

# PKC- $\eta$ mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways

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We previously demonstrated that protein kinase C- $\eta$  (PKC- $\eta$ ) mediates a phorbol 12-myristate-13-acetate (PMA)-induced proliferative response in human glioblastoma (GBM) cells. In this report, we show that PMA-stimulated activation of PKC- $\eta$  in U-251 GBM cells resulted in activation of both Akt and the mammalian target of rapamycin (mTOR) signaling pathways and an increase in cell proliferation. Expression of a kinase dead PKC- $\eta$  (PKC- $\eta$ KR) construct reduced the basal and PMA-evoked proliferation of PKC- $\eta$ -expressing U-251 GBM cells, as well as abrogated the PMA-induced activation of Akt, mTOR, and the mTOR targets 4E-BP1 and STAT-3. Treatment of cells with the PI-3 kinase inhibitor LY294002 (10  $\mu$ M) or the mTOR inhibitor rapamycin (10 nM) also reduced PMA-induced proliferation and cell-cycle progression. Expression of a constitutively active PKC- $\eta$  (PKC- $\eta$  $\Delta$ NPS) construct in a GBM cell line with no endogenous PKC- $\eta$  (U-1242) also provided evidence that PKC- $\eta$  targets the Akt and mTOR signaling pathways. Moreover, activation of 4E-BP1 and STAT-3 in both PMA-treated U-251 and PKC- $\eta$  $\Delta$ NPS-expressing U-1242 GBM cells was inhibited by rapamycin. However, activation of Akt, but not mTOR was inhibited by the PI-3 kinase inhibitor LY294002. This study identifies Akt and mTOR as downstream targets of PKC- $\eta$  that are involved in GBM cell proliferation.

*Oncogene* (2004) 23, 9062–9069. doi:10.1038/sj.onc.1208093  
Published online 18 October 2004

**Keywords:** PKC; glioblastoma; mTOR; Akt; proliferation

## Introduction

Glioblastoma multiforme (GBM) accounts for over half of all astrocytic tumors, the most frequent intracranial neoplasm in adults. GBM cells display a high rate of proliferation and an uncompromising propensity to infiltrate regional and remote brain structures. These

features result in a poor prognosis, with surgical resection of GBMs nearly impossible. Invasive astrocytic tumors differentially express integrins, and secrete a number of proteases, proteinase inhibitors, and growth factors (Rooprai *et al.*, 1999; Okada, 2000; Yamamoto *et al.*, 2002; Konopka and Bonni, 2003). Additionally, the activity and expression of protein kinase-C (PKC) family members are higher in malignant astrocytomas than in non-neoplastic astrocytes (Couldwell *et al.*, 1991; Todo *et al.*, 1991; Benzil *et al.*, 1992), suggesting that increased PKC activity may contribute to GBM tumorigenicity. Here, we demonstrate that activation of PKC- $\eta$  promotes GBM cell proliferation in part through the Akt and mammalian target of rapamycin (mTOR) signaling pathways.

The PKC family of calcium and/or lipid-activated serine–threonine kinases functions downstream of most membrane-associated signal transduction pathways (Molkentin and Dorn, 2001). Activities of PKC family members are associated with a number of cellular responses, including cell growth, differentiation, gene expression, hormone secretion, and motility (Nishizuka, 1984; Nishizuka, 1988; Gescher, 1992; Basu, 1993; Blobe *et al.*, 1994; Choe *et al.*, 2003). The classical PKC ( $\alpha$ ,  $\beta$ I,  $\beta$ II, and  $\gamma$ ) and novel PKC ( $\theta$ ,  $\epsilon$ ,  $\eta$ , and  $\delta$ ) isozymes are regulated by phorbol esters, diacylglycerols and phospholipids. The classical PKCs also require Ca<sup>2+</sup> for activity, whereas activities of novel and atypical ( $\iota$  and  $\zeta$ ) PKC isozymes are Ca<sup>2+</sup> independent (Nishizuka, 1995). Additionally, the atypical PKCs are not activated by diacylglycerol or phorbol esters (Resnick *et al.*, 1997). Although PKC isozymes and their related kinases have been shown to activate a number of signaling pathways in various cell types, those pathways involving PKCs in neural cell types are not well understood.

Upstream of PKCs, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, and ciliary neurotrophic factor help drive glial progression towards astrocytic and oligodendrocytic differentiation (Bogler *et al.*, 1990; McKinnon *et al.*, 1990; Mayer *et al.*, 1993, 1994; Rajan and McKay, 1998). Expression levels of genes encoding growth factors and growth factor receptors that control glial cell differentiation are frequently elevated in gliomas (Ekstrand *et al.*, 1991; Guha *et al.*, 1995; Weis *et al.*, 1999). This results in autocrine stimulation and increased activity of pathways downstream of growth factor receptors. Cell culture

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Received 28 June 2004; revised 18 August 2004; accepted 18 August 2004; published online 18 October 2004

experiments have shown that growth factor receptors activate several common signaling pathways, including the Akt signaling pathway.

Upon stimulation, Akt is activated through PI-3 kinase, which is dephosphorylated by the tumor suppressor phosphatase PTEN (Jiang *et al.*, 1999). Many GBMs show mutation and frequent loss of the PTEN gene (Fujisawa *et al.*, 1999; Sano *et al.*, 1999), resulting in an elevated Akt activity (Holland *et al.*, 2000). In its active form, Akt can increase GBM cell proliferation by enhancing metabolism through glycogen synthase kinase (GSK-3) and promoting transcription and protein synthesis through mTOR (Gingras *et al.*, 1998; Welsh *et al.*, 1998). Accordingly, it is thought that these and other growth factor-mediated signaling pathways have a critical role in the induction or progression of GBMs (McKinnon *et al.*, 1990; Ekstrand *et al.*, 1991; Hesselager *et al.*, 2003). Using constitutively active and kinase dead constructs in two different cell lines, our results indicate that PKC- $\eta$  mediates a mitogenic phenotype in GBMs by activating the mTOR pathway in parallel to the PI-3 kinase/Akt pathway. Inhibition of these pathways with a kinase dead PKC- $\eta$  construct, the PI-3 kinase inhibitor LY294002 (10  $\mu$ M), or rapamycin (10 nM) greatly reduced GBM cell proliferation.

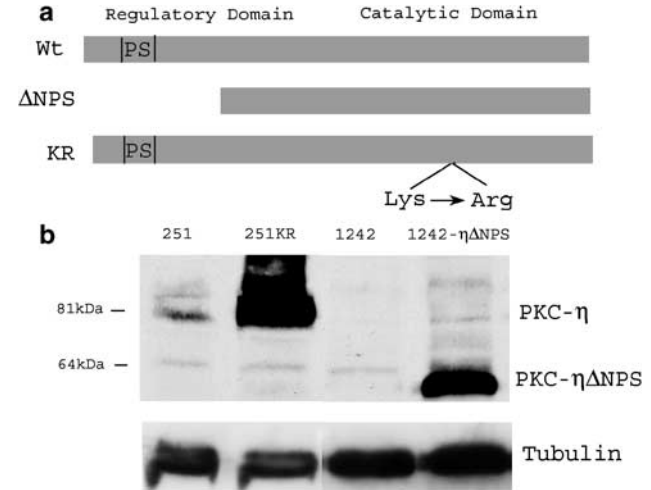
## Results

### Expression of PKC- $\eta$ mutants in astroglial tumor cells

We employed retrovirus delivery system to create several stable astroglial tumor cell lines that could help identify signaling pathways downstream of PKC- $\eta$ . Like other PKC family members, wild-type PKC- $\eta$  is composed of an N-terminal regulatory domain and a C-terminal catalytic domain (Figure 1a). In its inactive form, the region of the regulatory domain, termed the pseudosubstrate domain, is bound to and silences the catalytic domain. Mutations or deletions within these domains affect the kinase activity of PKC- $\eta$ . The constitutively active PKC- $\eta$  construct (PKC- $\eta$  $\Delta$ NPS) lacks a portion of the N-terminal domain containing the pseudosubstrate region, while the kinase dead construct (PKC- $\eta$ KR) has a mutation in the catalytic domain. The parental GBM cell lines differ in PKC- $\eta$  expression; U-251 cells endogenously express PKC- $\eta$ , while U-1242 cells do not express PKC- $\eta$  (Hussaini *et al.*, 2000). Western blots confirmed that U-1242 cells were deficient in PKC- $\eta$ , while expression levels of the PKC- $\eta$ KR and PKC- $\eta$  $\Delta$ NPS constructs were found to be several times greater than endogenous levels of PKC- $\eta$  in U-251 cells (Figure 1b).

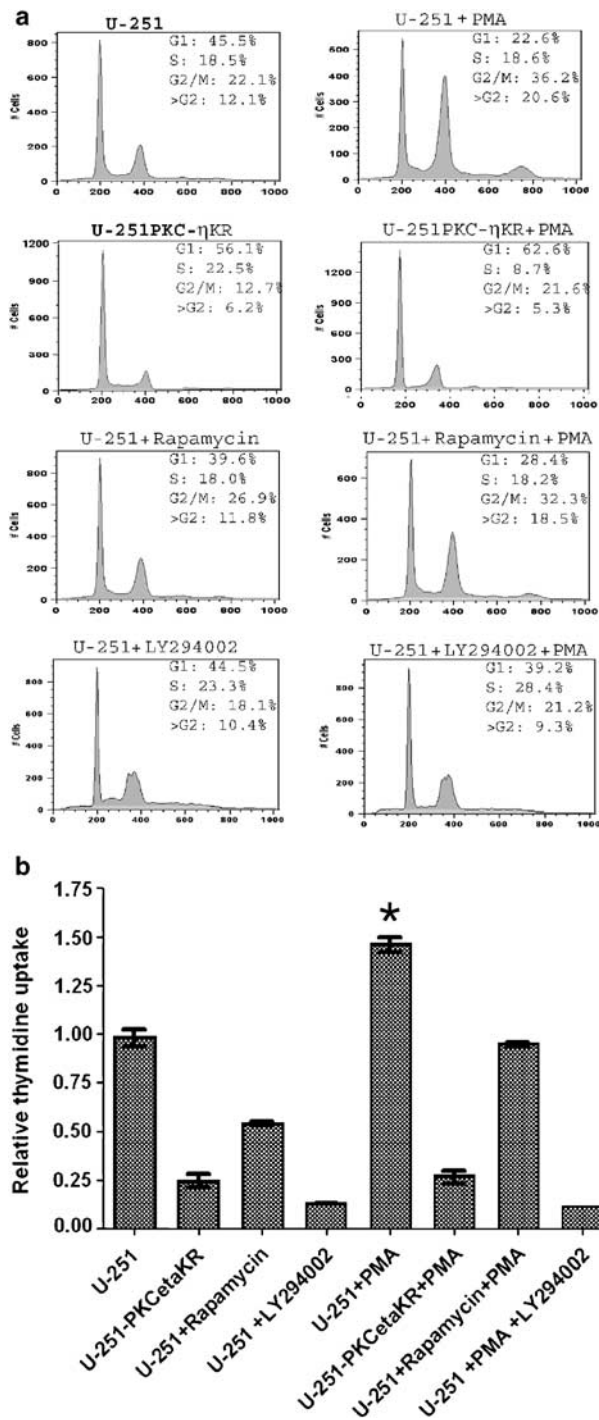
### PKC- $\eta$ KR, rapamycin, and LY294002 inhibit PMA-stimulated U-251 cell-cycle progression and $^3$ H-thymidine uptake

The Akt/mTOR pathway has been shown to regulate cell-cycle progression, as inhibition of this pathway can result in G1 cell-cycle arrest (Wiederrecht *et al.*, 1995;



**Figure 1** Western blot analysis of cytosolic PKC- $\eta$  in U-1242 and U-251 cells. (a) Wild type PKC- $\eta$  is composed of an N-terminal regulatory domain and a C-terminal catalytic domain. The constitutively active PKC- $\eta$  construct (PKC- $\eta$  $\Delta$ NPS) lacks a portion of the N-terminal domain containing the pseudosubstrate region (PS), while the kinase dead construct (PKC- $\eta$ KR) has a mutation in the catalytic domain. (b) NP-40 solubilized astrocytic tumor cell cytosolic proteins (200  $\mu$ g) were fractionated on 10% polyacrylamide gels and electroblotted onto nitrocellulose. Full-length PKC- $\eta$  shows an apparent molecular weight of 78 kDa, while PKC- $\eta$  $\Delta$ NPS appears to be 60 kDa

Hentges *et al.*, 2001). We have previously shown that U-251 cells treated with PMA for 24 h resulted in a striking increase in PKC- $\eta$  protein levels (Hussaini *et al.*, 2000). Concomitantly, U-251 cells treated with PMA for 24 h resulted in a decrease in total protein amounts of PKC- $\alpha$ , - $\beta$ 1, - $\delta$ , - $\epsilon$ , - $\theta$ , and - $\gamma$  (Hussaini *et al.*, 2000). To determine whether PKC- $\eta$  regulates GBM cell-cycle progression, we performed propidium iodide FACS analysis on U-251 cells treated with PMA (100 nM) (Figure 2a). The U-251 cells demonstrated a 50% decrease of cells in G1 and a 70% increase in polyploid cells 24 h after PMA treatment (100 nM). Expression of PKC- $\eta$ KR blocked both of these effects: stimulation of PKC- $\eta$ KR-expressing U-251 cells with PMA resulted in a 12% increase in cells in G1 and a 15% decrease in polyploid cells, as compared to untreated U-251 cells expressing PKC- $\eta$ KR. Moreover, PMA stimulation of U-251 cells in the presence of rapamycin (10 nM) led to a 28% decrease in cells in G1 and a 57% increase in polyploid cells, as compared to U-251 cells treated with rapamycin alone. Additionally, PMA stimulation of U-251 cells in the presence LY294002 (10  $\mu$ M) increased the number of cells in G1 by 12% and decreased the amount of polyploid cells by 11%, as compared to U-251 cells treated with LY294002 alone. Along with its effect on cell-cycle progression, a 24-h treatment with 100 nM PMA increased GBM cell proliferation, as measured by  $^3$ H-thymidine uptake in U-251 MG cells (Figure 2b; Hussaini *et al.*, 2000). The PMA-induced increase in proliferation required PKC- $\eta$  signaling, as it was completely blocked by PKC- $\eta$ KR expression.



**Figure 2** Rapamycin, LY294002, and PKC- $\eta$ KR inhibit PMA-stimulated U-251 GBM cell proliferation. (a) Empty-vector and PKC- $\eta$ KR-expressing U-251 cells were subjected to propidium iodide cell-cycle analysis. Those cultured with PMA (100 nM) were treated 18 h before samples were fixed. Those treated with rapamycin (10 nM) or LY294002 (10  $\mu$ M) were treated 19 h before fixation. (b) U-251 cells were measured for their ability to take up thymidine in the presence of PKC- $\eta$ KR or rapamycin (10 nM). Following PMA addition, cells were incubated for another 18 h. As with the cell-cycle analysis, those cultures treated with rapamycin (10 nM) were treated 1 h before PMA was added. The cultures were then pulsed with  $^3$ H-thymidine (2  $\mu$ Ci/ml) for 2 h and harvested for radioactivity determination. \* $P$ <0.05 compared to all other samples

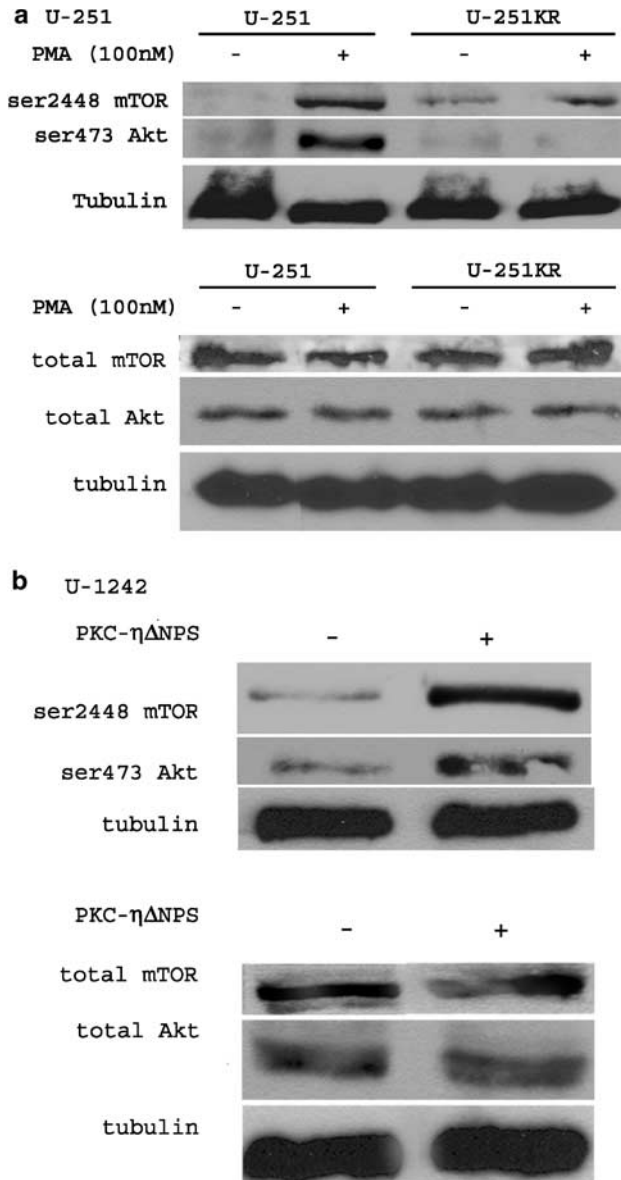
Additionally, pretreatment with rapamycin (10 nM) or the PI-3 kinase inhibitor LY294002 (10  $\mu$ M) completely inhibited PMA-stimulated U-251 cell proliferation.

*PKC- $\eta$  signals to Akt and mTOR*

To determine whether the Akt/mTOR pathway could be activated by PKC- $\eta$ , we expressed PKC- $\eta$ KR in U-251 cells. Previously, it had been shown that Akt could be activated by phospholipid binding and activation loop phosphorylation at thr-308 by PDK1, and by phosphorylation within the carboxy-terminus at ser-473 (Alessi *et al.*, 1996). Likewise, activation of mTOR has been shown to occur through phosphorylation of ser-2448 residues (Nave *et al.*, 1999). After stimulating classical and novel PKCs in U-251 cells for 30 min with PMA (100 nM), ser-2448 phosphorylation of mTOR increased 43  $\pm$  10-fold and ser-473 Akt phosphorylation levels increased 85  $\pm$  14-fold, compared with untreated controls (Figure 3a). However, expression of PKC- $\eta$ KR significantly reduced PMA-stimulated ser-473 phosphorylation of Akt by 95  $\pm$  5% ( $P$ <0.03) and ser-2448 phosphorylation of mTOR by 54  $\pm$  12% ( $P$ <0.05) above unstimulated levels. These data show that PMA-stimulated U-251 cells activate Akt and mTOR through PKC- $\eta$ . In U-1242 cells, which do not express PKC- $\eta$ , PKC- $\eta$  $\Delta$ NPS expression increased phosphorylation on ser-473 of Akt by 208  $\pm$  97% ( $P$ <0.03) and phosphorylation on ser-2448 of mTOR by 322  $\pm$  58% ( $P$ <0.03), as compared to empty-vector controls (Figure 3b). These data demonstrate that PKC- $\eta$  can activate Akt and mTOR in GBM cells.

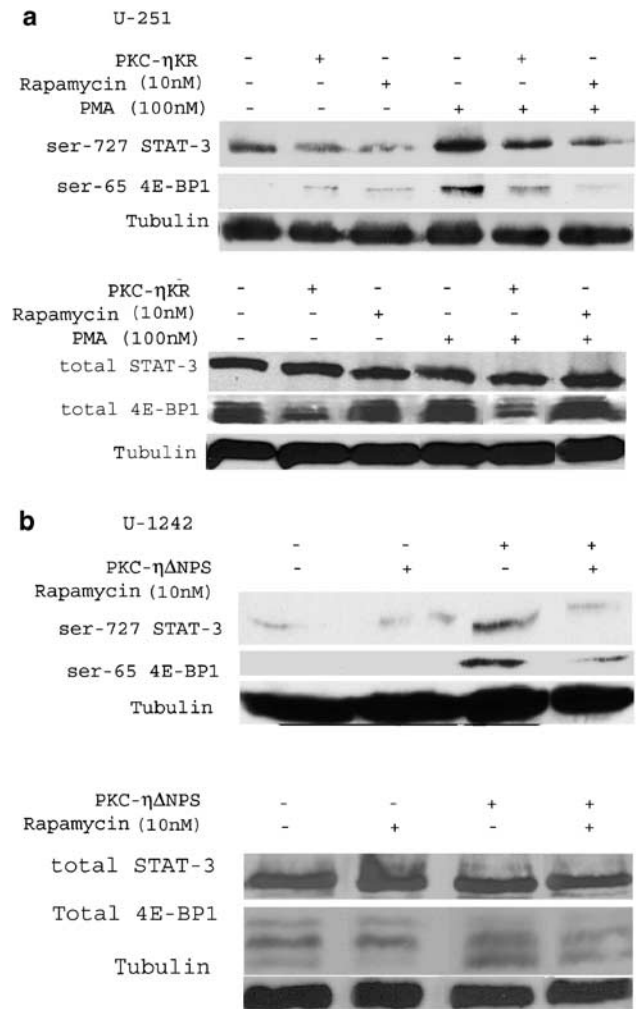
*PKC- $\eta$  mediates activation of STAT-3 and 4E-BP1 through mTOR*

mTOR has been shown to regulate cell growth and proliferation at the level of both gene transcription and protein synthesis. Upon activation, mTOR can stimulate cell growth, protein synthesis, and transcription by phosphorylating eukaryotic initiation factor, 4E-BP1, and the transcription factor STAT-3 (Brunn *et al.*, 1997; Fadden *et al.*, 1997). Phosphorylation of STAT-3 at ser-727 is indicative of a high activation state, and phosphorylation of 4E-BP1 at ser-65 blocks the inhibitory role of 4E-BP1 on translation (Pause *et al.*, 1994; Yokogami *et al.*, 2000). A 30-min treatment of U-251 cells with PMA increased phosphorylation on ser-727 of STAT-3 by 34  $\pm$  4% ( $P$ <0.05) and brought phosphorylation on ser-65 of 4E-BP1 to detectable levels, as compared to untreated controls (Figure 4a). However, this increase was abrogated in the presence of either PKC- $\eta$ KR or 10 nM rapamycin. Moreover, U-1242 cells expressing PKC- $\eta$  $\Delta$ NPS increased phosphorylation on ser-727 of STAT-3 by 158  $\pm$  88% ( $P$ <0.03) and raised phosphorylation on ser-65 of 4E-BP1 to detectable levels, as compared to empty-vector expressing controls (Figure 4b). Accordingly, these increases were blocked by 10 nM rapamycin. These data demonstrate that activation of STAT-3 and 4E-BP1 by PKC- $\eta$  depends on mTOR activation.



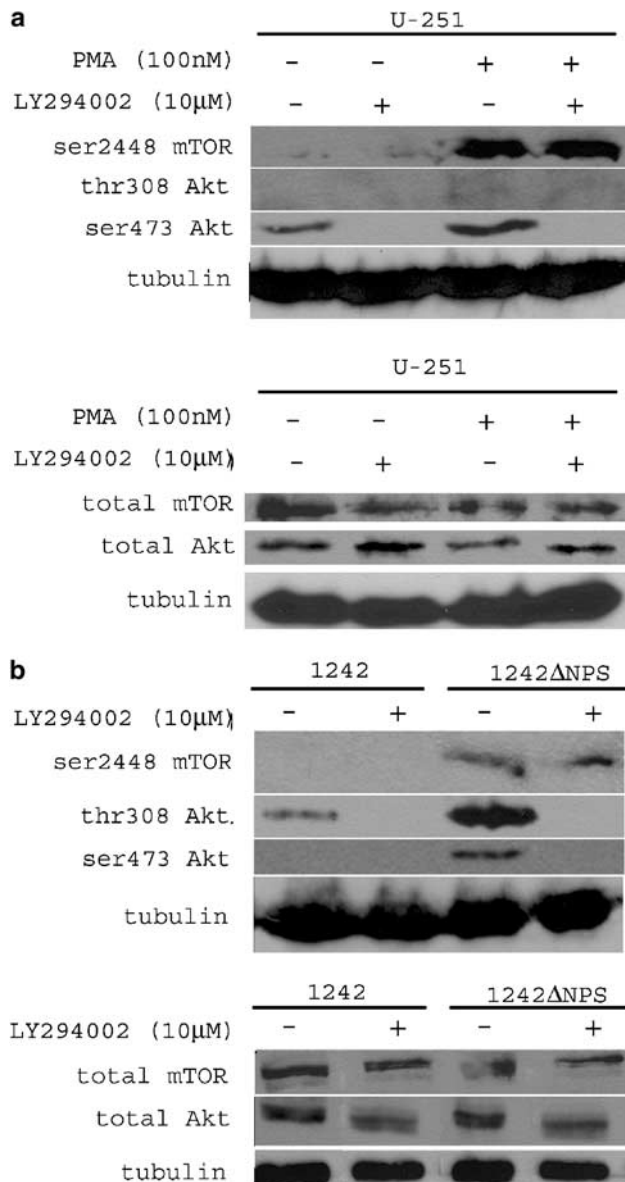
**Figure 3** PKC- $\eta$  mediates stimulation of Akt and mTOR in GBM cells. (a) Western blot analysis (200  $\mu$ g/lane) was performed on protein from empty-vector and PKC- $\eta$ KR-expressing U-251 cells in the presence or absence of a 30 min PMA treatment (100 nM). PMA greatly increased detection of both ser-473-Akt and ser-2448-mTOR in empty-vector U-251 cells, while PKC- $\eta$ KR expression resulted in a decrease in this affect. No significant change between samples was detected in total Akt or mTOR levels. (b) Western blot analysis (200  $\mu$ g/lane) performed on protein from empty-vector and PKC- $\eta\Delta$ NPS-expressing U-1242 cells demonstrates an increase in ser-473-Akt and ser-2448-mTOR. No significant change between samples was detected in total Akt or mTOR levels

Since the signaling pathways described in this paper can affect transcription and protein synthesis, total protein levels of mTOR, STAT-3, Akt, and 4E-BP1 were examined alongside each treatment (Figures 3–5). Aside from a significant decrease ( $P < 0.05$ ) in 4E-BP1 levels in samples from U-251 cells expressing PKC- $\eta$ KR as compared with those from empty-vector U-251 cells (Figure 4a), protein levels did not change with



**Figure 4** PKC- $\eta$  signals through mTOR to activate STAT-3 and 4E-BP1 in GBM cells. (a) Solubilized lysates from empty-vector or PKC- $\eta\Delta$ NPS-expressing U-1242 cells (200  $\mu$ g/lane) were fractionated on polyacrylamide gels and electroblotted onto nitrocellulose. Samples incubated with rapamycin were treated 2 h before lysis. Samples incubated with PMA were treated for 30 min prior to lysis. The blots were then probed for ser-727 STAT-3, ser-65 4E-BP1, and tubulin. Total protein levels of 4E-BP1 were significantly less in PKC- $\eta$ KR samples ( $P < 0.05$ ), as compared to empty-vector controls. (b) Lysates from U-251 cells (200  $\mu$ g/lane) were fractionated on polyacrylamide gels and electroblotted onto nitrocellulose. The nitrocellulose was then reacted with ser-727 STAT-3, ser-65 4E-BP1, and tubulin. No significant change between samples was detected in total 4E-BP1 or STAT-3 levels

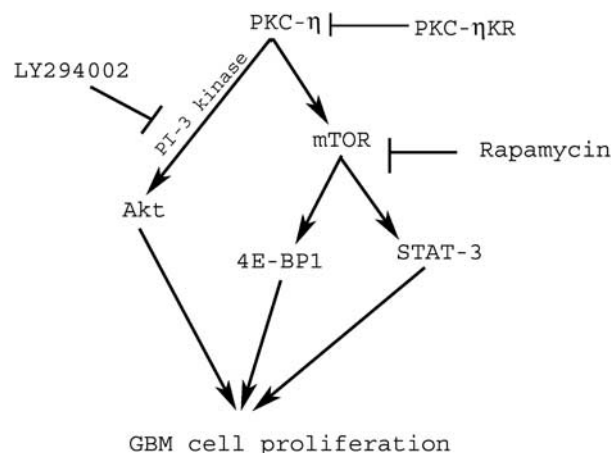
treatments that altered their phosphorylation states. The reduction in 4E-BP1 expression as a result of PKC- $\eta$ KR expression could be from an increase in P38 activity in these cells, as compared to empty-vector expressing controls. U-251 cells expressing PKC- $\eta$ KR show a significant increase in P38 activity and U-1242 cells expressing PKC- $\eta\Delta$ NPS show a significant decrease in P38 activity, as compared to empty-vector controls (Aeder *S et al.*, manuscript in preparation). Others have shown that P38 activity decreases 4E-BP1 expression in hematopoietic cell lines (Rolli-Derkinderen *et al.*, 2003).



**Figure 5** PKC- $\eta$  activates mTOR in the absence of Akt activity. **(a)** Empty-vector or PKC- $\eta$ KR-expressing U-251 cells were treated for 2 h with LY294002 as indicated. PMA-treated samples were incubated for 30 min in PMA. Solubilized lysates from empty-vector or PKC- $\eta$ KR-expressing U-251 cells (200  $\mu$ g/lane) were fractionated on polyacrylamide gels and electroblotted onto nitrocellulose. The nitrocellulose was then reacted with antibodies against ser-2448-mTOR, thr-308 Akt, ser-473 Akt, and tubulin. No significant change between samples was detected in total Akt or mTOR levels. **(b)** Empty-vector or PKC- $\eta$  $\Delta$ NPS-expressing U-1242 cells were treated for 2 h with LY294002, as indicated. Solubilized lysates (200  $\mu$ g/lane) from empty-vector or PKC- $\eta$  $\Delta$ NPS-expressing U-1242 cells were fractionated on polyacrylamide gels and electroblotted onto nitrocellulose. The nitrocellulose was then reacted with antibodies against ser-2448-mTOR, thr-308 Akt, ser-473 Akt, and tubulin. No significant change between samples was detected in total Akt or mTOR levels

#### PKC- $\eta$ activates mTOR and Akt in parallel

To determine whether PKC- $\eta$  is signaling through Akt to activate mTOR, we inhibited PKC- $\eta$ -stimulated Akt



**Figure 6** PKC- $\eta$  drives GBM cell proliferation by activating the Akt and mTOR signaling pathways. PKC- $\eta$  can independently activate Akt and mTOR in GBM cells, as the PI3-kinase inhibitor LY294002 is able to block PKC- $\eta$ -mediated activation of Akt but not mTOR. Inhibition of these pathways at any stage, with PKC- $\eta$ KR, LY294002, or rapamycin, inhibits PKC- $\eta$  mediated GBM cell proliferation

activation with the PI-3 kinase inhibitor LY294002 (10  $\mu$ M). As shown in Figure 5a, LY294002 blocked basal and PMA-stimulated Akt activation in U-251 cells. LY294002 also abolished Akt phosphorylation in wt U-1242 cells and U-1242 cells expressing PKC- $\eta$  $\Delta$ NPS (Figure 5b). However, activation of mTOR remained elevated in PMA-stimulated U-251 cells treated with LY294002 and U-1242 cells expressing PKC- $\eta$  $\Delta$ NPS treated with LY294002 (Figure 5a and b). These data support a model where separate pathways lead from PKC- $\eta$  to Akt activation and from PKC- $\eta$  to mTOR activation in GBM cells (Figure 6).

#### Discussion

Previous studies have clearly demonstrated that PKC and Akt activities are elevated in GBM cells, as compared to non-neoplastic astrocytes (Couldwell *et al.*, 1991; Todo *et al.*, 1991; Benzil *et al.*, 1992; Holland *et al.*, 2000). The increased Akt activity has been attributed to growth factor signaling and inactivation of PTEN, a phosphatase responsible for inhibiting PI-3 kinase signaling that is not expressed in the majority of GBM tumor cells (Fujisawa *et al.*, 1999; Sano *et al.*, 1999). In this study, we determined the roles of PKC- $\eta$  on the activation of mTOR and Akt, serine/threonine kinases that have been shown to control growth and proliferation in a number of cell types (Gingras *et al.*, 1998; Fingar *et al.*, 2002; Schalm *et al.*, 2003). mTOR can be regulated downstream of PI-3 kinase/Akt signaling, as well as independently of the PI-3 kinase/Akt pathway (Hara *et al.*, 1998; Patti *et al.*, 1998; Wang *et al.*, 1998; Kimball *et al.*, 1999).

In this report, mTOR and Akt were identified as downstream targets of PKC- $\eta$  by expressing PKC- $\eta$ KR

in U-251 (PKC- $\eta$ -expressing) cells and constitutively active PKC- $\eta$  (PKC- $\eta$  $\Delta$ NPS) in U-1242 (PKC- $\eta$ -deficient) cells. PKC- $\eta$ KR, the specific mTOR inhibitor rapamycin (10 nM), and the PI-3 kinase inhibitor LY294002 (10  $\mu$ M) significantly reduced basal and PMA-stimulated (100 nM) proliferation of PKC- $\eta$ -expressing GBMs. Cell-cycle analysis revealed that the inhibition was due to both a reduction in cells leaving G1 phase and a reduction in polyploid cells. Using phospho-specific antibodies, we found that activation of PKC- $\eta$  leads to the activation of Akt and the mTOR signaling pathway. Using the PI-3 kinase inhibitor LY294002, we were able to block PMA-stimulated activation of Akt, while mTOR activity remained elevated. These data provide a model where PKC- $\eta$  drives GBM cell proliferation by signaling in conjunction with PI-3 kinase to activate Akt, and independently of PI-3 kinase to activate the mTOR pathway (Figure 6). The results also indicate that PKC- $\eta$ KR could be used in a treatment aimed at blocking both Akt and mTOR-driven GBM cell proliferation.

In human and rat glioma cells, the differential expression of specific PKC isozymes accompanies alterations in both cell proliferation and differentiation. Expression patterns of PKCs may explain the conflicting data as to how specific PKC isozymes affect cell proliferation. For example, most studies have focused on the role of classical PKC isozymes, with conflicting reports on the roles of these PKCs increasing proliferation or blocking apoptosis in malignant astrocytic tumor cells. Taking into account other PKC isozymes, it is thought that differential expression of novel PKC isozymes may account for contradictory effects of phorbol ester treatments (Guizzetti *et al.*, 1998; Hussaini *et al.*, 2000). The effects of PKC- $\eta$  on proliferation appear to be cell type specific. In this study using GBM cells, and another performed with epithelial breast adenocarcinoma MCF-7 cells (Fima *et al.*, 2001), PKC- $\eta$  enhanced proliferation and cell-cycle progression. However, studies using NIH3T3 cells and keratinocytes demonstrated inhibitory effects of PKC- $\eta$  on cell-cycle progression (Livneh *et al.*, 1996; Ohba *et al.*, 1998; Kashiwagi *et al.*, 2000). Cell type specificity is not unique for PKC- $\eta$ , as other PKC isoforms have been shown to modulate proliferation in a cell-type specific manner. For example, PKC- $\alpha$  has been shown to suppress proliferation of intestinal epithelial cells (Frey *et al.*, 1997), F9 teratocarcinoma cells (Kindregan *et al.*, 1994), B16 and A-375 melanoma cells (Gruber *et al.*, 1992; Krasagakis *et al.*, 2004), CHO cells (Yamaguchi *et al.*, 1995), 3Y1 fibroblasts (Nakaigawa *et al.*, 1996), but enhanced growth in Swiss 3T3 cells and MCF-7 cells (Eldar *et al.*, 1990; Ways *et al.*, 1995).

Downstream of mTOR, 4E-BP1 and STAT-3 have been shown to promote cell proliferation through transcriptional and translational mechanisms (Gingras *et al.*, 1998; Kijima *et al.*, 2002). 4E-BP1 (also known as PHAS-1) normally binds eIF4E, inhibiting cap-dependent translation. Hyperphosphorylation of 4E-BP1 disrupts this binding, activating cap-dependent

translation (Pause *et al.*, 1994). A subset of these transcripts encodes proteins involved in cell growth and proliferation (Kozak, 1991). Studies have shown 4E-BP1 activity regulates cell growth, as expression of mutated 4E-BP1 constructs results in a reduction in cell size (Fingar *et al.*, 2002; Schalm *et al.*, 2003). Coordinated with cell division, an increase in cell size is required for cellular proliferation. Like 4E-BP1, STAT-3 is constitutively activated in a number of human tumors and possesses oncogenic potential and anti-apoptotic activities (Garcia and Jove, 1998; Bromberg *et al.*, 1999; Catlett-Falcone *et al.*, 1999; Aoki *et al.*, 2001). Transcriptional activation of STAT-3 is regulated by serine phosphorylation at Ser-727 via the MAPK or mTOR pathways (Yokogami *et al.*, 2000). In squamous cell carcinoma cells, expression of a constitutively active STAT-3 construct resulted in an increase in both proliferation and in the proportion of cells in the G2/M phases of the cell cycle (Kijima *et al.*, 2002). In this study, we expressed PKC- $\eta$  $\Delta$ NPS and PKC- $\eta$ KR constructs to show that PKC- $\eta$  can control the activities of these mTOR effectors in GBM cells, enabling them to drive expression of proteins that enhance cell proliferation. Moreover, identifying PKC- $\eta$ KR as an inhibitor of PKC- $\eta$ -mediated Akt activation, mTOR activation, and GBM cell proliferation indicates that regulation of this novel PKC with PKC- $\eta$ KR may prove useful for treating patients with highly aggressive and infiltrative brain tumors.

## Materials and methods

### Materials

Phorbol 12-myristate -13-acetate (PMA) and tubulin antibody (DM1A) were purchased from Sigma Company (St Louis, MO, USA). The polyclonal antibody specific for human PKC- $\eta$  was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The monoclonal antibody specific for ser-473 Akt and the polyclonal antibodies specific for thr-308 Akt, ser-2448 mTOR, ser-65 4E-BP1, ser-727 STAT-3, total Akt, total mTOR, total 4E-BP1, and total STAT-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The specific mTOR inhibitor rapamycin and the PI-3 kinase inhibitor LY294002 are products of Calbiochem (San Diego, CA, USA).

### Cell cultures

Human U-251 and U-1242 cell lines were generously supplied by Dr D.D. Bigner (Duke University) and Dr AJ Yates (Ohio State University), respectively. Both cell lines were originally isolated from astrocytic tumors that were designated as glioblastomas and their characteristics were described previously. Both lines were regularly determined to be free of mycoplasma with reagents from Gen-Probe, Inc. (San Diego, CA, USA). Cells were grown in minimal essential medium-alpha modification (MEM- $\alpha$ ) with 10% defined fetal bovine serum (Hyclone, Logan, UH). The cells were cultured to 100% confluence and passaged every 4–5 days in 4.8% CO<sub>2</sub>, 90% relative humidity. Prior to assays, cultures that were 80–100% confluent were in serum-free medium for 24 h.

### Retroviral expression

Retroviral delivery of genes allowed stable expression of PKC- $\eta$  constructs. Recombinant viruses were created by transfecting ecotropic Phoenix packaging cells, grown in DMEM supplemented with 10% FBS, with plasmid DNA carrying the gene of interest. Replication-defective retrovirus were harvested 48 h after transfection, sterile filtered to remove nonadherent producer cells, and used to infect U-1242 and U-251 human glioblastoma cells. Cells carrying the transgene were selected with media supplemented with 2  $\mu$ g/ml Blasticidin S 72 h postinfection. Finally, clones were assessed for expression by Western blotting (see below).

### Propidium iodide FACS analysis

Cell-cycle analysis of fixed cells was determined with propidium iodide staining. After treating serum-starved cells for 18 h, cells were rinsed two times in phosphate buffered saline (PBS). Cells were then fixed in 70% ethanol at  $-20^{\circ}\text{C}$  overnight. Following fixation, cells were centrifuged and resuspended in propidium iodide staining solution (0.1% Triton X-100, 0.2 mg/ml DNase-free RNase 0.02 mg/ml propidium iodide). Cell cycle was then analysed with a Becton Dickinson FACSVantage SE Turbo Sorter.

### $^3\text{H}$ -thymidine incorporation

Relative rates of DNA synthesis were assessed by determination of  $^3\text{H}$ -thymidine incorporation into trichloroacetic acid (TCA)-precipitable material. Serum-starved cells were treated with PMA and/or inhibitors, as indicated, for 24 h. Cells were then pulsed for 2 h with  $^3\text{H}$ -thymidine (2  $\mu\text{Ci/ml}$ ) and then washed with cold PBS. This was followed by 10 min washes with 10% TCA, first at  $4^{\circ}\text{C}$  and then at  $25^{\circ}\text{C}$ . Cells were then dissolved in 1 N NaOH and left overnight on a shaking platform. The samples were neutralized with an equivalent amount of 2 N HCL and placed in Ready-Safe scintillation fluid.  $^3\text{H}$ -thymidine incorporation was measured with a

Beckman Liquid scintillation counter. This assay was performed three times, with four replicates for each sample.

### Western blot analysis

For the detection of proteins, cells cultured for 24 h in serum-free conditions were first rinsed with ice-cold PBS (137 mM NaCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$  at pH 7.4) containing 0.2 mM sodium orthovanadate. PBS was then aspirated and cells were solubilized with 1.0% NP-40, 50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1  $\mu\text{g/ml}$  leupeptin, 2  $\mu\text{g/ml}$  aprotinin, 0.4 mg/ml sodium fluoride, 5 mg/ml dithiothreitol (DTT), and 0.2 mM sodium orthovanadate. The NP-40 extract was centrifuged at 14 000 g for 15 min. For each sample, 200  $\mu\text{g}$  of protein from the supernatant was then boiled for 5 min in sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) buffer and separated by SDS/PAGE on polyacrylamide slabs. The proteins were then electroblotted onto nitrocellulose-1 (GIBCO BRL) and reacted with polyclonal or monoclonal antibodies. The antibodies were detected with anti-rabbit or anti-mouse peroxidase conjugates and final detection was carried out with chemiluminescence enhancement (ECL, Amersham), as described by the manufacturer. All Western blots were performed at least three times, with representative blots displayed in the figures.

### Statistical analysis

Each FACS,  $^3\text{H}$ -thymidine, and Western blot assay was performed at least three times. A representative experiment is shown for each figure, while average changes and standard deviations from multiple experiments are mentioned in the text.

### Acknowledgements

We thank John B Schell and Alex M Ward for helpful advice on retrovirus construction. We also thank Janet V Cross and Stacey A Trotter for helpful discussions. This work was supported by Grant CA90851 (National Cancer Institute).

### References

- Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P and Hemmings BA. (1996). *EMBO J.*, **15**, 6541–6551.
- Aoki M, Blazek E and Vogt PK. (2001). *Proc. Natl. Acad. Sci. USA*, **98**, 136–141.
- Basu A. (1993). *Pharmacol. Ther.*, **59**, 257–280.
- Benzil DL, Finkelstein SD, Epstein MH and Finch PW. (1992). *Cancer Res.*, **52**, 2951–2956.
- Blobe GC, Obeid LM and Hannun YA. (1994). *Cancer Metast. Rev.*, **13**, 411–431.
- Bogler O, Wren D, Barnett SC, Land H and Noble M. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 6368–6372.
- Bromberg JF, Wizeszczynska MH, Devgan G, Zhao Y, Pestell RG and Albanese C Darnell Jr JE. (1999). *Cell*, **98**, 295–303.
- Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, Lawrence Jr JC and Abraham RT. (1997). *Science*, **277**, 99–101.
- Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS and Jove R. (1999). *Immunity*, **10**, 105–115.
- Choe Y, Jung H, Khang I and Kim K. (2003). *J Neuroendocrinol.*, **15**, 508–515.
- Couldwell WT, Uhm JH, Antel JP and Yong VW. (1991). *Neurosurgery*, **29**, 880–887.
- Ekstrand AJ, James CD, Cavenee WK, Seliger B, Pettersson RF and Collins VP. (1991). *Cancer Res.*, **51**, 2164–2172.
- Eldar H, Zisman Y, Ullrich A and Livneh E. (1990). *J. Biol. Chem.*, **265**, 13290–13296.
- Fadden P, Haystead TA and Lawrence Jr JC. (1997). *J. Biol. Chem.*, **272**, 10240–10247.
- Fima E, Shtutman M, Libros P, Missel A, Shahaf G, Kahana G and Livneh E. (2001). *Oncogene*, **20**, 6794–6804.
- Fingar DC, Salama S, Tsou C, Harlow E and Blenis J. (2002). *Genes Dev.*, **16**, 1472–1487.
- Frey MR, Saxon ML, Zhao X, Rollins A, Evans SS and Black JD. (1997). *J. Biol. Chem.*, **272**, 9424–9435.
- Fujisawa H, Kurer M, Reis RM, Yonekawa Y, Kelihues P and Ohgaki H. (1999). *Am. J. Pathol.*, **155**, 387–394.
- Garcia R and Jove R. (1998). *J. Biomed. Sci.*, **5**, 79–85.
- Gescher A. (1992). *Br. J. Cancer*, **66**, 10–19.
- Gingras AC, Kennedy SG, O'Leary MA, Sonenberg N and Hay N. (1998). *Genes Dev.*, **12**, 502–513.
- Gruber JR, Ohno S and Niles RM. (1992). *J. Biol. Chem.*, **267**, 13356–13360.
- Guha A, Dashner K, Black PM, Wagner JA and Stiles CD. (1995). *Int. J. Cancer*, **60**, 168–173.
- Guizzetti M, Wei M and Costa LG. (1998). *Eur. J. Pharmacol.*, **359**, 223–233.

- Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C and Avruch J. (1998). *J. Biol. Chem.*, **273**, 14484–14494.
- Hentges KE, Sirry B, Gingeras AC, Sarbassov D, Sonenberg N, Sabatini D and Peterson AS. (2001). *Proc. Natl. Acad. Sci. USA*, **98**, 13796–13801.
- Hesselager AG, Uhrbom L, Westermark B and Nister M. (2003). *Cancer Res.*, **63**, 4305–4309.
- Holland EC, Celestino J, Dai C, Schaefer L, Sawaya RR and Fuller GN. (2000). *Nat. Genet.*, **25**, 55–57.
- Hussaini IM, Karns LR, Vinton G, Carpenter JE, Redpath GT, Sando JJ and VandenBerg SR. (2000). *J. Biol. Chem.*, **275**, 22348–22354.
- Jiang BH, Aoki M, Zheng JZ, Li J and Vogt PK. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 2077–2081.
- Kashiwagi M, Ohba M, Watanabe H, Ishino K, Kasahara K, Sanai Y, Taya Y and Kuroki T. (2000). *Oncogene*, **19**, 6334–6341.
- Kijima T, Niwa H, Steinman RA, Drenning SD, Gooding WE, Wentzel AL, Xi S and Grandis JR. (2002). *Cell Growth Differ.*, **13**, 355–362.
- Kimball SR, Shantz LM, Horetsky RL and Jefferson LS. (1999). *J. Biol. Chem.*, **274**, 11647–11652.
- Kindregan HC, Rosenbaum SE, Ohno S and Niles RN. (1994). *J. Biol. Chem.*, **269**, 27756–27761.
- Konopka G and Bonni A. (2003). *Curr. Mol. Med.*, **3**, 73–84.
- Kozak M. (1991). *J. Biol. Chem.*, **266**, 19867–19870.
- Krasagakis K, Lindschau C, Fimmel S, Eberle J, Quass P, Haller H and Orfanos CE. (2004). *J. Cell Physiol.*, **199**, 381–387.
- Livneh E, Shimon T, Bechor E, Doki Y, Schieren E and Weinstein IB. (1996). *Oncogene*, **12**, 1545–1555.
- Mayer M, Bhakoo K and Noble M. (1994). *Development*, **120**, 143–153.
- Mayer M, Bogler O and Noble M. (1993). *Glia*, **8**, 12–19.
- McKinnon RD, Smith C, Behar T, Smith T and Dubois-Dalcq M. (1990). *Glia*, **7**, 245–254.
- Molkentin JD and Dorn GW. (2001). *Ann. Rev. Physiol.*, **63**, 391–426.
- Nakaigawa N, Hirai S, Mizuno K, Shuin T, Hosaka M and Ohno S. (1996). *Biochem. Biophys. Res. Commun.*, **222**, 95–100.
- Nave BT, Ouwens M, Withers DJ, Alessi DR and Shepherd PR. (1999). *Biochem. J.*, **1**, 427–431.
- Nishizuka Y. (1984). *Nature*, **308**, 693–698.
- Nishizuka Y. (1988). *Nature*, **334**, 661–665.
- Nishizuka Y. (1995). *FASEB J.*, **9**, 484–496.
- Ohba M, Ishino K, Kashiwagi M, Kawabe S, Chida K, Huh N and Kuroki T. (1998). *Mol. Cell. Biol.*, **18**, 5199–5207.
- Okada Y. (2000). *Verh. Dtsch. Ges. Pathol.*, **84**, 33–42.
- Patti ME, Brambilla E, Luzi L, Landaker EJ and Kahn CR. (1998). *J. Clin. Invest.*, **101**, 1519–1529.
- Pause A, Methot N, Svitkin Y, Merrick WC and Sonenberg N. (1994). *EMBO J.*, **13**, 1205–1215.
- Rajan P and McKay RD. (1998). *J. Neurosci.*, **18**, 3620–3629.
- Resnick MS, Luo X, Vinton G and Sando JJ. (1997). *Cancer Res.*, **57**, 2209–2215.
- Rolli-Derkinderen M, Machavoine F, Baraban JM, Grolleau A, Beretta L and Dy M. (2003). *J. Biol. Chem.*, **278**, 18859–18867.
- Rooprai HK, Vanmeter T, Panou C, Schnull S, Trillo-Pazos G, Davies D and Pilkington GJ. (1999). *Int. J. Dev. Neurosci.*, **(5–6)**, 613–623.
- Sano T, Lin H, Chen X, Langford LA, Koul D, Bondy ML, Hess KR, Myers JN, Hong YK, Yung WK and Steck PA. (1999). *Cancer Res.*, **59**, 1820–1824.
- Schalm SS, Fingar DC, Sabatini DM and Blenis J. (2003). *Curr. Biol.*, **13**, 797–806.
- Todo T, Shitara N, Nakamura H, Takakura K and Ikeda K. (1991). *Neurosurgery*, **108**, 11–16.
- Wang X, Campbell LE, Miller CM and Proud GC. (1998). *Biochem. J.*, **183**, 261–267.
- Ways DK, Kukoly CA, deVente J, Hooker JL, Bryant WO, Posekany KJ, Fletcher DJ, Cook PP and Parker PJ. (1995). *J. Clin. Invest.*, **95**, 1906–1915.
- Weis J, Schonrock LM, Zuchner SL, Lie DC, Sure U, Schul C, Stogbauer F, Ringelstein EB and Halfter H. (1999). *J. Neurooncol.*, **44**, 243–253.
- Welsh GI, Miller CM, Loughlin AJ, Price NT and Proud CG. (1998). *FEBS Lett.*, **421**, 125–130.
- Wiederrecht GJ, Sabers CJ, Brunn GJ, Martin MM, Dumont FJ and Abraham RT. (1995). *Prog. Cell Cycle Res.*, **1**, 53–71.
- Yamaguchi K, Ogita K, Nakamura S and Nishizuka Y. (1995). *Biochem. Biophys. Res.*, **210**, 639–647.
- Yamamoto M, Ueno Y, Hayashi S and Fukushima T. (2002). *Anticancer Res.*, **6C**, 4265–4268.
- Yokogami K, Wakisaka S, Avruch J and Reeves SA. (2000). *Curr. Biol.*, **10**, 47–50.