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Inactivation and dephosphorylation of protein kinase Bα (PKBα) promoted by hyperosmotic stress

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To study the role of protein kinase B (PKB) in response to cellular stress, we examined PKBα activity following different stress treatments. Hyperosmotic but not chemical stress resulted in inactivation of PKBα and prevented activation by pervanadate and mitogens. Hyperosmotic shock did not affect the MAP kinase pathway, suggesting that this inhibitory effect was specific for PKB. Our data further indicate that downregulation occurs via dephosphorylation of Thr308 and Ser473, the major regulatory phosphorylation sites of PKBα. Indeed, calyculin A, which inhibits protein phosphatases 1 and 2A, effectively blocked hyperosmotic stress-mediated inactivation (dephosphorylation) of PKBα. High osmolarity did not affect phosphatidylinositol 3-kinase activity but led to a marked increase in PI(3,4,5)P_3 and a decrease in PI(3,4)P_2 formation after pervanadate stimulation, suggesting that hyperosmotic stress has an inhibitory effect on a phosphatidylinositol 5-phosphatase which converts PI(3,4,5)P_3 into PI(3,4)P_2. Immunofluorescence studies revealed that membrane translocation, a prerequisite for PKB activation, was not affected by hyperosmotic stress. Our results indicate that hyperosmotic stress can act at two levels: (i) inhibition of phosphorylation of Thr308 and Ser473 by upstream kinases and (ii) by promoting rapid dephosphorylation of these regulatory sites.

Keywords: osmotic shock/phosphorylation/protein kinase B/protein phosphatase 2A/signal transduction

Introduction

Protein kinase B (PKB), also termed RAC kinase (Jones *et al*., 1991) or Akt (Bellacosa *et al*., 1991), represents a family of 3-phosphoinositide-regulated serine/threonine kinases (Hemmings, 1997a). PKBα, β and γ become phosphorylated and activated in response to mitogens and survival factors in a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent manner (Burgering and Coffer, 1995; Franke *et al*., 1995; Alessi *et al*., 1996; Didichenko *et al*., 1996; Meier *et al*., 1997; Andjelković *et al*., 1997; Walker *et al*., 1998). Phosphorylation of PKBα and β occurs on the two residues Thr308 (Thr309 in PKBβ) in the activation loop and Ser473 (Ser474 in PKBβ) within the C-terminal activation domain. Whereas the upstream kinase phosphorylating Ser473 (Ser474 in PKBβ) is still unknown, phosphorylation of the A-loop site by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is dependent on the product of PI 3-kinase, phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3] and, to a lesser extent, phosphatidylinositol-3,4-bisphosphate [PI(3,4)P_2] (Alessi *et al*., 1997; Stokoe *et al*., 1997), which is produced from PI(3,4,5)P_3 by polyphosphate-5-phosphatase activities (Kavanaugh *et al*., 1996; Woscholski and Parker, 1997). Additionally, PI(3,4,5)P_3 and PI(3,4)P_2 are thought to provide docking sites for PKB and PDK1, which both become targeted to the plasma membrane mediated by their pleckstrin homology (PH) domains (Hemmings, 1997b).

A dynamic balance between positive and negative regulatory mechanisms is crucial to setting the threshold at which extracellular stimuli trigger signal transduction pathways. In insulin receptor signalling, changes in PKBα activity control glycogen synthesis by phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3) (Cross *et al*., 1995), increase in protein synthesis by indirect inactivation of 4E-BP1 (Gingras *et al*., 1998) or the regulation of glucose uptake by GLUT4 translocation (Kohn *et al*., 1996). Furthermore, PKBα is necessary for cell survival and the prevention of apoptosis (Ahmed *et al*., 1997; Dudek *et al*., 1997; Kauffmann-Zeh *et al*., 1997; Kennedy *et al*., 1997; Kulik *et al*., 1997), which may occur by phosphorylation of the Bcl-x inhibitor BAD (Datta *et al*., 1997; Del Peso *et al*., 1997).

In contrast to the activation of PKB, downregulation of this kinase is less well understood. Inactivation of PKBα has been described in cells which detach from the extracellular matrix (anoikis), implying that PI 3-kinase is a mediator of the aberrant survival of Ras-transformed epithelial cells which are no longer attached to the extracellular matrix (Khwaja *et al*., 1997). In the current study, we examined the effect of high osmolarity on the regulation of PKBα. We demonstrate that hyperosmotic stress not only activates the stress-induced Jun N-terminal kinase (JNK) cascade (Galcheva-Gargova *et al*., 1994) but also strongly inhibits PKBα activation and activity. Inactivation of PKBα was due to dephosphorylation of the regulatory phosphorylation sites by protein phosphatase 2A (PP2A).

Results

Cellular stresses do not activate PKBα

To gain further insights into PKB regulation, we transiently transfected HEK-293 and COS-1 cells with haemagglutinin (HA) epitope-tagged wild-type PKBα (HA-PKBα) and tested the effect of different stress conditions on kinase activation (Figure 1). In agreement with our previous results (Andjelković *et al*., 1996; Meier *et al*., 1997), pervanadate potently stimulated PKBα. However, we did not detect any PKBα activation by 0.5 M sorbitol

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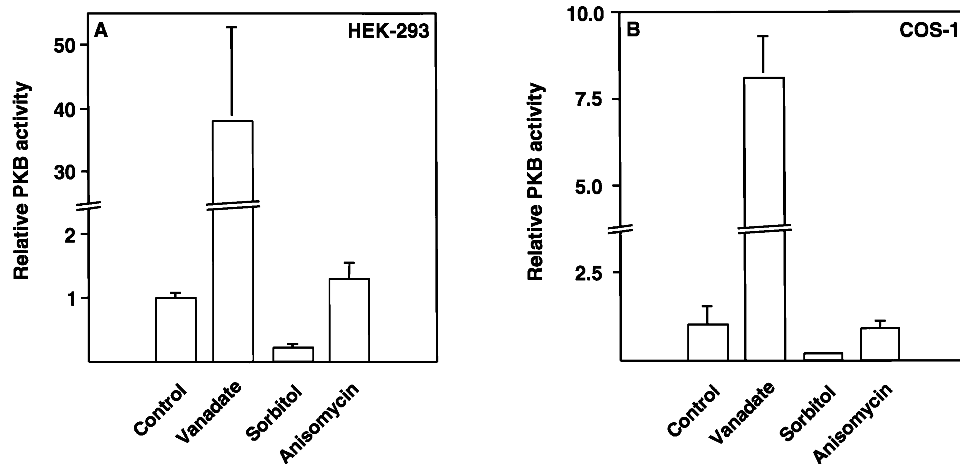

Fig. 1. Cellular stresses do not activate PKBα. HA-PKBα was transiently expressed in HEK293 (**A**) and COS-1 (**B**) cells. After serum starvation for 24 h, cells were stimulated with 0.1 mM pervanadate for 15 min, 0.5 M sorbitol for 30 min, 10 μg/ml anisomycin for 30 min or left untreated (control). After stimulation, cells were lysed and assayed for *in vitro* PKBα kinase activity (see Materials and methods). Activities are the average (± SD) of three independent experiments assayed in duplicate and are relative to the activities found in unstimulated cells.

(Figure 1), 0.5 M sodium chloride (data not shown) or 10 μg/ml anisomycin (Figure 1). Furthermore, in Swiss 3T3 cells, we did not detect PKBα activation when cells were treated by heat shock at 42°C for 30 min (M.Andjelković and B.A.Hemmings, unpublished data). In contrast, treatment with 0.5 M sorbitol (Figure 1) or 0.5 M sodium chloride (data not shown) reduced basal PKBα activity to 20–25% of the control in both HEK-293 and COS-1 cells. Basal PKBα activities were not reduced when cells were treated with either heat shock or anisomycin, indicating that osmotic shock selectively inhibits PKBα activity. We therefore decided to characterize the regulation of PKB in response to stress in more detail.

High osmolarity both prevents activation and inactivates PKBα

To test whether high osmolarity mediates inactivation of activated PKBα, we transiently transfected HA-PKBα into HEK-293 or COS-1 cells. Pervanadate-induced activation of PKBα was almost completely reversed when 0.5 M sorbitol or 0.5 M sodium chloride were added 15 min after stimulation (Figure 2A and B). Moreover, pre-treatment of the cells with 0.5 M sorbitol or 0.5 M sodium chloride prevented pervanadate-induced activation of PKBα. Similar results were obtained when platelet-derived growth factor (PDGF) was used to activate HA-PKBα (Figure 2C). Activation of HA-PKBα by PDGF was not detectable when cells were pre-treated with 0.5 M sorbitol. Furthermore, HA-PKBα activity was decreased dramatically when 0.5 M sorbitol was added to the cells after PDGF stimulation. To confirm the data obtained with overexpressed PKBα, we assayed endogenous PKBα from Swiss 3T3 cells immunoprecipitated with a polyclonal antibody raised against the C-terminus of PKBα (Figure 3). A 5-fold activation of endogenous PKBα was obtained after PDGF stimulation, which was similar to the activation of overexpressed PKBα. Sorbitol treatment moderately decreased the basal activity of endogenous PKBα but almost completely abolished PDGF-mediated stimulation (Figure 3).

Hyperosmotic and chemical stress are potent inducers of the stress kinase (JNK/p38) pathways (Han *et al.*, 1994; Kyriakis *et al.*, 1994; Meier *et al.*, 1996). We therefore looked for a link between activation of stress kinases and inactivation of PKBα in response to cellular stress by comparing the effects of sorbitol and anisomycin on both JNK and PKBα activation (Figure 2D). Pervanadate treatment caused JNK activation (2- to 3-fold over the basal level), which was enhanced further upon pre-treatment of HEK-293 cells with 10 μg/ml anisomycin or 0.5 M sorbitol (Figure 2D). Interestingly, whilst pre-treatment with sorbitol abolished PKBα activation by pervanadate, pre-treatment with anisomycin had no effect on PKBα activity after pervanadate stimulation (Figure 2A).

Inactivation of PKBα occurs by dephosphorylation of Thr308 and Ser473

We reasoned that the inhibition of activation and inactivation of PKB activity could be due to effects on the two key regulatory phosphorylation sites Thr308 and Ser473 (Alessi *et al.*, 1996). We therefore transiently transfected wild-type HA-PKBα and the constitutively active mutant HA-PKBα-T308D/S473D into HEK-293 cells and tested the effect of sorbitol upon PKBα activation (Figure 4A). Compared with HA-PKBα, the HA-PKBα-T308D/S473D mutant showed an 18-fold higher basal activity in unstimulated cells. Pervanadate treatment promoted a 25-fold stimulation of the wild-type activity but did not increase the activity of the HA-PKBα-T308D/S473D mutant. Moreover, pre-treatment with 0.5 M sorbitol for 15 min abolished the effect of pervanadate stimulation in HA-PKBα but had no effect on the HA-PKBα-T308D/S473D mutant. Immunoblotting of wild-type PKBα with an antibody specific for either phosphothreonine 308 or phosphoserine 473 revealed that both sites were phosphorylated in response to pervanadate but remained in a dephosphorylated state following pre-treatment of the cells with 0.5 M sorbitol for 15 min (Figure 4B). Taken together, these data suggest that dephosphorylation of both Thr308 and Ser473 was involved in the sorbitol-induced inactivation of PKBα.

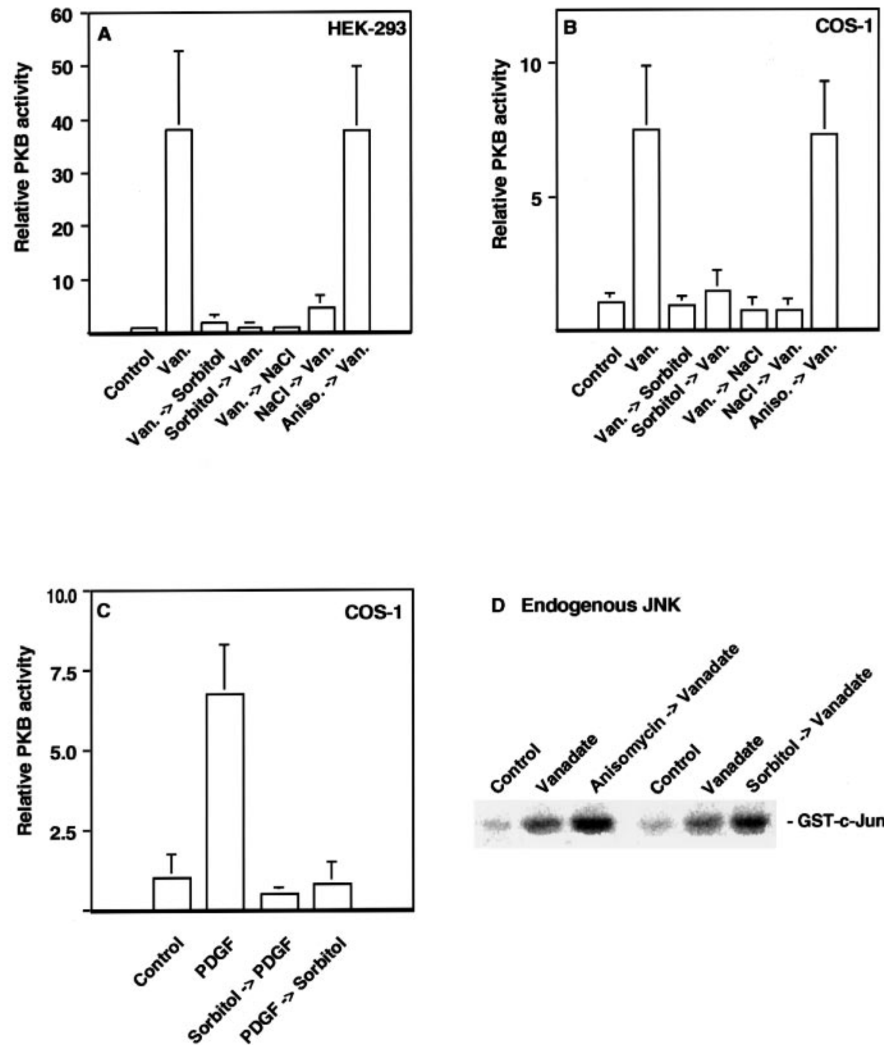

Fig. 2. Hyperosmotic but not chemical stress prevents activation and mediates inactivation of PKBα. (A and B) HEK-293 (**A**) and COS-1 (**B**) cells were transiently transfected with HA-PKBα, serum starved (24 h) and treated with 0.1 mM pervanadate for 15 min, 0.1 mM pervanadate for 15 min followed by 0.5 M sorbitol (0.5 M NaCl) for a further 15 min, pre-treated with 0.5 M sorbitol (0.5 M NaCl) for 15 min followed by 0.1 mM pervanadate for a further 15 min, or 10 μg/ml anisomycin for 15 min followed by 0.1 mM pervanadate for a further 15 min. (**C**) COS-1 cells transiently transfected with HA-PKBα and stimulated with 50 ng/ml PDGF for 15 min, pre-treated with 0.5 M sorbitol for 15 min followed by 50 ng/ml PDGF for 15 min, or treated with 50 ng/ml PDGF for 15 min before adding 0.5 M sorbitol (15 min). After stimulation, cells were lysed and assayed for *in vitro* PKBα kinase activity as described (Alessi *et al*., 1996). Activities are the mean (± SD) of two independent experiments assayed in duplicate. Relative activities are based on the activities found in unstimulated cells (control). (**D**) In parallel experiments, samples from HEK-293 cells (A) were used to assay endogenous JNK. JNK activity was measured in pull-down assays using GST–cJun(5–89) as substrate (Meier *et al*., 1996).

PP2A mediates osmotic shock-dependent dephosphorylation of PKBα

To understand the mechanism of Thr308/Ser473 dephosphorylation in the sorbitol-induced inactivation of PKBα, we predicted that PP2A dephosphorylates and inactivates PKBα *in vivo*, since PP2A inactivates PKBα *in vitro* whilst phosphatase 1 (PP1) does not (Andjelković *et al*., 1996). To test this, we used calyculin A, a cell-permeable inhibitor of PP1 and PP2A. HEK-293 cells transiently transfected with wild-type HA-PKBα were stimulated with pervanadate and sorbitol in the presence or absence of calyculin A (Figure 5A). As described previously for okadaic acid (Andjelković *et al*., 1996), treatment with calyculin A alone led to a 30-fold stimulation of PKBα. Pervanadate plus calyculin A resulted in a slightly higher activation of PKBα. In both cases, activation of PKBα was no longer inhibited by sorbitol. In addition, we performed a time-course of PKBα reactivation with calyculin A (Figure 5B). In the presence of sorbitol and pervanadate, inactive PKBα became reactivated 5 min after calyculin A addition, peaking at 20 min. Thus, calyculin A caused a robust activation of PKBα which was completely insensitive to sorbitol, indicating that PP2A is involved in mediating downregulation of PKBα activity through the dephosphorylation of the regulatory phosphorylation sites. However, activation of PKBα by calyculin A was sensitive to wortmannin (data not shown), showing that calyculin A not only activates PKBα by inactivating PP2A but also by the activation of upstream elements of PI 3-kinase. To gain further insight into this mechanism, we performed a time-course of PKBα activation with calyculin A in the presence or absence of

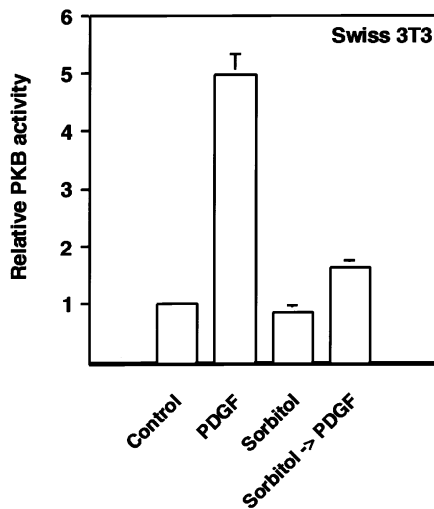

Fig. 3. PDGF-induced activation of endogenous PKBα in Swiss 3T3 cells is prevented by sorbitol. Swiss 3T3 cells were serum starved for 24 h, treated with 50 ng/ml PDGF or 0.5 M sorbitol for 15 min, pre-treated with 0.5 M sorbitol for 15 min followed by 50 ng/ml PDGF for 15 min, or left untreated (control), lysed and assayed for PKBα *in vitro* kinase activity. Kinase activity is the average (± SD) of two independent experiments assayed in duplicate and shown relative to the activities found in unstimulated cells.

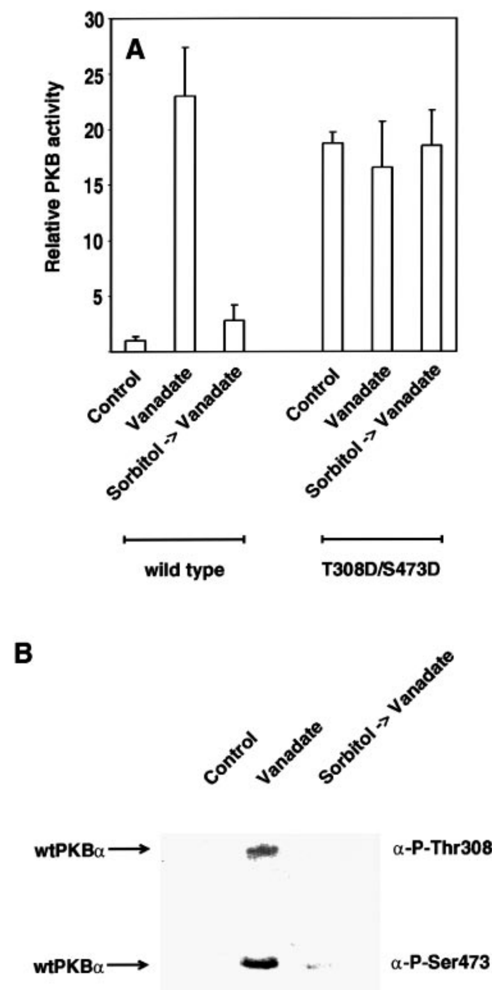

Fig. 4. Hyperosmotic stress-induced inactivation of PKBα involves the regulatory phosphorylation sites Thr308 and Ser473. (**A**) HEK-293 cells were transiently transfected with HA-PKBα or HA-PKBα-T308D/S473D mutants. After serum starvation for 24 h, cells were either left untreated (control) or were stimulated with 0.1 mM pervanadate for 15 min, or 0.5 M sorbitol for 15 min followed by 15 min stimulation with 0.1 mM pervanadate, and assayed for *in vitro* PKBα activity. (**B**) A 1 μg aliquot of total protein from overexpressed HA-PKBα from untreated, pervanadate- and sorbitol/pervanadate-stimulated HEK-293 cells (see A) was immunoblotted with phospho-specific polyclonal antibodies directed against PKBα-Thr308 or PKBα-Ser473.

sorbitol (Figure 5C). We found that during the first 10 min of calyculin A stimulation, sorbitol pre-treatment caused a lower PKBα activity compared with cells which were not treated with sorbitol. After 10 min of calyculin A stimulation, the PKBα activity of sorbitol-treated cells increased relative to untreated cells and slightly exceeded it after 20 min of calyculin stimulation. Thus, we conclude that sorbitol either activates PP2A directly or that an upstream element is inhibited by high osmolarity, thereby affecting PP2A activity or targeting. To investigate this idea, we first tested whether PP2A is upregulated by osmotic shock. HEK-293 cells, transiently transfected with HA-PP2Acα were stimulated and assayed for PP2A activity (Figure 5D). Sorbitol (0.5 M) did not affect PP2A activity in either unstimulated or pervanadate-treated cells, although pervanadate caused a 40% reduction of PP2A activity. Second, we tested whether sorbitol promotes targeting to PKBα. We were unable to detect any PP2A activity or protein associated with endogenous PKBα in immunoprecipitates from unstimulated, stimulated or sorbitol-pre-treated HEK-293 cell extracts (data not shown).

Hyperosmotic stress does not inhibit PI 3-kinase, PDK1 and MAP kinase

To study the specificity of osmotic stress-induced inactivation of PKBα, we examined the effect of sorbitol on PI 3-kinase and PDK1 (Burgering and Coffer, 1995; Franke *et al*., 1995; Alessi *et al*., 1997; Stokoe *et al*., 1997). Endogenous PI 3-kinase was assayed from HEK-293 (Figure 6A) and Swiss-3T3 (data not shown) cells stimulated either with pervanadate or PDGF. HEK-293 cells were pre-treated with or without 0.5 M sorbitol for 15 min followed by incubation with 0.1 mM pervanadate for 15 min, or were left untreated. PI 3-kinase activity was determined in anti-p85 immunoprecipitates as described by Whitman *et al*. (1985). Immunoprecipitates obtained from pervanadate-stimulated cell lysates showed a 7.5-fold increase of PI 3-kinase activity [as determined by phosphatidylinositol 3-phosphate (PI3P) production] compared with extracts from unstimulated cells (Figure 6A). The same extracts were also assayed in parallel for PKBα activation (Figure 6B, first three bars). As expected, stimulation with pervanadate resulted in a 3-fold activation of endogenous PKBα. Furthermore, sorbitol treatment before addition of pervanadate, which prevented activation of PKBα (Figure 6B), led to an 8-fold higher PI3P formation similar to pervanadate-stimulated cells, indicating that PI 3-kinase activity was unaffected by sorbitol treatment. The same experiments were repeated with Swiss 3T3 cells stimulated with 50 ng/ml PDGF for 15 min, with or without pre-treatment with 0.5 M sorbitol, or left untreated. Both, PKBα and PI 3-kinase activities were stimulated with PDGF. However, following pre-treatment with sorbitol, PDGF failed to activate PKBα but still

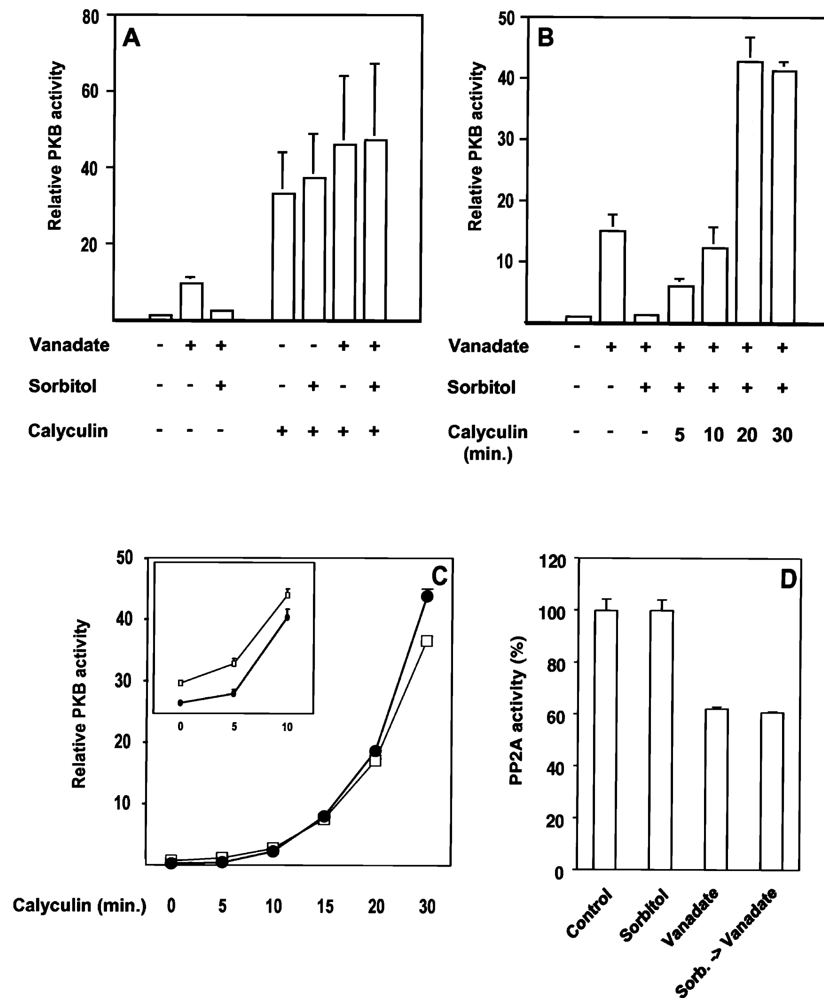

Fig. 5. Inhibition of PP2A/PP1 by calyculin A blocks sorbitol effect on PKBα. (**A**) Calyculin A blocks sorbitol-mediated inactivation of PKBα. HEK-293 cells were transiently transfected with HA-PKBα, serum starved and treated with 0.5 M sorbitol (15 min), followed by 0.1 mM pervanadate stimulation for a further 15 min (first three bars) as an internal control. Last three bars: in the presence of calyculin A (100 nM), cells were either not treated, stimulated with 0.5 M sorbitol for 15 min, or stimulated with 0.1 M pervanadate (15 min) or with pervanadate (15 min) and 0.5 M sorbitol (30 min). (**B**) Calyculin A reactivates sorbitol-induced inactivated PKBα. First three bars: internal control as described in (A). Next bars: cells pre-treated with 0.5 M sorbitol for 15 min then stimulated with 100 nM calyculin A in the presence of 0.1 mM pervanadate for the times indicated. (**C**) Time-course of PKBα activation in the presence or absence of 0.5 M sorbitol. HEK-293 cells were transiently transfected with HA-PKBα, pre-treated with 0.5 M sorbitol for 15 min (●) or left untreated (□) and then stimulated with calyculin A (100 nM) for the times indicated. The inset shows a better resolution of the first three time points. (**D**) HEK-293 cells were transiently transfected with HA-PP2Acα, stimulated with 0.5 M sorbitol for 30 min, 0.1 mM pervanadate (15 min) and 0.5 M sorbitol (15 min) prior to treatment with pervanadate for a further 15 min, or left untreated (control). After stimulation, HA-PP2Acα was immunoprecipitated and assayed as described in Materials and methods. The data shown are the average (± SD) of two independent experiments in duplicate.

activated PI 3-kinase (data not shown). We performed similar transfection experiments using the upstream activator PDK1. In agreement with previous results (Alessi *et al.*, 1997), PDK1 was constitutively active in HEK-293 cells and its activity did not change following sorbitol treatment (data not shown).

Next, we investigated whether osmotic stress results in a general inactivation of growth factor-induced signalling pathways. We focused our attention on the MAP kinase pathway involved in transmitting the mitogenic signals which result in cell growth but which is poorly activated in HEK-293 cells in response to cellular stress (Brunet and Pouysségur, 1996). HEK-293 cell extracts were assayed for MAP kinase activities (Figure 6B and C). Using the polyclonal antibody C-16, activation of p42 and p44 MAPK was detected either by mobility shifts on Western blots after SDS–PAGE or by *in vitro* kinase assays using myelin basic protein (MBP) as a substrate. As expected, both p42 and p44 MAP kinases were activated following pervanadate treatment. Moreover, treatment of HEK-293 cells with 0.5 M sorbitol had no effect on basal or pervanadate-stimulated p42 and p44 MAP kinase activities. The finding that sorbitol selectively inhibited PKBα but did not affect the MAP kinase pathway, PDK1 or PI 3-kinase activity suggests that the target of hyperosmotic stress is specific for the PKB signalling pathway acting downstream or independent of PI 3-kinase and PDK1.

High osmolarity elevates PI(3,4,5)P_3 and lowers PI(3,4)P_2 levels in pervanadate-stimulated HEK-293 cells

Because we found no correlation between PKBα and PI 3-kinase activity after hyperosmotic stress, we studied changes in 3′-phosphoinositide levels after hyperosmotic

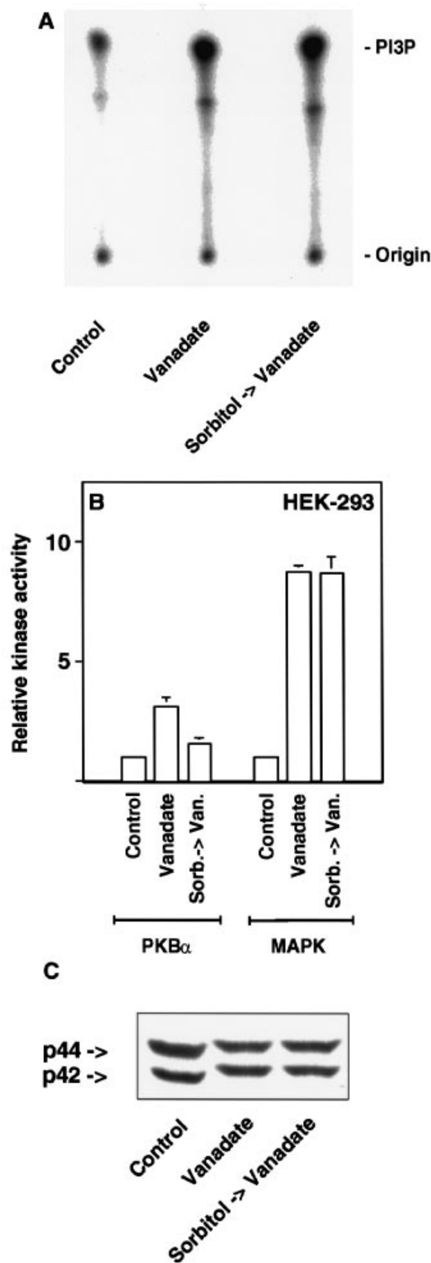

Fig. 6. Effect of hyperosmotic stress is PKB specific. HEK-293 cells were treated with 0.1 mM pervanadate for 15 min, pre-treated with 0.5 M sorbitol for 15 min before stimulation with 0.1 mM pervanadate for 15 min, or left untreated. Total cell lysates were assayed for (**A**) PI 3-kinase activities (**B**) PKBα, MAP kinase activities and (**C**) for p42 and p44 MAP kinase activation-associated band shifts.

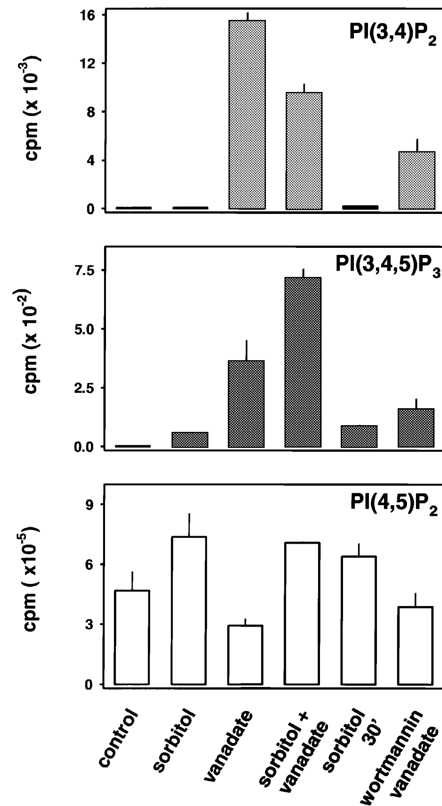

Fig. 7. Changes of phosphoinositide levels upon pervanadate/sorbitol stimulation in HEK-293 cells. HEK-293 cells were labelled with [^{32}P]orthophosphate for 120 min. After 15 min incubation in fresh medium, cells were stimulated with sorbitol (15 and 30 min), pervanadate (15 min) or were pre-treated with either sorbitol or wortmannin for 15 min before pervanadate was added for a further 15 min. After stimulation, [^{3}H]PI(4,5)P_2 was added to the combined material, and lipids were extracted as previously described (Didichenko *et al.*, 1996). Lipids were deacylated and the resulting glyceroinositides separated by HPLC.

stress-induced inactivation of PKBα. PI(3,4)P_2 and PI(3,4,5)P_3 have been shown to participate in the regulation of PKBα activity at the level of targeting PKB to the membrane and by direct binding to the PKBα-PH domain (Frech *et al.*, 1997; Stokoe *et al.*, 1997). As expected, 15 min pervanadate treatment caused an increase in both PI(3,4)P_2 and PI(3,4,5)P_3, which was accompanied by a decrease in the level of the PI 3-kinase substrate PI(4,5)P_2 (Figure 7). Pre-treatment of the cells with the PI 3-kinase inhibitor wortmannin (which blocks PKBα activation) for 15 min markedly inhibited the production of 3′-phosphoinositides whilst attenuating PI(4,5)P_2 consumption. Moreover, we found that sorbitol alone caused only modest changes in phosphoinositide levels. Pre-treatment with 0.5 M sorbitol for 15 min, which causes a marked ablation of pervanadate-stimulated PKBα activity, substantially inhibited pervanadate-stimulated PI(3,4)P_2 formation by 41% and surprisingly caused a 50% higher accumulation of PI(3,4,5)P_3.

Influence of PI(3,4,5)P_3 and PI(3,4)P_2 on PP2A activity in vitro

To test whether the sorbitol-induced decrease of PI(3,4)P_2 or increase of PI(3,4,5)P_3 affects PP2A activity, we assayed various PP2A heterodimers and trimers ($PP2A_2$, $PP2A_1$, $PP2A_3$ and $PP2A_0$) in the presence of different ratios of PI(3,4)P_2 and PI(3,4,5)P_3 using ^{32}P-labelled HA-ΔPH-PKBα as a substrate (Figure 8). To detect inhibition of phosphatase activity by phospholipids, we standardized the assays to achieve dephosphorylation of HA-ΔPH-PKBα in the absence of phospholipids which was fully reversed in the presence of 100 nM okadaic acid. With every form of PP2A tested, we were unable to detect activity changes in response to different ratios of PI(3,4)P_2 and PI(3,4,5)P_3, indicating that PKBα inactivation was not due to interactions and activity changes of PP2A with PI(3,4)P_2 or PI(3,4,5)P_3.

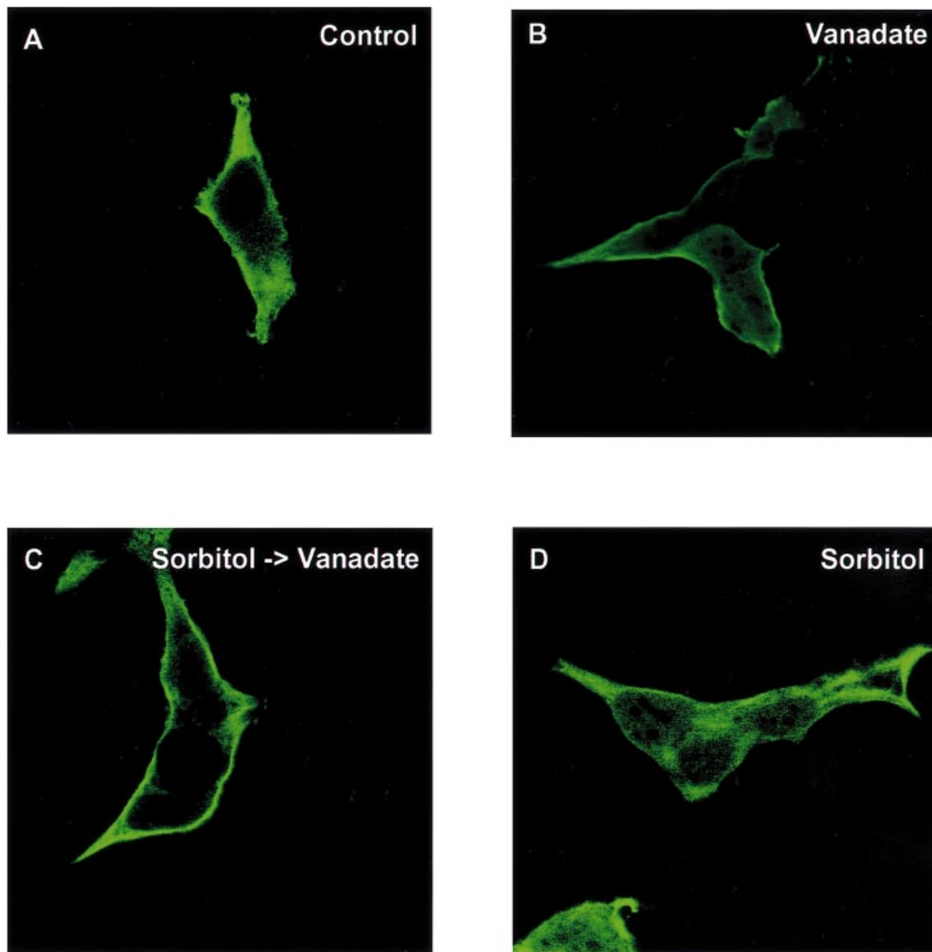

Fig. 8. Hyperosmotic stress does not block membrane translocation of PKBα. HEK-293 cells were transiently transfected with HA-PKBα and serum starved for 24 h. After starvation, cells were left untreated (**A**) or were stimulated with 0.1 mM pervanadate for 4 min (**B**), were pre-treated with 0.5 M sorbitol for 15 min and stimulated with 0.1 mM pervanadate for 4 min (**C**), or were treated with 0.5 M sorbitol for 30 min (**D**). The cells were stained and analysed as described in Meier *et al*. (1997).

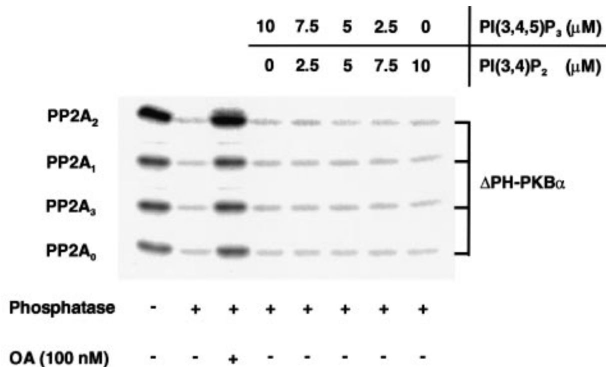

Fig. 9. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ do not change PP2A activity *in vitro*. ^{32}P-labelled HA-ΔPH-PKBα (see Materials and methods) was incubated with $PP2A_2$, $PP2A_1$, $PP2A_3$ and $PP2A_0$ in the presence or absence of 100 nM okadaic acid and with different ratios of $PI(3,4)P_2$ and $PI(3,4,5)P_3$. After 45 min, the reactions were stopped and ΔPH-PKBα phosphorylation was assessed by SDS–PAGE followed by quantification on a Storm 840/860 PhosphorImager.

Sorbitol does not inhibit PKBα membrane translocation

Membrane translocation is an important initial step in PKBα activation (Andjelković *et al*., 1997) but it is still unclear whether $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$ mediate membrane translocation. Our finding that $PI(3,4)P_2$ levels are reduced by hyperosmotic stress prompted us to study PKBα membrane translocation in response to 0.5 M sorbitol (Figure 9). HEK-293 cells transiently transfected with HA-PKBα were pre-treated with or without 0.5 M sorbitol for 15 min followed by stimulation with 0.1 mM pervanadate for 4 min, or were left untreated. Unstimulated cells showed a clear cytoplasmic distribution of HA-PKBα (Figure 9A). Incubation of the cells with 0.5 M sorbitol, which reduces basal PKBα activity, did not alter its subcellular localization (Figure 9D). As expected, pervanadate treatment resulted in PKB membrane translocation within 4 min of stimulation (Figure 9B) and, although pre-treatment of the cells with 0.5 M sorbitol prior to pervanadate stimulation prevents activation of PKB, translocation to the plasma membrane was unaffected (Figure 9C).

Discussion

The general mechanism for PKB activation is well established (Hemmings, 1997b). Stimulation of many receptor tyrosine kinases results in an increase in $PI(3,4,5)P_3$ and $PI(3,4)P_2$ levels, which in turn mediate targeting of PKBα, PDK1 and probably the not yet fully characterized Ser473

kinase to the plasma membrane by their PH domains (Andjelković *et al.*, 1997; Anderson *et al.*, 1998). PKBα then becomes phosphorylated and activated by PDK1 (and the Ser473 kinase) on Thr308 and Ser473. After activation, PKBα detaches from the plasma membrane and translocates to the nucleus (Andjelković *et al.*, 1997; Meier *et al.*, 1997). The new results presented in this report support the concept that negative regulatory signal transduction pathways also operate to modulate PKB activity.

We demonstrate here that hyperosmotic stress (0.5 M sorbitol or 0.5 M NaCl), but not chemical stress (anisomycin), inactivates endogenous and overexpressed PKBα in three different cell lines. This is in contrast to previous studies by Konishi *et al.* (1996), who reported activation of PKB by hyperosmotic shock. The probable explanation for this difference is that they used a general substrate which could, under certain circumstances, be phosphorylated by co-precipitating protein kinases. To avoid this, we and others (Cross *et al.*, 1995) took advantage of a specific peptide as a substrate for PKB. High osmolarity rapidly inactivates PKBα to a level of 20%. Similarly, it was reported that $p70^{S6K}$, a downstream effector of PDK1 and probably PKBα (Alessi *et al.*, 1998; Pullen *et al.*, 1998), becomes completely inactivated after the cells are treated with sorbitol (Chou and Blenis, 1996). The inhibitory effect of hyperosmotic stress on PKBα is selective, since JNK, a stress-activated protein kinase, and the classical MAP kinases (p42 and p44) remain upregulated in the presence of pervanadate and 0.5 M sorbitol. All tested stress conditions activated JNK, whereas only hyperosmotic stress inhibited PKBα, suggesting that the stress-activated protein kinases are not involved in the regulation of PKBα.

The measurement of PI 3-kinase *in vitro* and the *in vivo* levels of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ revealed no inhibition of PI 3-kinase activity in response to 0.5 M sorbitol. Instead, sorbitol treatment resulted in increased formation of $PI(3,4,5)P_3$ accompanied by a reduction in $PI(3,4)P_2$ production, indicating that hyperosmotic stress may inhibit the conversion of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ by a phosphatidylinositol 5-phosphatase.

PKBα was shown to be dephosphorylated at Thr308 and Ser473 after treatment with sorbitol. Since PP2A dephosphorylates PKBα *in vitro* and calyculin A prevented inactivation of pervanadate-activated PKBα by hyperosmotic shock, we reasoned that hyperosmotic stress may also regulate PP2A targeting or activity. However, upon sorbitol treatment, we detected no direct binding of PP2A to PKBα or activation of PP2A, although pervanadate led to a significant decrease of PP2A activity. This result is similar to the observation of Srinivasan and Begum (1994) who showed a decrease in PP2A activity in rat skeletal muscle cells upon insulin stimulation. Further evidence that PP2A activity is not upregulated directly by high osmolarity comes from the fact that the MAP kinase pathway, which is sensitive to PP2A (Alessi *et al.*, 1995), is not downregulated by osmotic shock. Furthermore, we were unable to find an effect of phospholipids on various forms of PP2A, suggesting that $PI(3,4)P_2$, which is downregulated by sorbitol, does not act to inhibit PP2A.

The precise role of different 3-phosphoinositides in the regulation of PKBα is somewhat controversial. $PI(3,4)P_2$, which is downregulated by sorbitol, has been shown to activate PKBα *in vitro* and *in vivo* (Franke *et al.*, 1997; Frech *et al.*, 1997; Klippel *et al.*, 1997). Furthermore, $PI(3,4,5)P_3$, which accumulates in response to hyperosmotic stress, does not activate but even inhibits PKBα activity *in vitro* (Franke *et al.*, 1997; Frech *et al.*, 1997; Klippel *et al.*, 1997), and allows direct activation by PDK1 *in vitro* (Alessi *et al.*, 1997; Stokoe *et al.*, 1997). Consequently, $PI(3,4,5)P_3$ could mediate translocation of PKBα but is not sufficient for its activation in the presence of hyperosmotic stress, because the latter process requires $PI(3,4)P_2$ levels above a certain threshold. The requirement for $PI(3,4)P_2$ production may be at the level of PKB or upstream kinases. However, the PH domain of PKB does not appear to mediate this process, as hyperosmotic stress resulted in a complete loss of ΔPH-PKBα activity (data not shown). Although PDK1 activity appears not to be affected by hyperosmotic stress, we cannot exclude the possibility that its localization, as well as that of the Ser473 kinase, is sensitive to $PI(3,4)P_2$.

It has been shown that PKBα activation is important for the suppression of apoptosis (Datta *et al.*, 1997; Del Peso *et al.*, 1997). Furthermore, inactivation of PKBα following hyperosmotic stress appears to be similar to inactivation of PKBα upon detachment of epithelial cells from the extracellular matrix, generally known as anoikis (Khwaja *et al.*, 1997). However, in contrast to anoikis which mediates cell death through inhibition of PI 3-kinase activity, hyperosmotic stress inactivates the PKB pathway not through changes in PI 3-kinase activity and concomitant $PI(3,4,5)P_3$ production but presumably by a complex regulation of PKB phosphorylation and changes in upstream activation due to altered ratios of the second messengers $PI(3,4)P_2$ and $PI(3,4,5)P_3$.

Following hyperosmotic stress, PKBα is inactivated rapidly accompanied by inhibition of $PI(3,4)P_2$ production, whereas the stress kinase pathways (JNK, p38) are activated. Thus, hyperosmotic stress may promote apoptosis at two independent levels: first, through activation of the JNK pathway (Verheij *et al.*, 1996) and, second, by inhibition of PKBα, a major promoter of cell survival (Kennedy *et al.*, 1997) acting through phosphorylation of BAD on Ser136. Phosphorylation of BAD promotes its dissociation from Bcl-x_L and its association with 14-3-3. Released Bcl-x_L then suppresses cell death pathways such as the cytochrome *c*-activated caspase protease cascade (Datta *et al.*, 1997; Del Peso *et al.*, 1997; Hengartner, 1998). The elucidation of the inhibitory pathways leading to PKB inactivation will provide further insights into the control of apoptosis.

Materials and methods

Cell culture, transient transfection and stimulation conditions

Human embryonic kidney HEK-293, monkey kidney COS-1 and Swiss 3T3 mouse fibroblasts were maintained as described previously (Meier *et al.*, 1997). Plasmid DNA (10 μg) was transiently transfected into HEK-293 cells using the calcium phosphate method (Chen and Okayama, 1988). For transfection of COS-1 cells, 3 μg of plasmid DNA was introduced using a DEAE–dextran method (Seed and Aruffo, 1987). After transfection, cells were serum starved for 24 h and then stimulated with 0.1 mM pervanadate (Andjelković *et al.*, 1996), 10 μg/ml anisomycin (Sigma), 50 ng/ml PDGF-BB (Gibco-BRL) or combinations as described in the figure legends.

Construction of expression vectors

The cytomegalovirus (CMV)-based expression constructs encoding wild-type HA-PKBα, HA-T308D-PKBα, HA-S473D-PKBα and HA-T308D/S473D-PKBα have been described (Alessi *et al*., 1996). Human HA-PP2Acα was cloned into the pCMV5 vector (D.Evans and B.A. Hemmings, unpublished).

Immunoprecipitation and Western blotting

Cells were lysed in lysis buffer containing 50 mM Tris–HCl pH 7.4, 1% NP-40, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate pH 7.4, 1 μM microcystin-LR (Biomol), 0.1 mM sodium orthovanadate, 1 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Extracts were centrifuged at 12 000 *g* for 15 min at 4°C prior to Western blotting or immunoprecipitation. Immunoblotting of p42 and p44 MAP kinases was carried out with the polyclonal antibody C16 (Santa Cruz Biotechnology). Detection of PKBα phosphorylation on Thr308 or Ser473 was done with anti-phospho-specific (Thr308 or Ser473) PKBα antibodies (New England Biolabs). Bands were visualized by the enhanced chemiluminescence system (Amersham) or alkaline phosphatase-conjugated secondary antibodies. To normalize PKB expression levels, immunoblots were visualized using fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Sigma) as secondary antibody and bands were quantified by chemifluorescence on a Storm 840/860 using ImageQuant software (Molecular Dynamics).

Protein kinase assays

PKBα. HA-tagged PKBα protein was immunoprecipitated with the monoclonal antibody 12CA5 coupled to protein A–Sepharose (Pharmacia). Immunocomplexes were washed three times in lysis buffer containing 0.5 M NaCl, once with lysis buffer and once with kinase buffer (50 mM Tris–HCl pH 7.4, 1 mM benzamidine, 0.5 mM PMSF). PKBα *in vitro* kinase assays were performed as previously described (Cross *et al*., 1995) using Crosstide (GRPRTSSFAEG) as substrate. Quantification of PKBα expression was accomplished with FITC as described above.

JNK. Total cell extracts were incubated with 10 μg of GST–cJun(5–89) immobilized on glutathione–agarose beads for 2 h at 4°C, washed twice in lysis buffer, twice with phosphate-buffered saline (PBS) and once in JNK assay buffer (25 mM Tris–HCl pH 7.4, 20 mM β-glycerophosphate pH 7.4, 0.1 mM EGTA, 1 mM sodium orthovanadate, 10 mM $MgCl_2$). The bound JNK was assayed as described previously (Meier *et al*., 1996).

p42/p44 MAP kinase. Lysates were prepared and immunoprecipitated with the anti-MAP kinase antibody C16 (Santa Cruz Biotechnology). Immunocomplexes were washed three times in lysis buffer containing 0.5 M NaCl and once in assay buffer (25 mM Tris–HCl pH 7.4, 20 mM β-glycerophosphate pH 7.4, 0.1 mM EGTA, 1 mM sodium orthovanadate) and taken into 40 μl of assay buffer containing 20 μg of MBP (Gibco-BRL) and 2.5 μM PKI. The reaction was initiated with the addition of 10 μl of 50 mM $MgCl_2$–0.5 mM [γ-^{32}P]ATP (5×10^5 c.p.m./nmol). After 10 min, a 40 μl aliquot was withdrawn, spotted on to a 1.5×1.5 cm square of Whatman P81 phosphocellulose paper and immersed in 75 mM phosphoric acid. After washing the papers, ^{32}P radioactivity incorporated into MBP was measured.

PI 3-kinase. The p85/p110 PI 3-kinase complex was immunoprecipitated from cell lysates (0.3 mg total protein) with a polyclonal antibody directed against recombinant p85α (Didichenko *et al*., 1996). Immunoprecipitates were collected and washed once with cold PBS, three times with lysis buffer containing 0.5 M LiCl and finally with 10 mM HEPES–NaOH, pH 7.4. Immunoprecipitates were then assayed for phosphatidylinositol phosphorylation activity as described in Whitman *et al*. (1985).

Measurement of phosphoinositides

HEK-293 cells were grown in 4-well plates (Nunc) to 70% confluency. The cells were washed once with Dulbecco's modified Eagle's medium (DMEM) and kept overnight in serum-free DMEM. Serum-starved cells were rinsed twice with phosphate-free RPMI medium (Gibco-BRL) and then incubated with 0.15 mCi of [^{32}P]/ml (Amersham) in phosphate-free RPMI for 120 min at 37°C. Labelling was ended by changing the medium to DMEM. The cells were allowed to equilibrate for 15 min at 37°C before incubation with 200 μl of fresh DMEM with or without 0.5 M sorbitol and stimulation with 0.1 mM pervanadate as indicated.

The reactions were terminated by the addition of 100 μl of 3.6 M HCl containing 2 mM dithiothreitol (DTT). The cells were scraped and the wells washed twice with 250 μl of methanol. For recovery determination, 10 μCi of [^{3}H]PI(4,5)P_2 (Amersham) was added to the combined material and lipids extracted with 850 μl of CH_3Cl as previously described (Didichenko *et al*., 1996). Extracted lipids were deacylated with methylamine reagent at 53°C for 40 min (Clarke and Dawson, 1981) and the resulting glyceroinositides separated by HPLC on a Partisphere Sax column (Whatman, 4.6×125 mm). Aliquots (200 μl) of each fraction were mixed with 150 μl of 1 M HCl, 150 μl of ethanol and 2 ml of Ready Safe (Beckman Instruments), and the ^{32}P and ^{3}H contents were determined by liquid scintillation counting.

PP2A assays

HA-tagged PP2Acα was transiently expressed in HEK-293 cells, stimulated as stated in the appropriate figure legends, immunoprecipitated (from 100 μg of total protein) with the anti-HA antibody 12CA5 and assayed for PP2A activity using Kemptide (LRRASVA) as a substrate as described previously (Favre *et al*., 1994).

Preparation of ^{32}P-labelled ΔPH-PKBα

HA-ΔPH-PKBα and Myc-PDK1 were expressed individually in HEK-293 cells, and equal amounts were immunoprecipitated with either the anti-HA antibody 12CA5 bound to protein A–Sepharose or with the anti-Myc antibody 9E10 bound to protein G–Sepharose, as described above. HA-ΔPH-PKBα and Myc-PDK1 (both bound to beads) were incubated at 30°C in kinase buffer (50 mM Tris–HCl pH 7.4, 20 mM β-glycerophosphate pH 7.4, 0.1 mM EGTA, 0.03% Brij 35) in the presence of 10 mM $MgCl_2$ and 50 μM ATP (sp. act. 5000 c.p.m./pmol). After 45 min, the beads were washed four times in 50 mM Tris–HCl pH 7.4, 50 mM NaCl, 0.1 mM EDTA, and HA-ΔPH-PKBα was eluted with 1 mg/ml HA epitope peptide (AAYPYDVPDYA) for 60 min at 4°C.

Dephosphorylation of ΔPH-PKBα with PP2A and different ratios of PI(3,4)P_2 and PI(3,4,5)P_3

^{32}P-labelled HA-ΔPH-PKBα was incubated in phosphate buffer (see above) with various forms of rabbit PP2A (PP2A_2, PP2A_1, PP2A_3 and PP2A_0) in the presence of different ratios of dioctanoyl derivatives of PI(3,4)P_2 and PI(3,4,5)P_3 (Frech *et al*., 1997). After the addition of phospholipids, the reactions were equilibrated at 30°C for 10 min and initiated by the addition of [^{32}P]HA-ΔPH-PKBα. After 30 min, the reactions were terminated by the addition of SDS–PAGE sample buffer and immediately resolved on 10% SDS–PAGE. Phosphorylation of ΔPH-PKBα was quantified with the Storm 840/860 PhosphorImager using ImageQuant software (Molecular Dynamics).

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