Binding of protein kinase B to the plakin family member periplakin

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Summary

The serine/threonine kinase protein kinase B (PKB/c-Akt) acts downstream of the lipid kinase phosphoinositide 3-kinase (PI3K) and functions as an essential mediator in many growth-factor-induced cellular responses such as cell cycle regulation, cell survival and transcriptional regulation. PI3K activation generates 3′-phosphorylated phosphatidylinositol lipids (PtdIns3*P*) and PKB activation requires PtdIns3*P*-dependent membrane translocation and phosphorylation by upstream kinases. However PKB activation and function is also regulated by interaction with other proteins. Here we show binding of PKB to periplakin, a member of the plakin family of cytolinker proteins. Interaction between PKB and periplakin was mapped to part of the pleckstrin homology (PH) domain of PKB, which is probably not involved in lipid binding, and indeed binding to periplakin did not affect PKB activation. We therefore investigated the possibility that periplakin may act as a scaffold or localization signal for PKB. In cells endogenous periplakin localizes to different cellular compartments, including plasma membrane, intermediate filament structures, the nucleus and mitochondria. Overexpression of the C-terminal part of periplakin, encompassing the PKB binding region, results in predominant intermediate filament localization and little nuclear staining. This also resulted in inhibition of nuclear PKB signalling as indicated by inhibition of PKB-dependent Forkhead transcription factor regulation. These results suggest a possible role for periplakin as a localization signal in PKB-mediated signalling.

Key words: PKB/Akt, Periplakin, Vimentin, Mitochondria, Insulin

Introduction

Protein kinase B (PKB, also known as c-akt) is a pleckstrin-homology (PH) domain containing serine/threonine kinase. In mammalian cells PKB is activated upon treatment of cells with a wide variety of extracellular growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin (for a review, see Coffer et al., 1998). Activation of the cognate receptor for these factors results in the recruitment to the plasma membrane and activation of phosphoinositide 3-kinase (PI3K). Active PI3K produces 3′-phosphorylated phosphatidylinositol (PtdIns3*P*) lipids that act as second messengers to recruit, among others, PH-domain-containing proteins to the plasma membrane. Upon translocation to the cell membrane PKB becomes phosphorylated on two residues essential for activation, Thr308 and Ser473. Besides translocation and phosphorylation PKB activity can also be regulated by interaction with other proteins. For example it has been suggested that aPKCs can bind to PKB and by phosphorylation of an unidentified residue can negatively regulate PKB activity (Doornbos et al., 1999). More recently a novel small protein named CTMP has been shown to interact with the C-terminal region of PKB and binding of CTMP results in inhibition of PKB activity (Maira et al., 2001).

Once activated, PKB can phosphorylate a range of proteins on either serine or threonine residues contained within an RxRxxS/T motif. One of the first identified substrates for PKB is glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995). PKB phosphorylates Ser9 of GSK3β and Ser21 of GSK3α both in vitro (Cross et al., 1995) and in vivo (van Weeren et al., 1998), and this results in inactivation of GSK3 thereby regulating glucose utilization and activation of glycogen synthesis (Rylatt et al., 1980).

PKB can also regulate transcription factor activity. Upon activation PKB has been shown to translocate to the nucleus (Andjelkovic et al., 1997; Meier et al., 1997), where it can phosphorylate transcription factors such as the FOXO members of the Forkhead family (reviewed by Kops and Burgering, 1999). This leads to nuclear export and inactivation of these FOXO transcription factors. Regulation of FOXO transcriptional activity has been implicated in cell cycle and cell death control by PKB. However, besides FOXO regulation many other mechanisms have been described for PKB-mediated protection from apoptosis. For example, direct phosphorylation of proteins such as the pro-apoptotic Bcl-2 family member BAD (Datta et al., 1997), or caspase-9 (Cardone et al., 1998). Irrespective of the multitude of potential protein targets for PKB it is clear that PKB can protect from mitochondrial-dependent apoptosis (Kennedy et al., 1999). Thus PKB activity can maintain mitochondrial membrane

stability and prevent cytochrome-C leakage under conditions of stress (Gottlob et al., 2001; Plas et al., 2001).

Periplakin belongs to the plakin family of cytolinker proteins that also includes desmoplakin, envoplakin, plectin and bullous pemphigoid antigen 1 (BPAG1) (Ruhrberg et al., 1997). It has an approximate molecular mass of 195 kDa, contains a central rod dimerisation domain and its C-terminal region is involved in intermediate filament binding, whereas its N-terminus has been shown to interact with cortical actin (DiColandrea et al., 2000). Periplakin is expressed in epithelial cells (Aho et al., 1998), where it is found to be, together with envoplakin, the precursor of the epidermal cornified envelope (DiColandrea et al., 2000). Furthermore, periplakin has been shown to be a target antigen in paraneoplastic pemphigus (de Bruin et al., 1999), and in keratinocytes it localizes at desmosomes and the interdesmosomal plasma membrane (Simon and Green, 1984). Although these observations suggest a specified role of plakin family members in epidermal cornification there is substantial evidence that within other cell types plakins may be involved in a variety of cellular processes. For example, plectin, the best-studied member of the plakin family, has been suggested to play a role in reorganization of microfilaments in apoptosis when it is cleaved by caspase 8 (Stegh et al., 2000). A further role in this is suggested by the observation that in muscle cells it is found associated with mitochondria and intermediate filaments (Reipert et al., 1999). Also in endothelial cells plectin localizes to focal contacts and here a role in adhesion is suggested (Gonzales et al., 2001).

In order to investigate PKB regulation through binding to other proteins in more detail we performed a yeast two-hybrid screen with a part of the PH domain of PKB. We found that periplakin is an interaction partner of PKB and determined the binding side within PKB. Furthermore we also show binding of periplakin to the intermediate filament protein vimentin. In addition to intermediate filament structures, periplakin localizes to the cell membrane, nucleus and mitochondria. Localization to these cellular compartments is in part influenced by the presence or absence of vimentin. These and other observations suggest that periplakin may act as a localization signal for PKB signalling.

Materials and Methods

Plasmids

The yeast two-hybrid bait pPC97PKB was constructed by cloning a 534 nt *Pvu*II-*Sma*I fragment of bovine PKBα (Burgering and Coffer, 1995), into *Sma*I-digested pPC97. The C-terminal fragment of periplakin (pPC86-c-ppl) interacting with this bait contained the last 1357 nt of the mRNA (including 529 nt 3′ UTR). Myc-c-ppl was constructed by subcloning the insert (*Sma*I-*Not*I) from pPC86-c-ppl into Myc9.1 (F. Zwartkruis, personal communication). GST-c-ppl was created by cloning the insert (*Eco*RI-*Not*I) of pPC86-c-ppl into *Eco*RI-*Not*I-digested pRP261 (pGEX-3X derivative) (Smith and Johnson, 1988). A bait of the C-terminal part of periplakin (pPC97-c-ppl) was constructed by inserting the *Sma*I-*Not*I fragment of pPC86-c-ppl into *Sma*I-*Not*I-digested pPC97. The vimentin-expressing clones interacting with pPC97-c-ppl all contained vimentin, missing the first N-terminal 10 aa (pPC86-vim). HA-vimentin was constructed by cloning the insert (*Sal*I-*Not*I) from pPC86-vim into *Sal*I-*Not*I-digested pMT2-HA (Burgering and Coffer, 1995). HA-PKB and HA-PKBdead have been described (Burgering and Coffer, 1995). Deletion mutants of HA-PKB were created as follows: Δ30-130 and Δ30-214 were created by isolation of partially digested *Msc*I fragments of HA-PKB and religation. Δ130-311 was created by isolation of a partially digested *Ava*I fragment of HA-PKB and religation. Δ311-507 was created by digestion of HA-PKB with *Xma*I and *Bcl*I, blunt ending with T4-DNA polymerase and religation. Δ9 was created by digestion of HA-PKB with *Sac*II and *Pst*I followed by blunt ending by S1 nuclease and religation. Δ9/+9GLY was made by inserting a *Sac*II-*Pst*I oligo encoding 9 glycine residues in *Sac*II/*Pst*I-digested HA-PKB. All mutants were verified by sequencing. The following constructs that were used have been described: HA-MAPK and HA-p70s6k (Burgering and Coffer, 1995), HA-FOXO4 (Medema et al., 2000) HA-full-length-periplakin (DiColandrea et al., 2000). All tagged constructs contain the tag at the 5′ position.

Antibodies

Anti-periplakin C-terminal peptide antibody was raised by immunizing two rabbits with a peptide encoding the last 12 amino acids of periplakin (IQELAVLVSGQK) coupled to KLH (7445 and 7446). This peptide is fully conserved between human and mouse periplakin. The anti-GST-c-ppl sera were raised by immunizing two rabbits with purified GST-c-ppl (5117 and 5118). Anti-PKB (5179 and 5178) (Burgering and Coffer, 1995); anti-MAPK [124 (de Vries-Smits et al., 1992)]; anti-HA [12CA5 (Burgering and Coffer, 1995)] and anti-myc [9E10 (van Weeren et al., 1998)] were described previously. Anti-cytochrome c was kindly provided by F. J. T. Zwartkruis, (UMCU, Utrecht, Netherlands). Anti-vimentin was purchased from Oncogene Science and anti-cox4 was from Molecular Probes. MAB1273, which recognizes a 65 kDa mitochondrial protein, was obtained from Chemicon International, anti-pSer193 FOXO4 was from Cell Signaling, and anti-actin was from Santa Cruz.

Cell culture

Rat-1, A14 and COS-7 cells were grown in Dulbecco's modified Eagle's medium. 293T (human embryonic kidney 293 cells immortalised with SV40 large T antigen), MCF-7 and MCF-7/FR (MCF-7 cells stably expressing the Fas receptor) cells were grown in RPMI. Both types of media were supplemented with 10% fetal calf serum (Bio-Whittaker, Belgium), 1% penicillin/streptomycin (Bio-Whittaker) and 2 mM L-Gln (Bio-Whittaker). Cells were treated for 10 minutes with 1 μM insulin after overnight starvation in medium without serum, unless otherwise indicated. Cells were transfected using the $Ca(PO_4)_2$ procedure, except MCF-7 cells, which were transfected with Fugene6 (Roche).

Yeast two-hybrid screen

Yeast two-hybrid screens were performed as described previously (Wolthuis et al., 1996). An oligo-dT primed 13.5 day mouse embryo cDNA library cloned into the pPC86 yeast two-hybrid vector was used in all cases. To diminish background, 50 mM 3-amino-triazol was included in the screen and clones were picked after 3 and 4 days.

GST pull-down assay

For purification of GST-c-ppl, protein expression was induced in DH5α using 100 nM isopropyl-1-thio-β-D-galactopyranoside for 20 hours at room temperature. Bacteria were collected and lysed in ice-cold phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors. The lysates were sonicated three times for 20 seconds at 60 Hz (UP200S GmbH) and centrifuged at 10,000 ***g*** for 20 minutes to remove insoluble material. GST-c-ppl was purified from the cleared lysate by batchwise incubation with glutathione-agarose beads (Sigma), and after washing the protein was eluted from the beads in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol and 10 mM glutathione. The eluted protein was dialysed for 20 hours in the same buffer without glutathione.

GST or GST-c-ppl attached to glutathione sepharose were incubated for 1 hour with extracts of A14 cells transiently transfected with either wild-type or kinase-dead HA-tagged PKB and washed four times in solubilisation buffer. Subsequently, bound protein was removed by elution with 40 mM reduced glutathione. Samples were analysed for the presence of PKB by western blotting using the anti-HA antibody.

Immunoprecipitations

Non-confluent cells were lysed in 0.5 ml RIPA buffer (20 mM Tris pH 8.0, 1% Triton X-100, 0.5% Na-DOC, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 2 μg/ml aprotinin) except for the endogenous Ppl-PKB and vimentin co-immunoprecipitations (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mg/ml trypsin inhibitor, 1 μg/ml leupeptin and 2 μg/ml aprotinin). Lysates were incubated for 2 hours at 4°C with 10 μl antibody and 100 μl pre-washed protein-A beads. The immunoprecipitations were washed four times with the used lysis buffer before being taken up in Laemmli buffer.

Immunoblot analysis

Protein samples in Laemmli buffer were separated by SDS-PAGE on 6% (periplakin) or 10% (others) gels and transferred to PVDF membrane (NEN). Western blots were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 2% nonfat dried milk (Protifar, Nutricia) and 0.5% bovine serum albumin (Sigma). The western blots were then incubated for 2 hours with the indicated primary antibodies in PBS containing 0.1% Tween 20 using the dilutions recommended by the manufacturers or 1:8000 for the self-generated antibodies. After washing four times for 5 minutes with PBS/0.1% Tween-20 blots were incubated with secondary antibodies anti-mouse HRP and anti-rabbit HRP (1:10,000) for 1 hour at 4°C. Blots were washed again four times for 5 minutes with PBS/0.1% Tween-20 and analysed with chemiluminescence (ECL (NEN)).

Immunofluorescence staining

Cells on coverslips were fixed in 4% paraformaldehyde for 30 minutes at 4°C and then permeabilised with 0.1% Triton X-100 in the presence of 0.5% BSA for 30 minutes at 4°C. Before fixation, cells were incubated with 100 nM Mitotracker (Molecular Probes) for 30 minutes. Cells were incubated with primary antibodies for 2 hours at 4°C, washed in PBS with 0.1% Triton X-100 and 0.01% BSA, and then incubated further for 1 hour with the appropriate conjugated secondary antibody. After further washing, coverslips were mounted in Immu-mount (Shandon) and examined using a 63× planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, the Netherlands) interfaced with a Leica TCS4D confocal laser-scanning microscope (Leica, Heidelberg, Germany). Digital images were recorded using Leica TCS NT version 1.6.587.

As primary antibodies, the anti-vimentin (Oncogene), anti-periplakin anti-serum 5117 (3rd boost) or non-immune antiserum were used; FITC-conjugated anti-rabbit, anti-mouse or anti-goat antibodies were used as secondary antibodies.

Vimentin extraction

A14 cells were lysed with 0.5% Triton X-100 in CSK-buffer (10 mM Pipes pH 6.8, 250 mM sucrose, 3 mM $MgCl_2$, 150 mM KCl, 1 mM EGTA and 1 mM PMSF) for 10 minutes (fraction 1: total cells). Lysates were centrifuged at 14,000 ***g*** for 10 minutes (supernatant, fraction 2: membrane proteins, cytosol, tubulin). The pellet was further extracted with 0.3 M KI in CSK-buffer for 5 minutes and centrifuged at 14,000 ***g*** for 30 minutes (pellet, fraction 4: nuclei, intermediate filaments). Subsequently, fraction 3 (actin) was isolated by dialyzing the supernatant against CSK-buffer for 16 hours and centrifuging at 14,000 ***g*** for 30 minutes. The pellet was resuspended in 0.3 M KI in CSK-buffer and incubated for 10 minutes after which the same dialysis was performed.

Percoll gradient-based cell fractionation

MCF-7, Rat-1 and COS-7 cells were fractionated using a percoll gradient-based assay as described (Baumann et al., 2000).

Affinity purification of mitochondria

Mitochondria from MCF-7 and Rat-1 cells were isolated as described (Herrnstadt et al., 1999).

Results

PKB interacts with periplakin

To search for PKB-interacting proteins we performed a yeast two-hybrid screen using a C-terminal part of the PH domain of bovine PKB as bait, since it has been shown that the N-terminal part is predominantly involved in the interaction with PtdIns3*P* lipids (Ferguson et al., 2000). The bovine bait has 98% similarity and 96% identity with human PKB. From a screen of 10^6 transformants, positive clones were isolated and from these we identified the homologue of a recently cloned protein named periplakin as a potential PKB-binding protein. We determined the full-length sequence of this clone and sequence comparison indicates that this protein (accession number AF126834) is indeed the mouse homologue of human periplakin (Fig. 1A), which has 97% similarity and 88% identity with human periplakin. To further investigate binding of PKB to periplakin we performed a number of experiments. First, the C-terminal domain of periplakin was fused to GST (GST-c-ppl) and lysates of cells expressing transiently transfected HA-tagged wild-type or kinase-dead PKB were incubated with GST-c-ppl, or GST alone as a negative control. Both HA-PKB and HA-PKBdead were found to bind to GST-c-ppl (Fig. 1B). Second, the C-terminal domain of periplakin was fused to an N-terminal myc tag (myc-c-ppl), which was co-transfected in A14 cells with either HA-PKB or HA-p70S6K as a control. myc-c-ppl was found to coprecipitate with HA-PKB and not with HA-p70S6K (Fig. 1C). Finally, to determine association between endogenous PKB and periplakin we first developed two different polyclonal antisera against periplakin. One polyclonal was directed against GST-c-ppl and the other against a C-terminal peptide of periplakin coupled to KLH. Both types of antiserum recognized GST-c-ppl (Fig. 2A), immunoprecipitated transfected myc-c-ppl (Fig. 2B) and specifically recognized a protein with the expected approximate molecular weight of periplakin (~200 kDa) in MCF-7 cells (Fig. 2C). Thus we conclude that these antisera indeed recognize endogenous periplakin. To determine which cell line(s) was suitable for analysing binding between endogenous PKB and periplakin, we analysed several cell lines for periplakin expression and observed highest expression in cells of (neuro)epithelial origin (Fig. 2D and data not shown), in keeping with the expression observed for several other members of the plakin family. Therefore, we analysed in MCF-7 cells whether endogenous interaction between periplakin and PKB occurs and we could indeed observe

co-immunoprecipitation between endogenous PKB and periplakin, after immunoprecipitating for PKB and analyzing for periplakin (Fig. 2E). Thus from these results we conclude that PKB and periplakin can indeed interact in vivo in cells.

Binding of periplakin to PKB can influence PKB function in several ways. To examine the consequence of periplakin binding on PKB activation, myc-c-ppl and HA-PKB were co-expressed in A14 cells. A14 cells are NIH3T3 cells overexpressing the human insulin receptor and treatment of these cells with insulin leads to a rapid and strong increase in PKB activity (Burgering and Coffer, 1995). However, overexpression of myc-c-ppl did not affect activation of HA-PKB by insulin (Fig. 2F). This strongly suggests that binding of periplakin does not interfere in growth-factor-induced activation of PKB.

Binding interface of PKB with periplakin

Having established that PKB and periplakin interact, but that this interaction does not affect activation of PKB by growth factors, we next wanted to investigate in more detail the binding of PKB to periplakin. Therefore we analysed binding of a series of HA-PKB mutants to periplakin. HA-PKB mutants were co-transfected with myc-c-ppl, immunoprecipitated and binding was determined by immunoblotting. In keeping with the performed yeast two-hybrid, in which part of the PH domain was used as a bait, the binding site within full length PKB could be defined to a short stretch of nine amino acids within the N-terminal part of the PH domain (Fig. 3A,B), as deletion mutants encompassing this region (Δ30-130 and Δ30-214), and the mutant Δ9 no longer bound to myc-c-ppl. The results obtained by immunoblot analysis were confirmed by analyzing binding in the yeast two-hybrid system. The structure of PH domains present within a variety of different proteins has been determined and consists of seven β-sheets followed by one α-helix. The presence of the β-sheets and α-helix in the PH domain of PKB is indicated in Fig. 3C and shows that the Δ9 mutant lacks the last part of the 5th flexible loop and most of the 6th β-sheet. Therefore, this deletion is expected to have an effect on the 3D structure of the PH domain. To restore at least the spacing of the amino acid residues within the pH domain, we added back to the Δ9 mutant a stretch of nine glycine residues. However, this did not restore binding to myc-c-ppl. To see whether any distortion of the PH domain structure would result in loss of myc-c-ppl binding we also analysed binding of myc-c-ppl to the W99A mutant of HA-PKB. In this mutant the residue (W99) conserved in all known PH domains was mutated and this is likely to result in a structural change of the PH domain, as W99A is catalytically inactive (data not shown). Nevertheless, myc-c-ppl did bind to this mutant, suggesting that the loss of myc-c-ppl binding to the Δ9 mutant is not necessarily due to a conformational change of the PH domain, but that myc-c-ppl probably binds to the 5th flexible loop.

Periplakin binds the intermediate filament vimentin

As periplakin expression does not interfere in growth-factor-induced PKB activation, we started to address the possibility that binding between periplakin and PKB may serve as a localization signal for PKB. Therefore, we first studied the cellular localization of periplakin in detail. All members of the plakin family have been shown to bind cytoskeletal proteins. Thus, to investigate which cytoskeletal proteins periplakin binds to, we performed a yeast two-hybrid analysis with the C-terminal part of periplakin on a 13.5 day mouse embryo cDNA library. This way we identified the intermediate filament protein vimentin as a potential binding partner of periplakin. To further establish periplakin binding to vimentin we co-expressed myc-tagged c-ppl with HA-vimentin in COS-7 cells and observed co-immunoprecipitation when precipitating myc-c-ppl in a stringent buffer and analysing by immunoblotting for

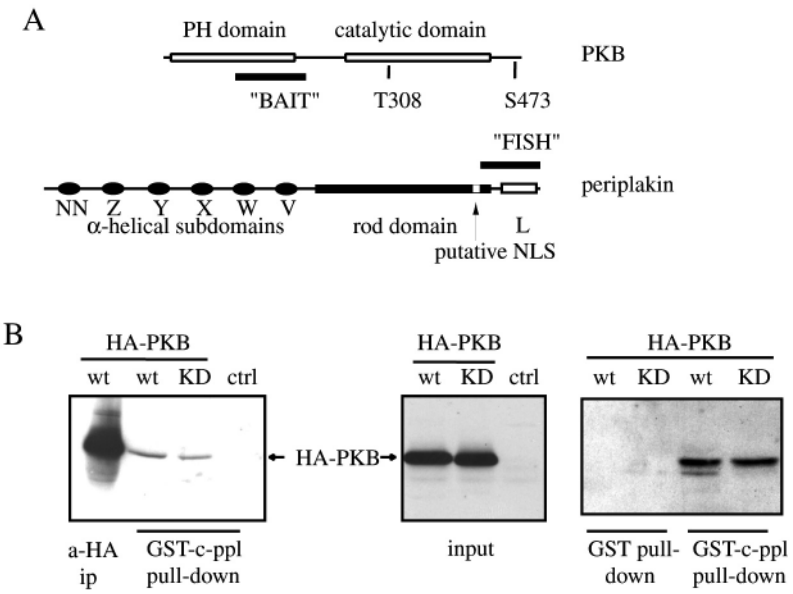

Fig. 1. PKB binds periplakin. (A) The yeast two-hybrid fragments of PKB (fish) and periplakin (bait). Periplakin consists of six α-helical subdomains (aa 16-920), a rod domain (aa 945-1554) and a linker domain (L; aa 1556-1756). (B) GST pull-down assay with GST-c-ppl. A14 cells were transiently transfected with either wild-type or kinase-dead HA-tagged PKB. Lysates were incubated for 1 hour with GST-c-ppl. Samples were analysed for the presence of PKB by western blotting using the anti-HA antibody. A direct immunoprecipitation of HA-PKB using anti-HA was used as a control. The experiment was also performed with either GST or GST-c-ppl (right panel). (C) COS-7 cells were transiently co-transfected with myc-tagged c-ppl and either HA-tagged PKB or p70S6K. Co-immunoprecipitations were performed with anti-HA and anti-myc antibodies and immunocomplexes were resolved with SDS-PAGE and immunoblotted with anti-myc and anti-HA antibodies, respectively.

the presence of vimentin (Fig. 4A). For reasons that are not entirely clear to us, we could only very faintly observe co-precipitation when precipitating vimentin and analysing for the presence of myc-c-ppl. Therefore, we also analysed co-localization by immunofluorescence in Rat-1 cells. In these experiments a clear co-localization is observed between myc-c-ppl and the endogenous vimentin network (Fig. 4B). A fractionation on A14 cells transiently transfected with myc-c-ppl was performed in which the vimentin network was solubilised and purified. Fractions were analysed for actin, vimentin and myc-c-ppl, which was found to be present in the vimentin fraction (Fig. 4C). Thus from these experiments we conclude that periplakin binds both PKB and vimentin through its C-terminus and is colocalised with vimentin. To determine whether PKB and vimentin bind to different or overlapping sites within the C-terminal part of periplakin we co-expressed, both in cells and in the yeast two-hybrid system, deletion mutants of the C-terminal part of periplakin together with either vimentin or PKB. However, in both cases we were unable to identify within periplakin a small peptide sequence responsible for vimentin or PKB binding. Although some difference was observed between PKB and vimentin binding (Fig. 4D) we could not establish whether PKB and vimentin bind different parts of periplakin, or whether binding of PKB and vimentin is mutually exclusive.

Periplakin localizes to different cellular compartments

In tumor cell development, loss of epithelial morphology and acquisition of mesenchymal characteristics is often correlated with increased expression of vimentin (Dandachi et al., 2001). As PKB function is also implicated in tumor development, we were interested to know where periplakin would localize in cells that lack vimentin expression and if differential localization, due to presence or absence of vimentin, may influence PKB function. In contrast to many other breast cancer cell lines, the breast epithelial carcinoma cell line MCF-7 expresses little or no vimentin (Stover et al., 1994; van de Klundert et al., 1992) but high levels of periplakin (Fig. 2), and therefore this cell line was used for confocal microscopy using the periplakin antibodies developed. Endogenous periplakin was shown to be co-localizing with the cell membrane, nucleus

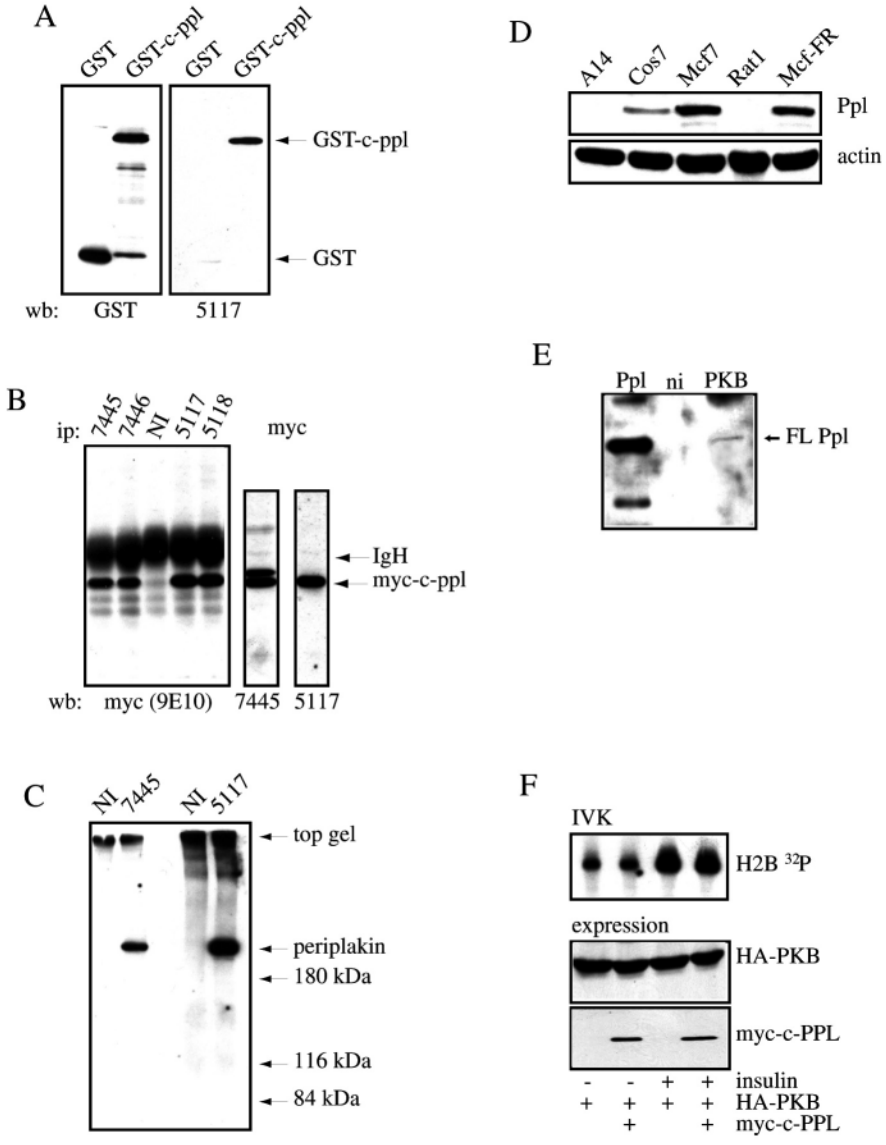

Fig. 2. Co-immunoprecipitation of endogenous PKB and periplakin. (A) Characterization of generated anti-periplakin antibodies. Purified GST and GST-c-ppl were separated by SDS-PAGE and immunoblotted with anti-GST or anti-GST-c-ppl (5117). (B) Cell lysates of COS-7 cells transfected with myc-c-ppl were either immunoprecipitated with anti-GST-c-ppl (5117 and 5118), anti-periplakin peptide serum (7445 and 7446; left panel), or anti-myc (9E10; two right panels). Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-myc (9E10; right panel), anti-periplakin peptide serum (7445) and anti-GST-c-ppl (5117; two left panels). (C) MCF-7 cells were lysed and periplakin was immunoprecipitated with anti-GST-c-ppl or anti-peptide serum. (NI) is an immunoprecipitation with non-immune serum as a control. Immunoprecipitates were separated by SDS-PAGE and immunoblotted for the presence of periplakin with 5117. (D) Epithelial cells express periplakin. Various cell lines were lysed and analyzed by western blotting using the anti-periplakin 5117 antibody. (E) Periplakin interacts with PKB endogenously. Periplakin and PKB were immunoprecipitated from MCF-7 cells using the anti-periplakin 5117 antibody and anti-PKB antibody, respectively, and the immune complex was resolved by SDS-PAGE and immunoblotted with the anti-periplakin 5117 antibody. Non-immune anti-serum was used as a negative control. (F) c-ppl expression does not affect insulin-induced PKB activation. A14 cells were transfected with HA-PKB either in the presence or absence of myc-c-ppl. After 36 hours cells were either treated with insulin for 7 minutes or left untreated. HA-PKB was immunoprecipitated and analysed for kinase activity as described (Burgering and Coffer, 1995).

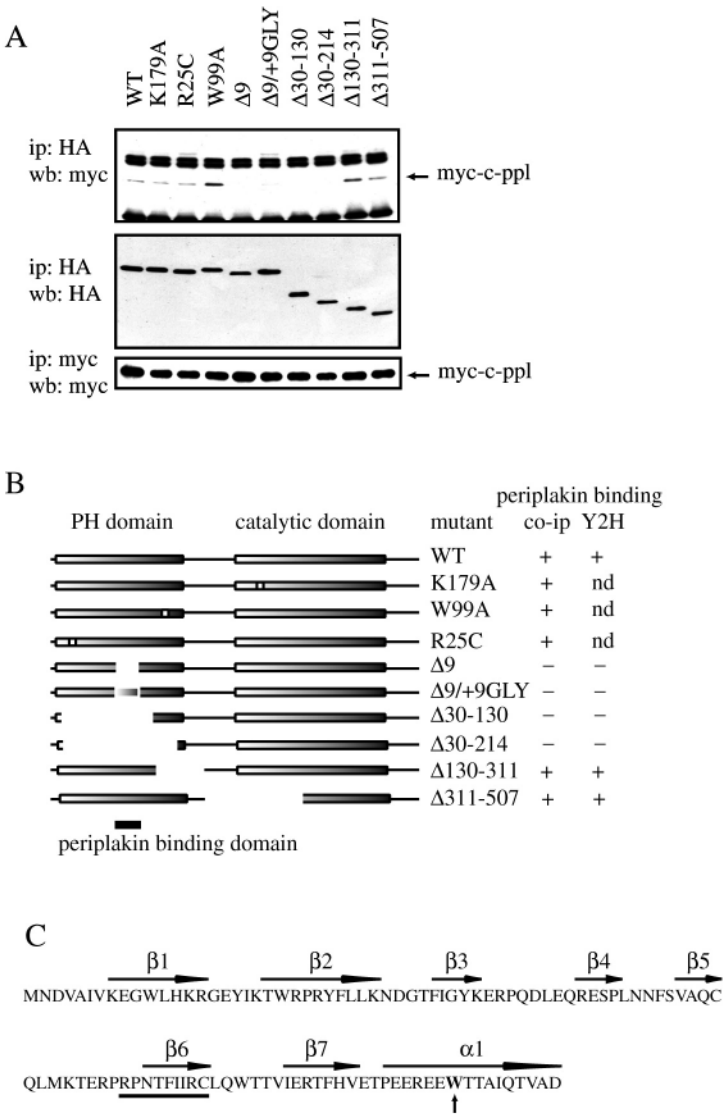

Fig. 3. Determination of the binding site of PKB to periplakin. (A) COS-7 cells were transiently transfected with myc-tagged c-ppl in combination with HA-tagged PKB constructs as indicated. HA-PKB was immunoprecipitated with anti-HA and analysed by western blotting for myc-c-ppl binding by immunoblotting with anti-myc (9E10) (upper panel). Expression of HA-PKB constructs and myc-c-ppl was checked by immunoblotting with anti-HA and anti-myc, respectively. (B) The outcome of the interaction studies by co-immunoprecipitation and yeast two-hybrid analysis. nd, not done. (C) Primary structure of the PH domain of PKB. The positions of the β-sheets and α-helix are indicated by arrows on top and were determined by sequence alignment with other PH domains (see also Ferguson et al., 2000). The nine amino acid deletion that results in loss of periplakin binding is indicated by a black line below the sequence. The conserved W99 residue is depicted in bold and indicated by an arrow.

and mitochondria. The latter was demonstrated by co-staining with a mitochondrial marker (Mitotracker). Also transfected HA-tagged periplakin was shown to be co-localizing with a mitochondrial marker (cytochrome c) in MCF-7 cells (Fig. 5A). Mitochondrial localization of periplakin was confirmed by biochemical fractionation methods. First, various cellular fractions including a mitochondrial fraction were isolated from MCF-7, COS-7 and Rat-1 cells by fractionation on a Percoll gradient (Fig. 5B). In a second approach we used sorting by magnetic beads (MACS) with a ferro-conjugated antibody (MAB1273, see Materials and Methods) that recognizes a 65-kDa mitochondrial membrane protein (Herrnstadt et al., 1999). This method allows rapid single step purification of organelles (Fig. 5C). Fractionation was monitored in both cases by using COX4 as a mitochondrial marker and MAPK as a non-mitochondrial marker. Both approaches revealed the presence of periplakin in the mitochondrial fraction, confirming the immunofluorescent data. Interestingly, we also demonstrated mitochondrial localization of periplakin in COS-7 cells, albeit that the fraction of periplakin localized to mitochondria appeared less compared with MCF-7. This observation suggests that in vimentin-containing cells, such as COS-7, periplakin is bound to vimentin and may function to recruit mitochondria to intermediate filament structures whereas, in non-vimentin-containing cells, periplakin appears to localize more clearly to mitochondria owing to the absence of clear filamentous staining.

Periplakin expression can affect PKB-signalling by sequestration

Since periplakin binds to PKB but does not interfere with activation of PKB and localizes to specific cellular compartments, we wanted to test whether periplakin expression may affect the ability of PKB to generate specific signalling outputs. Recently, we and others have shown that phosphorylation and inactivation of FOXO transcription factors by PKB occurs within the nucleus (Brownawell et al., 2001; Brunet et al., 2002). Consequently, inhibition of PKB nuclear transport should result in loss of FOXO regulation by PKB. To test this possibility we made use of the observation that in vimentin-expressing cells ectopically expressed myc-c-ppl localizes to intermediate filament structures, whereas little or no expression in the nucleus is observed (Fig. 4B). As expected and reported previously, co-expression of PKB resulted in inhibition of FOXO4 (AFX)-dependent transcription (Fig. 6A). Interestingly both full length and myc-c-ppl expression enhanced transcription by FOXO4. That this is likely to be due to sequestration of PKB within the cytosol is indicated by a lack of effect of myc-c-ppl expression on insulin-induced PKB activity (Fig. 2F) and a decrease of phosphorylation of the PKB site S193 of FOXO4 (Fig. 6B). Thus these results suggest that periplakin can indeed act as a scaffold, as it can modulate PKB-dependent signalling outputs without interfering with PKB activation itself.

Discussion

PKB is a major player in various essential growth factor regulated cellular processes (Bevan, 2001; Brazil and Hemmings, 2001) and tumorigenesis (Kops and Burgering, 1999; Tsatsanis and Spandidos, 2000). To better understand how PKB exerts its function in these processes we performed a yeast two-hybrid screen to find PKB-interacting proteins.

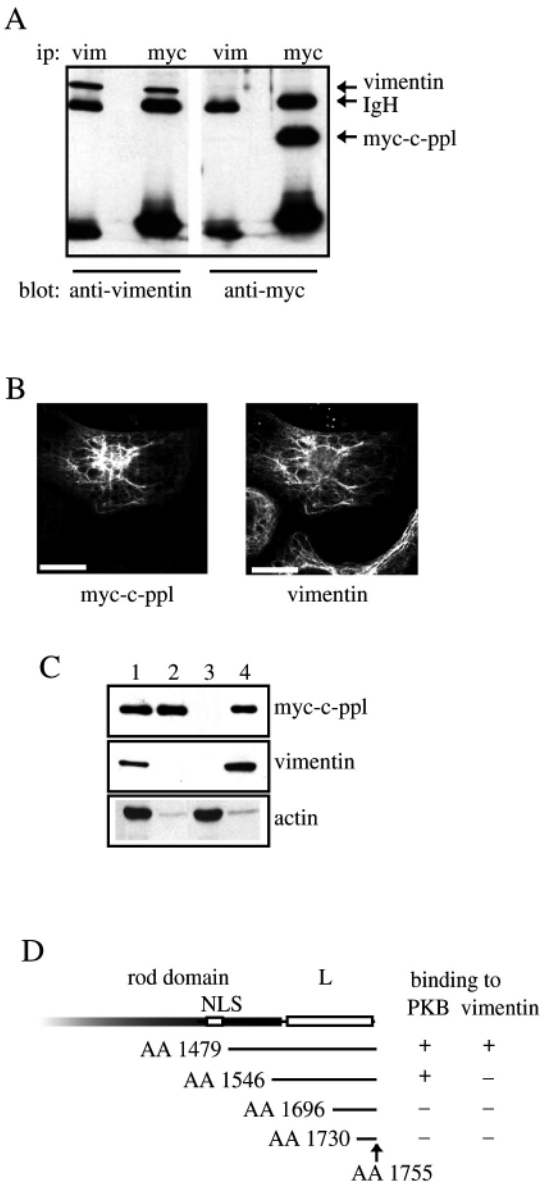

Fig. 4. Periplakin binds to the intermediate filament vimentin. (A) Periplakin interacts with vimentin. COS-7 cells were transiently transfected with myc-tagged c-ppl and co-immunoprecipitations were performed in PKB buffer using anti-myc and anti-vimentin antibodies. Samples were analysed by western blotting using either the anti-myc (anti-myc 9E10) or anti-vimentin (anti-vimentin, Oncogene Science) antibody. (B) Periplakin colocalises with vimentin. Rat-1 cells were transiently transfected with myc-tagged c-ppl and immunostained for myc-c-ppl and endogenous vimentin. Bars, 10 μm. (C) Myc-c-ppl is present in vimentin fractions. A14 cells were transiently transfected with myc-tagged c-ppl and fractionated using a KI-protocol. The fractions were immunostained for vimentin, actin and myc-c-ppl. Fraction 1, total; 2, cytosolic and membrane; 3, actin; and 4, vimentin. (D) Comparison of C-terminal deletion mutants and their binding to PKB and vimentin. The C-terminal part of periplakin is shown. Binding was determined by co-immunoprecipitation and yeast two-hybrid analysis.

Here we demonstrate that PKB binds to periplakin, a member of the plakin family, and we provide evidence that periplakin may function as a localization signal for PKB to bind to its correct cellular compartments.

PKB binding to periplakin could be demonstrated by yeast two-hybrid analysis as well as co-immunoprecipitations of ectopically and endogenously expressed proteins. The binding region within PKB that is responsible for the interaction with periplakin was narrowed down to a small region in the C-terminal part of the PH domain (Fig. 3B). Alignment of the PH domain of PKB with PH domains of which the 3D structure has been resolved indicates that binding to periplakin is most likely mediated by the 5th flexible loop of the PH domain. (Fig. 3C). Although it remains possible that binding to the Δ9 mutant is lost owing to general distortion of the 3D structure of the PH domain, we consider this unlikely for several reasons. First, restoring at least the spacing by replacing the deleted amino acids by glycine residues (Δ9-gly9) does not restore binding. Second, as one can still argue that also Δ9-gly9 is no longer properly folded, the presence of the binding site within a flexible loop would at least suggest that the primary sequence rather than its folding determines binding. In keeping with the latter conclusion would be the observations that the yeast two-hybrid interaction was isolated with a truncated PH domain construct and that myc-c-ppl still binds to the W99A mutant. With respect to this mutant it should then be noted that this tryptophane residue is likely to be an essential structural determinant for PH domains, since between all known PH domains this residue is the only one that is actually conserved (Musacchio et al., 1993). Indeed the W99A mutation results in a catalytically inactive mutant (not shown) and on SDS-PAGE this mutant shows aberrant migration behavior suggesting that this mutation distorts PH domain structure but does not affect binding to myc-c-ppl.

Finally, as the residues involved in binding PtdIns3*P* lipids are primarily located within the first half of the PH domain, binding of periplakin to the 5th flexible loop would be expected to have little effect on PtdIns3*P* binding. Although not measured directly, the fact that we do not observe inhibition of insulin-induced activation of HA-PKB when co-expressing myc-c-ppl (Fig. 2F) corroborates this suggestion.

Plakin family members have been shown to bind or colocalize with intermediate filaments (Ruhrberg et al., 1997; DiColandrea et al., 2000) and indeed we also found in our yeast two-hybrid screen that c-ppl binds to the intermediate filament vimentin. This interaction was confirmed by co-immunoprecipitation and co-localization studies. The binding to and colocalisation with vimentin in fibroblasts is in agreement with the observations of DiColandrea et al., who also observe periplakin colocalisation with vimentin in COS-7 cells. In addition, they observe in keratinocytes that full-length and the C-terminal part of periplakin partially colocalise with keratin filaments (DiColandrea et al., 2000). Thus, it appears that, depending on the cell type, periplakin may localize to different intermediate filament networks. Intermediate filaments are considered to function as scaffolding that structures the cytoplasm and resists extracellular stresses (Fuchs and Cleveland, 1998). The function of the binding of periplakin to vimentin is unclear and needs to be further investigated, but we hypothesize that periplakin, through binding to vimentin, correctly localizes PKB and other proteins within the cell. Recently it was

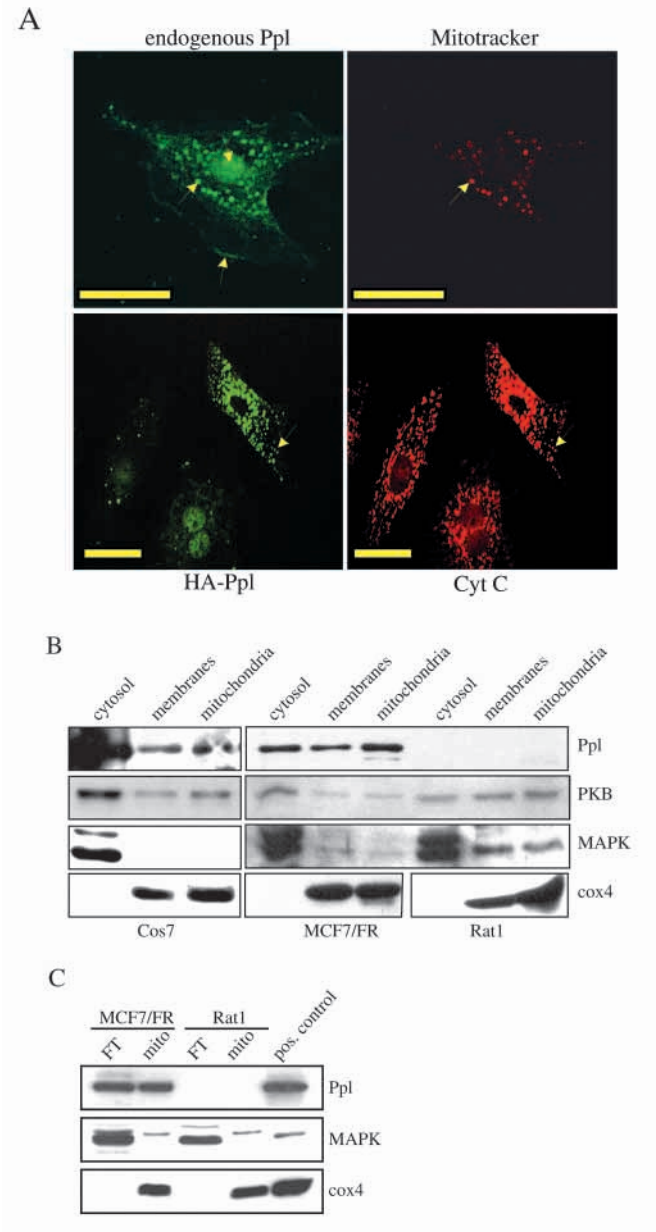

Fig. 5. Periplakin colocalises with mitochondria. (A) Confocal immunostaining for periplakin. MCF-7 cells were pre-incubated with Mitotracker and co-immunostained for periplakin using the anti-periplakin 5117 antibody (upper panel). Similar staining was observed with the other antibodies (5118, 7445 and 7446) and with affinity-purified 5117. No staining was observed using pre-immune serum from the rabbits (5117 and 5118) used for immunization with GST-c-ppl. MCF-7 cells were transiently transfected with HA-Ppl and immunostained for HA-Ppl using the HA antibody and for cytochrome c (lower panel). Bars, 10 μm. (B/C) Periplakin is found in mitochondrial fractions. (B) COS-7, MCF-7/FR and Rat-1 cells were fractionated using a Percoll gradient and the fractions were analysed by western blotting using anti-periplakin 5117, anti-PKB, anti-cox4 and anti-MAPK antibodies. (C) MCF-7/FR and Rat-1 cells were fractionated using the mitochondria affinity purification protocol. Fractions were analysed by immunoblotting, using the same antibodies as in B.

reported that PKB could interact with another intermediate filament, keratin K-10, and that K-10 binding results in PKB inhibition and keratin-induced cell cycle arrest (Paramio et al., 2001). As this study did not identify the nature of the interaction between K-10 and PKB, our results indicate the possibility that periplakin functions to bridge this interaction. Also, our observation that periplakin expression enhances FOXO4 transcriptional activity is consistent with the role of intermediate filaments in inducing cell cycle arrest as proposed by Paramio et al. (Paramio et al., 2001). Previously we have shown that FOXO transcription factors can cause cell cycle arrest in a p27kip-dependent manner (Medema et al., 2000). Since activated PKB translocates to the nucleus, where it inactivates FOXO transcription factors, sequestering PKB within the cytosolic compartment through binding to periplakin/intermediate filaments would enhance FOXO transcriptional activity and consequently stimulate cell cycle arrest.

In many cell types and under many conditions PKB signaling has been shown crucial for providing cellular protection against apoptosis. Apoptosis is often initiated by, or requires mitochondrial damage and leakage of, cytochrome C (Adrain and Martin, 2001). Recent reports have shown that PKB signalling can maintain mitochondrial membrane stability under conditions of stress (Gottlob et al., 2001; Kennedy et al., 1999; Plas et al., 2001). Therefore, the clear colocalisation of periplakin with mitochondria combined with its interaction with PKB suggests a possible role for periplakin in mediating PKB-dependent protection. In keeping with such a model are reports that vimentin is a substrate for caspase-9 and is cleaved at an early stage within the apoptosis process (Byun et al., 2001; Nakanishi et al., 2001) and that caspase-resistant vimentin delays apoptosis (Belichenko et al., 2001). Also, plectin is a substrate for caspase-8 and is cleaved early in apoptosis (Stegh et al., 2000). This caspase cleavage site of plectin is conserved in periplakin. However attractive, we have not been able to obtain clear evidence to support a role for periplakin in PKB-mediated apoptosis protection. This could be due to, for instance, redundancy in apoptosis protection signalling. Many cell types do not, or only slightly, express periplakin and still depend on PKB-mediated protection. Interestingly in this respect, plectin, a family member of periplakin, has been shown to associate with mitochondria as well (Reipert et al., 1999). It is possible that plectin and periplakin may act redundant in PKB-dependent protection since plectin has been shown to have a ubiquitous expression pattern (Wiche et al., 1983). Further, our observation that PKB localization to mitochondria, as determined by biochemical fractionations, appears independently of periplakin and/or vimentin expression indicates either redundant or alternative means of PKB localization to this compartment. However, these biochemical fractionations do not exclude the possibility that the presence of PKB is due to contamination of this fraction. Unfortunately, with respect to PKB localization, we could not complement the biochemical fractionation data with immunofluorescence studies, as the quality of all different PKB antibodies we tested thus far precluded this.

In addition to acting as a localization signal, periplakin may function as a shuttle for delivery of PKB to the various cellular compartments. This possibility derives primarily from the observation that periplakin localizes to all sites where PKB is

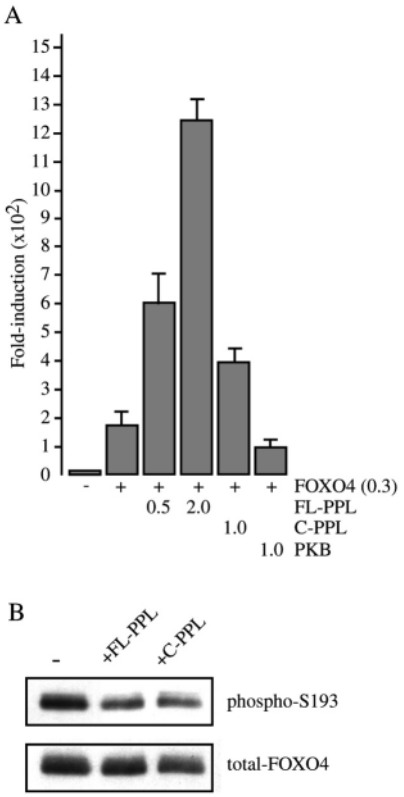

Fig. 6. Periplakin expression modulates PKB-dependent FOXO4 regulation. (A) 293T cells were transfected with a TK-renilla-luciferase (internal control) and 6xDBE luciferase reporter construct (Medema et al., 2000), in the absence or presence of FOXO4. Different amounts (μg) of full-length periplakin, myc-c-ppl or HA-PKB were cotransfected with FOXO4. 36 hours after transfection lysates were prepared and luciferase counts were measured and normalized against renilla luciferase counts. 6xDBE activity in the absence of FOXO4 was set at 1. Results represent the averages of three independent experiments. (B) Periplakin expression reduces PKB-dependent FOXO4 phosphorylation. 293T cells were transfected with the 0.5 μg full-length periplakin or 1.0 μg c-ppl and 1 μg HA-FOXO4. HA-FOXO4 was immunoprecipitated and the level of PKB-dependent phosphorylation was determined by immunoblotting using a phospho-specific antibody against pSer193.

either observed or suspected to localize. Periplakin localizes to the plasma membrane and PKB becomes activated at the plasma membrane by PI 3-kinase-mediated production of phosphoinositol lipids (Bos, 1995; Burgering and Coffer, 1995). Following activation, PKB should be able to translocate to other cellular compartments such as the nucleus and the vicinity of mitochondria, since substrates for PKB present within these cellular domains have been identified. For example, a fraction of PKB has been shown to translocate to the nucleus (Andjelkovic et al., 1997; Brownawell et al., 2001). Within the nucleus PKB is thought to phosphorylate substrates such as the FOXO family of transcription factors. We observe nuclear localization of periplakin which, along with other members of the plakin family, contains a putative bipartite nuclear localization (NLS). As other reports suggested that this NLS sequence in other plakins [e.g. plectin (Nikolic et al., 1996)] is also a part of the intermediate filament-binding region, it could be that (induced) loss of intermediate filament binding unmasks the NLS and results in nuclear translocation. These possibilities are currently under investigation. Finally, although our experiments only start to delineate such a possibility, it is interesting to note that such a function for periplakin and possibly plakins in general is very similar to the function of other large cytolinkers such as APC.

In conclusion, this study shows binding of periplakin to PKB and vimentin. The differential cellular localization of periplakin and its ability to affect PKB signalling when targeted to a specific localization suggest a role in determining cell-type-specific signalling by the PI3K/PKB pathway.

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