Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B*

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Protein kinase B lies “downstream” of phosphatidylinositol (PtdIns) 3-kinase and is thought to mediate many of the intracellular actions of insulin and other growth factors. Here we show that FKHR, a human homologue of the DAF16 transcription factor in Caenorhabditis elegans, is rapidly phosphorylated by human protein kinase Ba (PKBα) at Thr-24, Ser-256, and Ser-319 in vitro and at a much faster rate than BAD, which is thought to be a physiological substrate for PKB. The same three sites, which all lie in the canonical PKB consensus sequences (Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr)), became phosphorylated when FKHR was cotransfected with either PKB or PDK1 (an upstream activator of PKB). All three residues became phosphorylated when 293 cells were stimulated with insulin-like growth factor 1 (IGF-1). The IGF-1-induced phosphorylation was abolished by the PtdIns 3-kinase inhibitor wortmannin but not by PD 98059 (an inhibitor of the mitogen-activated protein kinase cascade) or by rapamycin. These results indicate that FKHR is a physiological substrate of PKB and that it may mediate some of the physiological effects of PKB on gene expression. DAF16 is known to be a component of a signaling pathway that has been partially dissected genetically and includes homologues of the insulin/IGF-1 receptor, PtdIns 3-kinase and PKB.

The conservation of Thr-24, Ser-256, and Ser-319 and the same three sites, which all lie in the canonical PKB consensus sequences (Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr)), became phosphorylated when FKHR was cotransfected with either PKB or PDK1 (an upstream activator of PKB). All three residues became phosphorylated when 293 cells were stimulated with insulin-like growth factor 1 (IGF-1). The IGF-1-induced phosphorylation was abolished by the PtdIns 3-kinase inhibitor wortmannin but not by PD 98059 (an inhibitor of the mitogen-activated protein kinase cascade) or by rapamycin. These results indicate that FKHR is a physiological substrate of PKB and that it may mediate some of the physiological effects of PKB on gene expression. DAF16 is known to be a component of a signaling pathway that has been partially dissected genetically and includes homologues of the insulin/IGF-1 receptor, PtdIns 3-kinase and PKB.

The conservation of Thr-24, Ser-256, and Ser-319 and the sequences surrounding them in DAF16 therefore suggests that DAF16 is also a direct substrate for PKB in C. elegans.

In recent years evidence has accumulated that many of the metabolic actions of insulin may be mediated by a protein kinase cascade that lies “downstream” of phosphatidylinositol (PtdIns) 3-kinase and the second messengers PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (reviewed in Refs. 1 and 2). A central player in this cascade is protein kinase B (PKB, also called c-Akt). This enzyme is activated when it becomes phosphorylated at Thr-308 and Ser-473 (3) by 3-phosphoinositide-dependent protein kinases 1 and 2 (PDK1, PDK2), respectively (4–7). The activation of PKB by PDK1 in vitro has an absolute requirement for PtdIns(3,4,5)P3 or PtdIns(3,4)P2 (4), and these mediators facilitate activation by binding to the pleckstrin homology domains of both PKB (5, 7) and PDK1 (8). Consistent with these observations, the phosphorylation of PKB at Thr-308, induced by either insulin or insulin-like growth factor 1 (IGF-1) is prevented by inhibitors of PtdIns 3-kinase (3). PDK2 has not yet been characterized although, like the phosphorylation of Thr-308, the insulin or IGF-1-induced phosphorylation of Ser-473 is prevented by inhibitors of PtdIns 3-kinase (3).

PKB mediates the metabolic actions of insulin by phosphorylating regulatory proteins at serine or threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr) motifs (9), of which the best characterized are the cardiac isoform of 6-phosphofructo-2-kinase (PFK2) (2, 10), the protein kinase glycogen synthase kinase 3 (GSK3) (11, 12), and the mammalian target of rapamycin (mTOR) (13), as well as the proapoptotic protein BAD (reviewed in Ref. 2). Phosphorylation by PKB activates cardiac PFK2, and this is thought to underlie the insulin-induced stimulation of glycolysis in the heart. Phosphorylation inhibits GSK3 and is thought to contribute to the stimulation of glycogen synthesis and global protein synthesis by insulin (1, 2). Phosphorylation by PKB activates mTOR, allowing it to catalyze several phosphorylation events that enhance the translation of specific proteins. The overexpression of PKB has also been shown to mimic other metabolic actions of insulin, such as the stimulation of glucose (14) and amino acid transport (15).

When cells are stimulated with IGF-1, PKB is initially translocated to the plasma membrane where it becomes activated by PDK1 and PDK2, but it subsequently accumulates in the nucleus (16). This raises the question of whether PKB mediates some of the effects of insulin on specific gene transcription, and several pieces of evidence would appear to support this contention. For example, the overexpression of constitutively active mutants of PKB mimics the effects of insulin in stimulating the transcription of the obesity gene product leptin (17) and in inhibiting the transcription of IGF-binding protein 1 (IGFBP-1) (18). The insulin-induced suppression of phosphoenolpyruvate carboxykinase (19) and IGFBP-1 (18) is prevented by inhibitors of PtdIns 3-kinase (wortmannin, LY 294002) and unaffected by the drugs that inhibit mTOR (rapamycin) or the classical mitogen-activated protein (MAP) kinase cascade (18, 19). Studies with constitutively active and dominant negative forms of PKB have shown that PKB may mediate transcriptional effects of insulin through a conserved insulin response sequence present in a number of genes known to be inhibited by insulin in the liver, such as IGFBP-1 and PEPCk (18). This suggests that PKB may indeed play an important role in mediating the...
effects of insulin on hepatic gene expression

The insulin/IGF-1-stimulated PKB cascade has also been identified in Caenorhabditis elegans, where it is known to stimulate metabolism, to inhibit dauer arrest and to shorten the life span of this nematode (reviewed in Ref. 20). In this pathway, which has been partially dissected by genetic techniques, the DAF2 gene encodes a homologue of the IGF-1 receptor and lies “upstream” of the AGEI gene that encodes a PtdIns 3-kinase homologue and the AKT1 and AKT2 genes that encode homologues of PKB. Downstream of PKB is the transcription factor DAF16; mutations in DAF16 return life span to normal that has been lengthened by inactivating mutations in AGEI or AKT1/2 (21, 22). Whether DAF16 is phosphorylated directly by AKT1/AKT2 is unknown, but we noticed that it possesses three consensus sequences for phosphorylation by PKB, all of which are highly conserved in several mammalian DAF16 homologues, namely the “forkhead” family members FKHR, FKHRL1, and AFX (23). Two of the three sites are conserved in a further DAF16 homologue encoded by the AFG21 gene (24). Here we establish that FKHR is phosphorylated at these three sites by PKB in vitro and in cotransfection experiments and that the same sites become phosphorylated in response to IGF-1 in 293 cells via a PtdIns 3-kinase-dependent pathway that is independent of mTOR or the classical MAP kinase cascade. The accompanying paper (25) demonstrates that FKHR-stimulated reporter gene expression is dependent on an intact insulin response sequence (IRS) and that transactivation by FKHR is inhibited by insulin via the phosphorylation of Ser-256 (25). The conservation of Thr-24, Ser-256, and Ser-319 and the sequences surrounding them in DAF16 suggests that DAF16 is likely to be a direct substrate for PKB in C. elegans. Taken together, these results indicate that PKB regulates the ability of FKHR to stimulate transcription.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture reagents and IGF-1 were purchased from Life Technologies, Inc. Laboratories (Paisley, Scotland) and protein G-Sepharose from Amersham Pharmacia Biotech (Milton Keynes, United Kingdom). PKI, the specific peptide inhibitor of the catalytic subunit of PKA (PP'YDFFASGRTGRRNAI), was synthesized by F. B. Caudwell in the MRC Protein Phosphorylation Unit and other peptides by Dr. G. Bloomberg (University of Bristol). Wortmannin was from Sigma (Poole, UK), PD 98059 and rapamycin from Calbiochem (Nottingham, UK), and the PCR cloning vectors pCR2.1 TOPO and pCR2.1 from Invitrogen (NV Leek, Holland). Restriction enzymes were purchased from NEB (UK Ltd.) (Hitchin, UK) and MBI (Vilnius, Lithuania). GST-BAD expressed in Escherichia coli was provided by Dr. Takayasu Kobayashi of this Unit. Vectors expressing Myc-tagged PKD1 (5) and GST-PKB(308D/S473D) (3) were provided by Dr. Maria Deak in the MRC Unit at Dundee.

Cell Culture, Transient Transfections, and Cell Lysis—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) fetal calf serum. Transfection of cells was carried out using the calcium chloride precipitation method, using 10 μg of DNA per 10-cm diameter dish. Prior to lysis, cells were serum-starved for 12 h. Cells were lysed in 1 ml ice-cold Buffer A (50 mM Tris acetate (pH 7.5), 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) β-mercaptoethanol). The lysates were centrifuged at 13,000 × g, and the supernatants were removed, frozen immediately in liquid nitrogen, and stored at −80°C until use.

Alignment, Cloning, Expression, and Purification of FKHR—Alignment, Cloning, Expression, and Purification of FKHR—Inspection of the public EST data bases identified 41 clones from 14 different tissues encoding vectors of FKHR. Alignment of the clones showed 100% identity with the nucleotide sequence of the second FKHR sequence to be reported (GenBank™ accession number AF032885 (26)). An epitope-tagged, full-length human FKHR construct was generated as follows. A fusion was generated in which the oligonucleotide 5′-GGCGG GATCC CGCCA CCATG GAGTT CATGC CCATG GAGCT-3′ encoding the epitope Glu-Phe-Met-Pro-Met-Glu (termed EE-tag) preceded the first 300 base pairs of FKHR (EST 1061191). This 350-base pair oligonucleotide produced the EE-FKHR fusion in a PCR reaction with a second oligonucleotide: 5′-GGCGG GCGGG CCCGG CGCGGC CGCCGC CCACC CCGGC CGCAG CGAG C3′ corresponding to bases 259–300 of the FKHR coding sequence. The PCR product was cloned into the TA cloning vector pCR2.1 TOPO. The remaining 1665 base pairs of FKHR (EST 166558) were incorporated into pCR2.1 by a non-PCR TA cloning method using the NotI and BspM restriction sites (27). A vector containing a construct of full-length FKHR was then generated by ligating the two halves of FKHR in pCR2.1. This construct was then subcloned into pCMV-5 and pEBG27 to overexpress recombinant FKHR in mammalian cells. Finally, the FKHR construct was subcloned into a pGEX4T-3 vector for expression of a GST-FKHR fusion protein in E. coli. Expressed GST-FKHR was purified by glutathione-Sepharose affinity chromatography and stored at −80°C in 50 mM Tris- HCl, pH 7.5 (20°C), 0.2 mM glutathione at a concentration of 2 mg/ml (4).

Generation of Phosphospecific Antibodies for FKHR—Phosphopeptides and dephosphopeptides were synthesized corresponding to residues 19–31 (RPRLCtwpGLPRFE), 248–262 (KSPRRRAaspMNDNSK), and 311–324 (TTFFRPSpSNASVS) of FKHR, where p indicates the site of phosphorylation (Thr-24, Ser-256, and Ser-319). The peptides were conjugated to both keyhole limpet hemocyanin and bovine serum albumin and injected into sheep at the Scottish Antibody Production Unit (Carluke, UK). Six weeks later, antiserum was passed through a CH-Sepharose column to which a dephosphopeptide had been coupled, followed by affinity chromatography on CH-Sepharose to which the corresponding phosphate antigen had been attached covalently. Phospho-specific antibodies were eluted with 0.1 M glycine, pH 2.4, immediately adjusted to pH 8 with Tris base and stored at 4°C. A further antibody was generated toward residues 636–651 of FKHR (LPNQSPFKVVTITTTrH) that recognizes the dephosphorylated as well as the phosphorylated form of the protein.

Activation of Protein Kinase B and Phosphorylation of FKHR and BAD—PKB (0.15–5.0 units/ml) that had been expressed in Sf9 cells and activated by phosphorylation with PKD1 (4) was incubated for 30 min at 30°C with FKHR (0.1 mg/ml) or BAD (0.05 mg/ml) in 50 mM Tris-HCl, 7.5% (v/v) EGTA, 2.5 μM PKI, 10 mM magnesium acetate, 0.1 mM ATP (30°C/pmol). One unit of PKB activity was that amount that catalyzed the phosphorylation of 1 nmol of the peptide GRPRTTSSFAEG in 1 min (11).

To determine the stoichiometry of phosphorylation of FKHR and BAD, aliquots of the reaction were denatured in SDS and subjected to polyacrylamide gel electrophoresis. After staining with Coomassie Blue and destaining, the gels were dried and the concentrations of GST-FKHR and GST-BAD were determined by densitometry using a Fuji-las LAS-1000 luminescent image analyzer calibrated with different concentrations of bovine serum albumin run in parallel on the same gel. The molecular masses of GST-FKHR and GST-BAD were taken as 97 kDa and 56 kDa, respectively.

RESULTS

Phosphorylation of FKHR by PKB in Vitro—The observation that FKHR contains three residues, located at Thr-24, Ser-256, and Ser-319, that lie in consensus sequences for phosphorylation by PKB (see introduction) led us to study the phosphorylation of this protein by PKB in vitro. FKHR was phosphorylated by PKB to a stoichiometry of >1 mol of phosphate/mol of protein (Fig. 1A). The initial rate of phosphorylation was much faster than that of the pro-apoptotic protein BAD (Fig. 1B), which is thought to be an in vivo substrate for PKB (29).

To identify the sites of phosphorylation, we raised phosphospecific antibodies that only recognize FKHR if it is phosphorylated at Thr-24, Ser-256, or Ser-319, that lie in consensus sequences for phosphorylation by PKB (see introduction) led us to study the phosphorylation of this protein by PKB in vitro. FKHR was phosphorylated by PKB to a stoichiometry of >1 mol of phosphate/mol of protein (Fig. 1A). The initial rate of phosphorylation was much faster than that of the pro-apoptotic protein BAD (Fig. 1B).

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In this study we have demonstrated a rapid phosphorylation of FKHR by PKB at Thr-24, Ser-256, and Ser-319. This occurs both in vitro (Figs. 2 and 3) and in cotransfection experiments (3). IGF-1 also stimulated the phosphorylation of FKHR at Thr-24, Ser-256, and Thr-319 (Fig. 5), maximal phosphorylation occurring within 10 min (data not shown). Consistent with the IGF-1-induced phosphorylation of FKHR being mediated by PKB, phosphorylation of Thr-24, Ser-256, and Ser-319 was prevented if the cells were incubated with the PtdIns 3-kinase inhibitor wortmannin (100 nM) prior to stimulation with IGF-1 (Fig. 5). The basal level of phosphorylation was also abolished by wortmannin.

Residues 24, 256, and 319 all lie in Arg-Xaa-Arg-Xaa-Xaa-(Ser/Thr) sequences that are not only consensus sequences for phosphorylation by PKB but also for phosphorylation by MAP kinase-activated protein kinase 1 (MAPKAP-K1, also called p90rsk) and p70 S6 kinase (9, 30). MAPKAP-K1 (which lies immediately downstream of MAP kinase) and p70 S6 kinase (which lies downstream of mTOR) are both activated in response to insulin or IGF-1, and their activation is also inhibited by wortmannin (31, 32). To investigate which of these protein kinases mediates the IGF-1-induced phosphorylation of FKHR in vivo, we therefore carried out additional experiments in which 293 cells were incubated with either rapamycin (which prevents the activation of p70 S6 kinase by inhibiting mTOR (33)) or PD 98059 (which prevents the activation of MAP kinase kinase-1 and hence the activation of MAPKAP-K1) (34). Neither of these drugs affected the basal or IGF-1-induced phosphorylation of FKHR at Thr-24, Ser-256, and Ser-332 (Fig. 5) of these drugs affected the basal or IGF-1-induced phosphorylation of FKHR at Thr-24, Ser-256, and Ser-332 (Fig. 5) of these drugs affected the basal or IGF-1-induced phosphorylation of FKHR at Thr-24, Ser-256, and Ser-332 (Fig. 5). The assignment of phosphorylation at Thr-24, Ser-256, and Thr-319 was also abolished by wortmannin.

**DISCUSSION**

In this study we have demonstrated a rapid phosphorylation of FKHR by PKB at Thr-24, Ser-256, and Ser-319. This occurs both in vitro (Figs. 2 and 3) and in cotransfection experiments (3).
We have also shown that all three sites on FKHR become rapidly phosphorylated when 293 cells are stimulated with IGF-1 (Fig. 5). The IGF-1-induced phosphorylation is prevented by inhibitors of PtdIns 3-kinase but not by inhibitors of the activation of the MAP kinase cascade or p70 S6 kinase. These experiments, together with the in vitro studies and cotransfection experiments, indicate that the IGF-1-induced phosphorylation of FKHR is mediated by PKB or a closely related enzyme.

A pathway in C. elegans has been partially dissected by genetic techniques in which DAF16 (an FKHR homologue) is a downstream component of a signaling cascade that includes homologues of the insulin/IGF-1 receptor, PtdIns 3-kinase, and PKB (see introduction). PDK1 has not yet been identified as a component of this pathway, perhaps because its inactivation has other (lethal) consequences stemming from its role in activating additional protein kinases that lie in distinct protein kinase cascades. This may explain why disruption of the two PDK1 homologues in Saccharomyces cerevisiae is also lethal (28). We have noticed that, as expected, the genome of C. elegans does indeed encode a PDK1 homologue located on the X chromosome (genomic clone number H33H01).

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Note Added in Proof—While this paper was under review, similar results to those presented here and in the following paper (25) have been found by three other laboratories. In analysis of Forkhead family members namely FKHR1 (36), AFX (37), and FKHR1 (38), phosphorylation was accompanied by nuclear export (36, 37) as a result of Ser-256 to alanine mimics it. Interestingly Ser-256 is the residue that has other (lethal) consequences stemming from its role in disrupting the kinase cascade leading to the regulation of DAF16 is present in mammalian cells. The finding that phosphorylation of FKHR inhibits its ability to stimulate transcription is consistent with genetic evidence in C. elegans that has shown that mutations in the DAF16 gene have the opposite phenotype to mutations in either the AGE1 (PtdIns 3-kinase) or AKT1/2 (PKB) genes (21–23). These findings raise the possibility that phosphorylation of FKHR (or its homologues) underlies the regulation of at least some of the genes whose transcription is inhibited by insulin.

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