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PKB/Akt modulates TGF-β signalling through a direct interaction with Smad3

Ingrid Remy[1], Annie Montmarquette[1] and Stephen W. Michnick[1,2]

Transforming growth factor β (TGF-β) has a major role in cell proliferation, differentiation and apoptosis in many cell types. Integration of the TGF-β pathway with other signalling cascades that control the same cellular processes may modulate TGF-β responses. Here we report the discovery of a new functional link between TGF-β and growth factor signalling pathways, mediated by a physical interaction between the serine-threonine kinase PKB (protein kinase B)/Akt and the transcriptional activator Smad3. Formation of the complex is induced by insulin, but inhibited by TGF-β stimulation, placing PKB–Smad3 at a point of convergence between these two pathways. PKB inhibits Smad3 by preventing its phosphorylation, binding to Smad4 and nuclear translocation. In contrast, Smad3 does not inhibit PKB. Inhibition of Smad3 by PKB occurs through a kinase-activity-independent mechanism, resulting in a decrease in Smad3-mediated transcription and protection of cells against TGF-β-induced apoptosis. Consistently, knockdown of the endogenous *PKB* gene with small-interfering RNA (siRNA) has the opposite effect. Our results suggest a very simple mechanism for the integration of signals arising from growth-factor- and TGF-β-mediated pathways.

TGF-β modulates gene transcription through receptor-mediated activation of the Smad proteins — transcriptional activators that transmit the signal from the cell surface to the nucleus[1,2]. The TGF-β receptor consists of a heteromeric typeI–typeII receptor complex (TBRI and TBRII) with serine-threonine kinase activity[3]. Activated receptor phosphorylates Smad2 and Smad3, which then form heteromeric complexes with Smad4 and translocate to the nucleus where they regulate the transcriptional response to TGF-β[4,5]. It has been shown that TGF-β-mediated apoptosis can be inhibited by insulin in hepatocytes and that the phosphatidylinositol-3-OH kinase (PI(3)K) pathway (which is activated by insulin stimulation) is involved in this protective effect conferred by insulin[6–8]. A downstream effector of PI(3)K, the serine-threonine protein kinase PKB/Akt, has been demonstrated to be crucial for a number of cellular responses to growth factors and insulin, including cell growth, protein synthesis and anti-apoptotic/survival signals[9]. Thus, we sought to identify a mechanism by which the PI(3)K–PKB pathway can interfere with TGF-β-induced apoptosis, initially by identifying new physical and functional links between TGF-β- and PI(3)K-mediated signalling pathways.

We reasoned that the simplest explanation for cross-talk between the TGF-β and PI(3)K–PKB pathways could be a direct interaction between pathway-specific proteins. Thus, a systematic screen for interactions between PKB and TGF-β signalling proteins was performed using protein-fragment complementation assays (PCA) based on β-lactamase and yellow fluorescent protein (YFP)[10–14]. The principle of the PCA strategy is that complementary fragments (F1 and F2) of a reporter protein (enzyme) will fold into an active form only if fused to two proteins that interact and bring the complementary fragments of the reporter protein into close proximity. Using the β-lactamase PCA in HEK293T cells, we identified a unique and previously unidentified interaction between PKB and Smad3 (Fig. 1a). These results were confirmed by co-immunoprecipitation of PKB and Smad3 in the human hepatoma Hep3B cell line (Fig. 1b). As TGF-β-induced apoptosis is mediated primarily by Smad3, these results suggested a potentially direct and specific mechanism through which PKB could modulate TGF-β-mediated apoptosis[15–18].

We then tested whether the PKB–Smad3 interaction is specific and regulated by TGF-β- and growth factor-mediated pathways. Using YFP PCA and co-immunoprecipitation assays of overexpressed or endogenous proteins in Hep3B cells, we observed that the PKB–Smad3 interaction is induced by insulin and inhibited by TGF-β (Fig. 1c–e). Furthermore, treatment with wortmannin — an inhibitor of PI(3)K that prevents the phosphorylation of PKB — blocked the effect of insulin, suggesting that Smad3 binds preferentially to the phosphorylated form of PKB. Treatment of cells with a combination of insulin and TGF-β demonstrated that the activation of the TGF-β pathway partially reverses the effect of insulin (Fig. 1c–e). Disruption of the interaction by TGF-β treatment suggests that the phosphorylated form of Smad3 does not bind to PKB. In addition, the interaction between PKB and Smad3 occurs specifically between the carboxy-terminal domain (amino acids 409–480) of PKB and the MH2 domain (oligomerization domain) of Smad3 (see Supplementary Information, Fig. S1a, b). Point mutations in the C-terminal domain of PKB (W413A or D415A), within a short α-helix, almost completely abolished binding to Smad3 (Fig. 2d and data not shown)[19].

After stimulation of insulin or growth-factor receptors, PKB is activated at the plasma membrane by phosphorylation at two key residues

[1]Département de Biochimie, Université de Montréal, C.P. 6128, Succursale centre-ville, Montréal, Québec, H3C 3J7, Canada.
[2]Correspondence should be addressed to S.W.M. (e-mail: stephen.michnick@umontreal.ca)

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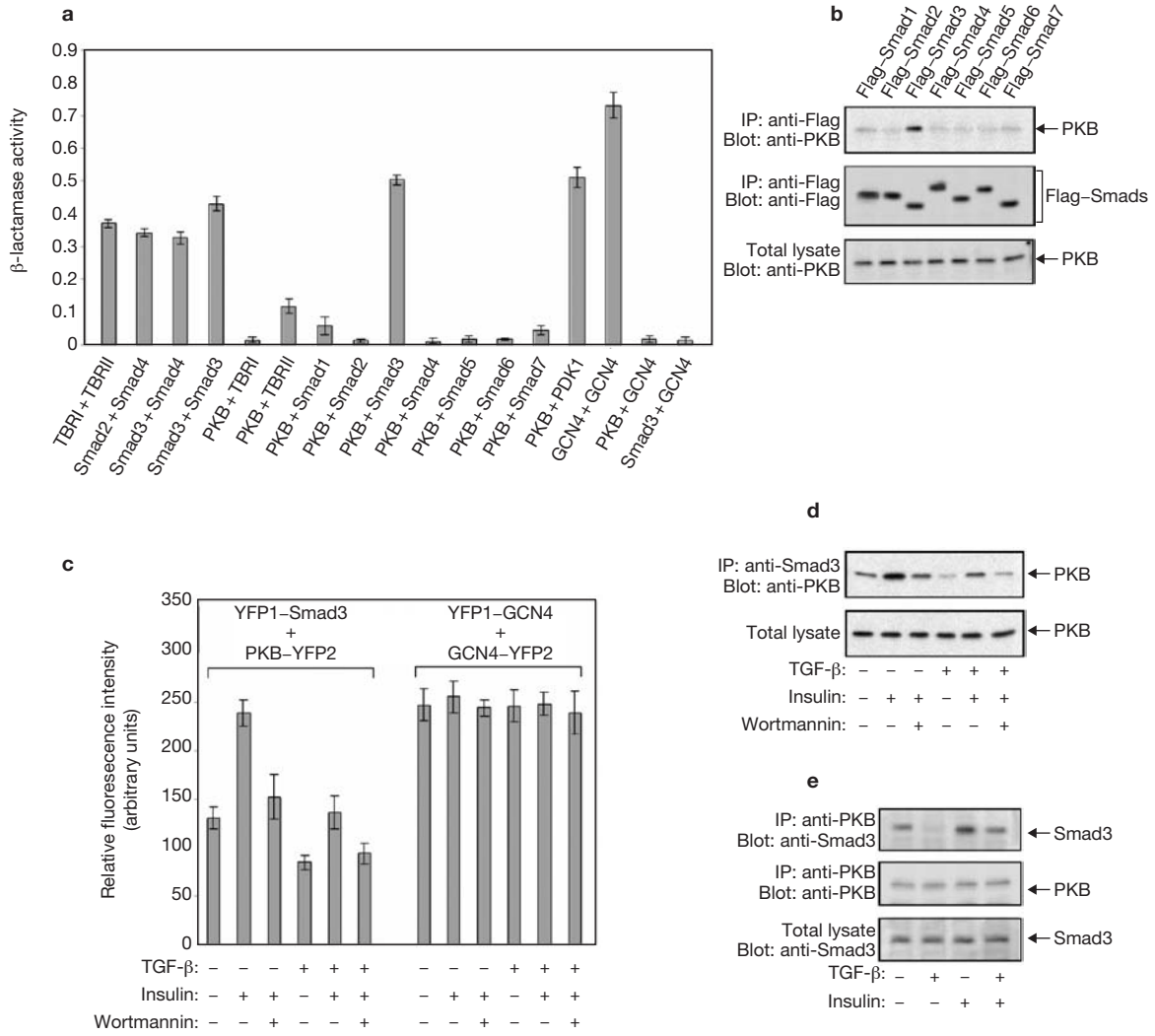

Figure 1 Identification and characterization of the PKB–Smad3 interaction. (**a**) Potential interacting partners for PKB in the TGF-β signalling pathway were tested using the β-lactamase PCA in HEK293T cells. Full-length cDNAs corresponding to known (positive controls; for example, PKB–PDK1 and dimerization of GCN4 leucine zippers), not probable (negative controls) or potential interacting proteins were fused to the F1 or F2 fragment of β-lactamase, and complementary pairs (F1 fusion and F2 fusion) were transiently co-expressed in cells. Reconstituted β-lactamase enzymatic activity is given in relative absorbance units (*y* axis). (**b**) Co-immunoprecipitation of PKB and Smad3. Hep3B cells were cotransfected with PKB and Flag-tagged Smad proteins. Smads were immunoprecipitated (IP) with an anti-Flag antibody, and the presence of PKB in the immune complexes was detected by immunoblotting with an anti-PKB antibody (top). The amount of Smad proteins in the immune complexes and expression level of PKB in total lysates were determined by immunoblotting with anti-Flag (middle) and anti-PKB (bottom) antibodies, respectively. (**c**) Pharmacological modulation of the PKB–Smad3 interaction was analysed by using the YFP PCA in Hep3B cells. Hep3B cells expressing the indicated fusion proteins were either left untreated or were treated with insulin, TGF-β and wortmannin. The relative amount of reconstituted YFP (a measure of the interaction between the fused protein partners) was detected by fluorometry of intact cells. The constitutive dimerization of GCN4 leucine zippers was used as a negative control. (**d**) The interaction of PKB with Smad3 is regulated by insulin and TGF-β. Hep3B cells were cotransfected with PKB and Smad3, and were either left untreated or were treated with TGF-β, insulin or wortmannin, as indicated. Smad3 was immunoprecipitated (IP), and the amount of PKB bound to Smad3 was determined by immunoblotting with an anti-PKB antibody. (**e**) Co-immunoprecipitation of endogenous PKB and Smad3 in Hep3B cells. Cells were either left untreated or were treated with TGF-β and insulin. PKB was immunoprecipitated (IP) and the amount of Smad3 bound to PKB was determined by immunoblotting with an anti-Smad3 antibody.

located in the activation loop (Thr 308 by PDK1) and in the C-terminal non-catalytic domain (Ser 473)[20]. To determine if the localization of PKB–Smad3 complexes is modulated by insulin- or TGF-β-mediated signalling pathways, we used YFP PCA with fluorescence microscopy to monitor the interactions in Hep3B cells (Fig. 2a). Formation of the PKB–Smad3 complexes were clearly induced by insulin (compared with starved cells) and observed both at the plasma membrane and in the cytosol in stimulated cells. However, no PKB–Smad3 complexes were found in the nucleus. The complexes were disrupted after wortmannin treatment, leaving some fluorescence in the cytosol but none at the plasma membrane. Simultaneous treatment with insulin and TGF-β partially reversed the stimulating effect of insulin (Fig. 2a). In contrast, TGF-β treatment increased the quantity of Smad3–Smad4 complexes co-incident with its nuclear translocation. Total Smad3 (detected by immunofluorescence microscopy) was found predominantly in the nucleus after TGF-β treatment

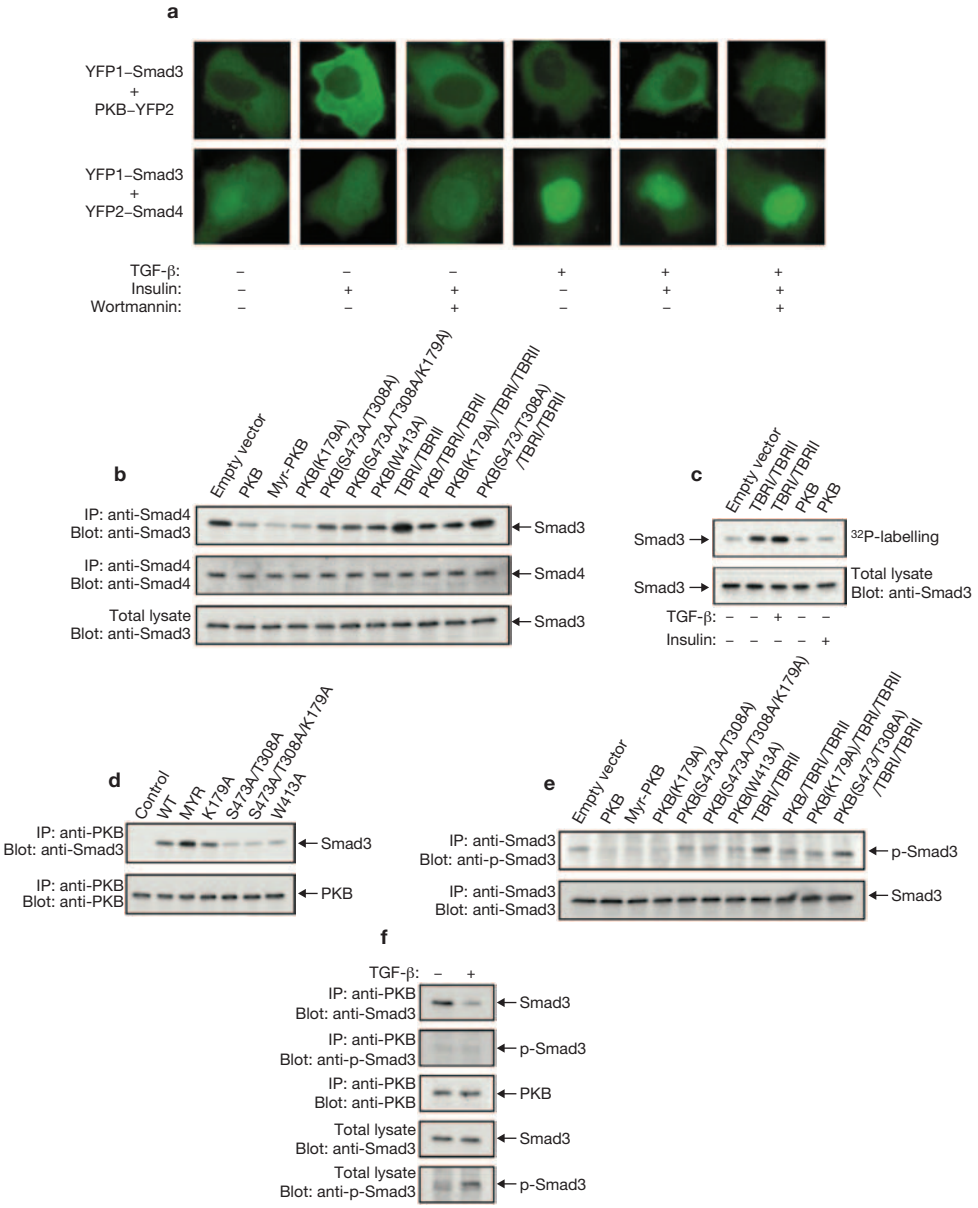

Figure 2 PKB inhibits Smad3 function. (**a**) PKB prevents the nuclear translocation of Smad3 in response to TGF-β. Localization of PKB–Smad3 and Smad3–Smad4 complexes was performed with YFP PCA. Hep3B cells were cotransfected with YFP1–Smad3 and PKB–YFP2, or with YFP1–Smad3 and YFP2–Smad4, and then either left untreated or treated with insulin, TGF-β and wortmannin, as indicated. Fluorescence microscopy was performed on live cells. Intensity of fluorescence was proportional to the number of complexes formed in cells (see Fig. 1c). (**b**) PKB prevents the formation of Smad3–Smad4 complexes. Hep3B cells were transfected with Smad3, Smad4 and the indicated forms of PKB, with or without TBRI and TBRII. Cells were then treated with TGF-β in the presence of serum before immunoprecipitation (IP) of Smad4. The amount of Smad3 bound to Smad4 was detected by immunoblotting with an anti-Smad3 antibody (top). The amount of Smad4 in the immune complexes and expression level of Smad3 in total lysates were determined by immunoblotting with anti-Smad4 (middle) and anti-Smad3 (bottom) antibodies, respectively. (**c**) Smad3 is not a substrate of PKB. Hep3B cells were co-transfected with Smad3 and PKB, Smad3 and TBRI–TBRII, or empty vector. Cells were labelled *in vivo* with γ-^{32}P-ATP and stimulated with TGF-β, insulin or left untreated. After immunoprecipitation with anti-Smad3 antibodies, the amount of phosphorylated Smad3 was detected by autoradiography. (**d**) Co-immunoprecipitation of Smad3 and different forms of PKB. Hep3B cells were cotransfected with Smad3 and the indicated forms of PKB before immunoprecipitation (IP) of PKB. The amount of Smad3 bound to the different forms of PKB was detected by immunoblotting with an anti-Smad3 antibody. (**e**) PKB attenuates TGF-β-induced phosphorylation of Smad3. Hep3B cells were cotransfected with Smad3 and the indicated forms of PKB, with or without TBRI–TBRII. Cells were treated with TGF-β in the presence of serum before immunoprecipitation (IP) of Smad3. The phosphorylation status of Smad3 was analysed by immunoblotting with anti-phospho-Smad3 (p-Smad3; top). Total levels of Smad3 in immunoprecipitates were detected by immunoblotting with anti-Smad3 (bottom). (**f**) PKB binds to the unphosphorylated form of Smad3. Hep3B cells were cotransfected with PKB and Smad3. Cells were then left untreated or were treated with TGF-β before immunoprecipitation (IP) of PKB. The amount of total Smad3 or phosphorylated Smad3 bound to PKB was determined by immunoblotting with anti-Smad3 or anti-phospho-Smad3 antibodies (p-Smad3), respectively.

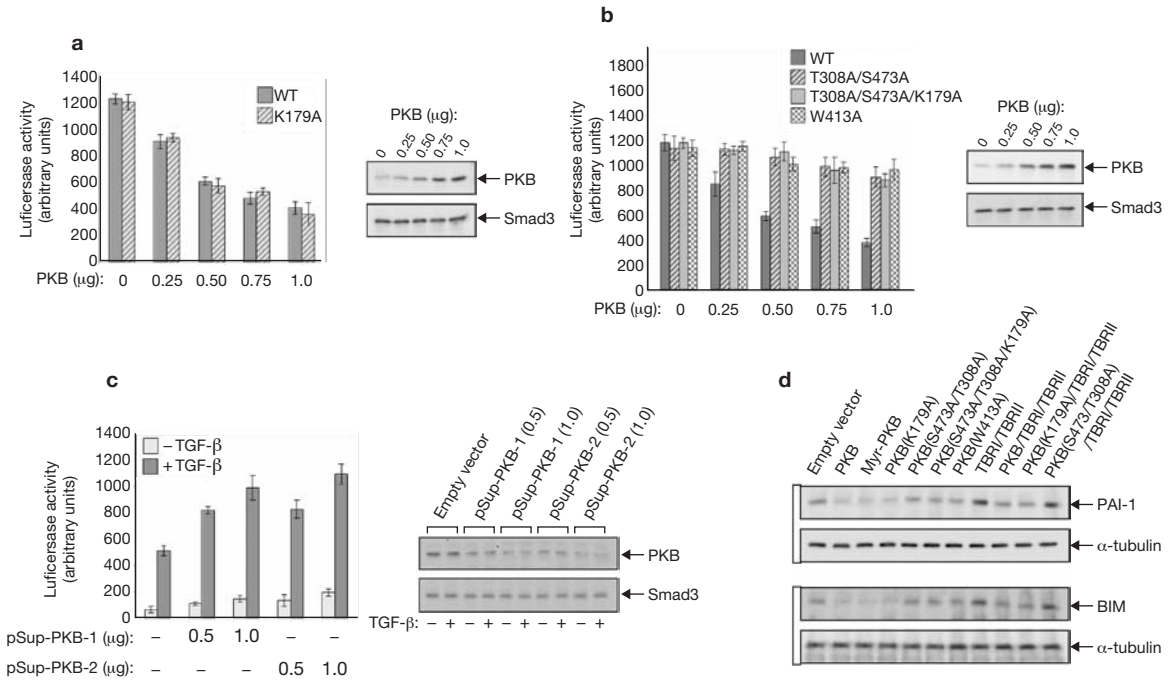

Figure 3 PKB inhibits the Smad3-induced activation of transcription. (**a**) Inhibition of Smad3-mediated transcription by PKB is independent of its kinase activity. Hep3B cells were transfected with the reporter vector p3TPlux, Smad3, Smad4 and increasing amounts of PKB- or PKB(K179A)-expressing vectors. Luciferase activity was measured 48 h after transfection. Expression levels of PKB and Smad3 in each sample were analysed by immunoblotting. (**b**) The phosphorylated form of PKB is required for maximal inhibition of Smad3-mediated transcription. Hep3B cells were transfected with the reporter vector p3TPlux, Smad3, Smad4 and increasing amounts of PKB, PKB(T308A/S473A)- PKB(T308A/S473A/K179A)- or PKB(W413A)-expressing vectors. Luciferase activity was measured 48 h after transfection. (**c**) siRNA-mediated suppression of endogenous PKB enhances TGF-β-induced Smad3-mediated transcription. Hep3B cells were transfected with the reporter vector p3TPlux and two different amounts of each of the pSuppress-PKB-1 and pSuppress-PKB-2 siRNA expression vectors. Cells were treated with TGF-β in the presence of serum, and luciferase activity was measured 48 h after transfection. Expression levels of endogenous PKB and Smad3 in each sample were analysed by immunoblotting. (**d**) PKB reduces the expression of endogenous PAI-1 and Bim proteins in Hep3B and BaF3 cells, respectively. Cells were transfected with the indicated forms of PKB, with or without TBRI–TBRII-expressing vectors, and treated with TGF-β in the presence of serum. The expression of PAI-1 and Bim were determined by immunoblotting cell lysates with anti-PAI-1 and anti-Bim antibodies. Immunoblotting with anti-α-tubulin was used as an internal control for loading.

(Supplementary Information, Fig. S1c). Our results suggest that the insulin-induced binding of PKB to Smad3 prevents the normal translocation of Smad3 to the nucleus after treatment of cells with TGF-β, whereas activation of the TGF-β pathway can partially inhibit the insulin-induced interaction between PKB and Smad3.

We then attempted to determine how the interaction of Smad3 with PKB reduces its nuclear translocation. The simplest explanation would be that PKB prevents the binding of Smad3 to Smad4. Thus, we examined the binding of Smad3 to Smad4 in the presence of different forms of PKB in Hep3B cells. Expression of wild-type, constitutively active (Myr, myristylated) and kinase-dead (K179A) forms of PKB all reduced levels of Smad3–Smad4, but not Smad2–Smad4 complexes, even when the TBRI and TBRII receptors were overexpressed (Fig. 2b and Supplementary Information, Fig. S1d). Furthermore, a phosphorylation-resistant form of PKB (T308A/S473A) was incapable of preventing the binding of Smad3 to Smad4, showing that phosphorylation of PKB-regulatory sites is required for maximal association to Smad3 (Fig. 2b; also see Supplementary Information, Fig. S2a).

We next asked whether PKB could inhibit Smad3 through direct phosphorylation. *In vivo* labelling of Hep3B cells with γ-^{32}P-ATP showed a significant increase in Smad3 phosphorylation when cells were cotransfected with the TBRI and TBRII receptors and stimulated with TGF-β; however, overexpression of PKB did not result in Smad3 phosphorylation, with or without insulin treatment (Fig. 2c). These results suggest that Smad3 is not a substrate of PKB. Moreover, the wild-type and kinase-dead (K179A) forms of PKB interact equally well with Smad3, suggesting that the catalytic activity of PKB is not involved in the formation of PKB–Smad3 complexes (Fig. 2d). However, the constitutively active (Myr, myristylated) form bound more efficiently to Smad3, whereas the phosphorylation-resistant PKB mutant (T308A/S473A) bound to a lesser degree, again indicating that phosphorylation of PKB is necessary for maximal association with Smad3. Furthermore, the specific Smad3-interaction disruption mutant PKB(W413A) also bound less to Smad3, showing that the association of PKB with Smad3 is specific (Fig. 2d and Supplementary Information, Fig. S2b–e). As Smad3 is not a substrate of PKB and because we have shown that PKB (and its kinase-dead form) cause a reduction in the observed binding of Smad3 to Smad4, our results suggest that PKB inhibits Smad3 activity by physically sequestering it.

We then examined whether the binding of PKB to Smad3 affects the phosphorylation of Smad3 in response to TGF-β treatment. TGF-β-induced phosphorylation of Smad3 was reduced in Hep3B cells transfected with the wild-type, kinase-dead and myristylated forms of PKB, even when the TBRI and TBRII receptor components were overexpressed (Fig. 2e), whereas PKB mutants W413A and T308A/S473A

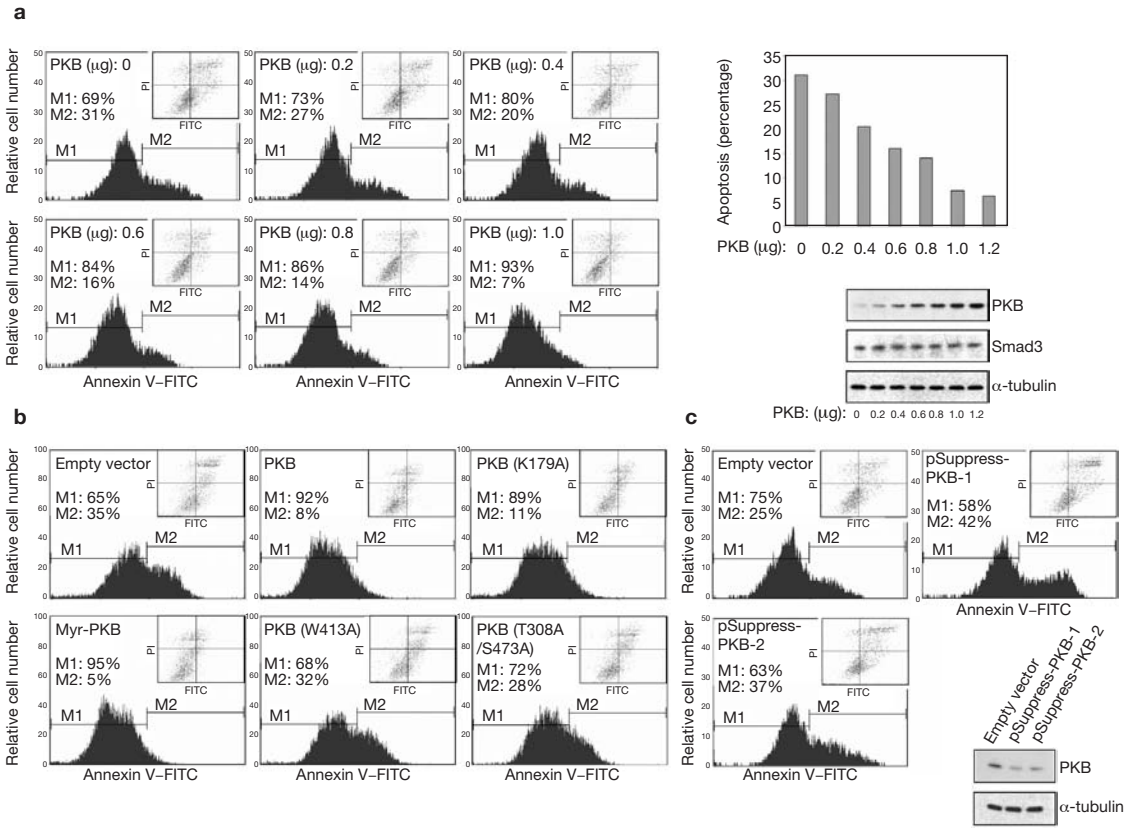

Figure 4 Wild-type and kinase-dead PKB protect cells against TGF-β-induced apoptosis. (**a**) Hep3B cells were transfected with increasing amounts of PKB-expressing vector, treated with TGF-β for 60 h in the presence of serum, stained with Annexin V–FITC and propidium iodide (PI), and analysed by flow cytometry. Cells that stain positive for Annexin V–FITC are undergoing apoptosis (M2 region, *x* axis). Cells that stain positive for both Annexin V-FITC and PI (top-right square) are either in the late stages of apoptosis or are already dead. Corresponding percentages of apoptotic cells are represented as histograms on the right. A fraction of each cell sample was lysed and the expression level of PKB and Smad3 were detected by immunoblotting with anti-PKB and anti-Smad3 antibodies. Anti-α-tubulin was used as an internal control for loading. (**b**) The protective effect of PKB against TGF-β-induced apoptosis is independent of its kinase activity, but requires its phosphorylation. Hep3B cells were transfected with the indicated forms of PKB, treated with TGF-β for 60 h in the presence of serum, stained with Annexin V–FITC and PI, and analysed by flow cytometry. (**c**) siRNA-mediated reduction of endogenous PKB enhances TGF-β-induced apoptosis. Hep3B cells were transfected with pSuppress-PKB-1 or pSuppress-PKB-2 vectors, designed to express siRNAs directed against *PKB* mRNA. Control cells were transfected with empty vector. Cells were treated with TGF-β for 60 h in the presence of serum, stained with Annexin V–FITC and PI, and analysed by flow cytometry. A fraction of each cell sample was lysed and the expression level of PKB was detected by immunoblotting with anti-PKB antibodies. Anti-α-tubulin was used as an internal control for loading.

had no effect, consistent with their reduced binding to Smad3 (Fig. 2d). Furthermore, the fact that PKB does not interact with Smad3 in cells treated with TGF-β (Fig. 1c–e) suggests that PKB binds to the unphosphorylated form of Smad3. Indeed very little of the phosphorylated form of Smad3 bound to PKB (Fig. 2f).

As PKB inhibits all aspects of Smad3 function (including phosphorylation, binding to Smad4 and nuclear translocation), we next examined the effect of increasing amounts of PKB on Smad3-induced activation of transcription using a luciferase-reporter system in Hep3B cells (p3TP-lux vector)[21,22]. Wild-type and kinase-dead PKB (K179A) decreased Smad3-mediated transcription to a similar extent (Fig. 3a), whereas the phosphorylation-resistant form of PKB (T308A/S473A) had no significant inhibitory effect (Fig. 3b), again suggesting that phosphorylation of PKB is required for optimal association to Smad3. Furthermore, reducing the amount of endogenous PKB in cells with sequence-specific siRNAs resulted in a significant increase of TGF-β-induced Smad3-mediated transcriptional activation that was proportional to the amount of siRNA transfected (Fig. 3c)[23]. Both the wild-type and kinase-dead forms of PKB reduced the Smad3-dependent expression of endogenous PAI-1 (plasminogen activator inhibitor type 1) and Bim (Bcl-2-interacting mediator of cell death) proteins in Hep3B and BaF3 cells, respectively (Fig. 3d)[21,24-26]. PKB was also found not to affect transcription mediated by other Smads (Smads 1, 5 and 8) in experiments using a reporter vector containing the BMP (bone morphogenetic protein)-responsive region of the type-X collagen promoter[27] (see Supplementary Information, Fig. S3a). Finally, PKB and PKB (K179A) inhibited GAL4 transcription mediated by a fusion of the MH2 domain of Smad3 (the domain that binds to PKB) to the GAL4 transcription factor, reinforcing the hypothesis that PKB physically sequesters Smad3 independently of its kinase activity (see Supplementary Information, Fig. S3b).

We then examined the consequences of PKB-mediated inhibition of Smad3 activity on TGF-β-induced apoptosis in Hep3B cells by annexin V–FITC and propidium iodide staining followed by flow cytometry. PKB had a protective effect against TGF-β-induced apoptosis that was proportional to the level of PKB expression (up to a four-

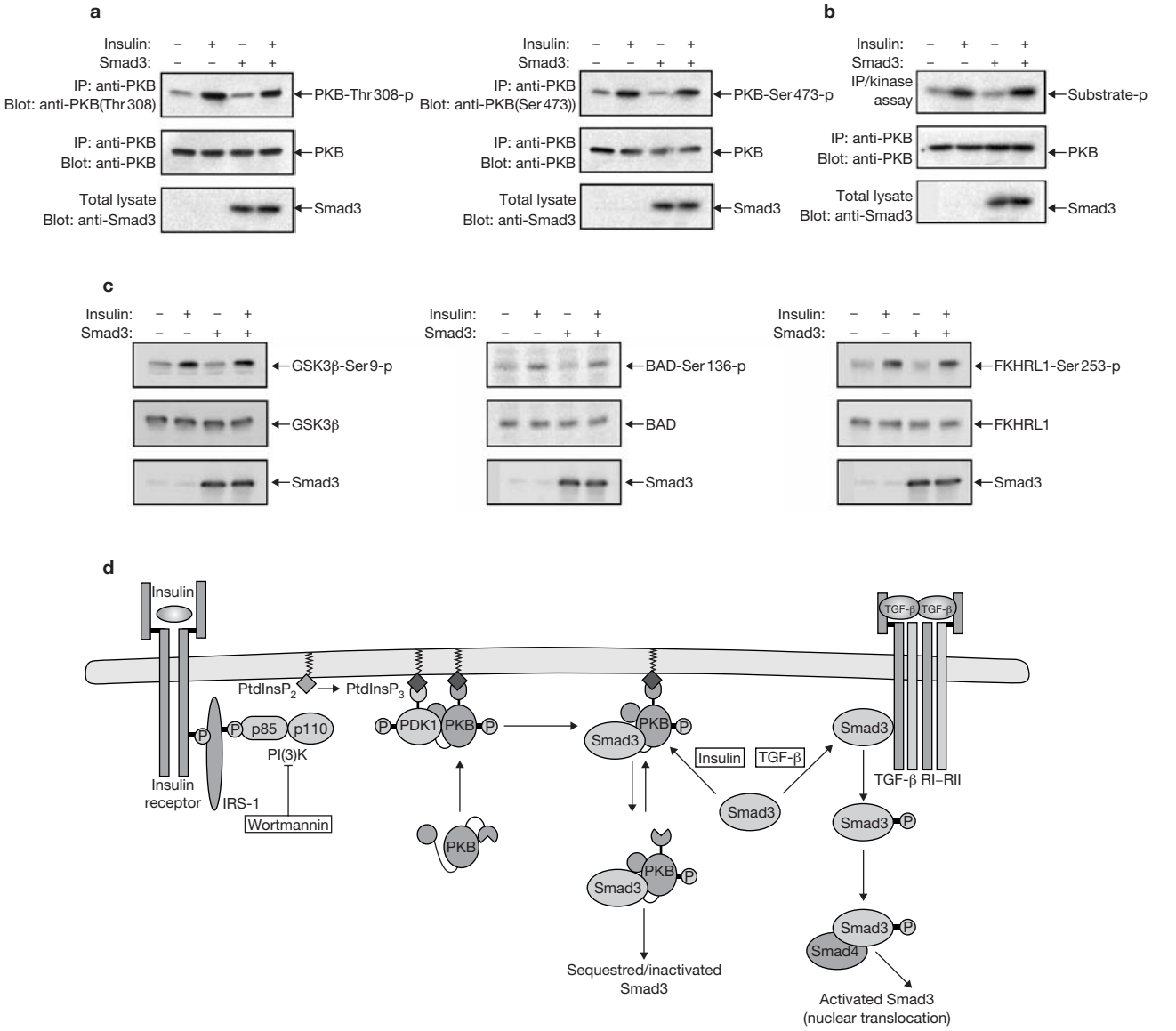

Figure 5 The binding of Smad3 to PKB does not prevent phosphorylation-mediated activation of PKB. (**a**) Hep3B cells were cotransfected with PKB- and Smad3-expressing vectors, or with PKB and an empty vector, and either stimulated (+) or not stimulated (–) with insulin. Cells were lysed and PKB was immunoprecipitated. The phosphorylation status of PKB on both of its regulatory sites was analysed by immunoblotting using the corresponding phospho-specific antibodies (top). The amount of PKB in the immune complexes and expression level of Smad3 in total lysates were determined by immunoblotting with anti-PKB (middle) and anti-Smad3 (bottom) antibodies. (**b**) An *in vitro* kinase assay to verify that Smad3 overexpression does not disrupt PKB kinase activity. HEK293T cells were cotransfected and treated as in **a**. PKB was immunoprecipitated and its kinase activity was measured using a pure substrate. Phosphorylation of the substrate (substrate-p) was detected by immunoblotting with anti-GSK3α/β-Ser21/9-p antibodies. (**c**) Smad3 does not prevent the phosphorylation of PKB substrates. Hep3B cells were cotransfected with PKB- and GSK3β-, BAD- or FKHRL1-expressing vectors, with or without Smad3. The phosphorylation status of GSK3β, BAD and FKHRL1 were analysed with phospho-peptide-specific antibodies. (**d**) A proposed model for the role of PKB in TGF-β signalling. After activation of the type I–type II TGF-β receptor complex, phosphorylated Smad3 dissociates from the receptor and forms a heteromeric complex with Smad4. The Smad3–Smad4 complexes translocate to the nucleus where they regulate the transcription of target genes. PKB inhibits the function of Smad3 by preventing its phosphorylation, binding to Smad4 and nuclear translocation, resulting in a decrease in Smad3-mediated transcription. Activation of the insulin pathway stimulates the interaction of PKB with Smad3, whereas TGF-β prevents the formation of PKB–Smad3 complexes. This mechanism is independent of PKB kinase activity, suggesting that PKB inhibits Smad3 function by physically sequestering it.

fold reduction in the percentage of cells undergoing apoptosis in the population; Fig. 4a). This process could be reversed by wortmannin treatment (see Supplementary Information, Fig. S4a). Furthermore, the kinase-dead form of PKB (K179A) protected against TGF-β-induced apoptosis as efficiently as the wild-type form (Fig. 4b). In contrast, the phosphorylation-resistant (T308A/S473A) and the Smad3-interaction disruption (W413A) PKB mutants had only a slight protective effect (Fig. 4b). Similar results were obtained when cells were treated with TGF-β and insulin instead of serum (see Supplementary Information, Fig. S4b), and the effects of both wild-type and kinase-dead PKB on TGF-β-induced apoptosis correlated with an increased viability of the cell population (see Supplementary

Information, Fig. S4c). Reducing the amount of endogenous PKB with sequence-specific siRNAs caused a significant increase in TGF-β-induced apoptosis (Fig. 4c). In addition, the kinase-dead form of PKB (K179A) was incapable of protecting Hep3B cells against apoptosis induced by other stimuli (TNFα–IFNγ[28]), demonstrating that in this case the protective effect of PKB depends on its kinase activity, in contrast to the inhibition of Smad3 by PKB in TGF-β signalling (see Supplementary Information, Fig. S4d).

Finally, although PKB inhibits the function of Smad3, Smad3 does not affect the phosphorylation-mediated activation of PKB. Overexpression of Smad3 in Hep3B cells did not disrupt stimulus-induced phosphorylation of PKB on both of its regulatory sites (Thr 308 and Ser 473), nor its kinase activity *in vitro* and *in vivo* (Fig. 5a–c).

TGF-β, insulin and growth factors have central and reciprocal roles in normal and pathological developmental–homeostatic processes, and growth factor- and insulin-mediated signalling pathways have been shown to modulate TGF-β signaling[6–8]. However, the molecular details of how these pathways control TGF-β signalling are not clear. Our results suggest a very simple mechanism by which growth and anti-apoptotic signalling, mediated by PI(3)K–PKB, thwarts TGF-β signalling by inducing an interaction between PKB and Smad3, thus reducing the pool of Smad3 available for TGF-β signalling. This mechanism is unique in being independent of PKB kinase activity, unlike parallel pathways through which PKB protects against apoptosis[17,26]. A model depicting the role of PKB in TGF-β signalling is presented in Fig. 5d.

The model may explain the unusual ability of TGF-β to mediate cell-cycle arrest in some contexts and apoptosis in others. First, relative levels of expression of PKB and Smad3 may determine the susceptibility to TGF-β-mediated apoptosis. A careful examination of the relative expression levels of PKB versus Smad3 may provide an indication of transitions in sub-populations of cells from growth and proliferation to apoptosis. Second, the fact that activation of PKB can inhibit Smad3 could suggest that growth-survival pathways can be dominant over a pathway that induces the opposite cellular response in the absence of a compensatory event, such as overexpression of Smad3 or strong activation of the TGF-β pathway. This may be particularly true in cancer cells where oncogenic mutants of PKB, or of the tumour suppressor PTEN that regulates PKB activity, could thwart the ability of TGF-β to induce apoptosis in these cells[29,30] □

METHODS

DNA constructs. Full-length cDNAs encoding PKB, PDK1, Smads 1–7, TGF-β receptor type I (TBRI) and type II (TBRII) were amplified by PCR and sub-cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), 5′ or 3′ of the F1 and F2 fragments of the enzyme TEM-1 β-lactamase (EC: 3.5.2.6). F1 corresponds to amino acids 26–196 (the amino-terminal signal sequence was removed and a M182T mutation was introduced). F2 corresponds to amino acids 197–286 of β-lactamase. PKB, Smad3 and Smad4 were also sub-cloned 5′ or 3′ of the F1 (amino-acids 1–158) and F2 (amino acids 159 to 239) fragments of YFP (Clontech, Palo Alto, CA), into the pcDNA3.1 vector. In all cases, a 10-amino-acid (for the YFP PCA) or 15-amino-acid (for the β-lactamase PCA) flexible linker consisting of $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$ was inserted between the cDNA and the PCA fragments. The PKB (W413A) and (T308A/S473A) point mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Co-immunoprecipitation, immunoblot analysis and kinase assay. Hep3B and HEK293T cell lines were transfected using Lipofectamine reagent (Invitrogen), according to the manufacturer's instructions. For the modulation assays, cells were serum-starved and either left untreated or treated with 500 nM wortmannin (Calbiochem, San Diego, CA) for 45 min. Next, cells were stimulated for 15 min with 3 μM insulin (Roche Diagnostics, Indianapolis, IN) and/or 400 pM TGF-β1 (Calbiochem) before lysis (lysis buffer: 1% NP-40, 0.1% SDS, 150 mM NaCl, 20 mM Tris at pH 8.0, 20 μg ml^{-1} aprotinin, 5 mM AEBSF, 10 mM NaF and 5 mM sodium vanadate). Flag–Smads were immunoprecipitated with an anti-Flag antibody (Chemicon, Temecula, CA) or with specific non-cross-reactive antibodies (anti-Smad2: S-20 and anti-Smad4: H-552, Santa Cruz Biotechnology, Santa Cruz, CA; anti-Smad3, Zymed Laboratories, South San Francisco, CA). PKB was immunoprecipitated with an anti-PKB antibody (N-19; Santa Cruz Biotechnology). Immunoblotting were performed using anti-PKB (Cell Signaling, Beverly, MA), anti-Smad2 (S-20), anti-Smad3 (I-20) or anti-Smad4 (H-552) antibodies (Santa Cruz Biotechnology). For the Smad2–Smad4 and Smad3–Smad4 co-immunoprecipitations, and for the analysis of Smad3 phosphorylation, non-starved Hep3B cells were treated with 400 pM TGF-β1 for 15 min before immunoprecipitations. Phosphorylation of Smad3 was analysed using an anti-Smad2/3-Ser433/435-p antibody (Santa Cruz Biotechnology). For the analysis of endogenous PAI-1 and Bim expression, Hep3B and BaF3 cells were treated with 400 pM TGF-β1 for 16 h (in the presence of serum), before cell lysis and immunoblotting with anti-PAI-1 (C-9; Santa Cruz Biotechnology) or anti-Bim (BD Pharmingen, San Jose, CA) antibodies. Anti-α-tubulin was used as an internal control for loading (Sigma). For the *in vitro* PKB kinase assay, HEK293T cells were serum-starved and stimulated for 15 min with 3 μM insulin before lysis. PKB was immunoprecipitated with immobilized anti-PKB antibodies (Cell Signaling) and the *in vitro* kinase assay was performed using a pure protein substrate (paramyosin fused to GSK3α/β crosstide corresponding to 20 residues surrounding GSK-3α/β-Ser21/9; Akt kinase assay kit, Cell Signaling). Phosphorylation of the substrate was detected by immunoblotting using anti-GSK3α/β-Ser21/9-p antibodies (Cell Signaling). The phosphorylation status of PKB was analysed using anti-PKB-Thr308-p and anti-PKB-Ser473-p antibodies (Cell Signaling). Phosphorylation of PKB substrates was analysed using anti-GSK3β-Ser9-p, anti-BAD-Ser136-p and anti-FKHRL1-Ser253-p (Cell Signaling). Anti-FKHRL1 (H-144; Santa Cruz Biotechnology), anti-BAD (C-7; Santa Cruz Biotechnology), anti-Smad3 (I-20; Santa Cruz Biotechnology) and anti-GSK3β (BD Pharmingen) were also used for immunoblotting.

β-lactamase PCA colorimetric assay. HEK293T cells were cotransfected at approximately 60% confluence in 12-well plates with 0.5 μg of each of the β-lactamase PCA fusion expressing vectors (1 μg total DNA per well), using Lipofectamine reagent (Invitrogen). Measurement of β-lactamase activity in cell lysates was performed as previously described[12]. β-lactamase enzymatic activity was measured with the chromogenic substrate nitrocefin and results are given in relative absorbance units (*y* axis). Error bars represent standard errors of the mean calculated for three independent samples.

YFP PCA fluorometric analysis. Hep3B cells were cotransfected at approximately 60% confluence in 12-well plates with the YFP PCA fusion expression vectors (1 μg total DNA per well), using Lipofectamine reagent (Invitrogen). 24 h after transfection, cells were serum-starved overnight and either left untreated or treated with 500 nM wortmannin (Calbiochem) for 60 min. Next, cells were stimulated for 30 min with 3 μM insulin (Roche) or 400 pM TGF-β1 (Calbiochem), washed with PBS, gently trypsinized and resuspended in 200 μl of PBS. The total cell suspensions were transferred to 96-well black microtitre plates (Dynex; VWR Scientific, Mississauga, Ontario) and subjected to fluorometric analysis (Spectra MAX GEMINI XS; Molecular Devices, Sunnyvale, CA). Next, the data were normalized to total protein concentration in cell lysates (protein assay; BioRad, Hercules, CA). The background fluorescence intensity corresponding to non-transfected cells was subtracted from the fluorescence intensities of all of the samples. Error bars represent standard errors of the mean calculated for three independent samples. Identical hormone, serum and wortmannin treatment protocols were used in the fluorescence microscopy experiments (Nikon TE2000U; 100× objective lens).

Immunofluorescence microscopy. Hep3B cells were serum-starved overnight and either left untreated, or treated with 3 μM insulin (Roche) or 400 pM TGF-β1 (Calbiochem) for 30 min. Cells were then fixed, permeabilized, blocked and incubated with anti-PKB or anti-Smad3 antibodies. Cells were stained with Alexa fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR).

***In vivo* ^{32}P labelling.** Hep3B cells were incubated in medium without phosphate and serum for 15 h, followed by a 5-h labelling with 0.2 mCi of γ-^{32}P-ATP ml^{-1} (Perkin Elmer-Life Sciences, Boston, MA). Cells were then stimulated with 400 pM TGF-β1 (Calbiochem) or 3 μM insulin (Roche Diagnostics) for 15 min before lysis. Smad3 was immunoprecipitated with anti-Smad3 antibodies (Zymed Laboratories) and the incorporation of radioactive phosphate (phosphorylated Smad3) was detected by autoradiography.

MTT assay. Hep3B cells were transfected with increasing amounts of PKB-expressing vectors and treated with 400 pM TGF-β1 for 60 h in the presence of serum. Cell viability was determined using a colorimetric MTT assay (Chemicon) according to the manufacturer's instructions. The reduction of yellow MTT into a purple formazan product by active mitochondria was measured at 600 nm on a Spectra MAX 190 (Molecular Devices) plate reader.

Apoptosis detection. Hep3B cells were transfected at approximately 60% confluence in 12-well plates with increasing amounts of a PKB-expressing vector, using Lipofectamine reagent (Invitrogen). The total amount of DNA per well was kept constant by adding empty vector (1.2 μg total DNA per well). Cells were treated with 400 pM TGF-β1 for 60 h in the presence of 10% serum (with simultaneous addition of 300 nM wortmannin in one case: see Supplementary Information, Fig. S4a), stained with Annexin V–FITC and propidium iodide according to the manufacturer's instructions (BD Pharmingen) and analysed by flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ). Supplementary experiments were also performed by treating serum-starved cells with 3 μM insulin and 400 pM TGF-β1 for 60 h, or by treating cells with a combination of TNFα (10 ng ml^{-1}) and IFNγ (10 U ml^{-1}) in the presence of 10% serum for 60 h.

siRNA-mediated down-regulation of the *PKB* gene. The production of siRNAs in mammalian cells was achieved using the pSuppress vector. Hep3B cells were transfected with pSuppress-PKB-1 or pSuppress-PKB-2 using the Lipofectamine reagent (Invitrogen). pSuppress-PKB-1 and pSuppress-PKB-2 produce siRNAs derived from 19-nucleotide sequences in the N terminus and catalytic domain of PKB, respectively. For apoptosis experiments, transfected cells were treated with 400 pM TGF-β1 for 60 h in the presence of 10% serum, stained with Annexin V–FITC and propidium iodide (BD Pharmingen), and then analysed by flow cytometry (FACScalibur, Becton Dickinson).

Luciferase reporter assays. Hep3B cells were transfected with p3TPlux (0.5 μg), Smad3 (0.25 μg)- and Smad4 (0.25 μg)-expressing vectors and increasing amounts of PKB expressing vectors (0–1 μg). The total amount of DNA per well was kept constant by adding empty vector. To measure the effect of removing endogenous PKB on Smad3-mediated transcription, Hep3B cells were transfected with p3TPlux (0.5 μg) and the pSuppress-PKB-1 or pSuppress-PKB-2 siRNA-producing vectors (0, 0.5 or 1 μg), or an empty vector, and treated with 200 pM TGF-β1 for 20 h. Luciferase activity was measured using a Bright-Glo Luciferase Assay System (Promega, Madison, WI). Expression levels of PKB and Smad3 in each sample were analysed by immunoblotting. Control experiments using a reporter vector containing the BMP-responsive region of the type-X collagen promoter instead of the 3TP-lux promoter were also performed using W20-17 cells treated with 50 ng ml^{-1} BMP-2 (Alpha Diagnostic, San Antonio, TX) for 20 h.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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