Partial purification and characterization of a wortmannin-sensitive and insulin-stimulated protein kinase that activates heart 6-phosphofructo-2-kinase

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A wortmannin-sensitive and insulin-stimulated protein kinase (WISK), which phosphorylates and activates cardiac 6-phosphofructo-2-kinase (PFK-2), was partially purified from perfused rat hearts. Immunoblotting showed that WISK was devoid of protein kinase B (PKB), serum- and glucocorticoid-regulated protein kinase and protein kinase C ζ (PKC ζ). Comparison of the inhibition of WISK, PKC α and PKC ζ by different protein kinase inhibitors suggested that WISK was not a member of the PKC family. In addition, WISK contained no detectable phosphoinositide-dependent protein kinase-1 (PDK1) activity. WISK phosphorylated recombinant heart PFK-2 in a time-dependent manner to the extent of 0.4 mol of phosphate incorporated/mol of enzyme subunit, and increased the $V_{\max}$ of PFK-2 twofold, without affecting the K_m for fructose 6-phosphate. WISK phosphorylated Ser-466 to a greater extent than Ser-483 in recombinant heart PFK-2, and both sites were demonstrated to be

phosphorylated to the same extent by PKB. Gel filtration and in-gel kinase analysis indicated that WISK was a monomer with a M_r of 56 500. Treatment of WISK with protein phosphatase 2A (PP2A) catalytic subunits reversed the effect of insulin, suggesting the involvement of an upstream activating kinase. Indeed, PDK1 was able to partially reactivate the PP2A-treated WISK and this reactivation was not enhanced by PtdIns(3,4,5) P_3 -containing vesicles. Moreover, a single 57 000- M_r band was labelled on incubation of the dephosphorylated WISK preparation with PDK1 and [γ -³²P]ATP. These findings provide evidence for the existence of a new protein kinase in the insulin signalling pathway, probably downstream of PDK1.

Key words: fructose 2,6-bisphosphate, glycolysis, phosphoinositide-dependent protein kinase 1.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6- P_2) is a potent positive allosteric effector of 6-phosphofructo-1-kinase [1]. The stimulation of heart glycolysis by insulin is due to a stimulation of glucose transport and an increase in Fru-2,6- P_2 concentration, which in turn results from the activation of 6-phosphofructo-2-kinase (PFK-2), the enzyme which catalyses the formation of Fru-2,6- P_2 . Indeed, insulin injection into rats increases the $V_{\max}$ of heart PFK-2 twofold. The insulin-induced activation persists after partial purification, suggesting that it results from covalent modification of the enzyme [2]. The effect of insulin to activate PFK-2 has also been observed in perfused rat hearts [3] and in isolated rat cardiomyocytes [4].

Insulin binding to its receptor initiates signalling through at least two pathways. One pathway leads to the activation of the mitogen-activated protein kinase (MAPK) cascade which, in turn, activates the MAPK-activated protein kinase-1 (MAPKAPK-1, also known as p90 ribosomal S6 kinase, RSK-2 or p90^{rsk}) [5]. Another pathway involves the activation of the lipid kinase, phosphoinositide 3-kinase [6]. This lipid kinase produces mainly PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 in the plasma

membrane, which can bind to the pleckstrin homology domains of at least two different serine/threonine protein kinases, namely phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB). PDK1 phosphorylates and activates PKB [7], p70 ribosomal S6 kinase (p70^{s6k}) [8], protein kinase C ζ (PKC ζ) [9] and the so-called serum- and glucocorticoid-regulated protein kinase (SGK) [10].

PKB, p70^{s6k}, PKC ζ and SGK belong to the AGC (PKA, PKG, PKC) protein kinase family. The AGC kinases possess a conserved threonine residue situated between domains VII and VIII of the kinase catalytic domain. This conserved threonine residue is phosphorylated by PDK1 and corresponds to Thr-308 in PKB α [7]. The full activation of PKB α requires phosphorylation of Thr-308 by PDK1 and phosphorylation at a second site, Ser-473, which corresponds to the PDK2 site [7]. The PDK2 motif surrounding Ser-473 of PKB is also conserved in p70^{s6k} and SGK [8,10]. In fact, PDK1 and PDK2 are probably the same enzyme. PDK1 can acquire PDK2 activity through its interaction with other protein(s) [11]. The binding of 3-phosphoinositides to the pleckstrin homology domain of PKB allows phosphorylation of Thr-308 by PDK1. However, the phosphorylation of the PDK1 site in p70^{s6k} and SGK is not

Abbreviations used: BAD, Bcl-2/Bcl-XL-antagonist, causing cell death; DTT, dithiothreitol; Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6-P, fructose 6-phosphate; GST, glutathione S-transferase; Glc-6-P, glucose 6-phosphate; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; MAPKAPK-1, MAPK-activated protein kinase-1; p70^{s6k}, p70 ribosomal protein S6 kinase; PDK1, phosphoinositide-dependent protein kinase-1; PFK-2, 6-phosphofructo-2-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PP2A, protein phosphatase 2A; SGK, serum- and glucocorticoid-regulated protein kinase; WISK, wortmannin-sensitive and insulin-stimulated PFK-2 kinase.

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directly dependent on 3-phosphoinositides. By contrast, the phosphorylation of the PDK2 site in PKB, p70^{s6k}, and SGK is dependent on 3-phosphoinositides [7,8,10,11].

Heart PFK-2 is phosphorylated and activated *in vitro* by the insulin-stimulated protein kinases, MAPKAPK-1, p70^{s6k} and PKB [12]. These protein kinases phosphorylate both Ser-466 and Ser-483 to about the same extent [12]. In isolated rat cardiomyocytes, the use of inhibitors indicated that the insulin-induced activation of PFK-2 did not involve MAPKAPK-1 and p70^{s6k}, but could be mediated by PKB [4]. However, our recent work in transfected human embryonic kidney (HEK)-293 cells suggested that PKB might not be required for PFK-2 activation by insulin [13]. Therefore, we purified the insulin-stimulated protein kinase(s) from perfused rat hearts using recombinant heart PFK-2 as a substrate. Interestingly, we found a protein kinase, distinct from PKB, SGK and PKC ζ , which phosphorylates and activates PFK-2 and which probably lies downstream of PDK1.

EXPERIMENTAL

Materials

Activated MAPKAPK-1 [14], catalytic subunits of PKA [15], and catalytic subunits of protein phosphatase 2A (PP2A) [16], were purified as cited. PKC α and PKC ζ were from Calbiochem and Biomol (Plymouth Meeting, PA, U.S.A.) respectively. PKB α was kindly provided by A. Paterson (University of Dundee) [17]. Glutathione S-transferase (GST)-PKB α [18], GST-SGK [19], polyHis-tagged PDK1 [20], GST-p70^{s6k} lacking the C-terminal 104 residues [8], and a truncated, activated form of SGK (lacking the 60 N-terminal residues and in which Ser-422 was mutated to Asp, [10]) were purified as cited. Recombinant polyHis-tagged bovine heart PFK-2/fructose 2,6-bisphosphatase (wild-type and mutants) was expressed and purified as described [13]. Bcl-2/Bcl-XL-antagonist, causing cell death (BAD) was cloned from a cDNA mouse T-lymphocyte library (prepared by the Dundee group), subcloned into the PGEX-4T3 vector and expressed in *Escherichia coli*. Forkhead was prepared as described [21]. Antibodies against PKC ζ (residues 3–23), PKB α (residues 1–176) [17], PKB β (residues 455–469) [17], and SGK (residues 419–431) [10] were raised in sheep. A rabbit polyclonal antibody [13] raised against the C-terminus of the PKB isoforms (called BAK and raised against residues 469–481) was used for immunoprecipitation and assay of PKB in perfused rat hearts. Phospholipid vesicles containing 0.1 mM Ptdcholine, 0.1 mM Ptdserine and 10 μ M sn-1-stearoyl-2-arachidonyl-D-PtdIns(3,4,5)P₃ were prepared by sonication [18]. Peptides termed 'MR6' (PVRMRRNSFT), 'MR25' (TPVRMRRNSFTPLSSNTIRR-PRNYSVG) and 'Crosstide' (GRPRTSSFAEG) were synthesized by V. Stroobant (Ludwig Institute, Brussels, Belgium). Polylysine (average M_r 56000), *N*-(ethylmaleimidocaproyloxy)-succinimide and 2-iminothiolane were from Sigma. All other biochemicals were obtained from the sources described in [4,12,18].

Time-course of insulin-induced PFK-2 and PKB activation in perfused rat hearts

Hearts from fed male Wistar rats were perfused by the Langendorff method [3] and freeze-clamped. For PKB measurements, samples were homogenized (Ultra-Turrax; Janke & Kunkel, Staufen, Germany) for 2 $\times$ 30 s in 5 vol. (v/w) of buffer A containing 50 mM Hepes (pH 7.5), 2 mM EGTA, 2 mM EDTA, 0.2% (w/v) Triton X-100, 150 mM NaCl, 50 mM NaF, 50 mM sodium β -glycerol phosphate, 5 mM sodium pyrophosphate, 0.5 mM Na₃VO₄, 0.1% (v/v) β -mercaptoethanol,

1 mM benzamidine hydrochloride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin, and 0.2 mM PMSF. After centrifugation (20000 g, 15 min), the supernatants were stored at -80°C . PKB was immunoprecipitated with the BAK antibody and assayed as described in [13]. PFK-2 measurements were performed essentially as described in [2], with slight modifications. Samples were homogenized (Ultra-Turrax) for 2 $\times$ 30 s in 5 vol. (v/w) of buffer B containing 20 mM Hepes (pH 7.5), 30 mM KCl, 20 mM NaF, 5 mM EGTA, 100 nM microcystine-LR, 1 mM dithiothreitol (DTT), 0.1 mM fructose 6-phosphate (Fru-6-P), 0.3 mM glucose 6-phosphate (Glc-6-P), 2 mM benzamidine hydrochloride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 0.1 mM PMSF. After centrifugation (27000 g, 30 min), supernatants were mixed with 1 vol. of a solution of 40% (w/v) poly(ethylene glycol) (average M_r 6000) in buffer B devoid of PMSF, DTT, Fru-6-P and Glc-6-P. After stirring for 30 min at 4 $^\circ\text{C}$, the precipitates were collected by centrifugation (12000 g, 15 min) and resuspended in 1 vol. of buffer B lacking Fru-6-P and Glc-6-P. Extracts (10 μ l) were incubated at 30 $^\circ\text{C}$ in a final volume of 0.2 ml containing 50 mM Hepes (pH 7.1), 100 mM KCl, 20 mM KF, 5 mM potassium phosphate, 1 mM DTT, 50 μ M Fru-6-P, 0.15 mM Glc-6-P, 10 μ M Mg citrate and 5 mM MgATP. The reactions were incubated for up to 10 min and then stopped for Fru-2,6-P₂ measurement [2].

Purification of PFK-2 kinases

Hearts from fed male Wistar rats were perfused with 0.25 μ M rapamycin and 5 μ M PD98059 in the presence or absence of 0.3 μ M wortmannin for 15 min, and further perfused for 5 min with 0.1 μ M insulin [3]. Hearts (20 for each condition) were freeze-clamped and homogenized (Ultra-Turrax) for 2 $\times$ 30 s in 5 vol. (v/w) of buffer C containing 50 mM Hepes (pH 7.5), 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 5 mM sodium β -glycerol phosphate, 5 mM sodium pyrophosphate, 0.1 mM Na₃VO₄, 0.1% (v/v) β -mercaptoethanol, 2 mM benzamidine hydrochloride, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin and 20% (v/v) glycerol. The extracts were centrifuged (40000 g, 60 min) and equal amounts of supernatant proteins from each condition were filtered through a 0.45 μ m membrane (Millex SLHV; Millipore, Bedford, MA, USA) and applied on a HiLoad 16/10 Q-Sepharose column equilibrated with buffer C for FPLC (Pharmacia). The column was extensively washed with buffer C and then eluted with a 400 ml linear salt gradient (0–0.5 M NaCl in buffer C) at a flow rate of 2 ml/min. Fractions (10 ml) were collected and those containing PFK-2 kinase activity were pooled and concentrated by dialysis against 5 vol. of buffer C containing 20% (w/v) poly(ethylene glycol) (average M_r 20000). The concentrated Q-Sepharose fractions were applied on a Blue Sepharose CL-6B column (1 $\times$ 10 cm) at a flow rate of 0.75 ml/min. Fractions (10 ml) were collected. After washing extensively with buffer C, the column was eluted with a linear salt gradient (0–1 M NaCl in buffer C) and fractions (3.5 ml) were collected. The Blue-Sepharose flow-through fractions, which contained most PFK-2 kinase activity, were pooled and loaded on a Mono Q HR 5/5 column equilibrated in buffer C at a flow rate of 1 ml/min. The column was eluted with a 20 ml linear salt gradient (0–0.5 M NaCl in buffer C). Fractions (0.5 ml) containing PFK-2 kinase activity were pooled and stored at -80°C . For gel filtration, a Superdex 200 HR column equilibrated with buffer C was loaded with 0.1 ml of the PFK-2 kinase preparation and eluted at a flow rate of 0.2 ml/min. Fractions (0.1 ml) were then assayed for the phosphorylation of Ser-466 present in the MR6 peptide. The column was calibrated with gel filtration standards (Bio-Rad).

PFK-2 kinase assay

PFK-2 kinase samples (5 μ l) were incubated in a final volume of 20 μ l with 1 μ M PFK-2 and 100 μ M [γ - 32 P]ATP (1000 cpm/pmol) in phosphorylation buffer containing 10 mM Mops (pH 7), 0.5 mM EDTA, 10 mM magnesium acetate, 0.1% (v/v) β -mercaptoethanol, 5 μ M PKA inhibitor peptide. After 5 min, the reactions were stopped by adding 10 μ l of 5% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) Bromophenol Blue, 100 mM DTT, and 65 mM Tris/HCl at pH 6.8 and heated for 5 min at 90 °C for SDS/PAGE [22] in 10% (w/v) acrylamide mini-gels. 32 P-incorporation was estimated by phosphorimaging [12]. Changes in kinetic properties of PFK-2 induced by phosphorylation were assessed as described in [12]. The phosphorylation of synthetic peptides by purified protein kinases was performed as described in [12].

Dephosphorylation by PP2A

To reduce the concentration of phosphatase inhibitors, PFK-2 kinase was diluted 5-fold in phosphorylation buffer (containing 50 mM magnesium acetate). Dephosphorylation was carried out by incubation with 4 units of PP2A [16] in a final volume of 50 μ l at 30 °C. After 30 min, the reaction was stopped with 1 μ M microcystin-LR.

Phosphorylation and activation by PDK1

Phosphorylation of protein kinases by PDK1 was carried out in two steps. Firstly, protein kinases were incubated in 10 μ l of phosphorylation buffer with or without PDK1 (5 μ g/ml) in the presence of 100 μ M unlabelled ATP, with or without PtdIns(3,4,5) P_3 -containing vesicles [18]. After incubation for 30 min at 30 °C, PFK-2 kinase was measured following incubation for a further 10 min in a final volume of 20 μ l (see above). 32 P-incorporation was estimated as described in [12].

In-gel kinase analysis

A peptide (MR25) corresponding to residues 457–485 of bovine heart PFK-2, containing the phosphorylation sites Ser-466 and Ser-483, was used as a substrate to detect PFK-2 kinase activity. A free thiol group was introduced at the N-terminus of the peptide by overnight incubation of 10 mM MR25 with 20 mM 2-iminothiolane in 0.5 ml of 0.1 M triethanolamine/HCl (pH 8.5) at room temperature [23]. The modified peptide was purified by HPLC in an acetonitrile gradient [24] and the chemical modification was confirmed by matrix-assisted laser-desorption ionization–time-of-flight MS. The peptide was dried under vacuum and coupled to polylysine using *N*-(ethylmaleimidocaproyloxy)-succinimide [25]. The peptide conjugate was purified by gel filtration [25] and added to a final concentration of 25 μ M prior to polymerization of a 10% (w/v) acrylamide mini-gel [22]. The gel was loaded with the amounts of protein kinases indicated in the legend to Figure 5. Following electrophoresis, protein kinases were detected after renaturation [26].

Enzyme assays

PFK-2 was assayed as described in [27]. One unit of PFK-2 activity corresponds to the formation of 1 μ mol of Fru-2,6- P_2 per min. PP2A was assayed with 4-nitrophenyl phosphate as substrate [16]. One unit of protein kinase or protein phosphatase activity corresponds to the (de)phosphorylation of 1 nmol of substrate per min.

Other methods

Protein was estimated by the method of Bradford [28], using γ -globulin as a standard. Kinetic constants were calculated by computer fitting non-linear least-squares regression analysis of the data to a hyperbola describing the Michaelis-Menten equation (Ultrafit; Biosoft, Cambridge, U.K.).

RESULTS

Time-course of the insulin-induced activation of PFK-2 and PKB in perfused rat hearts

PFK-2 activity was increased 2-fold after 2 min of perfusion with insulin (Figure 1), consistent with our previous results on the activation of PFK-2 by insulin in isolated rat cardiomyocytes [4]. The increase in PFK-2 activity was concomitant with PKB activation. The increase in PKB activity was transient, as already observed in skeletal muscle [17].

Purification of the protein kinases responsible for the insulin-induced activation of PFK-2

Rat hearts were perfused with rapamycin and PD98059 to prevent the activation of p70^{S6k} and MAPKAPK-1, either in the presence or absence of wortmannin. The hearts were then perfused with insulin for 5 min, the time-frame within which the activation of PFK-2 was maximal. Extracts were applied on Q-Sepharose and the column was eluted with a salt gradient. An insulin-stimulated and wortmannin-sensitive PFK-2 kinase peak eluted between 100 and 250 mM NaCl (Figure 2A). This peak contained PKB, as assessed by immunoblotting (Figure 2A, inset) and was pooled, dialysed and applied on Blue Sepharose. Most of the insulin-stimulated and wortmannin-sensitive PFK-2 kinase activity was recovered in the flow-through fractions. By contrast, PKB was retained by the column and could be eluted in a salt gradient as a very broad peak (Figure 2B). The insulin-stimulated and wortmannin-sensitive PFK-2 kinase in the Blue

Figure 1 Time-course of the activation of PFK-2 and PKB by insulin in perfused hearts

After 20 min of perfusion with 5 mM glucose, the perfusion was continued with 0.1 μ M insulin for the indicated times. The values for each time point are the means $\pm$ S.E.M. for four different heart perfusions. * Indicates a statistically significant effect ($P < 0.05$) of insulin (filled symbols) compared with controls (open symbols).

Figure 2 Chromatography of PFK-2 kinases from hearts perfused with insulin in the presence or absence of wortmannin

Hearts were perfused with 0.25 μ M rapamycin and 5 μ M PD98059 for 15 min in the presence (○) or absence of 0.3 μ M wortmannin (●), and for 5 min with 0.1 μ M insulin. Hearts from each condition were freeze-clamped and homogenized as described in the Methods section. After centrifugation, the supernatants were applied on Q-Sepharose (A). Fractions 14–21, containing PFK-2 kinase activity, were pooled, concentrated, dialysed and applied on Blue Sepharose (B). Finally, the flow-through Blue Sepharose fractions were pooled and loaded on Mono Q (C). PFK-2 kinase activity was measured by 32 P-incorporation into recombinant bovine heart PFK-2. PKB was detected by immunoblotting with anti C-terminal PKB antibody (BAK).

Sepharose flow-through was applied on a Mono Q column, to which a salt gradient was applied. PFK-2 kinase was eluted in two peaks at approx. 200 and 300 mM NaCl respectively (Figure 2C). The first peak (fractions 14–17), containing insulin-stimulated and wortmannin-sensitive PFK-2 kinase activity, was pooled and concentrated for characterization.

For two separate preparations, the specific activities of the partially purified PFK-2 kinase were 36 or 40 m-units/mg of

protein (insulin) and 3.0 or 3.7 m-units/mg of protein (insulin + wortmannin). The effect of insulin to increase PFK-2 kinase activity in a wortmannin-sensitive manner was amplified from a 3-fold increase in the pooled Q-Sepharose fractions to approx. 10-fold at the end of the purification (Table 1). This presumably reflects the removal of PFK-2 kinases not stimulated by insulin. Attempts at further purification by affinity chromatography, hydrophobic interaction chromatography and gel filtration were all unsuccessful, mainly because of substantial losses of activity. This would also partly explain the low yield (Table 1). Hereafter, we refer to the wortmannin-sensitive and insulin-stimulated PFK-2 kinase preparation as 'WISK'.

Attempts to identify known insulin-stimulated protein kinases in WISK

PKB α , PKB β and PKB γ could not be detected in WISK by immunoblotting (Figures 3A, 3B and 3C), whereas similar amounts of purified PKB α , based on PFK-2 kinase activity, were easily recognized. SGK and PKC ζ , which are insulin-stimulated protein kinases [9,10], were likewise undetectable in WISK (Figures 3D and 3E). In addition, PDK1 activity could not be detected in WISK when GST-PKB was used as a substrate in incubations with PtdIns(3,4,5) P_3 -containing vesicles (results not shown). A comparison of the IC_{50} values of different protein kinase inhibitors, namely Gö6976, Gö6983 and staurosporin, for the inhibition of WISK, PKC α and PKC ζ (Table 2), suggested that WISK was not a member of the PKC family.

Phosphorylation of heart PFK-2 by WISK

WISK phosphorylated recombinant bovine heart PFK-2 in a time-dependent manner up to 0.43 ± 0.07 ($n = 5$) mol of phosphate/mol of enzyme subunit (Figure 4A). This stoichiometry of phosphorylation corresponds to about half that observed for the *in vitro* phosphorylation of recombinant heart PFK-2, previously observed for PKA, PKB and p70^{s6k}, which tended towards 1 mol of phosphate incorporated/mol of enzyme [12,13]. Figure 4(A) also shows that the time-course of phosphorylation correlated with PFK-2 activation, measured under V_{max} conditions. After 60 min of phosphorylation by WISK, when the stoichiometry of phosphorylation had reached 0.4 mol of phosphate/mol of enzyme subunit, the V_{max} of PFK-2 was increased by 2-fold (Table 3). For the control PFK-2 kinase preparation (hearts perfused with wortmannin and insulin), the rate and extent of heart PFK-2 phosphorylation and the increase in V_{max} of PFK-2 were markedly lower (Figure 4A and Table 3).

We previously demonstrated that PKB phosphorylated recombinant heart PFK-2 to an equal extent and rate on Ser-466 and Ser-483 [12,13]. Indeed, for recombinant heart PFK-2, in which either Ser-466 or Ser-483 was mutated to glutamic acid (S466E or S483E), the extent of phosphorylation was reduced from 0.7 mol of phosphate incorporated/mol of enzyme subunit to about 0.35 mol of phosphate/mol of subunit after 80 min of incubation with PKB ([13] and Figure 4B). As expected, the heart PFK-2 double mutant S466E/S483E was barely phosphorylated by PKB (Figure 4B).

We compared the phosphorylation of the recombinant wild-type and mutant preparations by WISK (Figure 4C) with that observed for PKB (Figure 4B). Mutation of Ser-466 to glutamic acid reduced the initial rate and extent of phosphorylation of recombinant heart PFK-2 by WISK compared with the wild-type, indicating that Ser-483 phosphorylation was less than that of Ser-466. Mutation of Ser-483 to glutamic acid only slightly decreased the extent of phosphorylation by WISK (Figure 4C).

Table 1 Purification of PFK-2 kinases

PFK-2 kinase activity was measured as described in the Methods section with recombinant bovine heart PFK-2 as a substrate. Protein was estimated by the method of Bradford [28] with γ -globulin as a standard. The results summarize the purification of PFK-2 kinases from one of two separate preparations. N. M., not measurable.

Fraction	Wortmannin	Volume (ml)	Protein (mg)	Total activity (m-units)	Specific activity (m-units/mg)	Yield (%)
Extract	—	67	1500	N. M.	-	-
	+	67	1430	N. M.	-	-
Q-Sepharose	—	67	90	3150	35	100
	+	67	91	1005	11	100
Blue-Sepharose	—	60	41	1140	28	36
	+	60	41	276	7	27
Mono Q	—	2	4.5	160	36	5
	+	2	3.8	12	3	1.2

Figure 3 Immunoblot analysis of WISK

The indicated amounts of protein kinase measured with PFK-2 as a substrate were subjected to SDS/PAGE. Immunoblots were screened with anti-PKB α antibody (A), anti-PKB β antibody (B), an antibody against the C-terminus of PKB (BAK) (C), anti-SGK antibody (D), or anti-PKC ζ antibody (E). The positive controls correspond to the indicated amounts of PKB α (A–C), SGK (D) and PKC ζ (E).

As for PKB, the S466E/S483E double mutant was not a substrate for WISK. The results indicate that Ser-466 and Ser-483 are the only phosphorylation sites and that WISK preferentially phosphorylates Ser-466.

The effect of phosphorylation of PFK-2 by WISK was to increase the $V_{\max}$ of PFK-2 twofold, without affecting the affinity of PFK-2 for Fru-6-P (Table 3). Our previous mutagenesis studies indicated that Ser-466 phosphorylation was responsible for the increase in $V_{\max}$ of PFK-2, whereas the phosphorylation of both Ser-466 and Ser-483 was required to decrease the K_m of PFK-2 for Fru-6-P [13]. Therefore, the fact that WISK preferentially phosphorylated Ser-466 is in agreement with the effects of WISK on the kinetic properties of PFK-2. Like PKB, WISK phosphorylated the peptide MR6, which contains Ser-466, and Crosstide (results not shown), a peptide containing Ser-9 of glycogen synthase kinase-3 β . The affinity of WISK for other synthetic peptides, such as a peptide containing Ser-483 of PFK-2 (SNTIRRPKNYSVG) and the small ribosomal S6 peptide (KKRNRTLVA), was similar to that of PKB (results not shown). The protein 'BAD' and the transcription factor 'forkhead' were also substrates for WISK. However, as for the phosphorylation of heart PFK-2, the extent of phosphorylation of both BAD and forkhead by WISK was less than that observed with PKB, when equal amounts of PFK-2 kinase activity were used (results not shown).

Estimation of the molecular mass of WISK

The M_r of WISK was estimated by gel filtration on Superdex 200 HR. PFK-2 kinase activity eluted from the column was moni-

Table 2 Effect of protein kinase inhibitors on PFK-2 kinases

Protein kinases (between 20 and 60 m-units/ml) were assayed using the peptide MR6 (100 μ M) as substrate in a final volume of 50 μ l [12]. The concentrations of the inhibitors were varied from 0.1 to 10 000 nM. The concentration of inhibitor giving 50% inhibition of enzyme activity (IC_{50}) was calculated by computer fitting of the data to a hyperbola. The results are the means $\pm$ S.E.M. for three separate determinations, otherwise individual values are given.

Protein kinases...	IC_{50} (nM)			
	PKC α	PKC ζ	WISK	Control PFK-2 kinase
Gö6976	46 $\pm$ 9	> 10000	6590 $\pm$ 1330	2340 $\pm$ 300
Gö6983	12 $\pm$ 2	3530, 2093	> 10000	> 10000
Staurosporine	14 $\pm$ 4	690, 1025	145 $\pm$ 27	28 $\pm$ 10

Figure 4 Phosphorylation of recombinant wild-type and mutant bovine heart PFK-2 preparations by WISK

(A) Recombinant wild-type PFK-2 (0.033 mg/ml) was incubated with 0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{MgATP}$ and equal amounts, in terms of protein, of WISK (filled symbols, 20 m-units/ml) or the control PFK-2 kinase preparation from hearts perfused with wortmannin and insulin (open symbols, 1.6 m-units/ml) in a final volume of 50 μl . Aliquots were removed at the indicated times for the measurement of ^{32}P -incorporation ($n = 5$). In parallel incubations with 1 mM MgATP, aliquots were removed at the indicated times for PFK-2 assay with 1 mM Fru-6-P and 5 mM MgATP ($n = 2$). (B and C) The wild-type, S466E, S483E and S466E/S483E mutant heart PFK-2 preparations (0.015 mg/ml) were incubated as described above in a final volume of 25 μl with 11 m-units/ml of PKB (B) or 20 m-units/ml of WISK (C). The results are the means of 2–3 experiments.

tored by measuring the phosphorylation of MR6. WISK was eluted from the column as a single, rather broad, peak with a M_r of 44 000–66 000 (Figure 5A). The molecular mass of WISK was also estimated by in-gel kinase analysis, where a synthetic peptide (MR25) containing the phosphorylation sites, Ser-466 and Ser-483, was incorporated into an SDS/polyacrylamide gel. Following electrophoresis, renaturation, incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, gel drying and autoradiography, a single band with a M_r of $56\,490 \pm 480$ ($n = 5$) was detected (Figure 5B). As expected, the intensity of this band was less in the control PFK-2 kinase

Table 3 Effect of phosphorylation by WISK on the kinetic properties of PFK-2

PFK-2 (0.33 mg/ml) was incubated with 1 mM MgATP and equal amounts of protein of WISK (20 m-units/ml) or the control PFK-2 kinase preparation (1.6 m-units/ml), in a final volume of 50 μl . After 60 min, aliquots were removed for PFK-2 assay with 5 mM MgATP and concentrations of Fru-6-P up to 10 times the K_m . The results are the means $\pm$ S.E.M. of three separate determinations. * Indicates a significant difference ($P < 0.05$) with respect to the controls (unpaired t test).

Preparations	PFK-2 activity	
	K_m for Fru-6-P (μM)	$V_{\max}$ (m-units/mg)
Control PFK-2 kinase	55 ± 3	29.1 ± 0.2
WISK	43 ± 7	$63.0 \pm 9.0^*$

preparation (hearts perfused with wortmannin and insulin). Interestingly, the band disappeared after treatment of WISK with PP2A (Figure 5B), suggesting that the insulin-induced activation of WISK might be due to phosphorylation (see below). Indeed, when WISK was first dephosphorylated with PP2A and then incubated with ATP in the presence and absence of PDK1, the intensity of the 56 500- M_r band was increased in PDK1-treated WISK (Figure 5C), suggesting that WISK can be phosphorylated and activated by PDK1 (see below). No band was seen when PDK1 alone was subjected to the in-gel kinase analysis. The in-gel kinase analysis (Figure 5B) was also able to reveal bands corresponding to purified PKA ($M_r = 40\,300 \pm 780$, $n = 5$) and MAPKAPK-1 ($M_r = 83\,200$ or $76\,300$, $n = 2$), but not purified PKB or SGK, even when 100-fold greater amounts (based on PFK-2 kinase activity) were loaded onto the gel.

Inactivation of WISK by PP2A and reactivation by PDK1

The in-gel kinase analysis of WISK suggested that WISK might be activated by phosphorylation in response to insulin (Figure 5B). Therefore, we studied the effect of dephosphorylation by PP2A on WISK activity, measured with recombinant heart PFK-2 as a substrate. Incubation with PP2A inactivated WISK (Table 4), suggesting that phosphorylation was responsible for its activation. No reversal was observed when PP2A was blocked by preincubation with microcystin-LR (results not shown). Dephosphorylated WISK could be partially reactivated by incubation with PDK1. However, this reactivation was not greater in the presence of $\text{PtdIns}(3,4,5)\text{P}_3$ -containing vesicles (Table 4), as was the case for the activation of PDK1 substrates such as $\text{p}70^{\text{S}6\text{k}}$ and SGK, which do not interact with $\text{PtdIns}(3,4,5)\text{P}_3$ -containing vesicles [8,10]. Although $\text{PtdIns}(3,4,5)\text{P}_3$ -containing vesicles alone stimulated WISK, the stimulation could have been non-specific because it was also observed with other lipid vesicles, such as $\text{Ptdserine}/\text{Ptdcholine}$ (2–3-fold stimulation, results not shown). In control experiments, we verified that the activation of PKB by PDK1 was maximal in the presence of $\text{PtdIns}(3,4,5)\text{P}_3$ -containing vesicles and that the activation of SGK by PDK1 was independent of $\text{PtdIns}(3,4,5)\text{P}_3$ (Table 4). Table 4 also shows that heart PFK-2 is a substrate of SGK.

The fact that PDK1 partially reactivated WISK implies that WISK should be a substrate for PDK1. A Coomassie Blue-stained gel of the WISK preparation revealed that it contained several bands (results not shown). However, incubation of the WISK preparation with PDK1 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ led to the labelling

Figure 5 Estimation of the molecular mass of WISK

(A) Equal amounts (50 μ g) of the WISK (●) and the control PFK-2 kinase preparation (○) were applied onto a Superdex 200 HR column at a flow rate of 0.2 ml/min. Fractions (0.1 ml) were collected and 10 μ l of each fraction was assayed for protein kinase activity with MR6 (50 μ M) as a substrate [12] in a final volume of 50 μ l. Arrows indicate the elution positions of the molecular mass markers. (B) In-gel kinase analysis. Protein kinases were subjected to SDS/PAGE in acrylamide gels co-polymerized with the MR25 peptide (see the Methods section). The gel was incubated to renature protein kinases and was further incubated with 50 μ M [γ - 32 P]ATP (approx. 2000 cpm/pmol) in phosphorylation buffer. To compare the control PFK-2 kinase preparation (lane 1) with WISK (lane 2), equal amounts of protein were loaded onto the gel. Lane 1, control PFK-2 kinase (0.04 m-units); lane 2, WISK (0.5 m-units); lane 3, WISK (0.3 m-units) dephosphorylated by PP2A; lane 4, PKB α (50 m-units); lane 5, GST-SGK (50 m-units); lane 6, PKA (125 m-units); lane 7, MAPKAPK-1 (125 m-units); lane 8, WISK (1.1 m-units), which had been dialysed in buffer C devoid of protein phosphatase inhibitors, dephosphorylated by PP2A and incubated with 250 μ M MgATP (final volume 30 μ l); lane 9, WISK (1.1 m-units), treated as in lane 8 but incubated with PDK1 (50 μ g/ml); lane 10, 30 μ l of PDK1 (50 μ g/ml) incubated with 250 μ M MgATP; lane 11, PKA (50 m-units); lane 12, MAPKAPK-1 (50 m-units). (C) WISK (4.5 m-units) was dialysed against buffer C, which was devoid of protein phosphatase inhibitors and dephosphorylated with PP2A. Dephosphorylated WISK was then incubated with 250 μ M [γ - 32 P]MgATP (5000 cpm/pmol) in a final volume of 30 μ l with (lane 2) or without (lane 1) PDK1 (10 μ g/ml) for 5 min at 30 °C; lane 3 contains PDK1 alone (10 μ g/ml) incubated under the same conditions; lane 4, GST-PKB (1 μ g) incubated with PDK1 (10 μ g/ml), 250 μ M [γ - 32 P]MgATP (1000 cpm/pmol) and PtdIns(3,4,5) P_3 -containing vesicles for 5 min at 30 °C; lane 5, p70^{S6k} (1 μ g) incubated with PDK1 (10 μ g/ml) and 250 μ M [γ - 32 P]MgATP (1000 cpm/pmol) for 5 min at 30 °C. In this experiment the solvent front was allowed to run off the gel, in order to increase resolution in the M_r 50 000–60 000 region of the gel.

Table 4 Effects of treatment with PtdIns(3,4,5) P_3 and PDK1 on the PFK-2 kinase activities of WISK, dephosphorylated WISK, PKB and SGK

The protein kinases were incubated with unlabelled ATP in a final volume of 10 μ l of phosphorylation buffer with or without PDK1 and PtdIns(3,4,5) P_3 vesicles. After 30 min of incubation, PFK-2 kinase was assayed and the values are expressed as μ -units. WISK was dephosphorylated with PP2A as described in the Methods section. The results are the means $\pm$ S.E.M. of three separate experiments. * Indicates a significant difference ($P < 0.05$) with respect to the untreated controls (unpaired t test).

Addition	WISK	PP2A-treated WISK	GST-PKB	GST-SGK
None	39.8 $\pm$ 2.3	3 $\pm$ 0.4	25.9 $\pm$ 4.2	161.9 $\pm$ 15.3
PDK1	66.6 $\pm$ 6.0*	25.6 $\pm$ 3.3*	121.5 $\pm$ 30.4*	784.5 $\pm$ 31.2*
PDK1/PtdIns(3,4,5) P_3	65.7 $\pm$ 5.8*	21.6 $\pm$ 5.4*	525 $\pm$ 17.2*	763.4 $\pm$ 5.8*

of a single band (Figure 5C) with a M_r of 57240 ± 770 ($n = 6$), similar to that observed in the in-gel kinase analysis. Some labelling of this band was seen on incubation with ATP alone (Figure 5C), suggesting that WISK autophosphorylates, or that the preparation contains a contaminating kinase that phosphorylates WISK. As a control, incubation of GST-PKB and GST-p70^{s6k} with PDK1 and [γ -³²P]ATP led to the labelling of single bands with M_r of 71090 ± 2700 ($n = 3$) and 69640 ± 1730 ($n = 3$) respectively, as expected. In all the incubations with PDK1, the band migrating with a M_r of 62000 corresponds to autophosphorylated PDK1 [18].

Since WISK, like PKB, is a substrate of PDK-1, we tested whether WISK contained conserved sequences around the two phosphorylation sites found in PKB. The results indicated that anti-(phospho-Thr-308 PKB) and anti-(phospho-Ser-473 PKB) antibodies cross-react with WISK, however, with approx. 100 times less affinity than for PKB (results not shown).

DISCUSSION

We have partially purified a wortmannin-sensitive and insulin-stimulated protein kinase from rat hearts, that phosphorylates and activates heart PFK-2 *in vitro*. This protein kinase preparation, which we call WISK, phosphorylated Ser-466 to a greater extent than Ser-483. Both sites are phosphorylated *in vitro* by protein kinases of the insulin signalling cascades, including PKB [12], and in HEK-293 cells transfected with bovine heart PFK-2 and incubated with insulin [13]. Although WISK preferentially phosphorylated Ser-466 in heart PFK-2, the phosphorylation of synthetic peptides was similar to that observed with PKB. The proteins BAD and forkhead were phosphorylated to a somewhat lesser extent by WISK than by PKB. Our site-directed mutagenesis experiments show that the phosphorylation of Ser-466 is responsible for the increase in $V_{\max}$ of PFK-2 [13]. The 2-fold increase in $V_{\max}$ of PFK-2 induced by insulin, with no detectable change in K_m for Fru-6-P, seen in rat hearts *in vivo* [2], resembles the *in vitro* activation of heart PFK-2 after phosphorylation by WISK (Table 3).

In transfection experiments on HEK-293 cells, we showed that PKB was not required in the insulin signalling pathway leading to PFK-2 activation [13]. However, the transfection experiments did suggest a role for PDK1. These transfection experiments support the existence of a protein kinase, downstream of PDK1 and distinct from PKB, in the insulin signalling pathway for PFK-2 activation [13].

We believe that WISK is a hitherto unrecognized protein kinase in the insulin signalling pathway. Indeed, we were unable to detect any of the PKB isoforms, PKC ζ or SGK by immunoblot analysis of WISK and no PDK1 activity could be detected in the preparation. In addition, the pattern of inhibition of WISK by different protein kinase inhibitors indicates that WISK does not belong to the PKC family and differs from PKC ζ , which is a substrate of PDK1. Furthermore, WISK activation by PDK1 was not dependent on the presence of PtdIns(3,4,5) P_3 (Table 4 and see below) whereas the phosphorylation and activation of PKC ζ has been reported to be dependent on 3-phosphoinositides [9]. WISK was characterized as being a monomer with a M_r of approx. 57000.

The insulin-induced activation of WISK by insulin was reversed by treatment with PP2A, implying the involvement of an upstream activating kinase(s). Indeed the dephosphorylated WISK could be partly reactivated by treatment with PDK1. The

reactivation of WISK by PDK1 was not further enhanced by PtdIns(3,4,5) P_3 . The fact that full reactivation was not achieved suggests that site(s) other than those phosphorylated by PDK1 would be required for full activation. One site could correspond to the PDK2 site found in many AGC kinases (equivalent to Ser-473 of PKB, Thr-389 of p70^{s6k}, and Ser-422 of SGK) [7–10].

In conclusion, our findings demonstrate the existence of a protein kinase in the insulin signalling pathway downstream of PDK1, which differs from PKB, SGK and PKC ζ and which phosphorylates and activates heart PFK-2.

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REFERENCES

- Depré, C., Rider, M. H. and Hue, L. (1998) *Eur. J. Biochem.* **258**, 277–290
- Rider, M. H. and Hue, L. (1984) *FEBS Lett.* **176**, 484–488
- Depré, C., Rider, M. H., Veitch, K. and Hue, L. (1993) *J. Biol. Chem.* **268**, 13274–13279
- Lefebvre, V., Méchin, M.-C., Louckx, M. P., Rider, M. H. and Hue, L. (1996) *J. Biol. Chem.* **271**, 22289–22292
- Lavoine, A., Erikson, E., Maller, J. L., Price, D. J., Avruch, J. and Cohen, P. (1991) *Eur. J. Biochem.* **199**, 723–728
- Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) *Biochem. J.* **333**, 471–490
- Alessi, D. R. and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8**, 55–62
- Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. and Avruch, J. (1998) *Curr. Biol.* **8**, 69–81
- Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998) *Science* **281**: (5385) 2042–2045
- Kobayashi, T. and Cohen, P. (1999) *Biochem. J.* **339**, 319–328
- Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P. and Alessi, D. R. (1999) *Curr. Biol.* **9**, 393–404
- Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. and Rider, M. H. (1997) *J. Biol. Chem.* **272**, 17269–17275
- Bertrand, L., Alessi, D. R., Deprez, J., Deak, M., Viaene, E., Rider, M. H. and Hue, L. (1999) *J. Biol. Chem.* **274**, 30927–30933
- Sutherland, C., Campbell, D. G. and Cohen, P. (1993) *Eur. J. Biochem.* **212**, 581–588
- Reimann, E. M. and Beham, R. A. (1983) *Methods Enzymol.* **99**, 51–55
- Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P. and Tung, H. Y. (1988) *Methods Enzymol.* **159**, 390–408
- Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) *Biochem. J.* **331**, 299–308
- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reece, C. B. and Cohen, P. (1997) *Curr. Biol.* **7**, 261–269
- Casamayor, A., Torrance, P. D., Kobayashi, T., Thorner, J. and Alessi, D. R. (1999) *Curr. Biol.* **9**, 186–197
- Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D. et al. (1997) *Curr. Biol.* **7**, 776–789
- Rena, G., Guo, S., Cichy, S. C., Unterman, T. G. and Cohen, P. (1999) *J. Biol. Chem.* **274**, 17179–17183
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Alagon, A. C. and King, T. P. (1980) *Biochemistry* **19**, 4341–4345
- Rider, M. H., Van Damme, J., Vertommen, D., Michel, A., Vandekerckhove, J. and Hue, L. (1992) *FEBS Lett.* **310**, 139–142
- Kameshita, I. and Fujisawa, H. (1996) *Anal. Biochem.* **237**, 198–203
- Kameshita, I. and Fujisawa, H. (1989) *Anal. Biochem.* **183**, 139–143
- Crepin, K. M., Vertommen, D., Dom, G., Hue, L. and Rider, M. H. (1993) *J. Biol. Chem.* **268**, 15277–15284
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254