directed’ protein kinase, the mitogen-activated protein kinase homologue ‘ERK2’ (extracellular-signal-regulated protein kinase 2), phosphorylated a distinct Ser-Pro sequence in FKHR preferentially, although it also phosphorylated Ser³²⁹ at a lower rate (Y. L. Woods, unpublished work). DYRK1A phosphorylates other proteins at similar rates to FKHR, as described in the

Protein	Species	Sequence surrounding Ser ³²⁹
FKHR	Human	<u>T</u> <u>I</u> S <u>G</u> <u>R</u> <u>L</u> S [*] <u>P</u> <u>I</u> <u>M</u> <u>T</u> <u>E</u> <u>Q</u>
FKHRL1	Human	<u>T</u> <u>V</u> S <u>G</u> <u>R</u> <u>L</u> S <u>P</u> <u>I</u> <u>M</u> <u>A</u> <u>S</u> <u>T</u>
AFX	Human	<u>S</u> <u>V</u> S <u>T</u> <u>R</u> <u>L</u> S <u>P</u> <u>L</u> <u>R</u> <u>P</u> <u>E</u> <u>S</u>
DAF16	<i>C. elegans</i>	<u>G</u> <u>S</u> S <u>S</u> <u>R</u> <u>V</u> S <u>P</u> <u>A</u> <u>I</u> <u>G</u> <u>S</u> <u>D</u>

Figure 12 Amino acid sequences of FKHR homologues surrounding Ser³²⁹

Identities are shown in **boldface** type and conservative replacements are underlined. Ser³²⁹ is marked with an asterisk.

following paper [36], suggesting that this protein kinase is likely to have more than one role *in vivo*.

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