Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting

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The transcription factor, forkhead in rhabdomyosarcoma (FKHR), is phosphorylated at three amino acid residues (Thr-24, Ser-256 and Ser-319) by protein kinase B (PKB)α. In the present study, mutagenesis has been used to study the roles of these phosphorylation events in regulating FKHR function in transfected HEK-293 cells. We find that the overexpression of FKHR[S256A] (where Ser-256 → Ala) blocks PKB activity in cells, preventing phosphorylation of the endogenous substrates FKHRL1 and glycogen synthase kinase-3. Thus some reported effects of overexpression of this and other mutants may be indirect, and result from suppression of the phosphorylation of other sites on FKHR and/or other PKB substrates. For example, we have shown that Thr-24 phosphorylation alone is critical for interaction with 14-3-3 proteins, and that the substitution of Ser-256 with an alanine residue indirectly blocks 14-3-3 protein binding by preventing the phosphorylation of Thr-24. We also found that insulin-like growth factor (IGF)-1 and serum-induced nuclear exclusion of FKHR[S256A] depends on the degree of overexpression of this mutant. Our results indicated that the interaction of FKHR with 14-3-3 proteins was not required for IGF-1-stimulated exclusion of FKHR from the nucleus. We present evidence in support of another mechanism, which depends on the phosphorylation of Ser-256 and may involve the masking of a nuclear localization signal. Finally, we have demonstrated that the failure of IGF-1 to suppress transactivation by FKHR[S256A] is not explained entirely by its failure to bind 14-3-3 proteins or to undergo nuclear exclusion. This result suggests that Ser-256 phosphorylation may also suppress transactivation by FKHR by yet another mechanism, perhaps by disrupting the interaction of FKHR with target DNA binding sites and/or the function of the transactivation domain.

Key words: apoptosis, FKHR, insulin-like growth factor-1, protein kinase B, 14-3-3 proteins.

INTRODUCTION

It is now well established that protein kinase B (PKB, also called Akt) plays a critical role in mediating many of the effects of insulin and related growth factors, downstream from phosphatidylinositol (PI) 3-kinase (reviewed in [1–3]) and 3-phosphoinositide-dependent protein kinase-1 (PDK1). PKB mediates these effects by phosphorylating key regulatory proteins at serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-motifs [4], and well established substrates include glycogen synthase kinase (GSK)-3 [5,6], the type 3B cAMP phosphodiesterase [7] and several closely related members of the forkhead in rhabdomyosarcoma family of transcription factors (FKHR, FKHRL1 and AFX) [8–14].

FKHR, FKHRL1 and AFX are each phosphorylated by PKB at three residues *in vitro*, and, in co-transfection experiments with FKHR, these were shown to be Thr-24, Ser-256 and Ser-319 [9–14]. All three residues are conserved in the *Caenorhabditis elegans* homologue DAF16, and there is strong genetic evidence that DAF16 lies downstream of PKB homologues in a pathway that controls the life span of this invertebrate [15]. Phosphorylation of FKHR, FKHRL1 and AFX triggers nuclear exit [8,9,13,16,17], binding to 14-3-3 proteins [9] and inhibits transactivation in mammalian cells [9,10,18]. Transactivation by FKHR, FKHRL1 and AFX requires an intact insulin-response sequence (IRS) in the case of the insulin-like growth factor (IGF)-binding-protein-1 promoter [9,10,18], and the direct binding of FKHR to this sequence has been reported [19]. A similar, but not identical, sequence in the promoter of the Fas ligand, may be required for FKHRL1 to stimulate the transcription of this gene [9]. Overexpression of an FKHRL1 mutant, in which the amino acid residues corresponding to the PKB phosphorylation sites are substituted with an alanine residue, promotes apoptosis by a Fas-ligand-dependent mechanism. Therefore FKHRL1 may contribute to the PI 3-kinase-dependent, PKB-mediated inhibition of apoptosis that is triggered by survival factors [2]. Similarly, it has been reported that the overexpression of mutated AFX blocks cell cycle progression via the induction of p27kip1, suggesting that the PKB-mediated inactivation of AFX might promote oncogenesis [20].

It is unclear whether inhibition of the transactivation function of FKHR, FKHRL1 and AFX results from binding of 14-3-3 proteins and/or nuclear exit, or whether phosphorylation triggers inhibition directly by, for example, altering the interaction of these proteins with DNA or other *trans*-acting factors. In the present study, these problems have been addressed by mutating each of the three phosphorylation sites in FKHR and examining the effect on phosphorylation of the other sites, 14-3-3 binding,

nuclear exclusion and transactivation. Our results indicate that the regulation of these processes by IGF-1 and serum is more complex than has previously been supposed.

MATERIALS AND METHODS

Restriction enzymes were purchased from NEB (U.K.) Ltd (Hitchin, Herts, U.K.) and MBI (Vilnius, Lithuania). Antibodies, raised against 14-3-3 proteins (sc 628, sc629, sc731, sc732 and sc1019), were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and were used at $2\,\mu$g/ml final concentration. Mouse monoclonal glutathione S-transferase (GST) antibodies were from Sigma (Poole, Dorset, U.K.), and secondary anti-mouse antibodies coupled to fluorescein were from Stratech Scientific (Luton, Beds., U.K.). An FKHRL1 antibody and a phospho-specific antibody that recognizes FKHRL1 only when phosphorylated at Thr-32 were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A GSK-3α-specific antibody and a phosphospecific antibody that recognizes the Ser-21-phosphorylated form of GSK-3α was prepared by Dr Jane Leitch and Ms Carla Clark (MRC Unit, University of Dundee, Dundee, Scotland, U.K.). A phosphospecific antibody that recognizes PKB only when phosphorylated at Thr-308 was from NEB (Hitchin, Herts, U.K.). Sources of other materials have been given previously [12] and are also available from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

Cell culture, transient transfections and cell lysis

Human embryonic kidney, HEK-293, cells were cultured and transfected with FKHR as described previously [12]. Cells were serum starved for 20 h before stimulation with IGF-1 (100 ng/ml) alone or in combination with 10 % (v/v) foetal-calf serum (FCS). Cells were lysed in 1 ml of ice-cold buffer A [50 mM Tris/acetate (pH 7.5), 1 mM EGTA, 1 % (w/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM benzamidine, 0.2 mM PMSF and 0.1 % (v/v) 2-mercaptoethanol]. The lysates were centrifuged at 13 000 g, the supernatants were removed, frozen immediately in liquid nitrogen and stored at $-80\,°$C until use.

Expression, purification and phosphorylation of wild-type and mutated GST–FKHR

Mutations of Thr-24, Ser-256 and Ser-319 in FKHR were created by site-directed mutagenesis in pAlter.MAX (Promega), using single-stranded DNA templates and synthetic oligonucleotides, according to the manufacturer's instructions and as described previously [18]. Wild-type and mutant forms of FKHR in the vector pAlterMAX were subcloned into the TA cloning vector pCR2.1 to express haemagglutinin (HA)-tagged FKHR, in which the initiator methionine was removed. The DNA expressing HA–FKHR was then digested with *Bam*H1 and *Xba*1 at the cleavage sites in the poly-linker region and subcloned into the *Bam*H1 and *Spe*1 sites of the pEBG-2T vector. The final construct encoded GST immediately followed by HA–FKHR (hereafter termed GST–FKHR) and was used for expression in mammalian cells. Each construct was sequenced to establish the accuracy of the DNA construct.

Expressed GST–FKHR was batch-purified by glutathione–Sepharose affinity-column chromatography. Transfected cell lysate (200 μg of protein) was purified on 10 μl of a 1:1 slurry of glutathione–Sepharose beads in buffer A, then washed twice with buffer A containing 150 mM NaCl and twice in buffer A. Bound proteins were eluted with 0.2 M glutathione (pH 8) and then

prepared for SDS/PAGE and immunoblotting or overlay assays. The latter were performed using digoxygenin-labelled 14-3-3 in place of the primary antibody, followed by a digoxygenin–horseradish peroxidase secondary antibody.

In vitro phosphorylation of FKHR was performed as reported previously [12]. Each reaction was performed with 0.1 μM GST–FKHR in the presence of 1–3 units/ml PKB.

Confocal microscopy

HEK-293 cells, grown on coverslips, were transfected with wild-type or mutant forms of vectors encoding GST–FKHR. After 20 h of serum starvation, they were treated for 10 min with the PI 3-kinase inhibitor wortmannin (100 nM) or for 30 min with 10 % (v/v) FCS plus 100 ng/ml IGF-1 before fixing in methanol/2-N-morpholinoethane sulphonic acid (pH 6.9) (9:1, v/v), which had been cooled to $-20\,°$C. Cells were immunostained successively with a GST antibody and a secondary anti-mouse antibody coupled to fluorescein. Cell nuclei were counterstained for DNA with propidium iodide (1 μg/ml) and the coverslips were mounted in Hydromount [with 2.5 % (v/v) 1,4-diazobicyclo(2,2,2)octane ('DABCO') anti-fade]. The trans-fected cells were imaged using a Zeiss laser scanning confocal microscope 410 with a $\times 63$ plan-apo(NA1-4) objective.

Wild-type and mutant FKHR were also cloned into the pEGFP-N vector (Clontech) for expression of FKHR–green fluorescent protein (GFP) fusion proteins. Experiments with FKHR–GFP constructs were carried out, without fixation, in transfected living cells in 10 mM Hepes-buffered Dulbecco's modified Eagle's medium, in a chamber warmed to 37 $°$C.

Reporter gene studies

HEK-293 cells (1×10^6/60-mm dish) were plated in Opti-MEM with 5 % (v/v) bovine-calf serum, and transfected with calcium phosphate precipitates containing 10 μg/dish plasmid DNA and including 3 μg of a luciferase reporter-gene construct containing an array of three IRSs immediately upstream of the 81 bp thymidine kinase promoter (TK.IRS3), 3 μg of FKHR expression vectors, 2 μg of a cytomegalovirus (CMV)-driven vector ex-pressing β-galactosidase, and empty vector [18]. Cells were rinsed and re-fed with serum-free Dulbecco's modified Eagle's medium containing 1 mg/ml fatty-acid-free BSA 24 h before the addition of IGF-1 (100 ng/ml final concentration). Cell lysates were prepared 12 h later. Luciferase activity was measured using an Optocomp-I luminometer (MGM Instruments). Each experiment was performed in triplicate and repeated at least three times.

RESULTS

Mutation S256A blocks the phosphorylation of FKHR at Thr-24 and Ser-319 in HEK-293 cells and *in vitro*

Previous studies have shown that PKB can phosphorylate FKHR at Thr-24, Ser-256 and Ser-319 in cells [8,12], and that over-expression of FKHR[S256A] strongly suppresses the insulin-induced increase in overall phosphorylation of this mutant, although the sites affected were not identified [17]. In the present study, there was little phosphorylation of wild-type GST–FKHR in unstimulated cells and, as expected, all three sites became maximally phosphorylated in 15–30 min when transfected cells were stimulated with IGF-1 (Figure 1A). The half time for phosphorylation of each site was 2–5 min. In contrast, the mutation S256A completely prevented phosphorylation at Thr-24 and Ser-319 (Figure 1A, lanes 7 and 8, numbering from the left) but the mutation T24A did not prevent phosphorylation at
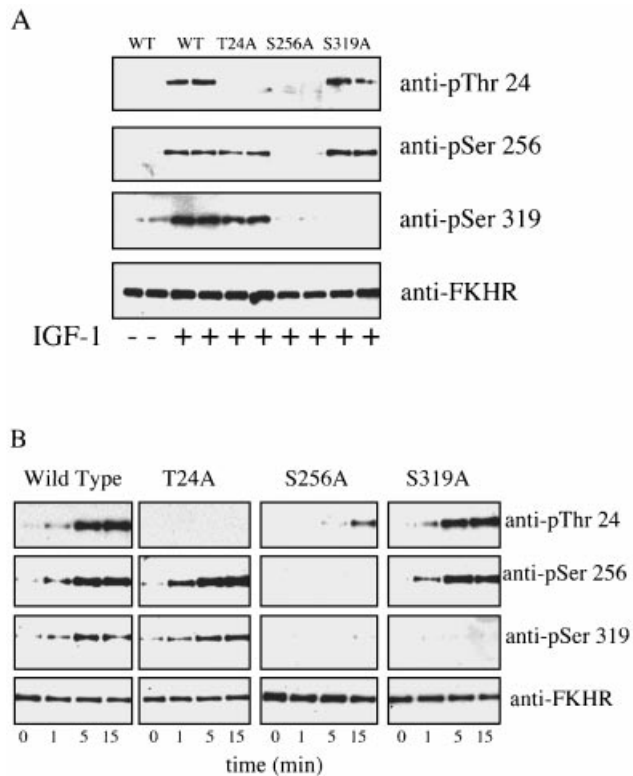
A

B

Figure 1   Phosphorylation of FKHR at Thr-24 and Ser-319 in IGF-1-stimulated HEK-293 cells and *in vitro* is blocked by mutation of Ser-256 to Ala

(**A**) Cells were transfected with 10 μg of vectors expressing wild-type (WT) or mutant GST–FKHRs, as shown above the panels. At 24 h post-transfection the cells were serum starved for an additional 20 h before treatment for 20 min with (+) or without (−) IGF-1. Cell lysates (10 μg) were separated by SDS/PAGE (10% gels), and gels were blotted with anti-peptide antibodies that recognize FKHR only when phosphorylated at Thr-24 (anti-pThr 24), Ser-256 (anti-pSer 256) or Ser-319 (anti-pSer 319). Samples were also immunoblotted with an antibody that recognizes dephosphorylated and phosphorylated FKHR equally well (anti-FKHR). (**B**) Cells were transfected with 10 μg of wild-type or mutant GST–FKHR, as shown above the panels. At 24 h the cells were serum starved for a further 20 h before treatment with 100 nM wortmannin for 10 min. The GST–FKHR was purified from lysates on glutathione–Sepharose and incubated with 1 unit/ml PKB and MgATP (see the Materials and methods section). Aliquots from the reaction mixture were taken at the times shown and separated by SDS/PAGE (10% gel); gels were immunoblotted with anti-peptide antibodies as described for (**A**). The characterization of the FKHR antibodies has been described in [12].

Ser-256 or Ser-319 (Figure 1A, lanes 5 and 6), and the mutation S319A did not prevent the phosphorylation of Thr-24 and Ser-256 (Figure 1A, lanes 9 and 10).

Similar results were obtained when each mutant was phosphorylated by PKB *in vitro* (Figure 1B). The mutation T24A did not affect the rate of phosphorylation at Ser-256 or Ser-319 and the mutation S319A did not prevent the phosphorylation of Thr-24 and Ser-256. However, the mutation S256A greatly reduced the rate of phosphorylation of Thr-24 and Ser-319 by PKB *in vitro*, demonstrating that this is an intrinsic property of the mutant protein. The protein is not likely to be misfolded, since it retains full transactivation activity (see below).

Mutants of FKHR are dominant-negative inhibitors of PKB

The observation that the mutation S256A prevented phosphorylation of Thr-24 and Ser-319 *in vitro* (Figure 1B) and in transfected HEK-293 cells (Figure 1A) could be explained in
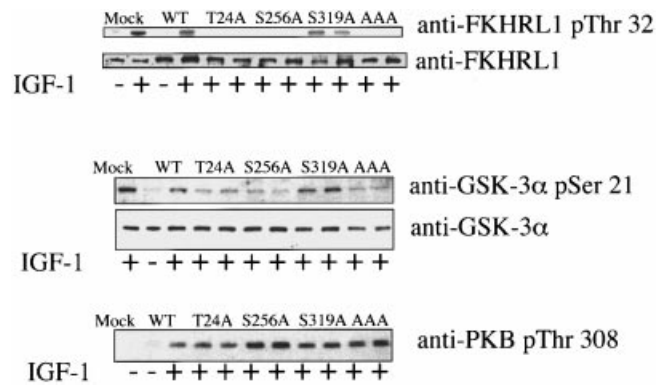

Figure 2   Dominant-negative effects of overexpressing GST–FKHR[T24A] or GST–FKHR[S256A]

Cells were mock-transfected without DNA (Mock) or transfected with wild-type (WT) or singly-mutated forms of FKHR, as shown above the panels, as well as with a vector expressing FKHR mutated at Thr-24, Ser-256 and Ser-319 (AAA). Cell lysates (20 μg protein) were separated by SDS/PAGE (10% gel), and gels were transferred to nitrocellulose and then immunoblotted with anti-peptide antibodies: (top panel) that recognize FKHRL1 only when phosphorylated at Thr-32 (anti-FKHRL1 pThr 32) or total FKHRL1 (anti-FKHRL1); (middle panel) GSK-3α phosphorylated at Ser-21 (anti-GSK-3α pSer 21) or total GSK-3α (anti-GSK-3α); (bottom panel) PKB phosphorylated at Thr-308 (anti-PKB pThr 308).

two ways. First, this mutation might prevent a conformational change, triggered by the phosphorylation of Ser-256, which is required in order to make the other sites accessible to PKB, as in the hierarchical model suggested previously [17]. Secondly, the S256A mutant might have inhibited PKB, preventing it from phosphorylating Thr-24 and Ser-319. This second explanation appears to be correct, because overexpression of GST–FKHR[S256A] or the GST–FKHR[T24A/S256A/S319A] triple mutant strongly suppressed the phosphorylation of endogenous FKHRL1 and endogenous GSK-3α, another established substrate of PKB [1,3] (Figure 2). Overexpression of the GST–FKHR[S256A] or the GST–FKHR[T24A/S256A/S319A] mutant did not alter endogenous levels of GSK-3α or FKHRL1, or block the phosphorylation of PKB at Thr-308 (Figure 2), a site that is essential for the activation of PKB by IGF-1 [1].

Overexpression of GST–FKHR[T24A] also prevented phosphorylation of endogenous FKHRL1 and GSK-3α (Figure 2). However, unlike GST–FKHR[S256A] (Figure 1B) and the GST–FKHR[T24A/S256A/S319A] triple mutant (results not shown), GST–FKHR[T24A] had no effect on the phosphorylation of Ser-256 and Ser-319 *in vitro* (Figure 1B) or in transfected HEK-293 cells (Figure 1A). Since Ser-256 and Ser-319 are both phosphorylated efficiently by PKB in GST–FKHR[T24A], overexpression of this mutant may prevent the phosphorylation of endogenous FKHRL1 and GSK-3α in cells by competing as an alternative substrate. Overexpression of GST–FKHR[S319A] did not inhibit the phosphorylation of endogenous FKHRL1 or GSK-3α.

In order to study the effects of mutation on the subcellular localization of FKHR, we prepared wild-type and mutant forms of FKHR fused to GFP. Surprisingly, and in contrast to GST–FKHR, there was no suppression of phosphorylation of the endogenous FKHRL1 at Thr-32 or GSK-3α at Ser-21 in HEK-293 cells transfected with phosphorylation site mutants of FKHR–GFP (results not shown), indicating that these mutants do not act as dominant-negative inhibitors of PKB. This may be explained by the far lower level of expression of FKHR–

GFP proteins in HEK-293 cells relative to that of GST–FKHR proteins.

Binding of wild-type and mutated FKHR to 14-3-3 proteins following IGF-1 stimulation

It has been reported that phosphorylated FKHRL1 binds to 14-3-3ζ when both proteins are overexpressed in HEK-293T cells [9]. In that study, the surprising observation was made that the mutation S256A, which does not itself conform to either of the canonical 14-3-3 binding motifs, Arg-Ser-Xaa-pSer/pThr-Xaa-Pro- or Arg-Xaa-Tyr/Phe-Xaa-pSer/pThr-Xaa-Pro- [21–23], decreased 14-3-3 binding equally as well as the mutation T24A, a residue that lies within a 14-3-3 binding consensus motif. In the present work, we examined whether the inhibitory effect of the S256A mutation on Thr-24 phosphorylation could be the mechanism by which this mutant blocks 14-3-3 binding. We expressed transfected GST–FKHR into HEK-293 cells and performed 'pull-down' studies to examine interactions between phosphorylated FKHR and the endogenous 14-3-3 proteins in these cells. As shown in Figure 3(A), the binding of all 14-3-3 proteins to FKHR was weak under basal conditions, but was strongly stimulated by IGF-1. Binding was greatly decreased in cells that had been preincubated with wortmannin (Figure 3B), a PI 3-kinase inhibitor that suppresses the phosphorylation of FKHR at all three sites [12]. Immunoblotting with antibodies specific for particular isoforms of 14-3-3 showed similar binding of 14-3-3γ/η and 14-3-3ζ (Figure 3B), as well as 14-3-3β and 14-3-3θ (results not shown). The binding of FKHR to endogenous 14-3-3 proteins was abolished by the mutations T24A or S256A. The effect of the S256A mutation was presumably indirect, resulting from the dominant-negative effect of this mutant to block Thr-24 phosphorylation (Figure 1). In contrast, the mutation S319A had minimal effect on 14-3-3 binding (Figure 3A).

In order to confirm our results using an independent method, we measured the binding of digoxygenin-labelled yeast 14-3-3 isoforms using the same FKHR samples in gel overlay experiments. In this more sensitive assay, the mutations T24A and S256A also blocked 14-3-3 binding (Figure 3C). These results confirm that the inability of FKHR[S256A] and FKHR[T24A] to bind 14-3-3 proteins in cells is an intrinsic property of these proteins and cannot be explained by other effects, such as the localization of the mutants. Taken together with results obtained previously by others [9], our data demonstrate that the mutations T24A and S256A block the binding of 14-3-3 to FKHR.

Since Thr-24 is the only 14-3-3 binding consensus in FKHR, we asked whether phosphorylation of Thr-24 alone was sufficient to allow 14-3-3 binding in vitro, using a S256D mutant of FKHR, which cannot be phosphorylated at position 256. Like wild-type FKHR, FKHR[S256D] showed slight binding to digoxygenin-labelled 14-3-3 proteins in vitro, which was greatly increased after phosphorylation by PKB in vitro. The level of binding was similar to that of wild-type FKHR that had been phosphorylated by PKB (Figure 3D). No binding of 14-3-3 proteins to FKHR[256D] was observed in unstimulated HEK-293 cells. Stimulation with IGF-1 induced 14-3-3 binding, but to a lesser extent than with wild-type FKHR (Figure 3C). This is consistent with the decreased phosphorylation of FKHR[S256D] at Thr-24 (relative to wild-type FKHR), under these conditions (results not shown). Our results demonstrate that phosphorylation of Thr-24 is necessary and sufficient for binding of FKHR to 14-3-3 proteins, and strongly suggests that the effect of the mutation S256A is indirect, because of the dominant-negative inhibition of Thr-24 phosphorylation.
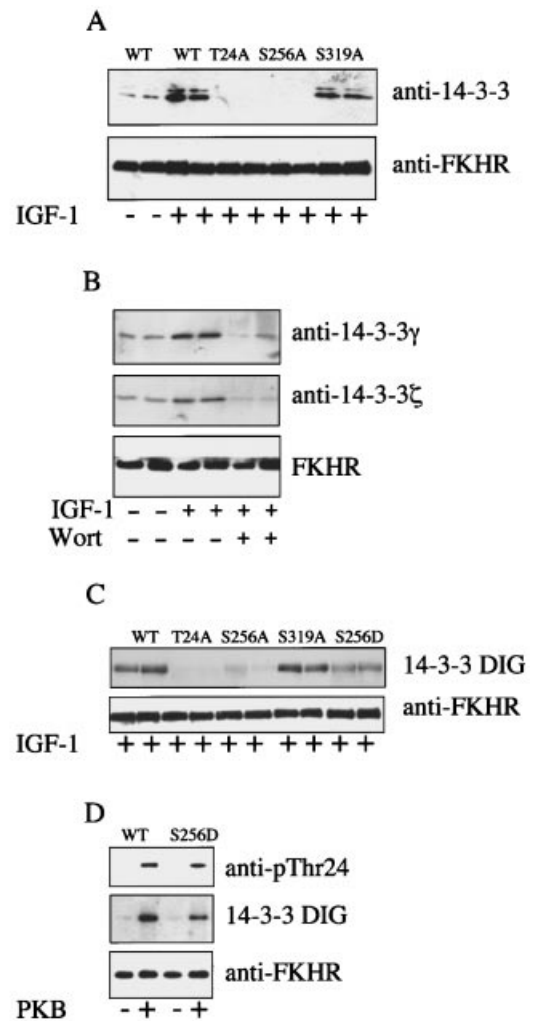

Figure 3   Binding of wild-type and mutant forms of FKHR to 14-3-3 isoforms in HEK-293 cells and in vitro

(**A**) Cells were transfected with 10 μg of wild-type (WT) or mutant GST–FKHR (T24A, S256A, S319A). At 24 h post-transfection the cells were serum starved for a further 20 h, then stimulated for 20 min with IGF-1 (100 ng/ml) (+). The GST–FKHR was purified from cell lysates on glutathione–Sepharose (see the Materials and methods section), separated by SDS/PAGE (10% gel) and gels were immunoblotted with antibodies that recognized all 14-3-3 isoforms (anti-14-3-3), as well as anti-FKHR. (**B**) As for (**A**), except that dishes were treated for 10 min with or without 100 nM wortmannin (Wort) and immunoblotted with antibodies raised against 14-3-3γ (anti-14-3-3γ) or 14-3-3ζ (anti-14-3-3ζ) or with anti-FKHR. (**C**) As for (**A**), except that after separation of the purified GST–FKHR proteins by SDS/PAGE and transfer to nitrocellulose, the gels were overlaid with a digoxygenin-labelled 14-3-3 binding peptide probe (14-3-3 DIG). A second gel was run in parallel and the membranes were probed with an anti-FKHR antibody. (**D**) As for (**C**), except that GST–FKHR proteins were purified on glutathione–Sepharose and incubated with MgATP for 1.5 h without or with 3 units/ml PKB before 14-3-3 overlay. The results shown are representative of those obtained in several similar experiments.

Role of phosphorylation in controlling the subcellular localization of FKHR

It has been reported previously that epitope-tagged FKHR [8,17] and FKHRL1 [9] are present in the nucleus of unstimulated cells and excluded from the nucleus when the cells are stimulated with growth factors [17], serum [8] or serum and growth factors used in combination [9]. In the present study, we confirmed these results in HEK-293 cells transfected with either
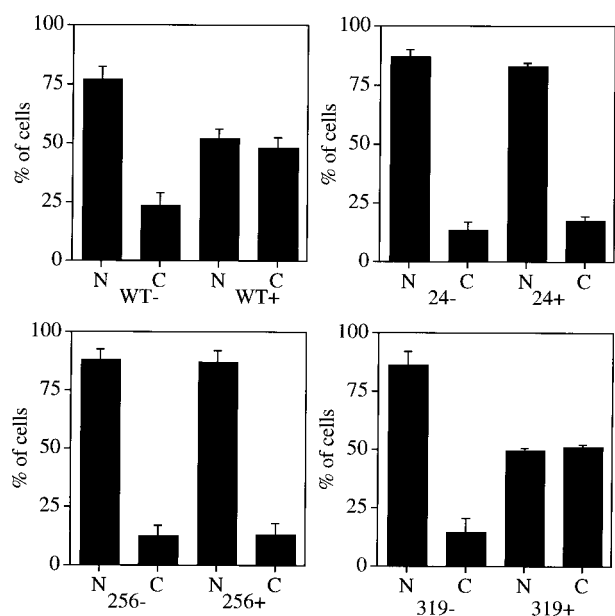
Figure 4   Quantification of the proportion of wild-type and mutant forms of FKHR in the nucleus and cytosol of HEK-293 cells before and after stimulation with IGF-1 and serum

Cells were transfected as described for Figure 1, except that, after 20 h of serum starvation, they were treated for 10 min with 100 nM wortmannin or for 30 min with 100 ng/ml IGF-1 plus 10% (v/v) FCS before fixation. 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 (WT) and mutant FKHR (24, 256, 319) in the wortmannin-treated (−) and IGF-1/serum-stimulated state (+). The values are the means ± S.E.M. of three determinations.

GST-tagged (Figure 4) or GFP-tagged wild-type FKHR (Figure 5). In studies with GST–FKHR, we compared the distribution of tagged proteins in cells treated with the PI 3-kinase inhibitor wortmannin (to block PKB activation) to cells treated with IGF-1 plus serum. In contrast, the GFP constructs enabled us to examine the effect of IGF-1 and serum on the distribution of tagged FKHR in living cells. For this reason, we did not pretreat the cells with wortmannin in these experiments and examined the localization of FKHR–GFP by repeated scanning of the same field. The images presented in Figure 5(A) may be viewed at http://www.BiochemJ.org/bj/354/bj3540605add.htm

Wild-type FKHR, tagged by either method, was largely nuclear in unstimulated cells but underwent translocation to the cytosol within 30 min of stimulation with serum and IGF-1 (Figure 4, upper left panel and Figure 5, topmost two panels). However, when we studied the localization of the FKHR mutants, there were striking differences between the GST-tagged and GFP-tagged proteins. GST–FKHR[S256A] and GST–FKHR[T24A] (Figure 4), were almost entirely nuclear in unstimulated cells and underwent little translocation in response to serum plus IGF-1 (Figure 4). In contrast, the corresponding FKHR–GFP mutants were already partially (S256A) or largely (T24A) cytosolic in unstimulated live cells and, in both cases, further translocation to the cytosol was stimulated by IGF-1 and serum (Figure 5). We suggest that the inability of GST–FKHR[S256A] and GST–FKHR[T24A] to be excluded from the nucleus following growth-factor stimulation is a dominant-negative effect, because of the high level of expression of these proteins. The mutation S319A did not prevent the ability of IGF-1 to suppress translocation, indicating that phosphorylation of this residue is not essential for

IGF-1-stimulated nuclear exit, although the mutation S319A also resulted in an increase in the proportion of GST–FKHR in the nucleus in the absence of IGF-1 (Figure 4).

Role of Ser-256 in nuclear exclusion

Previous studies have shown that the signal responsible for nuclear targeting is located somewhere within the DNA binding domain of forkhead/winged-helix proteins [8,24]. Many nuclear localization signals comprise sequences that are enriched in basic residues [25]. In FKHR, three consecutive arginines (residues 251–253) that are part of this putative nuclear location site also lie within the Ser-256 PKB consensus phosphorylation motif (Arg-Arg-Arg-Ala-Ala-Ser-) as well as the FKHR DNA binding domain. We therefore considered the possibility that these three residues may be critical for nuclear targeting of FKHR and that phosphorylation of Ser-256 may disrupt this function. As shown in Figure 5(B, right panel), the substitution of these three arginine residues with neutral amino acids completely disrupted nuclear targeting of FKHR–GFP in unstimulated cells. Placing an aspartate residue at Ser-256, to mimic phosphorylation, also induced substantial cytoplasmic localization, even in unstimulated cells (Figure 5B, middle panel). Together, these results provide the first direct evidence that residues within the basic region of the FKHR DNA binding domain are essential for nuclear targeting, and that the introduction of a negative charge at this site is sufficient to disrupt this function.

Effect of mutation on transactivation by GST–FKHR and FKHR–GFP constructs

We compared the ability of IGF-1 to inhibit transactivation by wild-type and mutant GST–FKHR and FKHR–GFP proteins. In these studies we used a transactivation reporter gene construct in order to differentiate between dominant-negative effects (which would be seen only with GST–FKHR constructs) and the direct effects of these mutations (which would be seen in GST–FKHR and FKHR–GFP constructs). In these studies we performed co-transfection with appropriate expression vectors and luciferase reporter gene constructs containing an array of three IRSs immediately upstream of the 81 bp thymidine kinase promoter (TK81.IRS3) or the thymidine kinase promoter alone (TK81). The TK81 and TK81.IRS3 constructs had negligible basal activity in HEK-293 cells and co-transfection with wild-type GST–FKHR or FKHR–GFP stimulated promoter activity by more than 20-fold (results not shown). IGF-1 treatment decreased GST–FKHR-stimulated transcription by 60% and, in agreement with our previous results in HepG2 cells [18], this effect was blocked by an inhibitor of PI 3-kinase (LY 294002), but not by rapamycin or an inhibitor of the classical mitogen-activated protein kinase pathway (PD 98059) (results not shown). IGF-1 suppressed transactivation by GST–FKHR and FKHR–GFP to a similar extent (Figure 6).

Interestingly, the mutation T24A disrupted the effect of IGF-1 on transactivation by GST–FKHR but not by FKHR–GFP, indicating that this is a dominant-negative effect of this mutation, and that 14-3-3 binding is not a pre-requisite for inhibition of transactivation. In contrast, the mutation S256A disrupted the effect of IGF-1 on transactivation by both GST–FKHR and FKHR–GFP, indicating that this mutation blocks IGF-1 signalling to FKHR directly, independently of any dominant-negative effects. Presumably, a sufficient amount of FKHR–GFP remains in the nucleus, even after IGF-1 stimulation to saturate the IRSs of the reporter gene.

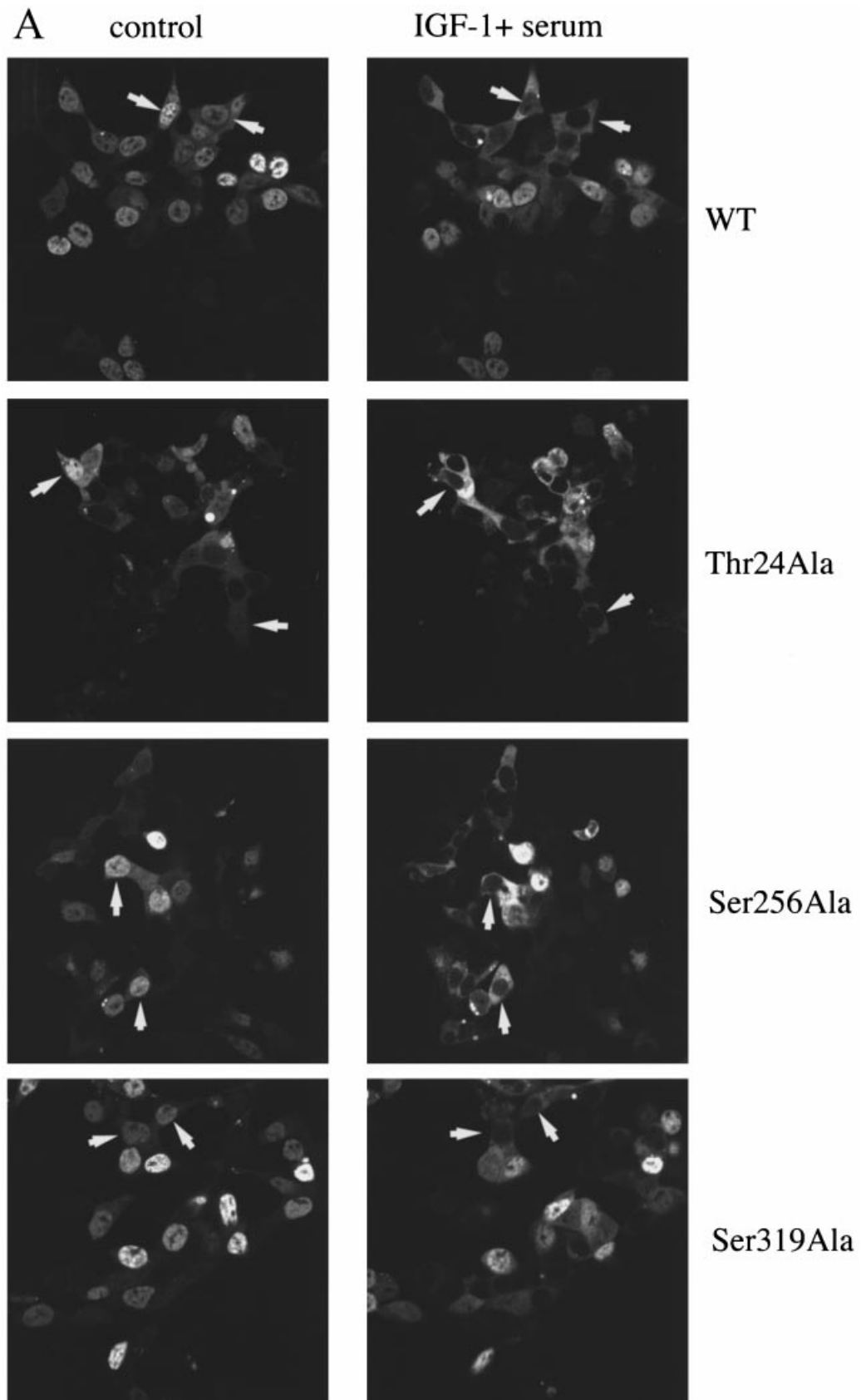The mutation S319A did not disrupt the ability of IGF-1 to suppress transactivation.

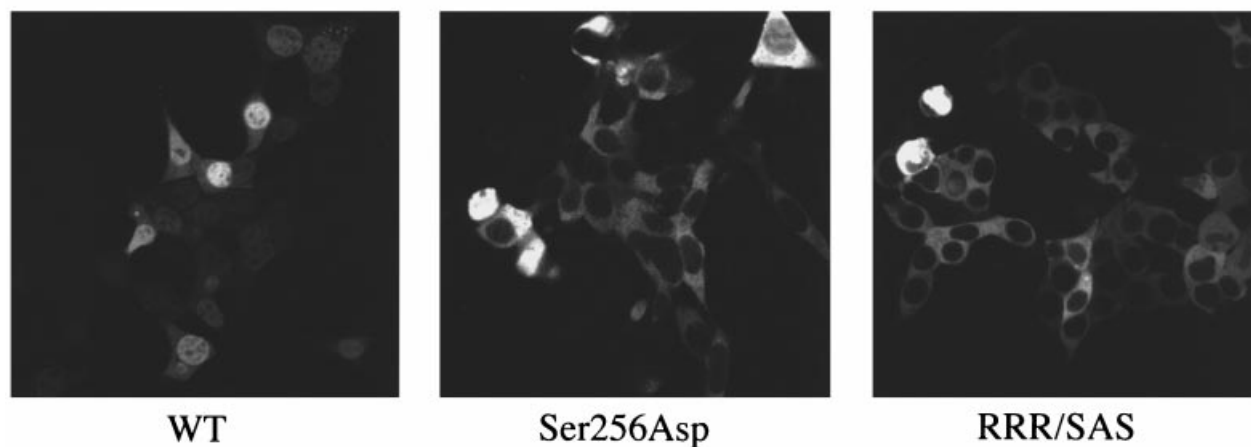**Figure 5 For legend see facing page**

Figure 5   Imaging of wild-type and mutant forms of FKHR–GFP in living cells

(**A**) Cells were transfected with FKHR–GFP as described for Figure 1, except that after 20 h of serum starvation they were treated for 90 min with 100 ng/ml IGF-1 plus 10% (v/v) FCS, and observed by confocal microscopy on a heated stage. FKHR fluorescence is shown for wild-type (WT), FKHR[T24A] (Thr24Ala), FKHR[S256A] (Ser256Ala) and FKHR[S319A] (Ser319Ala). Arrows indicate the same cells before and after stimulation with IGF-1 plus serum. These images may be viewed at http://www.BiochemJ.org/bj/354/bj3540605add.htm (**B**) Cells were transfected as in (**A**), except that fluorescence is shown in unstimulated cells transfected with wild-type (WT), FKHR[S256D] (Ser256Asp) or the FKHR[R251S/R252A/R253S] triple mutant (RRR/SAS).

DISCUSSION

In the present study, we demonstrate that the GST–FKHR[S256A] mutant acts as a dominant-negative inhibitor of PKB in transfected HEK-293 cells and thereby prevents the phosphorylation of PKB substrates, including the other sites on FKHR. This would appear to explain how the mutation S256A blocks phosphorylation of Thr-24 and Ser-319, without having to invoke an earlier idea [17] that the phosphorylation of Ser-256 induces a conformational change in FKHR which is required to make Thr-24 and Ser-319 accessible for phosphorylation. Moreover, our results also demonstrate that the phosphorylation of Thr-24 is necessary and sufficient for the binding of FKHR to 14-3-3 proteins (Figure 3) and strongly suggest that the effect of the S256A mutation in preventing 14-3-3 binding is indirect and is due to dominant-negative inhibition of Thr-24 phosphorylation. It has been reported that a 60 residue fragment of FKHR, immediately C-terminal to Ser-256, is necessary in order for the mutation S256A to prevent the phosphorylation of Thr-24 and Ser-319 [17]. It will therefore be interesting to examine whether this portion of FKHR is essential for the dominant-negative effect of the S256A mutation, or whether phosphorylation of Ser-256 also results in a conformational change in FKHR required for phosphorylation of Thr-24 and Ser-319.

Catalytically inactive and/or phosphorylation-site mutants of PKB are frequently used as 'dominant negative' mutants of this enzyme but, in most cases, are probably acting as dominant-negative inhibitors of PDK1, one of the 'upstream' activators of PKB. However, PDK1 activates many other protein kinases of the AGC subfamily *in vivo* [1,3], so that the observed effects may be explained by the inhibition of protein kinases other than PKB. The GST–FKHR[S256A] or GST–FKHR[T24A/S256A/S319A] mutants therefore may be useful as more specific inhibitors of PKB activity in cells. For example, it has been reported that at least half of the apoptotic effects caused by overexpressing FKHR[T24A/S256A/S319A] does not depend on the DNA binding activity of FKHR [14], indicating that indirect effects of FKHR mutants can readily be measured in functional readouts.

For these reasons, our observations with these FKHR mutants highlight the potential danger of using them to define roles for FKHR *in vivo*. The phenotypic effects of overexpressing phosphorylation site mutants of FKHR [8,14,16–18], FKHRL1 [9] or AFX [13,20] have suggested roles for these proteins in the
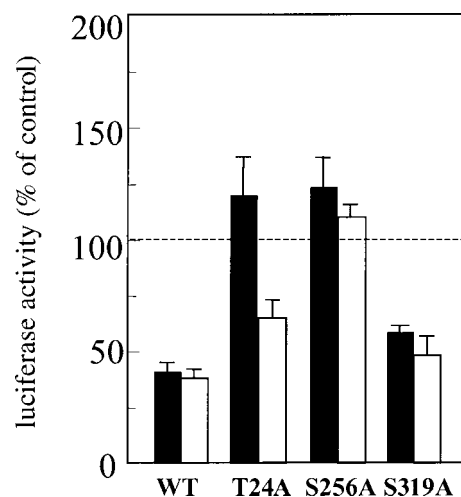

Figure 6   Effect of IGF-1 on transactivation by GST–FKHR and FKHR–GFP fusion proteins

HEK-293 cells were transfected with 10 μg/dish plasmid DNA, including 3 μg of a luciferase reporter gene construct containing an array of three IRSs immediately upstream of the 81 bp thymidine kinase promoter (TK.IRS3) [18], 3 μg of FKHR expression vector, 2 μg of a CMV-driven β-galactosidase vector and 2 μg of empty vector. Cells were re-fed with serum-free medium for 24 h then treated with/without 100 ng/ml IGF-1 for 12 h before harvesting. Basal TK.IRS3 promoter activity is negligible in HEK-293 cells, and luciferase activity largely reflects transactivation by FKHR. Luciferase activity is shown for wild-type (WT), T24A, S256A and S319A FKHR. Filled bars, GST–FKHR; open bars, FKHR–GFP. The values are the means $\pm$ S.E.M. of three determinations.

regulation of apoptosis [9,14] and cell cycle arrest [13,20]. It is possible that these mutants are able to suppress the phosphorylation of other PKB substrates. For example, the apoptotic effects of FKHR and FKHRL1 may simply result from blockade of the phosphorylation and inactivation of GSK-3, which has also been implicated in PKB-mediated regulation of apoptosis [26].

In contrast to the GST–FKHR mutants, FKHR–GFP[S256A] and FKHR–GFP[T24A] did not exhibit dominant-negative properties, because of the very low levels of expression. These mutants are capable of being excluded from the nucleus following stimulation with IGF-1 and serum (Figure 5A), demonstrating that at least one further mechanism(s), distinct from 14-3-3 binding, must contribute to nuclear exclusion. The inability of the GST–FKHR[T24A] and GST–FKHR[S256A] mutants to be excluded from the nucleus following growth-factor stimulation has been observed previously with myc-tagged mouse FKHR[S256A] and FKHR[T24A] [17]. This suggests that these mutants, like the corresponding GST mutants, might affect localization indirectly by exerting a dominant-negative effect.

In summary, our results indicate that the mechanism of nuclear exclusion of FKHR and its regulation by growth factors may be more complex than previously supposed and may involve more than one regulatory mechanism. We have shown that phosphorylation of Ser-256 may contribute to this process by masking a nuclear localization signal, and others have proposed that phosphorylation of Thr-24 may contribute to nuclear exclusion [9,17]. The inability of the dominant-negative GST–FKHR[S256A] mutant to be excluded from the nucleus suggests that phosphorylation of other PKB targets may also be required and that PKB activity is essential for nuclear exclusion of FKHR. Finally, our results indicate that the phosphorylation of Ser-256 may also suppress transactivation by FKHR by a mechanism that is independent of 14-3-3 binding or nuclear exclusion, perhaps by disrupting the function of the trans-activation domain *per se* [18] or by altering the interaction of FKHR with target DNA binding sites [27].

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