Heart 6-Phosphofructo-2-kinase Activation by Insulin Results from Ser-466 and Ser-483 Phosphorylation and Requires 3-Phosphoinositide-dependent Kinase-1, but Not Protein Kinase B*

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Luc Bertrand‡‡, Dario R. Alessi*, Johan Deprez‡, Maria Deak‡, Eric Viaene‡, Mark H. Rider‡‡**, and Louis Hue‡ *‡

From the *Hormone and Metabolic Research Unit, Université catholique de Louvain, and the Institute of Cellular Pathology, Avenue Hippocrate, 75, B-1200 Brussels, Belgium and the ‡Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, University of Dundee, Dundee, DD1 4HN, Scotland

Previous studies have shown that (i) the insulin-induced activation of heart 6-phosphofructo-2-kinase (PFK-2) is wortmannin-sensitive, but is insensitive to rapamycin, suggesting the involvement of phosphatidylinositol 3-kinase; and (ii) protein kinase B (PKB) activates PFK-2 in vitro by phosphorylating Ser-466 and Ser-483. In this work, we have studied the effects of phosphorylation of these residues on PFK-2 activity by replacing each or both residues with glutamate. Mutation of Ser-466 increased the $V_{max}$ of PFK-2, whereas mutation of Ser-483 decreased citrate inhibition. Mutation of both residues was required to decrease the $K_m$ for fructose 6-phosphate. We also studied the insulin-induced activation of heart PFK-2 in transfection experiments performed in human embryonic kidney 293 cells. Insulin activated transfected PFK-2 by phosphorylating Ser-466 and Ser-483. Kinase-dead (KD) PKB and KD 3-phosphoinositide-dependent kinase-1 (PDK-1) co-transfectants acted as dominant negatives because both prevented the insulin-induced activation of PKB as well as the inactivation of glycogen-synthase kinase-3, an established substrate of PKB. However, the insulin-induced activation of PFK-2 was prevented only by KD PDK-1, but not by KD PKB. These results indicate that the insulin-induced activation of heart PFK-2 is mediated by a PDK-1-activated protein kinase other than PKB.

The stimulation of heart glycolysis by insulin results from the stimulation of glucose transport and the activation of 6-phosphofructo-2-kinase (PFK-2), the enzyme that synthesizes fructose 2,6-bisphosphate (1, 2). Fructose 2,6-bisphosphate is itself a potent stimulator of 6-phosphofructo-1-kinase and hence of glycolysis. The insulin-induced stimulation of glucose transport and PFK-2 activation is mediated by phosphatidylinositol 3-kinase, a component of one of the insulin signaling pathways (3). Phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, products of phosphatidylinositol 3-kinase, in turn, induce the phosphorylation of PKB, which leads to its activation. 3-Phosphoinositide-dependent kinase-1 (PDK-1) phosphorylates PKBα on Thr-380, as well as on Ser-473, when complexed to a targeting subunit (4, 5). PKB has been proposed to mediate one of the metabolic effects of insulin, namely the inactivation of glycogen synthase kinase-3 (GSK-3), leading to stimulation of glycogen synthesis in skeletal muscle (6). Heart PFK-2 was the second substrate of PKB to be recognized, and PKB activates heart PFK-2 in vitro by phosphorylating Ser-466 and Ser-483 (7). The phosphorylation site sequences surrounding Ser-466 and Ser-483 resemble those found in the other PKB targets, namely GSK-3 and BAD (4). The activation of heart PFK-2 due to phosphorylation by PKB resulted from a 2-fold increase in both $V_{max}$ and affinity for Fru-6-P, one of the substrates of PFK-2. One of the aims of this work was to study three heart PFK-2 mutants (S466E, S483E, and S466E/S483E) in which each or both serine residues in the PKB phosphorylation sites were replaced with Gln to mimic their phosphorylation. The relative contribution of each phosphorylation site to the changes in activity of PFK-2 was assessed by kinetic measurements.

In isolated cardiomyocytes, the insulin-induced activation of PFK-2 was insensitive to rapamycin and PD98059 (8), which inhibit p70 ribosomal S6 kinase and mitogen-activated protein kinase activation, respectively. However, the effect of insulin to activate PFK-2 was blocked by inhibitors of phosphatidylinositol 3-kinase (wortmannin and LY294002 (8)). To test the hypothesis that PKB is required for the insulin-induced activation of heart PFK-2, we carried out transfection experiments in human embryonic kidney (HEK)-293 cells, which contain the components of the insulin signaling pathways and have been used in mechanistic studies (9–11). HEK-293 cells were transfected with cDNA constructs coding for recombinant wild-type protein kinase B; PDK-1, 3-phosphoinositide-dependent kinase-1; GSK-3, glycogen synthase kinase-3; HEK, human embryonic kidney; BH1(His)6, polyhistidine-tagged recombinant bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; EE-GSK-3, EFMPME epitope-tagged GSK-3; HA-PKB, hemagglutinin-tagged PKB; KD, kinase-dead; GST, glutathione S-transferase; Mops, 4-morpholinepropanesulfonic acid; MALDI-MS, matrix-assisted laser desorption-ionization mass spectrometry; HPLC, high performance liquid chromatography.

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bovine heart PFK-2 (BH1) and mutants. In these cells, we studied the effect of insulin on the extent of phosphorylation and changes in PFK-2 activity, and we identified the phosphorylation sites in the wild-type and the three mutants. Finally, cotransfection of heart PFK-2 together with different PKB and PDK-1 constructs (wild-type or dominant negatives) was performed to evaluate the role of PKB in the insulin-induced activation of heart PFK-2. Our findings suggest the possible participation of PDK-1-activated protein kinase(s), other than PKB, in the insulin signaling pathway that activates PFK-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—C-terminally polyhistidine-tagged bovine heart PFK-2/ fructose-2,6-bisphosphatase (BH1(Has)), cDNA, cloned in pBluescript II KS+ phagemid (7), was used to create two single mutations (S466E and S483E) by polymerase chain reaction with the mutant oligonucleotides 5'-GATGAGAAGGAACGAATTCACGCCTCTG-3' for S466E and 5'-CGAATACCTAGACGTCCAAGAAATTACGAAGTTGGGAGC-3' for S483E. The mutated polymerase chain reaction fragments were introduced into the original phagemid to create pBluescript II KS+ (for S483E) and pBluescript II KS+/BH1(Has)/S466E. One unique restriction site (BstI) is located between the two mutation sites, and the other (BanHI) is located at the end of the cDNA. The BstBI/BanHI fragment from pBluescript II KS+/BH1(Has)/S483E, containing the S483E mutation, was introduced into pBluescript II KS+/BH1(Has)/S466E to create the double mutant pBluescript II KS+/BH1(Has)/S466E/S483E. The different mutants were verified by sequencing and introduced into the bacterial expression vector pET3a as described (7). The wild-type and mutant BH1(Has) cDNAs were also introduced into the pCMV5 eukaryotic expression vector (12) as follows.

An optimal Kozak consensus sequence (TCCACC) was first added at the beginning of the BH1(Has) cDNA by polymerase chain reaction using pBluescript II KS+/BH1(Has), as a template (the oligonucleotides were 5’-CATCGATTCCACATTGCAGGAAATTCGCCC-3’ and 5’-CGAATTCTTCCGCTGGCGAAG-3’). The polymerase chain reaction product was finally reintroduced into the same phagemid using the Stratagene and EcoRI unique restriction sites to form a complete BH1(Has) cDNA including the Kozak consensus sequence. The Clal/BamHI fragment, containing this complete BH1(Has) cDNA, was then introduced into the pCMV5 vector using the same sites to form the pCMV5/BH1(Has) vector. The different mutations were introduced into this vector at the SstI and BanHI unique restriction sites.

**Activated PKB, used for the in vitro phosphorylation experiments, was prepared as follows.** N-terminally His-tagged PKBα (provided by A. Paterson, Dundee University) was activated in vitro by incubation with PKD-1, MgATP, and phosphatidylinositol 3,4,5-trisphosphate as described previously for His-tagged PKBα (13). PKD-1 was then removed from His-tagged PKBα by chromatography on heparin-Sepharose. pCMV5 vectors containing the cDNA coding human GSK-3β with the EFPMEP epitope tag at the N-terminus (EE-GSK-3) and hemagglutinin-tagged PKBα (HA-PKB) have been described (14). pCMV5 vectors containing the cDNA coding the triple mutant kinase-dead (KD) PKBα (called AAA-PKB, in which Lys-179 involved in ATP binding and Thr-308 and Ser-473 required for phosphorylation-induced activation have been mutated to Ala) and the double mutant KD PKD-1 (in which Lys-111 and Asp-223 involved in catalysis have been mutated to Ala) were obtained using the Quickchange kit (Stratagene) following instructions provided by the manufacturer. Anti-His antibody (CLON-TECH), anti-glutathione S-transferase (GST) antibody (Pharmingen), and anti-HA and anti-c-Myc antibodies (Roche Molecular Biochemicals) were from the indicated sources. A rabbit polyclonal antibody (which we call BAK) was raised against the C-terminal peptide of PKBα (FFQF-SYSAASSTA) and purified on protein A-Sepharose. This antibody was used for endogenous PKBα assay. All other materials were from sources previously cited (7, 13–15).

**In Vitro Phosphorylation of Recombinant Heart PFK-2 by PKB—** Wild-type BH1(Has) and the S466E and S466E/S483E mutants were expressed in Escherichia coli strain BL21(DE3) pLysE, and the S483E mutant was expressed in BL21(DE3) pLysE. Culture, lysis, and purification were carried out as described (15). For measurement of the changes in kinetic properties induced by phosphorylation, the wild-type and mutant BH1(Has) preparations were incubated with PKBα (see table legends for details) in phosphorylation buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM MgATP, 0.05% (v/v) β-mercaptoethanol, 5 mM magnesium acetate, 1 μM microcystin, 1 μM cAMP-dependent protein kinase inhibitor peptide, and 50 μM EGTA. After 30 min, the reactions were quenched, the reactions were stopped, and aliquots were taken for the measurement of PKF-2 activity. For the determination of $K_m$, the concentration of the substrate understudy was varied up to 10 times the $K_m$, whereas the concentration of the other substrate was saturating (5 mM for MgATP and 2 mM for Fru-6-P). For the $IC_{50}$, for magnesium citrate, the concentrations of Fru-6-P and MgATP were 100 μM and 5 mM, respectively. The results are the means ± S.E. of three separate experiments; otherwise, individual values are given.

### TABLE I

**Stoichiometry and kinetic properties of the phosphorylation of BH1(Has) preparations by PKB**

For measurements of the stoichiometry of $^{32}$P incorporation, wild-type and mutant BH1(Has) preparations (all at 0.1 mg/ml) were phosphorylated with $\gamma$-$^{32}$P-MgATP (0.1 mM) and PKB (100 milliunits/ml) in a final volume of 25 μl until a plateau was reached (80 min). $^{32}$P incorporation was measured by phosphoinaging after SDS polyacrylamide gel electrophoresis. To study the kinetic properties of phosphorylation, wild-type and mutant BH1(Has) preparations (10 mM to 3 μM) were phosphorylated with MgATP (1 mM) and PKB (5 milliunits/ml) in a final volume of 40 μl for 5 min. Following SDS polyacrylamide gel electrophoresis, $^{32}$P incorporation was also measured by phosphoinaging. The results are the means ± S.E. for the number of determinations shown in parentheses.

| BH1/His preparation | $^{32}$P incorporation | PKB preparation |
|---------------------|------------------------|----------------|
|                     | mol/mol subunit | $K_m$ (μM) | $V_{max}$ (μM/min per mg protein) | NM |
| Wild-type            | 0.82 ± 0.04 (4) | 167 ± 25 (3) | 106 ± 7 (3) | NM |
| S466E               | 0.43 ± 0.03 (4) | 144 ± 6 (3) | 43 ± 2 (3) | NM |
| S483E               | 0.40 ± 0.05 (4) | 141 ± 19 (3) | 37 ± 2 (3) | NM |
| S466E/S483E         | 0.05 ± 0.01 (4) | NM | NM |

* $p < 0.01$ compared with the wild-type value.

### TABLE II

**Changes in the kinetic properties of wild-type and mutant BH1(Has) preparations induced by phosphorylation or phosphorylation by PKB**

BH1(Has) preparations (0.1 mg/ml) were incubated with 500 milliunits/ml PKB at 30 °C. After 30 min, the reactions were stopped, and aliquots were taken for the measurement of PKF-2 activity. For the determination of $K_m$, the concentration of the substrate understudy was varied up to 10 times the $K_m$, whereas the concentration of the other substrate was saturating (5 mM for MgATP and 2 mM for Fru-6-P). For the $IC_{50}$, for magnesium citrate, the concentrations of Fru-6-P and MgATP were 100 μM and 5 mM, respectively. The results are the means ± S.E. of three separate experiments; otherwise, individual values are given.

| BH1/His preparation | $K_m$ for Fru-6-P (μM) | $V_{max}$ (μM/min per mg protein) | $K_m$ for MgATP (μM) | $IC_{50}$ for Mg citrate (μM) |
|---------------------|------------------------|----------------------------------|----------------------|---------------------------|
| Wild-type Control   | 121 ± 10               | 31 ± 2                           | 588,600              | 24,33                     |
| +PKB                | 61 ± 3*                | 71 ± 1*                          | 661,587              | 56,66                     |
| S466E Control       | 143 ± 8                | 64 ± 4                           | 824,719              | 29,36                     |
| +PKB                | 76 ± 3*                | 66 ± 3*                          | 743,701              | 59,75                     |
| S483E Control       | 103 ± 3                | 35 ± 2                           | 658,667              | 59,49                     |
| +PKB                | 68 ± 5*                | 67 ± 3*                          | 601,683              | 59,79                     |
| S466E/S483E Control | 66 ± 4*                | 65 ± 4                           | 714,659              | 52,71                     |
| +PKB                | 73 ± 3*                | 69 ± 3*                          | 601,723              | 59,61                     |

* $p < 0.01$ compared with the wild-type (non-phosphorylated) value.

$^{a}$ $p < 0.01$ compared with the non-phosphorylated value.
were stopped by diluting 10-fold in 20 mM Hepes (pH 7.5), 50 mM KCl, 0.5 mM EGTA, 5 mM EDTA, 1 mM potassium phosphate, 20% (v/v) glycerol, and 0.1% (v/v) β-mercaptoethanol and chilled in ice. Aliquots were taken for PFK-2 activity. Kinetic measurements were made when phosphorylation had reached a maximum. *In vitro* measurements of 32P incorporation into purified PFK-2 were performed as described (7).

Transfection of HEK-293 Cells—HEK-293 cells were cultured in 10-cm diameter dishes in Dulbecco’s minimal essential medium containing 10% (v/v) fetal calf serum and transfected using a modified calcium phosphate procedure (11) with pCMV5 DNA constructs (wild-type or mutant BH1(His)₆, EE-GSK-3, wild-type HA-PKB, GST-AAA-PKB, and mutant PDK-1) and empty vector to reach a total of 10 μg of added DNA. In cotransfection experiments, the amount of cotransfected DNA was in 5–10-fold excess with respect to the transfected vector. Transfection was performed overnight at 37 °C, and then the medium was aspirated and replaced with fresh Dulbecco’s minimal essential medium containing 10% (v/v) fetal calf serum. After 10 h, the cells were

**FIG. 1.** Insulin-induced activation of transfected heart PFK-2 (A) and endogenous PKB (B) in HEK-293 cells. HEK-293 cells were transiently transfected with vectors expressing wild-type BH1(His)₆. The cells were stimulated with insulin as described under “Experimental Procedures.” The values are the means ± S.E. for at least three separate determinations on three different cell preparations. PFK-2 activity was measured at pH 7.1 with optimal substrate concentrations (5 mM MgATP and 2 mM Fruc-6-P) (16). Inset, PFK-2 activity measured in cells transfected with the indicated amounts of PFK-2 vector. Open bars, unstimulated cells; closed bars, cells stimulated with insulin for 10 min. The extent of insulin-induced activation of PFK-2 is indicated.

**FIG. 2.** Insulin-induced phosphorylation of wild-type PFK-2 in HEK-293 cells. Transfected BH1(His)₆ from 32P-labeled HEK-293 cells, which were incubated with rapamycin and PD98059 and with or without insulin (10 min) and wortmannin as indicated, was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis for autoradiography (11).

**FIG. 3.** HPLC profiles of 32P-labeled tryptic peptides of BH1(His)₆ from transfected HEK-293 cells (A–C) and of BH1(His)₆ phosphorylated *in vitro* by PKB (D). Immunoprecipitated 32P-labeled BH1(His)₆ was digested with trypsin. Peptides were purified by reverse-phase HPLC using a linear gradient of acetonitrile (dashed line). Tryptic peptide maps (A–C) are the means of two separate experiments. In all cases, HEK-293 cells were preincubated with rapamycin and PD98059. *In vitro* phosphorylation of purified recombinant BH1(His)₆ by PKB (D) had reached a plateau of 32P incorporation before digestion with trypsin. In all experiments, >95% of the radioactivity was eluted between 10 and 30% of acetonitrile.
The cells were then treated with or without wortmannin (100 nM, 10 min), rapamycin (100 nM, 30 min) and PD98059 (50 μM, 30 min) and further incubated with or without insulin (100 nM) for the indicated periods of time.

Measurements of PFK-2 and PKB Activities in Cell Extracts—After removing the incubation medium, the HEK-293 cells were frozen in liquid nitrogen. The cells were then lysed in 1 ml of ice-cold Buffer A (50 mM Hepes (pH 7.5), 50 mM KF, 1 mM potassium phosphate, 1 mM EDTA, 1 mM NaVO₄, 0.1% (v/v) β-mercaptoethanol, 1 mM phenylmethanesulfonfl fluoride, 1 mM benzamidine hydrochloride, and 1 μg/ml leupeptin) by passage through a syringe fitted with a fine needle. PFK-2 activity was measured in supernatants (15,000 × g for 5 min) as described (16). In the experiments reported in this paper, transfected HEK-293 cells contained between 20 and 100 times more PFK-2 activity (0.1–0.5 milliunits/mg of protein) than the non-transfected control cells (5 microunits/mg of protein), depending on the amount of transfected PFK-2 DNA (1–10 μg) and on transfection efficiency (30–80% of cells transfected after 40 h). Endogenous PKB activity was measured in immunoprecipitates as follows. Cell extracts (0.3 ml), corresponding to 10 μg of protein, were incubated for 2 h at 4 °C with agitation in a final volume of 0.5 ml of Buffer A and 60 μg of PKB antibody (BAK) coupled to 25 μl of protein A-Sepharose. The immune complexes were washed six times with lysis buffer (11) and three times with buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 1 μg/ml leupeptin by passage through a syringe fitted with a fine needle. PFK-2 activity was measured in supernatants (15,000 × g for 5 min) as described (16). In the experiments reported in this paper, transfected HEK-293 cells contained between 20 and 100 times more PFK-2 activity (0.1–0.5 milliunits/mg of protein) than the non-transfected control cells (5 microunits/mg of protein), depending on the amount of transfected PFK-2 DNA (1–10 μg) and on transfection efficiency (30–80% of cells transfected after 40 h). Endogenous PKB activity was measured in immunoprecipitates as follows. Cell extracts (0.3 ml), corresponding to ~5 mg of protein, were incubated for 2 h at 4 °C with agitation in a final volume of 0.5 ml of Buffer A and 60 μg of PKB antibody (BAK) coupled to 25 μl of protein A-Sepharose. The immune complexes were washed six times with buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol, 0.1% (v/v) Triton X-100, and 0.5 μM NuCl and twicewith buffer containing 50 mM Tris-HCl (pH 7.5), 0.03% (w/v) Brij-35, 0.1 mM EGTA, and 0.1% (v/v) β-mercaptoethanol. The beads were then resuspended in 20 μl of PKB assay buffer (10 mM Mops (pH 7), 0.5 mM EDTA, 10 mM magnesium acetate, and 0.1% (v/v) β-mercaptoethanol). PKB activity was measured at 30 °C in a final volume of 50 μl of PKB assay buffer in the presence of 2.5 μM cAMP-dependent protein kinase inhibitor peptide with 0.25 mM substrate peptide (RPRAAT (17)) and 0.1 μM [γ-32P]MgATP (specific radioactivity of 1500 cpm/pmol). After 20 min, 20-μl aliquots were removed for the measurement of 32P incorporation (18). Transfected HA-PKB activity was measured by the same method on immune complexes obtained from 100 μg of protein extract treated with 3 μg of anti-HA antibody.

Phosphorylation Site Identification—HEK-293 cells were transfected with 10 μg of plasmid containing wild-type or mutant BH1(His) cDNAs. The cells were washed with phosphate-free Dulbecco’s minimal essential medium and incubated for 4 h with [32P]orthophosphate (1 mCi/ml). The cells were pretreated with rapamycin (100 nM) and PD98059 (50 μM) for 30 min with or without wortmannin (100 nM) for 10 min and then incubated with or without insulin (100 nM) for 10 min. The cells were lysed (11), and PFK-2 was immunoprecipitated using 160 μg of anti-His monoclonal antibody/10-cm dish of cells. Cell extracts were incubated for 30 min at 4 °C on a shaker with antibody coupled to 20 μl of protein G-Sepharose. The immune complexes were washed six times with lysis buffer (11) and three times with buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.1% (v/v) β-mercaptoethanol. The beads were then resuspended in 20 μl of electrophoresis buffer (2% (w/v) SDS and 1.6% (v/v) β-mercaptoethanol) and alkylated with...
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RESULTS AND DISCUSSION

In Vitro Studies

The contribution of the C-terminal residues (Ser-466 and Ser-483) to the changes in the kinetic properties of PFK-2 induced by phosphorylation was studied by site-directed mutagenesis. Wild-type BH1(His)$_6$ and the three mutants (S466E, S483E, and S466E/S483E) were expressed in bacteria and purified by a two-step procedure including anion-exchange and metal affinity chromatography (7). The mutant preparations had the same chromatographic behavior as the wild-type. After SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining, a single band was observed for each preparation, which migrated with the expected $M_r$ of 61,000 (data not shown).

The stoichiometry of phosphorylation of recombinant wild-type BH1(His)$_6$ by PKB (Table I) was close to 1 mol of phosphate incorporated per mol of enzyme subunit (measured by the ninhydrin method), in agreement with previous work (7). PKB phosphorylated S466E and S483E with a stoichiometry corresponding to about half that of the wild-type. The double mutant (S466E/S483E) was not phosphorylated by PKB. Moreover, the $V_{\text{max}}$ of PKB for wild-type PFK-2 was twice that of the S466E and S483E mutants, whereas the affinity of PKB for all the preparations was the same (Table I). The difference in the rate of phosphorylation could be due to the incorporation of a negative charge, which could then act as a product inhibitor in the PKB reaction.

In agreement with previous results (7), phosphorylation of recombinant wild-type BH1(His)$_6$ by PKB increased the $V_{\text{max}}$ (2-fold) and decreased the $K_m$ of PFK-2 for Fru-6-P (2-fold) (Table II). In addition, phosphorylation of the wild type by PKB decreased the sensitivity toward magnesium citrate inhibition (21), without affecting the $K_m$ for ATP. The double mutation (S466E/S483E) had the same effects on the kinetic properties as those observed in the phosphorylated wild-type, suggesting that the double mutation indeed mimicked the effects of phosphorylation on activity. As expected, incubation of the S466E/S483E mutant with PKB had no further effect on its kinetic properties. In summary, our results show that introduction of a negative charge (by phosphorylation or mutation) at Ser-466 or Ser-483 doubled the activity of transfected PFK-2 (wild-type wild-type heart PFK-2 were $3^2$P-labeled and incubated with (Fig. 1A). The effect of insulin to double PFK-2 activity was consistently observed when the activity of transfected PFK-2 ranged between 100 and 500 microunits/mg of protein in unstimulated cells (Fig. 1A, inset). The insulin-induced PFK-2 activation was sensitive to LY294002 (data not shown) and wortmannin and was insensitive to rapamycin and PD98059 (Fig. 1A). Endogenous PKB activation correlated with the insulin-induced increase in PFK-2 activity (Fig. 1B).

To identify the phosphorylated residue(s) in transfected PFK-2 following insulin stimulation, HEK-293 cells expressing wild-type heart PFK-2 were $3^2$P-labeled and incubated with insulin for 10 min in the presence or absence of wortmannin. Rapamycin and PD98059 were present under all conditions. Immunoprecipitation of transfected PFK-2 and autoradiography after SDS-polyacrylamide gel electrophoresis revealed a single $3^2$P-labeled band with an expected $M_r$ of 61,000. The labeling of this band was increased 2-fold by insulin and blocked by wortmannin (Fig. 2). The $3^2$P-labeled bands were excised and digested with trypsin, and peptides were separated by reverse-phase HPLC. For wild-type PFK-2 from unstimulated cells (Fig. 1A). The effect of insulin on PFK-2 was assayed (16) under the conditions described in the figure and table legends. GSK-3 activity ratio (with and without protein phosphatase 2A treatment) was measured in immunoprecipitates as described (14). Kinetic constants were calculated by computer fitting of the data to a hyperbola describing the Michaelis-Menten equation by nonlinear least-squares regression. One unit of enzyme activity corresponds to the formation of 1 mol (PFK-2) or 1 nmol (protein kinases) of product/min under the assay conditions.

Studies in HEK-293 Cells

Activation and Phosphorylation of Transfected Heart PFK-2 by Insulin in HEK-293 Cells—Incubation of HEK-293 cells with insulin for 5–20 min had no effect on the activity of endogenous PFK-2 in non-transfected cells (data not shown), whereas it doubled the activity of transfected PFK-2 (wild-type wild-type heart PFK-2 ranged between 100 and 500 microunits/mg of protein in unstimulated cells (Fig. 1A, inset). The insulin-induced PFK-2 activation was sensitive to LY294002 (data not shown) and wortmannin and was insensitive to rapamycin and PD98059 (Fig. 1A). Endogenous PKB activation correlated with the insulin-induced increase in PFK-2 activity (Fig. 1B).

To identify the phosphorylated residue(s) in transfected PFK-2 following insulin stimulation, HEK-293 cells expressing wild-type heart PFK-2 were $3^2$P-labeled and incubated with insulin for 10 min in the presence or absence of wortmannin. Rapamycin and PD98059 were present under all conditions. Immunoprecipitation of transfected PFK-2 and autoradiography after SDS-polyacrylamide gel electrophoresis revealed a single $3^2$P-labeled band with an expected $M_r$ of 61,000. The labeling of this band was increased 2-fold by insulin and blocked by wortmannin (Fig. 2). The $3^2$P-labeled bands were excised and digested with trypsin, and peptides were separated by reverse-phase HPLC. For wild-type PFK-2 from unstimu-
HA-PKB activity was undetectable in non-transfected cells. Overexpression of HA-PKB (anti-HA antibody) and EE-GSK-3 (anti-EE antibody) was assessed by immunoblotting. (cotransfected with HA-PKB), 4.5 (cotransfected with GST-AAA-PKB), and 3.1 (cotransfected with KD Myc-PDK-1) milliunits/mg of protein. (three separate cell preparations. EE-GSK-3 is expressed as an activity ratio measured before and after treatment with protein phosphatase 2A. HA-PKB and EE-GSK-3 activities were measured in immunoprecipitates. The values are the means ± S.E. of at least three determinations on three separate cell preparations. EE-GSK-3 is expressed as an activity ratio measured before and after treatment with protein phosphatase 2A. In a representative experiment, EE-GSK-3 activity was undetectable (non-transfected (NT) cells) or 5.2 (non-cotransfected cells), 1.7 (cotransfected with HA-PKB), 4.5 (cotransfected with GST-AAA-PKB), and 3.1 (cotransfected with KD Myc-PDK-1) milliunits/mg of protein. HA-PKB activity was undetectable in non-transfected cells. Overexpression of HA-PKB (anti-HA antibody) and EE-GSK-3 (anti-EE antibody) was assessed by immunoblotting.

Effects of cotransfected PKB or PDK-1 mutants on activation of HA-PKB (A) and inactivation of EE-GSK-3 (B) by insulin in HEK-293 cells. HEK-293 cells were cotransfected with vectors expressing wild-type (WT) HA-PKB (A) or wild-type EE-GSK-3 (B) as well as with GST-AAA-PKB or KD Myc-PDK-1 as indicated. Cells were incubated with rapamycin and PD98059 and with or without insulin for 10 min. HA-PKB and EE-GSK-3 activities were measured in immunoprecipitates. The values are the means ± S.E. of at least three determinations on three separate cell preparations. EE-GSK-3 is expressed as an activity ratio measured before and after treatment with protein phosphatase 2A (PP2A). In a representative experiment, EE-GSK-3 activity was undetectable (non-transfected (NT) cells) or 5.2 (non-cotransfected cells), 1.7 (cotransfected with HA-PKB), 4.5 (cotransfected with GST-AAA-PKB), and 3.1 (cotransfected with KD Myc-PDK-1) milliunits/mg of protein. HA-PKB activity was undetectable in non-transfected cells. Overexpression of HA-PKB (anti-HA antibody) and EE-GSK-3 (anti-EE antibody) was assessed by immunoblotting.

Effect of cotransfected PKB or PDK-1 on PFK-2 Activation by Insulin in HEK-293 Cells—The potential role of PKB and PDK-1 in the insulin-induced activation of PFK-2 was studied in HEK-293 cells that had been cotransfected with wild-type BH1(His)6 PFK-2 and wild-type HA-PKB, dominant-negative GST-AAA-PKB, or dominant-negative KD Myc-PDK-1. Under all conditions, rapamycin and PD98059 were present. The effect of insulin was tested in experiments that lasted only 10 min and were therefore too short to affect the level of expression of PFK-2. This lack of change in enzyme content and expression was verified by immunoblotting (Figs. 6 and 7).

We first verified that GST-AAA-PKB and KD PDK-1 indeed behave as dominant negatives by measuring the effect of insulin on the activity of cotransfected PKB or GSK-3, two downstream components in insulin signaling (14). When wild-type HA-PKB was used as a reporter (Fig. 6A), overexpression of either GST-AAA-PKB or KD Myc-PDK-1 prevented the insulin-induced activation of HA-PKB. Moreover, in agreement with previous reports (14), insulin inactivated EE-GSK-3 as indicated by the decrease in its activity ratio (Fig. 6B). This ratio is a measure of the activation state of GSK-3 because it relates its activity to that obtained after full activation by treatment with protein phosphatase 2A. This index has been used to assess the extent of inactivation of GSK-3 by insulin or insulin-like growth factor-1 (14). The overexpression of wild-type HA-PKB inactivated EE-GSK-3, and as reported (14), this inactivation was even more pronounced than with insulin. Overexpression of GST-AAA-PKB or KD Myc-PDK-1 prevented the insulin-induced inactivation of EE-GSK-3 (Fig. 6B). Therefore, GST-AAA-PKB and KD Myc-PDK-1 acted as dominant-negative mutants, as described (22–24).

We then tested whether GST-AAA-PKB and KD Myc-PDK-1 could also prevent the insulin-induced activation of transfected PFK-2. As for GSK-3, we determined the activity ratio as an index of PFK-2 activation. Insulin activated endogenous PKB and transfected PFK-2 (Fig. 7), in agreement with the results in Fig. 1. Cotransfection of wild-type PKB resulted in little, if any, effect on PFK-2 activity ratio in control or insulin-treated cells. As expected, the activation of endogenous PKB was di-
were cotransfected with vectors expressing wild-type (WT) PKB, with GST-AAA-PKB or KD Myc-PDK-1) and activity was 10–30 (non-cotransfected cells as well as cotransfected cells the unstimulated cells. In a representative experiment, total PKB action was 3 (non-transfected cells), 310 (cotransfected with HA-PKB), 220 (cotransfected with GST-AAA-PKB), and 230 (cotransfected with KD Myc-PDK-1) microunits/mg of protein. The values are the means + S.E. of at least four separate determinations on five separate cell preparations. C, shown are immunoblots of transfected PFK-2 (anti-His antibody), HA-PKB (anti-HA antibody), GST-AAA-PKB (anti-GST antibody), and KD Myc-PDK-1 (anti-Myc antibody). NT, non-transfected.

In conclusion, insulin activates heart PFK-2 by inducing the phosphorylation of Ser-466 and Ser-483 in intact cells. Although these sites are known to be phosphorylated by PKB in vitro, our results indicate that, in intact cells, PKF-2 activation by insulin is mediated by PKD-1 and that PKB may not be essential. Therefore, other protein kinase(s), downstream of PKD-1, might mediate PKF-2 activation by phosphorylation. Examples of insulin-sensitive protein kinases, whose activation is dependent upon phosphatidylinositol 3-kinase 3-kinase and PDK-1, include protein kinase Cζ and serum- and glucocorticoid-regulated protein kinase (25, 26). Future work will focus on whether these kinases or a novel protein kinase mediates the activation of PFK-2 by insulin in intact cells.

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Figure 7. Effects of cotransfected PKB or PDK-1 on activation of PKB (A) and PFK-2 (B) by insulin in HEK-293 cells. HEK-293 cells were cotransfected with wild-type (WT) BH1(His)6PDK-1 microunits/mg of protein. A, PFK-2 is expressed as an activity ratio (measured at pH 7.1 with optimal substrate concentrations before and after treatment with protein phosphatase 2A (PP2A)).