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 (Thr208, Ser329 and Ser391) 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.

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**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), FKHRL1 and AFX (acute-lymphocytic-leukaemia-1 fused gene from chromosome X1). Subsequent work has established that PKB phosphorylates FKHR, FKHRL1 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, FKHRL1 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 FKHRL1 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...
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 Ser\(^{259}\) 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. Ser\(^{259}\) is conserved in FKHR1 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 (TYYADFIASGRTGRRNAIHD), was synthesized by Mr F. B. Cruddwell 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). Ni\(^{2+}\)-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 containing an array of three insulin response sequences im-

**Antibodies**

A phosphopeptide was synthesized corresponding to residues 324–334 of FKHR (ISGRL\(^{39}\)SPMTE, where \(^{39}\) indicates phosphoserine) and conjugated to both keyhole-limpet haemo-

**Cell culture, transient transfections and cell lysis**

HEK-293 cells were cultured at 37 °C in an atmosphere of 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) foetal-calf serum. Transfection of the cells was carried out using the CaCl\(_2\) precipitation method, employing 10 \(\mu\)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]/% (w/v) TritonX-100/1 mM EDTA/50 mM NaF/10 mM so-

**Luciferase reporter experiments**

HEK-293 cells were transfected with 10 \(\mu\)g of plasmid DNA/ dish, including 3 \(\mu\)g of a luciferase reporter gene construct containing an array of three insulin response sequences im-

**Immunoprecipitation of FKHR from HEK-293-cell extracts**

Cell lysate (500 \(\mu\)g of protein) was added to 10 \(\mu\)l of packed Protein G-Sepharose attached to 10 \(\mu\)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 lysates were then centrifuged at 13 000 g and the supernatants removed, frozen immediately in liquid nitrogen and stored at −80 °C until use.

**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\(\mu\)g/ml and, before use, phospho-specific antibodies were preincubated with the unphosphorylated form of the relevant phosphopeptide immunogen (10 \(\mu\)g/ml).

**Purification of Ser\(^{259}\) 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 Blender 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 10000 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 column equilibrated in 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 0.02, 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 collected and analysed as described in the Results section.

Protein kinases

Vectors encoding Myc-tagged PDK1 [17] and full-length GST–PKBz [18] (obtained from Dr M. Deak in the MRC Protein Phosphorylation Unit in Dundee) were expressed in HEK-293 cells and GST–PKBz was purified on GSH–Sepharose. The vector encoding GST–DYRK1A[1–500] [19] was expressed in Escherichia coli, and the protein purified on GST–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 PKBz 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 Ser177) [20] and His₆–Myc–PKD1[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 (KKISGRSLPIMTEQ). Assays were carried out at 30 °C using 50 μM Woodtide in 50 mM Tris/HCl (pH 7.5)/0.1 mM EGTA/2.5 μM PKI/10 mM magnesium acetate/0.1 mM [γ-32P]ATP (10 ε 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 μg/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-cyano-4-hydroxycinnamic 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/ucsfhtml3.4/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 μg of plasmid DNA encoding FKHR (in pcDNA3.1) and GFP–DYRK1A (in pEGFP-C1) [23], using the Fugene 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 NaVO₃ 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 Heps (pH 7.9)/1 mM EDTA/1 mM EGTA/20 mM NaF/1 mM NaVO₃/1 mM Na₃P₂O₅/1 mM dithiothreitol (DTT)/0.5 mM PMSF/1 mM benzamidine], 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 Heps (pH 7.9)/420 mM NaCl/1 mM EDTA/1 mM EGTA/20% (v/v) glycerol/20 mM NaF/1 mM Na₃P₂O₅/1 mM Na₃P₂O₅/1 mM DTT/0.5 mM PMSF/1 mM benzamidine]. 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 Ser²⁹⁹ as a novel phosphorylation site in FKHR

PKBz, that had been expressed as a His-tagged fusion protein in S/21 cells and affinity-purified by chromatography on 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 ²³⁵P-labelled tryptic peptides, namely T1, T2a and T3 (Figure 1A), consistent with the known phosphorylation of FKHR at three residues (Thr³¹, Ser²⁵⁶ and Thr³¹⁹, see the Introduction). The peptides were identified by Edman sequencing and MS. Peptide T1 (AASMDNNSKFASKSR), 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) …, revealed that it was a diphosphorylated derivative phosphorylated at Ser²⁵⁹ as well as at the expected Ser³¹⁹ (Figure 2).

The sequence surrounding Ser²⁵⁹ does not conform to the consensus for phosphorylation by PKB, which is Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr–, suggesting that Ser²⁵⁹ 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 Ser²⁵⁹ 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 ²³⁵P radioactivity by Cerenkov counting. The major ²³⁵P-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. ²³⁵P 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.

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In order to investigate whether Ser\(^{329}\) was a site of phosphorylation in vivo, we raised an antibody against a peptide comprising residues 324–334 of FKHR, phosphorylated at Ser\(^{329}\). 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 Thr\(^{24}\) 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-pSer\(^{329}\) antibody was further established by competition studies, which showed that it was neutralized by the phosphopeptide antigen used to raise it, but not by the corresponding unphosphorylated peptide or by phosphopeptides encompassing each of the three authentic PKB phosphorylation sites on FKHR (Figure 3B).

The anti-pSer\(^{329}\) antibody was then used to examine whether Ser\(^{329}\) is phosphorylated in transfected mammalian cells. These studies revealed that phosphorylation of GST–FKHR at Ser\(^{329}\) occurs under basal conditions, and that it is not increased by exposure to IGF-1 or after co-transfection with PDK1, the ‘upstream’ activator of PKB (Figure 4A). In contrast, stimulation with IGF-1 or co-transfection with PDK1 enhanced phosphorylation of the sites targeted by PKB, such as Thr\(^{24}\) (Figure 4A), in the same extracts. We also found that Ser\(^{329}\) phosphorylation of transfected FKHR was not altered by stimulation for up to 60 min with epidermal growth factor, carbachol, the calcium ionophore ionomycin, PMA, amino acid deprivation or exposure to UV-C radiation (results not shown).

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 Ser\(^{329}\) 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, re-fed 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.

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 (+).

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

We found that the Ser329 kinase contaminating His-tagged PKBz 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 shown).

The 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).
Kinase DYRK1A phosphorylates the transcription factor FKHR at Ser$^{329}$

Figure 7 Ser$^{329}$ kinase phosphorylates the synthetic peptide Woodtide

(A) The peptide KKISGLSPIMTEQ (50 \( \mu \)M) was phosphorylated for 10 min by a partially purified preparation of the Ser$^{329}$ kinase from rabbit skeletal muscle, chromatographed on a Vydac C$_{18}$ column and $^{32}$P radioactivity released after each cycle of Edman degradation was measured by solid-phase sequencing of the phosphopeptide as in Figure 2. (B, C) The Ser$^{329}$ 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 Ser$^{329}$ (●). Phosphorylation of GST–FKHR at Ser$^{329}$ was detected with the specific anti-pSer$^{329}$ antibody as described in Figures 3(A) and 4. The diagonal line shows the NaCl gradient. A$_{280}$ is 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 Ser$^{329}$ (Figure 4).

The nickel-NTA–agarose eluate was capable of phosphorylating the synthetic peptide Woodtide, corresponding to the amino acid sequence surrounding Ser$^{329}$ (see the Materials and methods section). Phosphorylation of this peptide occurred specifically at the position equivalent to Ser$^{329}$ and not at the shown). A total of 2500 g of muscle was used in this preparation.

Table 1 Purification of the Ser$^{329}$ kinase from rabbit skeletal muscle

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<th>Step</th>
<th>Volume (ml)</th>
<th>[Protein] (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Activity (units per mg)</th>
<th>(Total units)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<td>0.3</td>
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<td>5</td>
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other serine or the threonine residue (Figure 7A). The Woodtide kinase activity co-purified through several chromatographic steps with the Ser\(^{329}\) kinase activity (Figure 7B), which was detected by phosphorylation of the FKHR protein followed by immuno-blotting with the anti-pSer\(^{329}\) antibody (Figure 7C). The faster Woodtide kinase assay was therefore used to monitor the Ser\(^{329}\) kinase activity routinely during its purification.

The procedure for purifying the Ser\(^{329}\) 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 (NH\(_4\))\(_2\)SO\(_4\) fractions were passed through Ni-NTA–agarose established that the activity was largely precipitated at 35\% (w/v) (NH\(_4\))\(_2\)SO\(_4\). About 50 \(\mu\)g 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\(^{2+}\)\(\cdot\)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 major autophosphorylating band (Figure 8C). Finally, we demonstrated that the majority of the Woodtide kinase activity in peak fractions revealed 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 Ser\(^{329}\) 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 Ser\(^{329}\) (results not shown). Moreover, the specific activity of the bacterially expressed kinase (150 units/mg at 50 \(\mu\)M Woodtide) was similar to that of the rabbit muscle preparation, which ranged from 90 to 100 units/mg for several different preparations. Conversely, the Ser\(^{329}\) kinase from rabbit skeletal muscle phosphorylated DYRKtide (RRRFRPASPLRGPPK), a synthetic peptide shown previously to be phosphorylated by DYRK1A [19]. DYRKtide (50 \(\mu\)M) was phosphorylated 20% faster by Woodtide than by either bacterially expressed DYRK1A or the Ser\(^{329}\) kinase from skeletal muscle.

The Ser\(^{329}\) 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 C\(_{18}\) 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 Ser\(^{329}\) (Figures 9C and 9D). In conjunction with the results presented above, this establishes that the Ser\(^{329}\) 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 Ser\(^{329}\) 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–

<table>
<thead>
<tr>
<th>Table 2 Identification of the major autophosphorylating band in Figure 8(B) as DYRK1A</th>
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<tbody>
<tr>
<td>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 (RP66) and human (HP86) DYRKs (Swiss-Prot accession numbers Q61214, Q63470 and Q13627 respectively). The Table summarizes the peptides from the rabbit Ser(^{329}) kinase that matched the murine DYRK1A protein kinase. The asterisk (<em>) indicates a methionine sulphone derivative; the double (**) indicates an entry that matched the submitted peptide ions were the mouse (MP86), rat (RP66) and human (HP86) DYRKs (Swiss-Prot accession numbers Q61214, Q63470 and Q13627 respectively). The Table summarizes the peptides from the rabbit Ser(^{329}) kinase that matched the murine DYRK1A protein kinase. The asterisk (</em>) indicates a methionine sulphone derivative; the double (**) indicates an entry that matched the submitted peptide ions were the mouse (MP86), rat (RP66) and human (HP86) DYRKs (Swiss-Prot accession numbers Q61214, Q63470 and Q13627 respectively).</td>
</tr>
<tr>
<td>Mass</td>
</tr>
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<td>4107.12</td>
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Kinase DYRK1A phosphorylates transcription factor FKHR at Ser\(^{329}\)

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 Ser\(^{329}\) 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). Ser\(^{329}\) is conserved not only in all the mammalian FKHR homologues (FKHR, FKHR1L1 and AFX), but also in the C. elegans homologue DAF16 (Figure 12). The striking conservation of Ser\(^{329}\) 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 Ser\(^{329}\) 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\(^{\alpha}\) 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 Ser\(^{329}\) in vitro. 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 FKHR1L1 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–agarase and are not co-purified with DYRK1A. It would be interesting to know whether the other DYRK isoforms phosphorylate FKHR specifically at Ser\(^{329}\). 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
Y. L. Woods and others

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 antisera (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.

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.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Sequence surrounding Ser329</th>
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<tbody>
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<td>FKHR</td>
<td>Human</td>
<td><strong>T</strong> <strong>I</strong> <strong>S</strong> <strong>G</strong> <strong>R</strong> <strong>L</strong> <strong>S</strong> <strong>P</strong> <strong>I</strong> <strong>M</strong> <strong>T</strong> <strong>E</strong> <strong>Q</strong></td>
</tr>
<tr>
<td>FKHR-L1</td>
<td>Human</td>
<td><strong>T</strong> <strong>Y</strong> <strong>S</strong> <strong>G</strong> <strong>R</strong> <strong>L</strong> <strong>S</strong> <strong>P</strong> <strong>I</strong> <strong>M</strong> <strong>T</strong> <strong>E</strong> <strong>Q</strong></td>
</tr>
<tr>
<td>AFX</td>
<td>Human</td>
<td><strong>S</strong> <strong>Y</strong> <strong>S</strong> <strong>T</strong> <strong>R</strong> <strong>L</strong> <strong>S</strong> <strong>P</strong> <strong>I</strong> <strong>M</strong> <strong>T</strong> <strong>E</strong> <strong>Q</strong></td>
</tr>
<tr>
<td>DAF16</td>
<td>C. elegans</td>
<td><strong>G</strong> <strong>S</strong> <strong>S</strong> <strong>R</strong> <strong>V</strong> <strong>S</strong> <strong>P</strong> <strong>A</strong> <strong>I</strong> <strong>G</strong> <strong>S</strong> <strong>D</strong></td>
</tr>
</tbody>
</table>

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 Ser239 at a lower rate (Y. L. Woods, unpublished work). DYRK1A phosphorylates other proteins at similar rates to FKHR, as described in the

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

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REFERENCES


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