# β1-Integrin and PTEN control the phosphorylation of protein kinase C

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Phosphorylation of protein kinase C (PKC) provides an amplitude control that operates in conjunction with allosteric effectors. Under many conditions, PKC isotypes appear to be highly phosphorylated; however, the cellular inputs that maintain these phosphorylations are not characterized. In the present work, it is shown that there is a differential phosphorylation of PKCδ in adherent versus suspension cultures of transfected HEK-293 cells. It is established that integrin activation is sufficient to trigger PKCδ phosphorylation and that this signals through phosphoinositide 3-kinase (PI3-kinase) to stimulate the phosphorylation of two sites, T505 and S662. The loss of signal input to PKCδ in suspension culture is dependent on the tumour suppressor gene *PTEN*, which encodes a bi-functional phosphotyrosine/phosphoinositide 3-phosphate phosphatase. In the $PTEN^{-/-}$ UM-UC-3 bladder carcinoma cell line grown in suspension, transfected PKCδ no longer accumulates in a dephospho-form on serum removal. By contrast, in a UM-UC-3-derivative cell line stably expressing *PTEN*, PKCδ does become dephosphorylated under these conditions. Employing the PTEN $Gly^{129} \rightarrow Glu$ mutant, which is selectively defective in lipid phosphatase activity, it was established that it is the lipid phosphatase activity that controls PKCδ phosphorylation. The evidence indicates that PKCδ phosphorylation and its latent activity are maintained in serum-deprived adherent cultures through integrin–matrix interactions. This control acts through a pathway involving a lipid product of PI3-kinase in a manner that can be suppressed by PTEN.

Key words: adherence, phosphatidylinositol 3-kinase, phosphoinositide phosphatase, tumour suppressor.

## INTRODUCTION

The distinctions between the behaviour of cells in cell–cell and cell–matrix contact with those in isolation or suspension are profound. This is due, in part, to the permissive effects that integrin engagement can have upon growth factor signalling; for example, the ability of platelet-derived growth factor (PDGF) and fibroblast growth factor to stimulate proliferation can be determined by integrin–substrate interactions [1]. The nature of these integrin outputs and the relationship of such tonic signals to the acute ones observed on, for example, cells newly spreading on matrix [2], are not defined. Nevertheless, such tonic signals play a central role in the survival and responses of cells.

Protein kinase C (PKC) isotypes are effector-dependent protein kinases, which recently have been shown to require a series of phosphorylations within their catalytic domains in order to maintain optimum catalytic potential (for a review see [3]). One of these phosphorylation sites is located within a conserved motif (…TFCGT…) in the activation loops of the kinases and two are within the C-terminal variable regions (V5 domains); the more C-terminal of these is also in a conserved motif (…FXXFS/TY…). For the classical PKCs (cPKC), which are the best characterized, the activation-loop phosphorylations play a critical role in determining specific activity, whereas the C-terminal sites variably contribute to catalytic activity, stability and to the maintenance of a closed conformation, protein-phosphatase resistant state (for a discussion see [3]). Less detailed evidence indicates that similar properties are conferred by phosphorylation of novel PKC (nPKC) isotypes.

The pathways involved in PKC phosphorylation remain incompletely characterized; however, there is evidence for the involvement of the phosphoinositide 3-kinase (PI3-kinase)/phosphoinositide-dependent kinase-1 (PDK1) pathway in activation-loop phosphorylation [4–6]. Studies with nPKCs indicate that the hydrophobic site at the C-terminus is phosphorylated by an atypical PKC (aPKC) complex [7], but for cPKCs the evidence is for autophosphorylation at this site [8]. These observations have been made possible through the manipulation of culture conditions so that PKC isotypes accumulate in poorly phosphorylated states; upon stimulation with serum, phosphorylation increases. The involvement of signalling pathways in these acutely-induced serum responses has been established through combinations of pharmacology and manipulation of expression. However, under many cell-culture conditions the cPKC and nPKC isotypes remain in a highly phosphorylated state, so that, on isolation, they display a robust effector-dependent activity. The tonic signals involved in maintaining phosphorylated PKC under these conditions have not been elucidated.

In the present study, investigation of the optimum conditions for monitoring nPKC phosphorylation indicated that culture of cells in suspension resulted in a very low degree of basal nPKC phosphorylation. It is shown that this is a function of integrin disengagement and that the suppression of signals is under the control of the PtdIns-3-phosphatase activity of the tumour

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, foetal-calf serum; G129E, $Gly^{129} \rightarrow Glu$-substituted protein; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PDK1, phosphoinositide-dependent kinase-1; PI3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B (c-akt); PKC, protein kinase C; aPKC, atypical PKC; cPKC, classical PKC; nPKC, novel PKC.

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suppressor gene *PTEN*. Integrin activation is sufficient to trigger nPKC phosphorylation in a PI3-kinase dependent manner, and chronic inhibition of PI3-kinase in adherent cultures is sufficient to lead to the accumulation of dephosphorylated inactive nPKCs. The results demonstrate that PKC phosphorylation is capable of contributing to the permissive action of integrins through their cumulative phosphorylation in a process that is only slowly reversible.

## EXPERIMENTAL

### Cell culture and transfection

Cell culture and transfection procedures for HEK-293 cells were as described previously [9]. Wild-type UM-UC-3 cells or the UM-UC-3 PTEN-stable transfectants were grown on a 90-mm dish to approx. 70 % confluence in 10 % (v/v) foetal-calf serum (FCS) in Dulbecco's modified Eagle's medium (DMEM) in a humidified 10 % $CO_2$ incubator at 37 °C. The UM-UC-3 PTEN-stable transfectants were grown additionally in the presence of 400 $\mu$g/ml of G418 (neomycin). For transfections, the medium was removed from the dish and replaced with 5 ml of Optimem (Gibco BRL), and then left for 30 min in a humidified 10 % $CO_2$ incubator at 37 °C. For each transfection, 600 $\mu$l of Optimem was mixed with 18 $\mu$l of FuGene reagent (Stratagene) and left for 20 min. Following this period, 20 $\mu$g of DNA was added and the mixture was left for a further 10 min before it was added to the cells. The cells were then left in a humidified 10 % $CO_2$ incubator at 37 °C for 16 h. At the end of this time, the medium was replaced with 10 % FCS in DMEM and incubation was continued for 24 h. The cells were then either treated with trypsin and maintained in suspension in DMEM without serum or maintained as adherent cells in the presence or absence of serum or inhibitors, as indicated for the individual experiments and in the Figure legends.

For comparison of wild-type PTEN and the PTEN $Gly^{129} \rightarrow$ Glu (G129E) mutant defective in lipid phosphatase activity (for a review see [10]), expression constructs for the PTEN were co-transfected with PKC$\delta$ at a plasmid ratio of 3:1. Cells were cultured and treated as indicated above and detailed below and in the Figure legends.

### Activation of integrin signalling

Integrin signalling was stimulated through the use of specific substrates and through the cross-linking of selective monoclonal antisera. Poly-D-lysine (Sigma) and collagen (Sigma) were used as non-specific and specific matrices respectively. Stock solutions of 50 $\mu$g/ml in 3 ml of PBS, were added to 20-mm dishes and left overnight at 4 °C. The medium was then removed, the plate was gently washed with PBS and 2 ml of a suspension of approx. $1 \times 10^6$ cell/ml were added. At the times indicated, the cells were treated and then harvested by centrifugation at 10000 ***g*** for 1 min (benchtop microfuge) in 4 × SDS-sample buffer [1 × SDS-sample buffer = 0.25 M Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol, 1.55 % (w/v) dithiothreitol, 2 M urea, 10 mM EDTA, 1 % (w/v) Bromophenol Blue].

For integrin activation in suspension cultures, cells were serum starved in suspension for 24 h and then 500 $\mu$l of approx. $1 \times 10^6$ cell/ml suspension was incubated with 20 $\mu$g/ml of $\beta$1-integrin antiserum (see Figure legends) at 4 °C for 20 min. The cells were gently washed once with warm DMEM (37 °C), and then incubated with warm DMEM containing 10 $\mu$g/ml of anti-mouse IgG (Fc specific) cross-linking antisera (Sigma) for the times indicated. The cells were then harvested by centrifugation in 1 × SDS-sample buffer and analysed by immunoblotting.

### Growth factor stimulation

An aliquot of a 24 h serum-starved suspension culture was incubated with 10 $\mu$M lysophosphatidic acid (LPA; Sigma), 30 ng/ml recombinant PDGF B-chain homodimer (R&D Systems) or with both together for 30 min. The cells were then harvested in 4 × SDS-sample buffer and analysed for PKC$\delta$ protein and phosphorylation by immunoblotting.

### Generation of PTEN-expressing stable UM-UC-3 cell lines

The enhanced calcium phosphate method of Chen and Okayama [11] was used to transfect the UM-UC-3 cell line. Briefly, the UM-UC-3 cell culture was diluted 12-fold and allowed to grow in DMEM supplemented with 10 % FCS under humid conditions (5 % $CO_2$) at 37 °C for 8 h. At the time of transfection, the cells were approx. 40 % confluent. Supercoiled expression vector (20 $\mu$g/ml) was used in the transfection. The plasmid DNA was mixed with 500 $\mu$l of 0.25 M $CaCl_2$, 500 $\mu$l of 2 × BBS [1 × BBS = 25 mM *N*,*N*,-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid, 140 mM NaCl, 0.75 mM $Na_2HPO_4$, pH 6.95] was added and the mixture was incubated at room temperature for 20 min. After incubation, the DNA mixture was added dropwise to the culture medium. The cells were incubated for 24 h in the calcium phosphate transfection medium (DMEM containing 10 % FCS and the DNA/calcium phosphate solution) and were then washed twice with PBS and cultured in fresh medium (DMEM containing 10 % FCS). After two days, the confluent cell culture was diluted 10-fold and grown in selection medium (DMEM, 10 % FCS, 800 $\mu$g/ml neomycin). The cells were maintained in selection medium for 2 weeks and individual surviving colonies were transferred to 6-well plates. The colonies were grown to approx. 60 % confluence in DMEM containing 10 % FCS and 800 $\mu$g/ml neomycin. The individual clones were treated with trypsin and were cultured, in duplicate, on 6-well plates. Clones were subjected to immunoprecipitation and Western blotting analysis, using the Santa Cruz PTEN (A2B1) mouse monoclonal antibody to confirm expression of PTEN. To facilitate visualization of the PTEN band, the immune complexes were incubated in SDS/PAGE sample buffer at room temperature, which results in the formation of IgG heavy-chain dimers (approx. 100 kDa) and not of IgG heavy chain monomers (approx. 50 kDa) which would mask the PTEN protein. Three clones (1, 2 and 21) were selected for biochemical analysis. The Santa Cruz PTEN antibody (A2B1) was used at a concentration of 1 $\mu$g/ml for immunoprecipitation and 0.2 $\mu$g/ml for Western blotting. The secondary, conjugated anti-mouse-horseradish-peroxidase antibody was used at a concentration of 1:4000 (v/v).

### Phosphorylation and activation of PKC$\delta$

The Ser-(phosphate)657, site-specific polyclonal antiserum was raised against a phosphopeptide FKGFS(P)FVNP, based upon the region flanking the hydrophobic motif in PKC$\delta$. An antiserum for the equivalent site in PKC$\varepsilon$ was raised employing the sequence FKGFS(P)YFGE. Activation-loop phospho-site polyclonal antisera for PKC$\delta$ and PKC$\varepsilon$ [Thr-(phosphate)505 and Thr-(phosphate)566 respectively] were raised by immunizing with the peptides RAST(P)FCGT and TTTT(P)FCGT respectively. All the sera were tested against phosphorylated and dephosphorylated forms of the immunogen. The sera showed some cross-reactivity with the unphosphorylated peptide, therefore all Western-blot analyses were performed in the presence of the cognate dephosphorylated peptide (dephospho-peptide) at 1 $\mu$g/ml; this was sufficient to compete with the dephospho-proteins in the immunoreaction (results not shown). Transfected

cells were lysed in SDS-sample buffer and protein samples were separated by SDS/PAGE [12]. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and analysed for phosphorylation at the sites indicated in the Figures. Western blots were developed using horseradish-peroxidase-coupled donkey anti-rabbit IgG secondary serum (1:5000, v/v) (Amersham Pharmacia Biotech) and ECL® (Amersham Pharmacia Biotech).

### Other methods

Myc-immunoprecipitation of PKC$\delta$ and kinase assays were performed as described previously [9]. Caspase activation was assayed as described previously [13]. FACS analysis was by the method of [14,15].

## RESULTS

### PKC$\delta$ phosphorylation is supported by integrin engagement and PI3-kinase

During investigation of the phosphorylation of the nPKC isotypes, PKC$\delta$ and PKCε, it was noted that serum deprivation of adherent cultures produced a relatively modest reduction in activation-loop and C-terminal hydrophobic site phosphorylation. To define conditions where a more robust accumulation of dephosphorylated nPKC would occur, we tested the response to loss of cell-substrate interaction. As shown in Figure 1(A), serum starvation of suspension cultures of HEK-293 cells transfected with PKC$\delta$ led to an almost complete loss of T505/S662-phosphorylated PKC$\delta$, in contrast with its behaviour in serum-starved adherent cultures. A similar pattern of behaviour was observed for PKCε and its T566/S729 sites (results not shown). Much of the data shown in the present study are for PKC$\delta$ alone; however, similar responses for PKCε were observed, which, for clarity, are not included. Serum stimulation of the suspension cultures of PKC$\delta$ transfected cells induced a substantial increase in T505/S662 phosphorylation, whereas only a minor effect was seen in the adherent cultures. The serum-induced increase in T505/S662 phosphorylation was blocked by the PI3-kinase inhibitor, LY294002, if the inhibitor was added prior to stimulation. Once the sites were occupied, the inhibitor had no acute effect (see below).

The effect of these distinct phosphorylation states on PKC$\delta$ kinase activity was investigated by immunopurification of the protein from transfected HEK-293 cells. In line with previous observations [9], PKC$\delta$ activity mirrored the changes in phosphorylation at these sites. Thus PKC$\delta$ activity was barely detectable in immunocomplexes from serum-starved suspension cultures (Figure 1B). In contrast to adherent cultures, therefore, suspension culture of these cells produced a poorly phosphorylated, low activity form of PKC$\delta$.

To determine whether the effects of suspension culture represented a non-specific consequence of engagement of an apoptotic programme, we monitored commitment to apoptosis under suspension-culture conditions in PKC$\delta$ transfected cells, using a caspase activation assay [13]. After 24 h of suspension culture under serum-free conditions, no difference was observed in the activation of caspase when compared with serum-maintained adherent cultures; similarly, no differences were observed on monitoring cells for a sub-G1 DNA content by FACS (results not shown). Thus the observed effects on PKC$\delta$ were not a (potentially non-specific) consequence of engagement of an apoptotic programme but lay upstream prior to commitment.

In order to focus on the mechanism(s) involved in the suspension-dependent properties of PKC$\delta$, we sought to determine the nature of the parallel response of the PKC$\delta$ T505 and

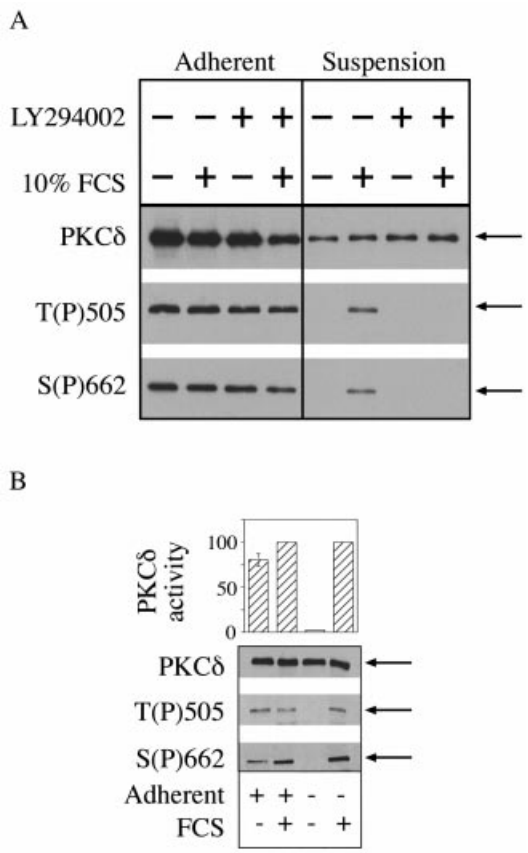


**Figure 1 Effect of adherence on PKC$\delta$ phosphorylation and catalytic activity**

Adherent and suspension cultures of HEK-293 cells, transiently overexpressing myc-tagged PKC$\delta$, were serum starved for 24 h. (**A**) HEK-293 cell cultures were then FCS stimulated for 30 min with or without pre-treatment with 10 $\mu$M LY294002 for 30 min. The proteins were analysed by immunoblotting with antisera raised to the sites indicated on the left. The blot is representative of six or more similar experiments. (**B**) Upper panel: PKC$\delta$ activity of immunoprecipitated myc-tagged PKC$\delta$ from adherent and suspension cultures with or without serum stimulation (lower panel) was determined. The data represents the average and range of two independent experiments, with specific basal activities (−FCS) normalized to the serum-induced (+FCS) activities for each condition.

S662 phosphorylation sites. The data given above suggest that there was either a relationship between the occupancy of these two sites or an independent requirement for some common upstream event, e.g. the accumulation of PtdIns(3,4,5)$P_3$. In order to test any relationship, we employed a PKC$\delta$ T505A mutant. In suspension cultures of HEK-293 cells transfected with this mutant, serum-induced S662 phosphorylation was observed only if the phosphatase inhibitor okadaic acid (or calyculin; results not shown) was included (Figure 2). This S662 response remained sensitive to LY294002, indicating that the control of this phosphorylation retained an independent input from PI3-kinase and was not simply dependent upon phosphorylation of T505 in the activation loop of the protein. For these acute responses, it would appear that dephosphorylation of the PKC$\delta$ S662 site dominates its phosphorylation in the absence of T505 phosphorylation, hence the requirement for the phosphatase inhibitor. This behaviour is consistent with the interrelationships defined for the equivalent sites in PKC$\alpha$ [16]. Thus, although there is a functional relationship between these two

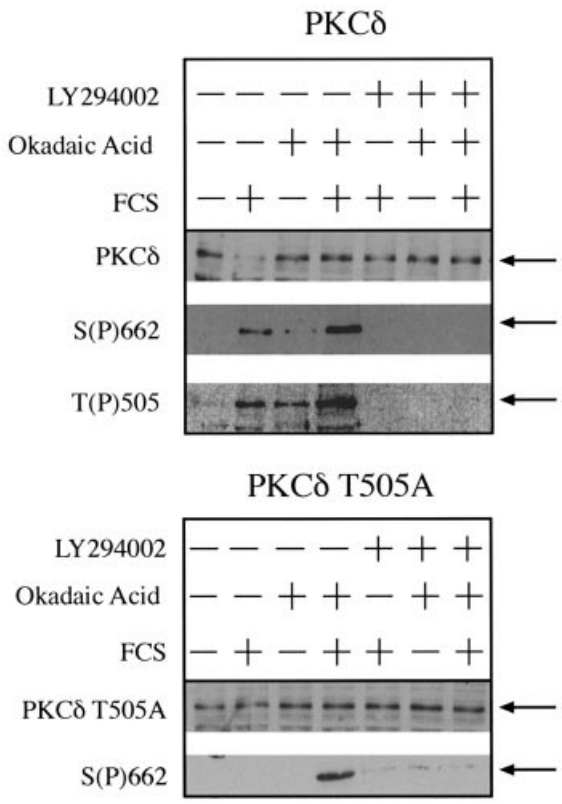


**Figure 2 Phosphorylation of the PKC$\delta$ T505 and S662 sites are independently sensitive to LY294002**

Suspension cultures of HEK-293 cells, transiently overexpressing PKC$\delta$ (upper panel) or the PKC$\delta$ T505A mutant (lower panel), were serum starved for 24 h while in suspension. Cultures were then treated with or without LY294002 for 30 min before stimulation with either FCS, 1 $\mu$M okadaic acid or both together, for a further 30 min. The whole-cell lysates were analysed by immunoblotting for PKC$\delta$ and phosphorylation as indicated on the left. The blot is representative of three independent experiments.

sites, there is also an independent upstream element in the form of an LY294002 sensitive pathway.

The distinctive behaviour of PKC$\delta$ phosphorylation in suspension and adherent cultures suggested that under conditions of serum deprivation, the necessary signals required to maintain phosphorylated PKC$\delta$ in adherent cultures were derived from integrin–substrate interactions. To assess this, we investigated whether integrin engagement and cross-linking at the cell surface of serum-starved suspension cultures of HEK-293 cells induced PKC$\delta$ phosphorylation. The binding and cross-linking of either of two distinct anti-$\beta$1 integrin antisera induced PKC$\delta$ phosphorylation (Figures 3A and 3B). This response showed the same pattern of sensitivity to inhibitors as that observed for a serum-induced response; pre-treatment with rapamycin selectively inhibited S662 phosphorylation, LY294002 blocked both T505 and S662 responses, and bisindolylmaleimide I led to loss of S643 phosphorylation selectively (Figure 3C). Treatment with these reagents following stimulation had no effect; the reduced phosphorylation of S662 shown following LY294002 addition post-treatment was not consistently observed (see below). This pattern of behaviour implies that the proximal controls acting to support acute serum responses are conserved for these integrin-dependent inputs; this would include the dominant influence

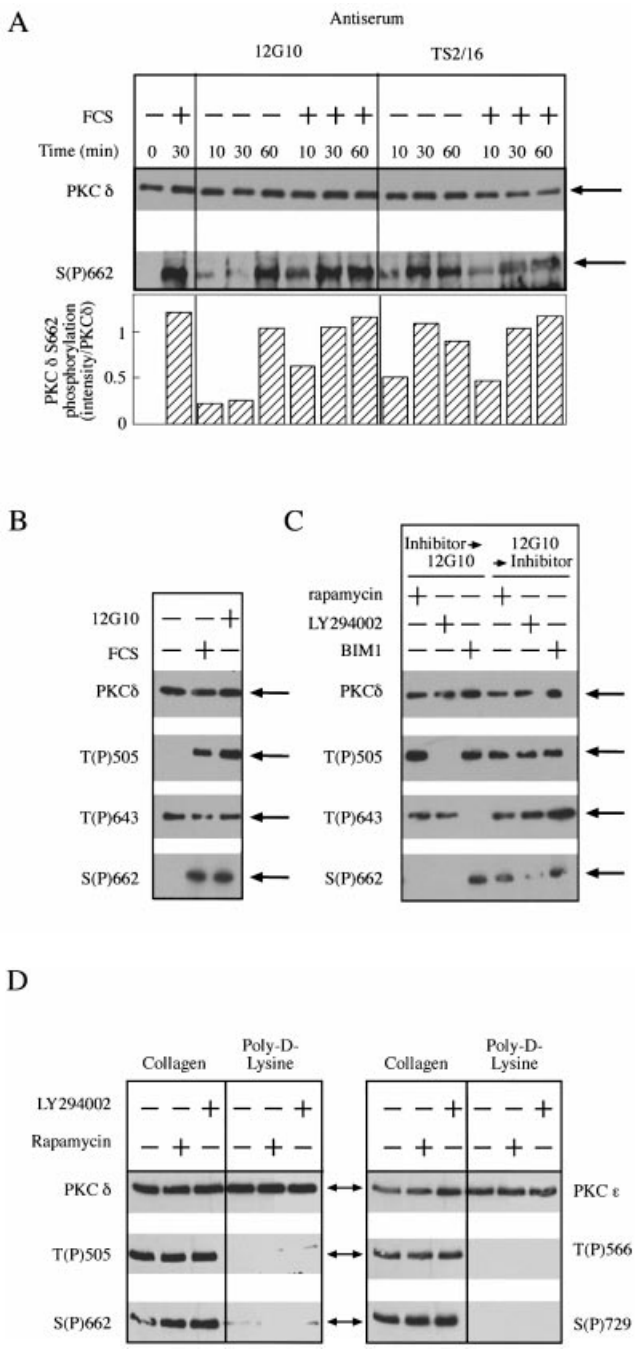


**Figure 3 PKC$\delta$ and PKC$\varepsilon$ phosphorylation following $\beta$1 integrin activation**

(**A**) HEK-293 cells transiently overexpressing PKC$\delta$ were deprived of serum while in suspension for 24 h. Cells were then incubated with 12G10 or TS2/16 anti-$\beta$1-integrin antisera, followed by crosslinking with (+) or without (−) concomitant FCS treatment for the times indicated. The S662 phosphorylation [S(P)662] was monitored by Western blotting and the immunoreactivity was quantified (arbitrary units) and expressed as a function of immunoreactive PKC$\delta$, and is shown as a histogram in the lower panel. The results are representative of three similar experiments demonstrating integrin-induced PKC$\delta$ phosphorylation. (**B**) Cells treated as in (**A**) were stimulated either with FCS or with 12G10 antiserum for 30 min. PKC$\delta$ phosphorylation at all three priming sites [T(P)505, T(P)643 and S(P)662] was monitored by Western blotting. (**C**) Cells transfected and starved as described in (**A**) were treated with 12G10 before or after treatment with 20 nM rapamycin, 10 $\mu$M LY294002 or 10 $\mu$M bisindolylmaleimide I (BIM1) for 30 min. PKC$\delta$ phosphorylation at the three priming sites indicated [T(P)505, T(P)643 and S(P)662] was monitored by Western blotting. (**D**) A serum-starved suspension culture of PKC$\delta$-transfected HEK-293 cells was plated either on to collagen or poly-D-lysine for 1 h. The phosphorylation of PKC$\delta$ was analysed by immunoblotting with antisera raised to the sites indicated (left panel); similar observations were made for PKC$\varepsilon$ (right panel).

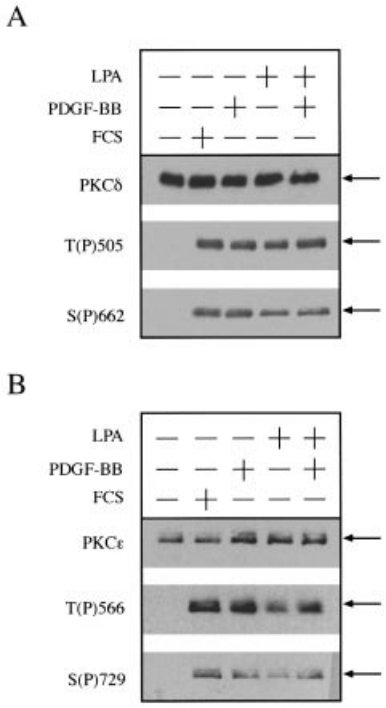


**Figure 4 PKC$\delta$ and PKC$\varepsilon$ phosphorylation following stimulation with purified growth factors**

Suspension cultures of serum-starved HEK-293 cells, transiently transfected with either (**A**) PKC$\delta$ or (**B**) PKC$\varepsilon$, were treated with either FCS, 30 ng/ml PDGF B-chain homodimer (PDGF-BB), 10 $\mu$M LPA, or both PDGF-chain homodimer and LPA together, for 30 min as indicated. The whole-cell lysates were analysed for PKC content and phosphorylation by immunoblotting.

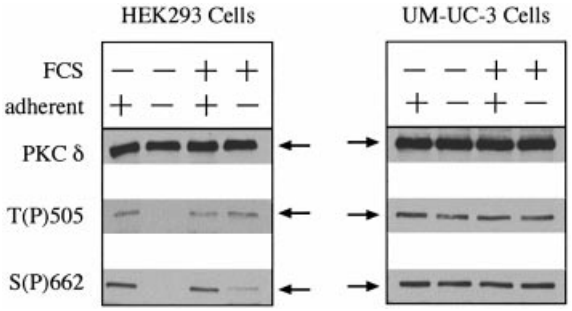


**Figure 5 PKC$\delta$ remains phosphorylated in suspension cultures of UM-UC-3 cells**

HEK-293 cells (left panel) or UM-UC-3 cells (right panel) were transiently transfected with PKC$\delta$. Cells were deprived of serum for 24 h, while either adherent or in suspension, and then FCS stimulated for 30 min. PKC$\delta$ phosphorylation at the priming sites indicated was monitored by Western blotting.

of the mammalian target of rapamycin ('mTOR'), which is responsible for conferring rapamycin sensitivity to the system [9].

Confirmation that integrin engagement was sufficient to induce this PKC$\delta$ phosphorylation was obtained by plating suspension cultures of serum-starved, PKC$\delta$ transfected HEK-293 cells on to collagen or the non-specific substrate poly-D-lysine. Permitting cells to adhere on to collagen for 60 min was sufficient to induce PKC$\delta$ phosphorylation; no equivalent response was observed for poly-D-lysine (Figure 3D). Administration of inhibitors for 30 min, after plating on to collagen for a 30 min period, was not sufficient to reverse this effect, which is consistent with the finding that, once occupied, these inhibitors have no acute effect on these phosphorylation sites in PKC$\delta$. Inhibitor pre-treatment is not informative as it is not possible to distinguish effects on adherence *per se* from those on integrin signalling.

These results indicate that integrin engagement is sufficient to account for the sustained phosphorylation of PKC$\delta$ in serum-starved adherent cultures. Since the serum-induced responses in suspension cultures might also be derived from the presence of integrin substrates, we investigated the effects of two purified agonists, PDGF B-chain homodimer and LPA. Stimulation of serum-deprived suspension cultures of transfected HEK-293 cells with either agonist induced PKC$\delta$ phosphorylation at the T505 and S662 sites (Figure 4A). To determine whether this effect was selective for this PKC isotype, a second novel isotype, PKC$\varepsilon$, was also monitored. Under similar conditions, phosphorylation of both the activation-loop (T566) and hydrophobic (S729) sites was observed in response to these agonists (Figure 4B). For PKC$\varepsilon$, but not PKC$\delta$, the effect of LPA was significantly weaker than either serum or PDGF, implying some specificity of action.

The results show that the activation-loop and hydrophobic sites in PKC$\delta$ are independently controlled by LY294002-sensitive pathways. This appears to require integrin engagement, which is sufficient to elicit phosphorylation of PKC$\delta$ under adherent conditions. Notably, other agonists produced a similar response.

## PTEN has a dominant role in controlling PKC$\delta$ phosphorylation in suspension culture

The tumour suppressor gene *PTEN* has been implicated in the control of integrin coupling to the PI3-kinase pathway and/or the catabolism of PtdIns(3,4,5)$P_3$ itself, through the action of its phosphotyrosine and PtdIns3*P* phosphatase activities (recently reviewed in [10] and [17]). To assess the consequences of loss of function of PTEN on PKC$\delta$ phosphorylation, we investigated the bladder carcinoma cell line, UM-UC-3, which has been shown to be *PTEN*$^{-/-}$ [18]. In contrast to HEK-293 cells, when PKC$\delta$-transfected UM-UC-3 cells were placed in suspension and serum starved for 24 h, there was no change in the level of T505/S662 PKC$\delta$ phosphorylation (Figure 5). This effect of *PTEN* loss does not appear to be mediated by distinct pathways, since the turnover of PKC$\delta$ phosphorylation in suspension cultures of UM-UC-3 cells does not observably differ from that in adherent HEK-293 cells; addition of inhibitors does not acutely influence the retention of phosphorylated PKC$\delta$ (results not shown). Similar analyses with PKC$\varepsilon$-transfected cells demonstrate that the loss of *PTEN* broadly affects nPKC isotypes (results not shown).

The above data imply that *PTEN* plays a dominant role in suppressing the pathway(s) necessary to maintain nPKC phosphorylation. To establish this point, we derived *PTEN*-expressing stable isolates of UM-UC-3 cells (see the Experimental section). Two of these lines (UM-UC-3–PTEN clones 1 and 2) were transfected with PKC$\delta$ and its phosphorylation was monitored (both *PTEN*-expressing lines behaved similarly; data for clone 1 is shown in Figure 6A). It is evident that in contrast to the parental UM-UC-3 line, this *PTEN*-expressing line did not retain PKC$\delta$ phosphorylation when cells were placed in suspension

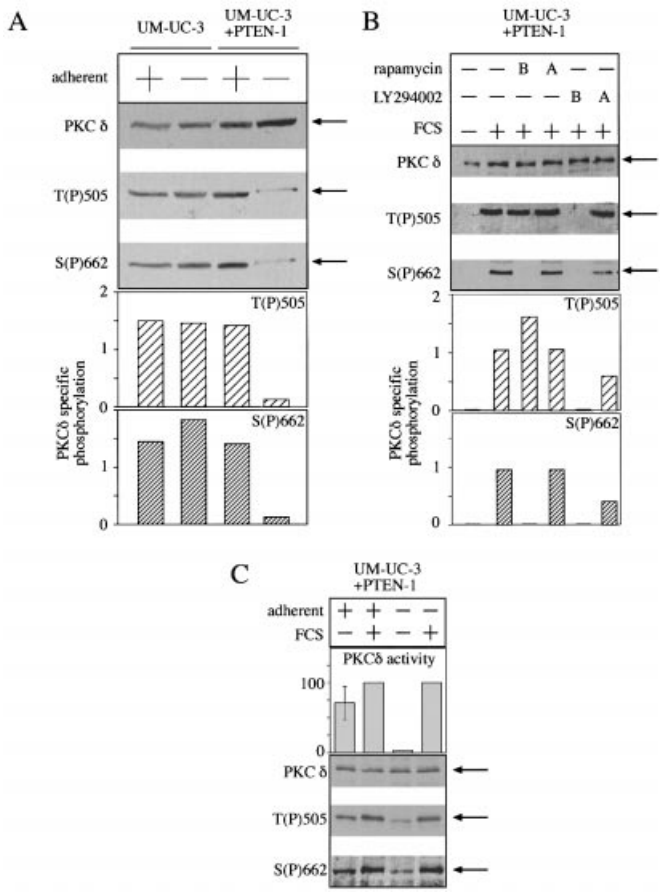


**Figure 6 Restoration of *PTEN* expression to UM-UC-3 cells renders PKC$\delta$ phosphorylation sensitive to culture in suspension**

(**A**) UM-UC-3 cells or UM-UC-3 cells stably expressing *PTEN* were transiently transfected with PKC$\delta$. Suspension or adherent cultures were serum starved for 24 h. The whole-cell lysates were analysed by immunoblotting for PKC$\delta$ and its phosphorylation. The extent of phosphorylation as a function of PKC$\delta$ protein was quantified and is shown in the lower panels. The results are representative of five similar independent experiments. (**B**) Suspension cultures of serum-starved *PTEN*-expressing UM-UC-3 cells, transiently overexpressing PKC$\delta$, were treated with either 20 nM rapamycin or 10 $\mu$M LY294002 for 30 min, before (B) or after (A) serum treatment. The whole-cell lysates were analysed by immunoblotting. PKC$\delta$ specific phosphorylation for the two sites [T(P)505 and S(P)662] is shown in the lower panels. (**C**) PKC$\delta$ transfected, adherent or suspension cultures of UM-UC-3 cells stably expressing *PTEN* were serum starved for 24 h. They were then either serum stimulated (+FCS) for 30 min or not stimulated (−FCS) as indicated. PKC$\delta$ was immunopurified and its catalytic activity was determined (histogram). The kinase data represents the average and range of two independent experiments, with basal activities (−FCS) normalized to the serum-induced (+FCS) activity. The Western blot shows the phosphorylation of the T505 [T(P)505] or S662 [S(P)662] sites from one of these two experiments.

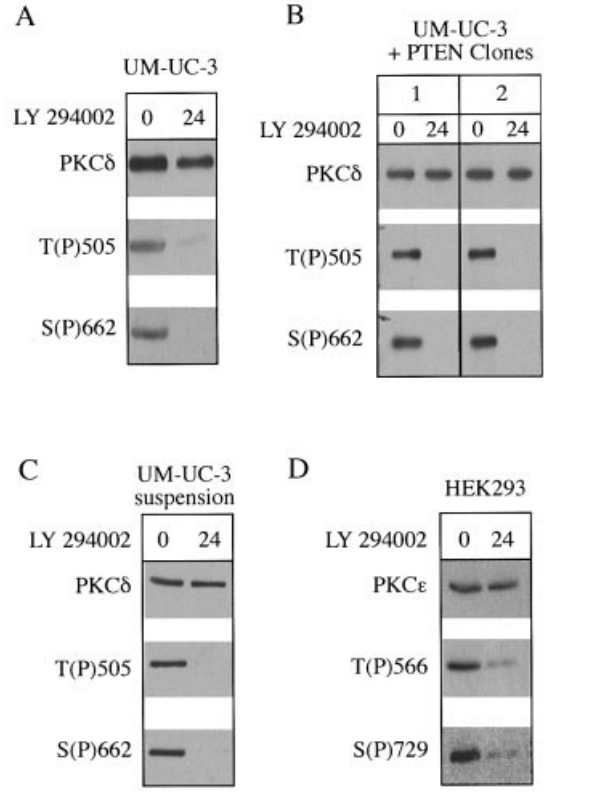


**Figure 7 Chronic PI3-kinase inhibition leads to loss of phosphorylation of PKC$\delta$ and PKC$\varepsilon$**

Adherent cultures of (**A**) UM-UC-3 cells or (**B**) UM-UC-3 cells stably expressing *PTEN* (two independent clones, 1 and 2) were transiently transfected with PKC$\delta$. Cells were then treated with or without LY294002 (10 $\mu$M) for 24 h. (**C**) UM-UC-3 cells were transfected as above, but then maintained in suspension while treated without or with LY294002 (10 $\mu$M). (**D**) HEK-293 cells, transiently transfected with PKC$\varepsilon$, were serum starved for 24 h with or without treatment with 10 $\mu$M LY294002. In each experiment, whole-cell lysates were prepared and analysed by immunoblotting. The phosphorylation of the T505 or S662 sites (PKC$\delta$) and the T566 or S729 sites (PKC$\varepsilon$) were determined by Western blotting, as indicated.

in the absence of serum. As noted for HEK-293 cells, adherent cultures of these *PTEN*-expressing lines retained phosphorylated PKC$\delta$. When suspension cultures of transfected UM-UC-3–PTEN cells were exposed to serum, PKC$\delta$ was acutely phosphorylated on the T505 and S662 sites (Figure 6B). These responses showed the same inhibitor sensitivity as those observed for serum-induced phosphorylations in suspension cultures of HEK-293 cells. Thus treatment with rapamycin before serum addition (B, in Figure 6B) selectively inhibited S662 phosphorylation, whereas treatment with LY294002 blocked both S662 and T505 phosphorylation. Drug treatments after 30 min of serum stimulation (A, in Figure 6B) had little effect. Consistent with the data above, immunopurified PKC$\delta$ activity reflected the changes in the phosphorylation state of the protein (Figure 6C).

### Chronic loss of PI3-kinase activity reverses the influence of *PTEN* loss

The prediction arising from the above results is that, in serum-deprived adherent cultures, integrin inputs to PKC are effected through PI3-kinase-derived lipid products and that this is switched off by culture in suspension through *PTEN* action. To test the chronic effects of PI3-kinase, we treated adherent cultures of PKC$\delta$ transfected cells with the PI3-kinase inhibitor, LY294002. Both adherent HEK-293 cells (results not shown) and UM-UC-3 cells showed sensitivity to this inhibitor on prolonged exposure, with almost complete loss of PKC$\delta$ T505- and S662-site phosphorylation after 24 h (Figure 7A). Similarly, *PTEN*-expressing clones of UM-UC-3 cells also displayed sensitivity to chronic inhibition of PI3-kinase (Figure 7B), as did UM-UC-3 cells in suspension (Figure 7C). These chronic effects of PI3-kinase inhibition were also observed for PKC$\varepsilon$ (Figure 7D). Thus integrin and PTEN do indeed appear to control nPKC

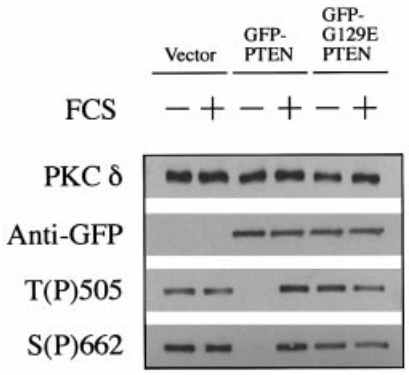


**Figure 8 Wild-type PTEN, but not PTEN G129E, exerts control on PKC$\delta$ phosphorylation**

UM-UC-3 cells were transfected with PKC$\delta$ and either vector or green fluorescent protein (GFP)–PTEN constructs as indicated. Cells were maintained in suspension culture for 24 h prior to stimulation with 10% (v/v) FCS. Extracts were prepared in sample buffer and the PKC$\delta$ content and phosphorylation [T(P)505 and S(P)662] was monitored by Western blotting.

phosphorylation at both their activation-loop and hydrophobic sites in a manner that requires the PI3-kinase pathway.

Both activities of PTEN have been implicated in integrin signalling to PI3-kinase through control of the phosphotyrosine-dependent recruitment of the latter to focal adhesion kinase, and also through degradation of the PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$ products [19]. The mechanism of PTEN action in controlling PKC phosphorylation in suspension cultures was investigated by employing the PTEN G129E mutant, which has been shown to retain its phosphotyrosine phosphatase activity while losing its PtdIns3*P* phosphatase activity [20]. Co-transfection of UM-UC-3 cells with PKC$\delta$ and either the control vector, wild-type PTEN or PTEN G129E, followed by culture in suspension in the absence of serum, led to a selective accumulation of dephosphorylated PKC$\delta$ only in the presence of wild-type PTEN (Figure 8). The absence of any influence of the PTEN G129E mutant on PKC$\delta$ phosphorylation indicates that it is the dephosphorylation of phosphatidylinositol at the 3-hydroxy position that is the key to controlling T505 and S662 phosphorylation.

## DISCUSSION

The evidence presented in the present study demonstrates that PKC$\delta$ phosphorylation and its effector-dependent activity can be decreased to very low levels by culture of cells in suspension in the absence of serum. Adherent cultures do not display a similarly robust response. The implication is that cell–matrix interactions are sufficient to maintain PKC$\delta$ phosphorylation, a conclusion that is supported by the demonstration that $\beta$1-integrin activation or plating on to collagen (a $\beta$1-integrin substrate), but not poly-D-lysine (non-specific adherence), is sufficient to induce phosphorylation of PKC$\delta$. This relationship is further supported by the finding that PTEN PtdIns3*P* phosphatase activity is required to uncouple the inputs to PKC$\delta$. The demonstration that chronic PI3-kinase inhibition mimics the effect of cell–matrix disengagement indicates that there is tonic signalling by integrins via PI3-kinase-derived lipid products and that this is the limiting factor in the control of PKC$\delta$ phosphorylation in serum-starved adherent cultures. It is shown, additionally, that similar controls appear to operate on PKC$\varepsilon$.

It has been established that members of the PKC family, like many other acutely phosphorylated (AGC) protein kinases, require a series of phosphorylations within their activation loops and C-termini in order for them to express optimum effector-dependent activity (see [3]). This dependence of PKC upon phosphorylation was not recognized until almost a decade after the discovery of PKC [21,22], principally because under many conditions PKC isotypes are present in phosphorylated, active, ligand-dependent forms. For example, baculovirus-expressed PKC$\beta$ splice variants show a high degree of phosphorylation in their three 'permissive' phosphorylation sites [23,24]. Nevertheless, recent studies *in vitro* and in intact cells have highlighted the importance of these phosphorylations in controlling PKC activity and also have elucidated some of the proximal elements involved in effecting phosphorylation. Thus PI3-kinase acting through PDK1 has been implicated in the activation-loop phosphorylation of all PKC isotypes [4–6]. There remains debate as to the events associated with the phosphorylation of the C-terminal hydrophobic sites in PKC [3,25]. The evidence for nPKC isotypes is consistent with the operation of an upstream kinase complex containing an aPKC isotype [7]. Further evidence that there are two independent pathways operating to control these two events is given in the present work. Thus, despite the apparent requirement for PKC$\delta$ activation-loop phosphorylation (T505) prior to hydrophobic site (S662) phosphorylation, the protein phosphatase inhibitor okadaic acid (or calyculin) reveals the serum-dependent phosphorylation of the S662 site in the PKC$\delta$ T505A mutant, i.e. in the absence of activation-loop phosphorylation. This suggests that the conformation of the T505-phosphorylated form of PKC$\delta$ favours the protection of S662 from dephosphorylation, similar to the situation for PKC$\alpha$ [16,26]. The T505 phosphorylation-independent, serum-induced S662 phosphorylation remains sensitive to PI3-kinase inhibition, consistent with the action of an aPKC (complex), which is also under PI3-kinase/PDK1 control [4,5]. How these observations on nPKCs relate to other members of the PKC (or indeed AGC) kinase family remains to be resolved, although it is notable that the recent studies on *PDK1*$^{-/-}$ embryonic stem cells demonstrate that insulin-like growth factor-1 can induce PKB S473 (hydrophobic site) phosphorylation in the absence of T308 (activation loop) phosphorylation [27].

Although there has been significant progress in unravelling the immediate upstream regulators for PKC, the basis of the high stoichiometry of phosphorylation observed in many circumstances has not been addressed. It has been documented that the phosphorylated, cytosolic forms of PKC (i.e. unliganded) are relatively resistant to dephosphorylation by protein phosphatases [28]. So, in part, the reason for accumulation of phosphorylated PKC is slow dephosphorylation. However, this does not define what tonic signals are required to permit the upstream kinase(s) to operate.

The action of integrins in providing survival and proliferative signals to cells, as well as operating in a permissive/synergistic fashion with growth factors, have been well documented (for recent reviews see [29,30]). Proximal signals include the Ras/Raf/MEK/MAP kinase cascade and the activation of PI3-kinase. The latter has been implicated in survival pathways associated with the phenomenon of anoikis, apoptosis associated with the loss of adherence (see [31]). Anoikis can be suppressed by the expression of PKB (c-akt) [32], consistent with the view that a critical PI3-kinase output is effected via PKB, e.g. through the phosphorylation and consequent sequestration of BAD (Bcl-2/Bcl-$X_L$-antagonist), causing cell death [33,34]. Whereas, this may, in part, contribute to the response, the behaviour of PKB suggests that other PI3-kinase targets may prove important. For

example, under serum-starved adherent conditions PKB can become essentially unphosphorylated at the S473 site; suspension culture appears to have no selective effect upon PKB (D. B. Parekh and P. J. Parker, unpublished work). The distinct phosphorylation of PKC$\delta/\epsilon$ under these two conditions suggests that these proteins may play a more important role in distinguishing these cellular states irrespective of whether or not they directly implement an anti-apoptotic programme. The previous observation that suppression of PKC phosphorylation in adherent culture will promote cellular apoptosis [35] indicates that these processes can, in fact, play a critical role in this decision making process.

The coupling of integrin signalling to PI3-kinase appears to be via the focal adhesion kinase ('FAK'); phosphorylation of this kinase, directly or indirectly, leads to the recruitment of a class I PI3-kinase and consequent production of PtdIns(3,4,5)$P_3$ (see [36]). The findings of the present study indicate that, although there is an acute stimulation of PtdIns(3,4,5)$P_3$ production on integrin triggering, there must be a tonic signal from integrin engagement in adherent cultures that contributes substantially to the basal level of PtdIns(3,4,5)$P_3$. It is only when this basal input is blocked by chronic inhibition of PI3-kinase that PKC$\delta$ accumulates in a dephosphorylated form.

In suspension culture there appears to be a reduction in the integrin-dependent accumulation of the PI3-kinase-derived lipid products, since PKC$\delta$ accumulates in a dephosphorylated form without pharmacological intervention. It is shown in the present study that this reduced signal is critically controlled by *PTEN*. The human bladder carcinoma cells, UM-UC-3, in which *PTEN* is no longer expressed, do not lose PKC$\delta$ phosphorylation on culture in suspension, despite this process remaining sensitive to chronic PI3-kinase inhibition. Re-introduction of *PTEN* into these cells re-sensitizes the PKC$\delta$ phosphorylation to suspension culture. The implication is that *PTEN* has a dominant role in shutting down PtdIns(3,4,5)$P_3$ accumulation in suspension. This contrasts with the behaviour of UM-UC-3 cells and UM-UC-3/PTEN cells in adherent culture, where neither shows overt sensitivity of PKC$\delta$ phosphorylation to the presence of PTEN. Whether this distinction reflects control of *PTEN* (i.e. signal termination) or strength of signal output is unclear; however, it is clear that PTEN exerts its effect through removal of 3-phosphorylated phosphoinositides, since it is the lipid 3-phosphatase activity that confers its properties in this context.

It has been established that both PKC and PKB are controlled via the PI3-kinase/PDK1 pathway [4,6,37,38]. However, the fact that PKC$\delta$ remains highly phosphorylated under serum-starved adherent culture conditions, when PKB displays undetectable phosphorylation, indicates that distinct controls operate on these different AGC family kinases. One likely distinction between these protein kinases is that there is a difference in their sensitivity to protein phosphatases. For PKC, once phosphorylated it is relatively resistant to dephosphorylation, unless maintained in an open, ligand-bound, active conformation [28]. Hence, PKC$\delta$ phosphorylation would effectively operate as a ratchet, in a manner well suited to slow decision making processes. Although this represents one distinction between PKC and other acutely phosphorylated (AGC) kinases, there is the additional specificity input afforded by the requirement of ligand binding for phosphorylation [9]. Thus, whereas basal diacylglycerol levels may be sufficient for the effective, dynamic recruitment of PKC to an appropriate membrane compartment and, similarly, basal PtdIns(3,4,5)$P_3$ levels may be sufficient for PDK1 recruitment, the same may not be true for PKB, which has a lower affinity for PtdIns(3,4,5)$P_3$ than PDK1 [39]. Since the steady-state phosphorylation of any of these protein kinases will reflect a balance of the on and off rates, it is inevitable that a combination of sensitivities for the two processes is responsible for the distinctions between them.

In conclusion, the results of the present study indicate that high levels of nPKC phosphorylation can be achieved through culture of cells on integrin substrates. This process is actively repressed by *PTEN* following integrin disengagement, and it will be of future interest to determine whether the loss of *PTEN* in tumours provides a selective advantage for (invading) cells through the retention of phosphorylated PKC isotypes.

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## REFERENCES

1 Elliott, B., Ostman, A., Westermark, B. and Rubin, K. (1992) Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. J. Cell. Physiol. **152**, 292–301
2 Price, L. S., Leng, J., Schwartz, M. A. and Bokoch, G. M. (1998) Activation of Rac and Cdc42 by integrins mediates cell spreading. Mol. Biol. Cell **9**, 1863–1871
3 Parekh, D. B., Ziegler, W. and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. EMBO J. **19**, 496–503
4 Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998) Protein kinase C isotypes controled by phosphoinositide 3-kinase through the protein kinase PDK1. Science **281**, 2042–2045
5 Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S. and Toker, A. (1998) Regulation of protein kinase C $\zeta$ by PI 3-kinase and PDK-1. Curr. Biol. **8**, 1069–1077
6 Dutil, E. M., Toker, A. and Newton, A. C. (1998) Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). Curr. Biol. **8**, 1366–1375
7 Ziegler, W. H., Parekh, D. B., Le Good, J. A., Whelan, R. D. H., Kelly, J. J., Frech, M. M., Hemmings, B. A. and Parker, P. J. (1999) Rapamycin-sensitive phosphorylation of PKC on a carboxyterminal site by an atypical PKC complex. Curr. Biol. **9**, 522–529
8 Behn-Krappa, A. and Newton, A. C. (1999) The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation. Curr. Biol. **9**, 728–737
9 Parekh, D., Ziegler, W., Yonezawa, K., Hara, K. and Parker, P. J. (1999) mTOR controls one of two kinase pathways acting upon nPKC$\delta$ and nPKC$\epsilon$. J. Biol. Chem. **2743**, 34758–34764
10 Maehama, T. and Dixon, J. E. (1999) PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell. Biol. **9**, 125–128
11 Chen, C. A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transfoming cells with plasmid DNA. Biotechniques **6**, 632–638
12 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
13 Martins, L. M., Kottke, T., Mesner, P. W., Basi, G. S., Sinha, S., Frigon, Jr., N., Tatar, E., Tung, J. S., Bryant, K., Takahashi, A. et al. (1997) Activation of multiple interleukin-1$\beta$ converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. J. Biol. Chem. **272**, 7421–7430
14 Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods **139**, 271–279
15 Gong, J., Traganos, F. and Darzynkiewicz, Z. (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal. Biochem. **218**, 314–319
16 Bornancin, F. and Parker, P. J. (1997) Phosphorylation of protein kinase C-$\alpha$ on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. J. Biol. Chem. **272**, 3544–3549
17 Tamura, M., Gu, J., Tran, H. and Yamada, K. M. (1999) PTEN gene and integrin signaling in cancer. J. Natl. Cancer Inst. **91**, 1820–1828
18 Hamilton, J. A., Stewart, L. M., Ajayi, L., Gray, I. C., Gray, N. E., Roberts, K. G., Watson, G. J., Kaisary, A. V. and Snary, D. (2000) The expression profile for the tumour suppressor gene PTEN and associated polymorphic markers. Br. J. Cancer **82**, 1671–1676
19 Tamura, M., Gu, J., Danen, E. H. J., Takino, T., Miyamoto, S. and Yamada, K. M. (1999) PTEN Interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. J. Biol. Chem. **274**, 20693–20703

20 Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P. and Tonks, N. K. (1998) The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc. Natl. Acad. Sci. U.S.A. **95**, 13513–13518
21 Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U. and Fabbro, D. (1989) Biosynthesis and post-translational modifications of protein kinase C in human breast cancer cells. J. Biol. Chem. **264**, 13902–13909
22 Pears, C., Stabel, S., Cazaubon, S. and Parker, P. J. (1992) Studies on the phosphorylation of protein kinase C-$\alpha$. Biochem. J. **283**, 515–518
23 Tsutakawa, S. E., Medzihradszky, K. F., Flint, A. J., Burlingame, A. L. and Koshland Jr., D. E. (1995) Determination of *in vivo* phosphorylation sites in protein kinase C. J. Biol. Chem. **270**, 26807–26812
24 Keranen, L. M., Dutil, E. M. and Newton, A. C. (1995) Protein kinase C is regulated *in vivo* by three functionally distinct phosphorylations. Curr. Biol. **5**, 1394–1403
25 Newton, A. C. (1997) Regulation of protein kinase C. Curr. Opin. Cell Biol. **9**, 161–167
26 Bornancin, F. and Parker, P. J. (1996) Phosphorylation of threonine-638 critically controls the dephosphorylation and inactivation of protein kinase C-$\alpha$. Curr. Biol. **6**, 1114–1123
27 Williams, M. R., Arthur, J. S., Balendran, A., Van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R. (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr. Biol. **10**, 439–448
28 Hansra, G., Bornancin, F., Whelan, R., Hemmings, B. A. and Parker, P. J. (1996) 12-*O*-Tetradecanoylphorbol-13-acetate-induced dephosphorylation of protein kinase C$\alpha$ correlates with the presence of a membrane associated protein phosphatase 2A heterotrimer. J. Biol. Chem. **271**, 32785–32788
29 Schwartz, M. A. (1997) Integrins, oncogenes, and anchorage independence. J. Cell. Biol. **139**, 575–578

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30 Kumar, C. C. (1998) Signaling by integrin receptors. Oncogene **17**, 1365–1373
31 Frisch, S. M. and Ruoslahti, E. (1997) Integrins and anoikis. Curr. Opin. Cell Biol. **9**, 701–706
32 Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H. and Downward, J. (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J. **16**, 2783–2793
33 Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91**, 231–241
34 del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R. and Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science **278**, 687–689
35 Whelan, D. H. and Parker, P. J. (1998) Loss of protein kinase C function induces an apoptotic response. Oncogene **15**, 1939–1944
36 Cary, L. A. and Guan, J. L. (1999) Focal adhesion kinase in integrin-mediated signaling. Front. Biosci. **15**, D102–D113
37 Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P. et al. (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science **279**, 710–714
38 Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. and Avruch, J. (1998) 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase *in vivo* and *in vitro*. Curr. Biol. **8**, 69–81
39 Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science **277**, 567–570