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Expression and purification of active PKB kinase from *Escherichia coli*

Shoshana Klein[a], Tamar Geiger[a], Inbal Linchevski[a], Mario Lebendiker[b], Anna Itkin[a], Karin Assayag[a], Alexander Levitzki[a,*]

[a] *Unit of Cellular Signaling, Department of Biological Chemistry, The Hebrew University of Jerusalem, Safra Campus, Jerusalem 91904, Israel*
[b] *Protein Purification Unit, Wolfson Center for Applied Structural Biology, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*

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Abstract

PKB/Akt is a protein involved in control of apoptosis, proliferation and cellular metabolism, and it has been found to be activated in many cancers. Activation of PKB involves recruitment of the enzyme by its PH domain to the cell membrane, and phosphorylation at two residues, T308 and S473. To produce active PKB kinase from *Escherichia coli*, we constructed a derivative of PKB lacking the PH domain and mutated to glutamate at residues S124, T450 and the activating residue S473 (ΔPH-PKB-EEE). ΔPH-PKB-EEE was expressed in *E. coli* together with PDK1, the kinase responsible for phosphorylating PKB at T308, which was expressed as a GST-fusion. Full-length ΔPH-PKB-EEE was obtained by using a double tag strategy: His6 at the N-terminus and FLAG at the C-terminus. The protein was purified by nickel affinity chromatography, followed by passage over an anti-FLAG column. The final purification step, anion exchange over a monoQ column, separated phosphorylated from unphosphorylated protein. Active recombinant PKB kinase was thus produced from *E. coli*, by a simple, reproducible procedure.
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Keywords: PKB; Co-expression; His-tag; FLAG-tag; *E. coli*; Kinase; Recombinant protein

Protein kinase B (PKB)/Akt kinase is a key protein involved in the control of apoptosis, proliferation, and cellular metabolism (reviewed in [1,2]). PKB exists as three highly homologous isoforms, PKBα, β, and γ. PKB has been found to be activated or over-expressed in a number of cancers, including breast, pancreatic, and ovarian cancers and melanoma [3–6]. Furthermore, PKB is negatively regulated by PTEN, a potent tumor suppressor (for review see [7]), which is mutated or deleted in many advanced cancers [8–12]. Thus, activation of PKB is associated with the most aggressive forms of cancer. Therefore, inhibition of PKB is an attractive approach to therapy for a wide variety of cancers [13–17].

In vivo, activation of PKB depends on the activity of phosphoinositide 3′ kinase (PI-3′K). The phosphoinositides (PIP_3, PIP_2) produced by PI-3′K bind to the N-terminal PH domain of PKB, anchoring PKB to the plasma membrane, where it is phosphorylated. If the PH domain is replaced with a myristoylation domain, PKB becomes constitutively activated [18]. At the membrane, the enzyme PDK1 (phosphoinositol-dependent kinase) activates PKB, by phosphorylation of PKBα threonine residue 308 (or its equivalent T309 in PKBβ, T305 in PKBγ) [19]. Serine residue 473 (S474 in PKBβ, S472 in PKBγ) must also be phosphorylated for maximal activity, although the kinase responsible for this is a subject of debate, the latest candidate being DNA-PK [20]. A

* Corresponding author. Fax: +972 2 6512958.
E-mail address: LEVITZKI@vms.huji.ac.il (A. Levitzki).

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number of other serine and threonine residues are apparently constitutively phosphorylated [21].

To aid us in our search for PKB inhibitors, we required a convenient source of purified, active PKB protein. We co-expressed GST-tagged PDK1 and His_6-tagged PKBα in *Escherichia coli*. To minimize heterogeneity due to partial phosphorylation, we mutated residues S124, T450, and S473 to glutamate. The mutation S473E was also expected to assist in activation of the protein, but we did not include a T308E mutation, because mutating T308 inactivated the protein. The PKB was active, whether or not the expression construct included the PH domain. Much of the protein, however, was truncated. To purify the full-length product, we double-tagged the construct, placing His_6 at the N-terminus and FLAG-peptide at the C-terminus. Here, we report on the purification and properties of active PKB kinase, produced from *E. coli*.

Materials and methods

Site-directed mutagenesis and plasmid construction

Plasmid pCMV5-HA-PKB (HA-tagged wild-type, human PKBα [21]) was given to us by D. Alessi. The *Eco*RI–*Kpn*I fragment encoding PKB was subcloned into vector pBlueScriptIIKS$^-$ (Stratagene), for ease of mutagenesis and sequencing. Unless stated otherwise, all DNA manipulations were performed using *E. coli* DH5α. All mutations were confirmed by DNA sequencing, and the sequenced fragments carrying the mutations were reintroduced into pCMV-HA-PKB by standard restriction digestion and ligation. DNA was sequenced by the Center for Genomic Technologies of The Hebrew University of Jerusalem.

Mutations to change T308 (not shown) or S124 were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Double-stranded mutagenic oligonucleotides (Gibco-BRL Life Technologies) were as follows (only the sense strand is shown; capital letters indicate changes from the wild-type sequence):

S124A: 5′ gaggaggagatggacttcAgAtcTggcGCCcccagtga caac (introduces *Bgl*II site for screening; TCA → GCC converts S124 to A).

S124E: 5′ gaggaggagatggacttcAgAtcTggcGAGcccagtga caactcag (introduces *Bgl*II site; TCA → GAG converts S124 to E).

Mutations to change T450 or S473 were introduced by PCR, as follows:

T450A: sense primer (mutagenic): 5′ cccagatgatcaccatc GcCccTccGgaccaagatg (ACA → GCC converts T450 to A; introduces *Bsp*EI and *Ava*II sites).

T450E: sense primer (mutagenic): 5′ cccagatgatcaccatc GAGccaccGgaccaagatg (ACA → GAG converts T450 to E; introduces *Ava*II site).

The antisense primer for T450 mutations was the "Reverse primer" from vector pBlueScriptIIKS$^-$. The PCR fragments were digested with *Bcl*I and *Xho*I, and cloned into pSK23, which had been propagated in the *dam*$^-$ strain, *E. coli* JM110.

S473E: Sense primer (non-mutagenic): 5′ gggcacattaa-gatcacagac (nucleotides 1054–1074 in the sequence of human PKBα, GenBank Accession No. M63167).

Antisense primer (mutagenic): 5′ ctgGGTACCtcag-gccgtgccgctggccgagtaTTCgaactggg (includes a *Kpn*I/*Asp*718 site for cloning; the mutation to glutamate also introduces a *Bst*BI site for screening). The PCR fragment was digested with *Hin*dIII and *Asp*718, to replace the wild-type fragment.

HA-PKB-S124E,T450E,S473E ("PKB-EEE") was constructed in stages. S473 was converted to E as described above, using the T450E mutant as the PCR template, to generate HA-PKB-T450E,S473E. The *Apa*I–*Bam*HI fragment from PKB-T450E,S473E, was used to replace the equivalent fragment in pCMV5-HA-PKB-S124A, generating the triple mutant.

The various mutant constructs were recloned into the mammalian expression vector, pCMV5, for transfection into HEK293 cells. Cell culture, transfection, and radioactive kinase assays were as previously described [14], except that PKB derivatives were immunoprecipitated from cell lysates using anti-HA monoclonal antibody (12CA5).

For expression in *E. coli*, *Eco*RI–*Xho*I fragments carrying HA-PKB or HA-PKB-EEE were subcloned into vector pET28a (Novagen), to generate pET-His_6-HA-PKB and pET-His_6-HA-PKB-EEE. This vector encodes resistance to kanamycin.

The PH domain was deleted from PKB by PCR of the C-terminal segment, which was subcloned as a *Bam*HI–*Sal*I fragment from an intermediate vector between the *Bam*HI–*Xho*I sites of pET28a, to generate pET-His_6-ΔPH-PKB. The *Bgl*II–*Xho*I fragment of pCMV5-HA-PKB-EEE (the *Bgl*II site had been introduced during mutagenesis of S124E) was subcloned between the *Bam*HI–*Xho*I sites of pET28a, to generate pET-His_6-ΔPH-PKB.

A C-terminal FLAG tag was added to pET-His_6-HA-PKB-EEE, by ligation of a double-stranded oligonucleotide, between the *Bst*BI site introduced with the S473E mutation and the *Xho*I site of the vector. The sequence of the FLAG oligonucleotide was:

Sense strand: 5′ cgaatactcggccagcggcactgcaggaggcgactac aaggatgacgatgacaagtgataac.

Antisense strand: 5′ tcgagttatcacttgtcatcgtcatccttgtagt cgcctcctgcagtgccgctggccgagtatt.

The *Pst*I site within this oligonucleotide was helpful for screening.

An *Nco*I–*Eag*I fragment that encodes most of the PDK1 gene was subcloned from pEBG2T-GST-PDK1, a gift from D. Alessi. The PDK1 fragment was cloned between the *Nco*I–*Hin*dIII sites of vector pGEX-KG [22], after "filling-in" of the *Eag*I and *Hin*dIII sites by Klenow fragment. This generated an in-frame GST-PDK1 fusion ("pGEX-PDK1"), in an *E. coli* expression plasmid that confers resistance to ampicillin. We also co-expressed both GST-PDK1 and His_6-ΔPH-PKB-EEE from a single plasmid, but the yield of phosphorylated PKB was not improved.

Protein expression and purification

For protein expression, plasmids were transformed into *E. coli* BL21(DE3), BL21(DE3, pLysS) or BL21-CodonPlus(DE3)-RP (Stratagene). First, either pGEX-KG or pGEX-PDK1 was introduced, followed by enrichment for transformed colonies by growth in LB supplemented with ampicillin (50 mg/L) and glucose (2%). For *E. coli* strains BL21(DE3, pLysS) or BL21-CodonPlus(DE3)-RP, selection for resistance to chloramphenicol (25 mg/L) was also maintained. A second round of transformation was performed using the pET-PKB constructs, followed by selection on LB-agar, supplemented with glucose (2%), ampicillin (100 mg/L), and kanamycin (30 mg/L) (as well as chloramphenicol, when relevant). The double transformants were grown overnight at 37 °C in LB supplemented with glucose (2%) and the appropriate antibiotics. The bacteria were subcultured the following morning, grown to $OD_{600} = 0.3$ at 37 °C, and then transferred to 23 °C. When the cultures reached $OD_{600} = 0.5$, IPTG (0.3 mM) was added. The best yield of full-length, phosphorylated protein was obtained when cultures were harvested 6 h after IPTG induction. Approximately, 7 g wet weight cells were obtained per liter of culture. The bacterial pellets were rinsed with buffer consisting of 50 mM Tris–HCl, pH 8, 300 mM NaCl, and 1 mM AEBSF (Roche), recentrifuged, snap-frozen in liquid nitrogen, and stored at −70 °C.

Escherichia coli BL21(DE3, pLysS) or BL21-CodonPlus(DE3)-RP was lysed by gentle agitation with lysozyme (3 mg/ml) for 20–30 min at 4 °C. The lysis buffer (15 ml buffer per 7 g bacterial pellet) consisted of 20 mM Hepes, pH 7.4, 150 mM NaCl, supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM β-glycerol phosphate, and 5 mM sodium pyrophosphate) and protease inhibitors (10 μg/ml leupeptin, 0.2 mM AEBSF, 1 μg/ml aprotinin, 0.5 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, and 3 mM of 2-mercaptoethanol). DNAseI (Roche) was added to a concentration of 40 U/ml, and the lysate was mixed gently for 20 min at 4 °C, followed by three freeze–thaw cycles in liquid nitrogen. Although for all three BL21-derived strains, sonication solubilized more anti-PKB reactive protein, it led to a decrease in the relative signal obtained with anti-phosphoT(308)-PKB antibody, indicating a reduction in kinase activity. We therefore preferred the more gentle lysis procedure. The lysate was either snap-frozen in liquid nitrogen and stored at −70 °C, or directly loaded onto 2 ml Ni–NTA–agarose beads (Qiagen), which had been pre-equilibrated with lysis buffer.

Before loading the lysate onto the Ni–NTA–agarose beads, imidazole (pH 8) was added to a concentration of 10 mM. The beads were rotated with the lysate for 2 h at 4 °C, unbound protein was collected, and the beads were washed with 20 mM Hepes, pH 7.4, 1 M KCl, 10 mM imidazole, 10% glycerol, and 0.2 mM AEBSF and phosphatase inhibitors. His_6-tagged protein was eluted with 24 ml of the same buffer, except that the concentration of KCl was 100 mM and that of imidazole was 200 mM.

The eluate from the Ni–NTA–agarose beads was loaded onto 2 ml of anti-FLAG M2 agarose beads (Sigma), which had been pre-equilibrated with 20 mM Hepes, pH 7.4, 150 mM NaCl, and 0.2 mM AEBSF and phosphatase inhibitors. The beads plus Ni–NTA eluate were rotated at 4 °C for at least 1 h, before collection of the unbound protein and washing of the beads in the same buffer. FLAG-tagged protein was eluted with 20 ml buffer to which FLAG peptide (100 μg/ml) and glycerol (10%) had been added. The eluate was snap-frozen in liquid nitrogen and stored at −70 °C, until the next step.

In the final purification step, the protein was diluted and loaded at a flowrate of 1.5 ml/min on a MonoQ HR 5 × 0.5 cm column equilibrated with buffer consisting of 25 mM Tris–HCl, pH 8.2, 3 mM of 2-mercaptoethanol, 0.1 mM EGTA, and 10% glycerol. The column was washed with five column volumes of equilibration buffer + 0.1 M NaCl and five column volumes of equilibration buffer + 0.15 M NaCl and then the bound protein was eluted with a linear gradient of 30 ml NaCl (0.15–0.3 M NaCl) at a flowrate of 1 ml/min. One milliliter fractions were collected and analyzed.

PAGE and immunoblotting

At every stage of the purification, samples were removed for activity assays, frozen, and stored at −70 °C. Samples for SDS–PAGE (10% polyacrylamide) and Western blotting were denatured by boiling for 5 min in Laemmli sample buffer. Polyacrylamide gels were stained using Gelcode-Blue (Pierce). Antibodies against phospho-Akt [Thr308 (Catalog #9275; used at 1:1000 dilution) or Ser473 (#9271; 1:1000)] were from Cell Signaling Technology; anti-PDK1 (#sc9118;

1:3000) and anti-His_6 (#sc8036; 1:1000) were from Santa Cruz Biotechnology; anti-GST (#05-311; 1:2000) was from Upstate Biotechnology; and anti-FLAG M2 (1:2500) was from Sigma. For detection of phospho-proteins, nitrocellulose blots were blocked with 5% BSA in TBST (10 mM Tris–HCl, pH 7.5, 170 mM NaCl, and 0.2% Tween 20) and antibodies were diluted into the same. Otherwise, the blocking solution consisted of low fat milk diluted 1:20 into TBST. Blots were incubated in the antibody solution overnight at 4 °C, and then with horseradish peroxidase-conjugated secondary antibodies for 40 min at room temperature. Immunoreactive bands were visualized using ECL. For reprobing, blots were stripped of antibody by incubation for 20 min in 62 mM Tris–HCl, pH 6.8, 2% SDS, and 100 mM of 2-mercaptoethanol, at 55 °C, followed by several washes with double-distilled water and a final wash with TBST.

PKB kinase assays

Kinase activity was assessed using the ELISA assay that we developed [14]. Specific activity was determined using the radioactive assay, as described earlier [14], except that, for the bacterially produced enzyme, no PKI (an inhibitor of PKA) was added to the reaction mixture and the concentration of ATP in the assay was 100–250 μM, depending on the experiment. IC_{50} values for PKB inhibitors were determined at 10 μM ATP.

Results and discussion

PKBα derivatives for expression in E. coli

In preliminary experiments (data not shown), we investigated the effect on PKB kinase activity of mutating various threonine or serine residues, by immunoprecipitating the mutant proteins from HEK293 cells. Full activation of PKB requires phosphorylation of T308 and S473 [21]. Mutations to aspartate (D) or glutamate (E) are often able to mimic the phosphorylated states of threonine (T) or serine (S). In our hands, the T308D,S473D double mutant of Alessi et al. [21], which had been reported to be constitutively active, was inactive. When S473 was mutated to either D or E, the PKB was partially activated, and could be fully activated by induction of the cells with IGF1. Mutation of T308 to either D or E resulted in inactive protein. Mutation of residues that had been reported to be constitutively phosphorylated in mammalian cells, namely S124 or T450, to A (to mimic the non-phosphorylated state), had no effect on enzyme activity, leading us to conclude that phosphorylation at these sites is not essential for enzyme activation. The triple mutant, S124E,T450E,S473E, had elevated basal activity and could be induced to full activity by IGF-1 stimulation.

We anticipated that the PH domain would not participate in activation of PKB by co-expressed PDK1 in *E. coli*, and we suspected that it would be easier to express a shorter protein in this heterologous system. We therefore constructed a series of four plasmids, with and without the S124E,T450E,S473E triple mutation, and with and without the PH domain, in the *E. coli* His_6-tag expression vector, pET28 (Fig. 1). These plasmids were introduced into *E. coli* BL21(DE3)/pLysS, which had been previously transformed with pGEX-KG or pGEX-PDK1. Selection for both the PDK1 and PKB-encoding plasmids was maintained. Expression of both GST-PDK1 and the PKB derivatives was induced using IPTG, and verified by immunoblotting.

In initial experiments, co-expression of each of the four constructs together with GST-PDK1 led to phosphorylation of T308, as detected by immunoblotting with antibody specific for the PKB phosphorylated at T308. In the absence of GST-PDK1, T308 was not phosphorylated (Fig. 2). Anti-S473 PKB antibody also gave a distinct signal with immunoblots, whether or not S473 had been mutated to E, and whether or not GST-PDK1 was expressed, indicating that the S473 residue was at least partially phosphorylated in *E. coli*. Weak PKB kinase activity was detected in crude lysates for all four activated constructs, using the ELISA PKB activity assay (data not shown). The shorter ΔPH

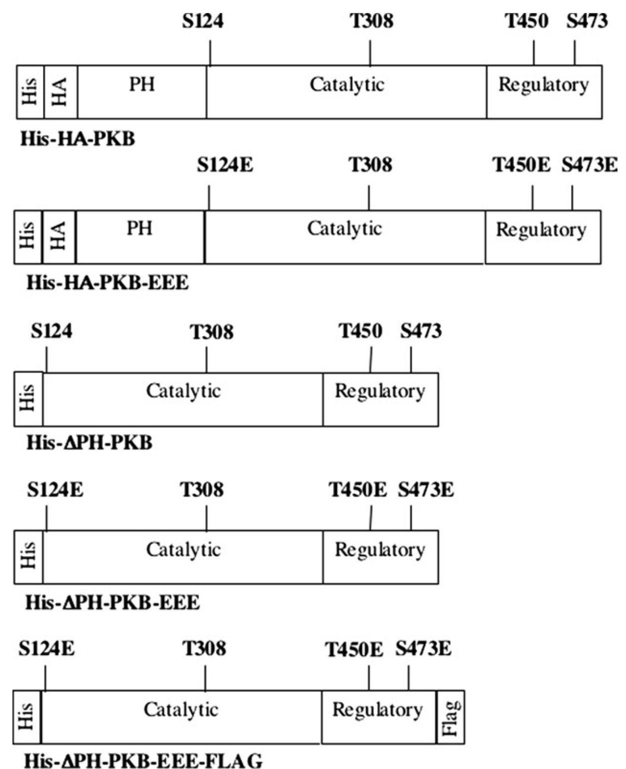

Fig. 1. Constructs for expression of PKB in *E. coli*.

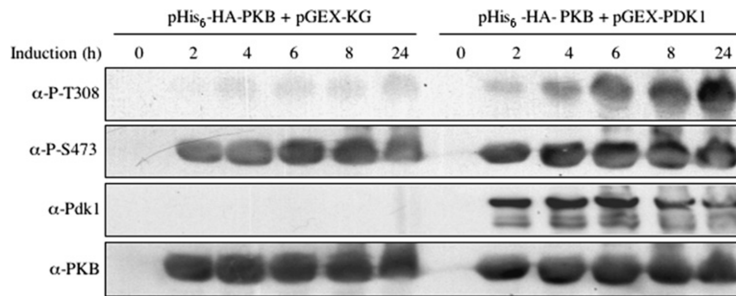

Fig. 2. Coexpression of GST-PDK1 and His_6-HA-PKB in *E. coli* BL21(DE3) leads to phosphorylation of PKB residue T308. Two Western blots were prepared in parallel, and incubated with anti-phospho(T308)PKB and anti-phospho(S473)PKB. The blots were then stripped and incubated with anti-PKB or anti-PDK1 antibody.

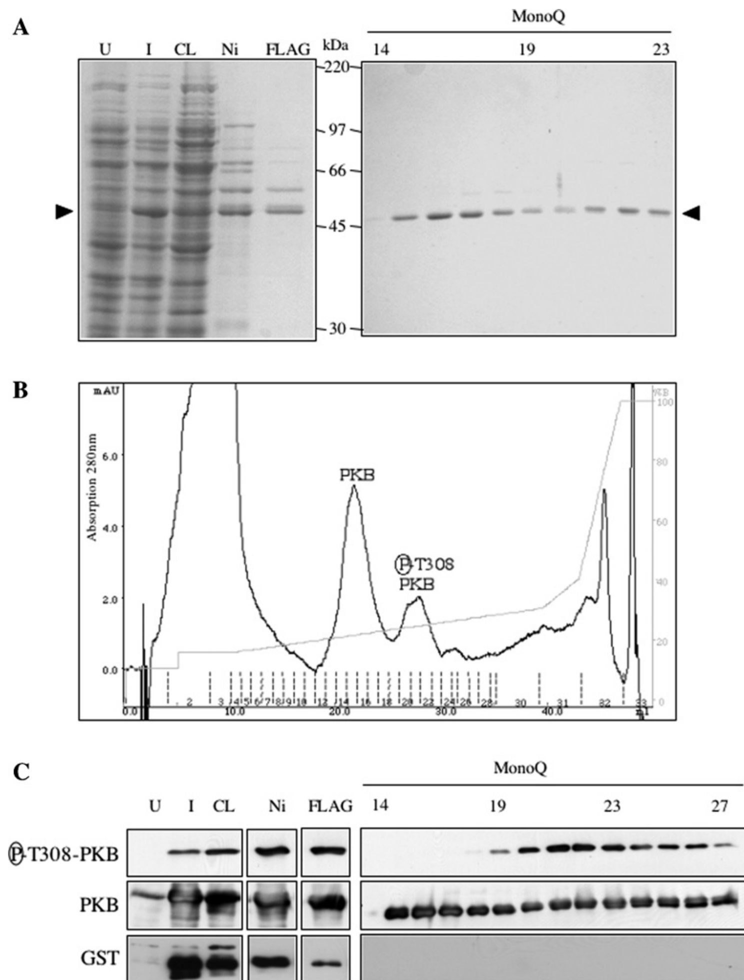

Fig. 3. Purification of His_6-ΔPH-PKB-EEE-FLAG. (A) Samples were removed at each stage of the purification, denatured in Laemmli buffer, and separated by SDS–PAGE. The gels were stained with Gelcode-Blue (Pierce). The right-hand side gel shows fractions 14–23 from the MonoQ column. The arrowheads show the position of His_6-ΔPH-PKB-EEE-FLAG. (B) MonoQ scan. (C) Western blot. Samples were treated as in (A). The gels were blotted onto nitrocellulose and probed with rabbit anti-phospho(T308)PKB. The blots were then probed with mouse anti-GST, to visualize GST-Pdk1. Finally, the blots were stripped and reprobed with rabbit anti-PKB. Lanes that were consecutive on the original gels are boxed together. [U, uninduced; I, induced with IPTG, for 6 h; CL, cleared lysate; Ni, after purification on Ni–NTA–agarose (Qiagen); FLAG, after purification on anti-FLAG-M2 agarose (Sigma)].

derivatives were expressed slightly better than the full-length proteins. In light of these results, we decided to concentrate our efforts on improving the expression and purification of the His_6-ΔPH-PKB-EEE derivative.

Purification of ΔPH-PKB-EEE protein

In the early experiments, we estimated that approximately 4 mg of anti-PKB reactive protein was expressed per liter of bacterial culture. In most experiments, more

than half of this protein remained in the insoluble pellet, after cell lysis. In attempts to increase the yield of active protein, we performed temperature and time course experiments, and also attempted to express the protein in a variety of *E. coli* strains (data not shown). The optimal conditions at which we arrived were: induction for 6 h with 0.3 mM IPTG, at 23 °C. Longer induction led to increased production of truncated products. Even after 6 h induction, truncated protein was clearly visible on Western blots. After purification on a Ni–NTA–agarose column, His_6-ΔPH-PKB-EEE comprised only ~30% of the Gelcode-Blue-stained protein. (See Fig. 3A, which shows the purification of His_6-ΔPH-PKB-EEE-FLAG. Ni–NTA purification of His_6-ΔPH-PKB-EEE protein gave analogous results.) Slightly more full-length protein was obtained using *E. coli* BL21-CodonPlus(DE3)-RP than *E. coli* BL21(DE3)/pLysS, so later experiments were performed using this background.

To improve the purification procedure, and concomitantly to overcome the problem of the truncated protein products, we decided to add a C-terminal tag to the protein. We hoped that the double purification procedure, using an N-terminal as well as a C-terminal tag, would yield full-length protein. We elected to add the FLAG peptide to the C-terminus of His_6-ΔPH-PKB-EEE, because proteins carrying this tag can be released from the anti-FLAG agarose purification column by competition with free FLAG peptide. Moreover, we knew from other studies that PKB could be labeled with the short His_6 tag at the C-terminus without compromising its activity (unpublished data). We therefore hoped that the small FLAG peptide would not affect PKB activity.

The double purification procedure (Table 1) indeed solved the problem of the truncated PKB products. Even after overexposure of Western blots, only full-length His_6-ΔPH-PKB-EEE-FLAG was detected (Fig. 3C), and this comprised ~80% of the total protein. This protein ran as a doublet on SDS–PAGE, the lower band representing protein that was not phosphorylated on T308. To separate the phosphorylated from the non-phosphorylated protein, a final step of anion exchange, utilizing a MonoQ column, was performed (Fig. 3B). This final step also removed all detectable traces of GST-PDK1 protein (Fig. 3C). The overall yield from this procedure was poor, because most of the PKB was insoluble and discarded after bacterial lysis, and because at each step we sacrificed yield in the interests of improved specific activity. A further major loss occurred during elution from the anti-FLAG beads, where up to 50% of the anti-PKB reactive protein remained bound to the column, despite intensive efforts to elute this (data not shown). We finally obtained 20 μg of phosphorylated PKB protein (from 1 L of bacterial culture; 7 g wet weight), which was >95% pure.

Biochemical characterization of ΔPH-PKB-EEE

The purified His_6-ΔPH-PKB-EEE-FLAG enzyme had a specific activity of >700 units/μg, where 1 unit was defined as 1 pmol/min phosphate transferred to 20 μM 7-mer specific substrate, RPRTSSF [23]. The FLAG tag affected neither the K_m for ATP nor for substrate. The K_m for ATP was 155 μM (Table 2; average of four experiments, using His_6-ΔPH-PKB-EEE and His_6-ΔPH-PKB-EEE-FLAG), higher than that obtained for full-length, wild-type His_6-HA-PKB from HEK293 cells (40 μM; [14]). The K_m for the 7-mer substrate was 3.5 μM (Table 2; average of four experiments, using His_6-ΔPH-PKB-EEE and His_6-ΔPH-PKB-EEE-FLAG), lower than that for full-length, wild-type His_6-HA-PKB from HEK293 cells (18 μM; [14]). We do not know whether these differences are due to the deletion of the PH domain, the triple S124E,T450E,S473E mutation, or both.

Table 1
Purification of His_6-ΔPH-PKB-EEE-FLAG (from 1 L bacterial culture)

Purification step	Total protein (mg)	Amount enzyme (mg)	Specific activity[a] (units/μg)	Approximate purity (%) [yield (%)]
1. Cleared lysate	120	<6	1.7	5
2. Ni–NTA–agarose	11	4.5	29.5	40–50 (23)
3. Anti-FLAG M2 agarose	0.7	0.5	108	75–85 (21)
4. MonoQ				
Phosphorylated fraction	0.02	0.02	120	>95 [0.3]
Non-phosphorylated	0.044	0.044	0	

[a] These values were determined at an ATP concentration of 100 μM. The specific activity of the MonoQ purified product when assayed at 250 μM ATP ($K_{m\,ATP}$ = 155 μM) was >700 units/μg. 1 unit = 1 pmol phosphate/min transferred to substrate peptide.

Table 2
Comparison of ΔPH-PKB-EEE and full-length PKB

		ΔPH-PKB-EEE from *E. coli*	Full-length PKB from HEK293 cells
K_m (μM)	7-mer peptide	3.5	18[a]
	ATP	155	40[a]
IC_{50} (μM)	NL-71-101	6	4[a]
	H89	0.6	0.8

[a] These values are from [14].

To verify that our protein is suitable for screening for PKB inhibitors, we compared the effects of known inhibitors of PKB on ΔPH-PKB-EEE. The PKA inhibitor, H89 (which inhibits PKB in addition to PKA, but less potently), had virtually the same IC_{50} when measured on full-length His_6-HA-PKB from HEK293 cells and on ΔPH-PKB-EEE from *E. coli* (Table 2). The IC_{50} of the PKB inhibitor NL-71-101 was $6 \pm 1.5\,\mu M$ (Table 2), which compares well with the published IC_{50} of $4\,\mu M$, using HA-PKB from HEK293 cells [14]. We therefore believe that ΔPH-PKB-EEE can be used to search for PKB inhibitors in large-scale in vitro screens.

Expression of kinases in *E. coli* is fraught with difficulties, including poor expression, degradation, and sequestration as insoluble protein in inclusion bodies. In this paper, we have described a simple, 3-step procedure for purification of active PKB kinase from *E. coli*. We activated the PKB kinase by co-expression with GST-PDK1. To ensure the isolation of full-length protein, we tagged our construct on both the N and C termini, and performed two rounds of affinity purification, one employing the N-terminal His_6 tag and the other using the C-terminal FLAG tag. The final purification step involved anion exchange over a MonoQ column. The double tag approach [24,25] was recently used to purify another difficult recombinant kinase, ERK3, from *E. coli* [26]. Indeed, this approach may be applicable wherever poor expression leads to truncated products that co-purify with the full-length protein.

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