Phosphoinositide 3-kinase-γ induces *Xenopus* oocyte maturation via lipid kinase activity

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Type-I phosphoinositide 3-kinases (PI3Ks) were characterized as a group of intracellular signalling proteins expressing both protein and lipid kinase activities. Recent studies implicate PI3Ks as mediators of oocyte maturation, but the molecular mechanisms are poorly defined. Here we used the *Xenopus* oocyte expression system as a model to investigate a possible contribution of the γ-isoform of PI3K (PI3Kγ) in the different pathways leading to cell-cycle progression by monitoring the time course of germinal vesicle breakdown (GVBD). Expression of a constitutive active PI3Kγ (PI3Kγ-CAAX) induced GVBD and increased the levels of phosphorylated Akt/protein kinase B and mitogen-activated protein kinase (MAPK). Furthermore, PI3Kγ-CAAX accelerated progesterone-induced GVBD, but had no effect on GVBD induced by insulin. The effects of PI3Kγ-CAAX could be suppressed by pre-incubation of the oocytes with LY294002, PD98059 or roscovitine, inhibitors of PI3K, MEK (MAPK/extracellular-signal-regulated protein kinase kinase) and cdc2/cyclin B kinase, respectively. Mutants of PI3Kγ-CAAX, in which either lipid kinase or both lipid and protein kinase activities were altered or eliminated, did not induce significant GVBD. Our data demonstrate that expression of PI3Kγ in *Xenopus* oocytes accelerates their progesterone-induced maturation and that lipid kinase activity is required to induce this effect.

Key words: cell cycle, germinal vesicle breakdown, PI 3-kinase-γ, progesterone.

INTRODUCTION

Xenopus oocytes are a widely used model system for the study of the cell cycle, development and signal transduction. Fully grown stage V and VI *Xenopus* oocytes are arrested at the G_2 stage of the first meiotic prophase [1,2]. The physiological trigger for maturation of oocytes is the steroid hormone progesterone, which initiates a multi-step signalling cascade involving a decrease in cytosolic cAMP levels, activation of mitogen-activated protein kinase (MAPK) and subsequent germinal vesicle breakdown (GVBD). A distinct pathway leading to MAPK activation and GVBD can be stimulated by insulin or insulin-like growth factor (IGF) acting on the IGF-1 receptor [3,4]. Since only external application of progesterone induced maturation, but injection did not, it was proposed that the putative oocyte progesterone receptor (PR) is a membrane-bound surface receptor [2,5]. However, two recent reports describe the cloning of an amphibian homologue of the human PR [6,7]. They present new evidence suggesting that the oocyte response to progesterone might be mediated by a cytosolic or nuclear PR, not associated with the plasma membrane and functioning in a novel, non-genomic fashion.

Although the complex mechanisms of maturation in *Xenopus* oocytes have been studied extensively (reviewed in [5]), many of the initial steps and downstream events occurring after progesterone or insulin stimulation remain to be elucidated. Previously the activation of phosphoinositide 3-kinases (PI3Ks) was implicated as a critical step in insulin-induced mitogenic signalling in *Xenopus* oocytes [4,8–10]. The PI3Ks are a large family of enzymes that catalyse the phosphorylation of phosphoinositides at their D3 position of the inositol ring. Subgroup I of the PI3Ks was shown to phosphorylate all phosphoinositides under *in vitro* conditions, but their major *in vivo* product is phosphatidylinositol 3,4,5-trisphosphate (PIP_3). In addition to their lipid kinase activity, all four species (α, β, γ and δ) of type-I PI3K also express *in vitro* protein kinase activity [11–14]. The isoforms best characterized are PI3Kα and PI3Kβ (class IA), which both form heterodimers with a regulatory adapter protein (p85). They are regulated via receptors with either an intrinsic or a receptor-associated tyrosine kinase activity (see [15]). The isoform PI3Kγ (class IB) can be activated by G-protein $\beta\gamma$ subunits (G$\beta\gamma$ subunits) [16] and was found to associate with a novel 101 kDa protein (p101). This regulatory subunit does not share any functional homology with any other known proteins [17]. Interestingly, when expressed in COS-7 cells, PI3Kγ seems to utilize the two different enzymic activities to initiate two separate downstream signals. The lipid product PIP_3 activates the protein Ser/Thr kinase Akt/protein kinase B (PKB) [18] and the protein kinase activity participates in the stimulation of MAPK [12].

Previous reports revealed that resumption of meiosis in *Xenopus* oocytes induced by insulin, but not progesterone, can be blocked by the PI3K inhibitors wortmannin and LY294002 [4,10]. Klippel et al. [9] described a constitutive active p85-binding PI3K that is targeted to the membrane and which induced GVBD by itself. Several lines of evidence also suggest Ras proteins as essential components of insulin-induced oocyte maturation [10] and place Ras downstream of PI3K [19]. A constitutive active protein Ser/Thr kinase Akt/PKB, which itself

Abbreviations used: GVBD, germinal vesicle breakdown; MAPK, mitogen-activated protein kinase; P-MAPK, phosphorylated MAPK; MEK, MAPK/extracellular-signal-regulated protein kinase kinase; MPF, mitosis-promoting factor; PI3K, phosphoinositide 3-kinase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PR, progesterone receptor; xPR, amphibian PR; PKB, protein kinase B; P-Akt, phosphorylated Akt/PKB; G$\beta\gamma$ subunits, G-protein $\beta\gamma$ subunits; SIP, signalling inositol phosphatase; SHIP, SH_2-containing inositol phosphatase; FRAP, FKBP12-rapamycin-associated protein.

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can be activated by class-I PI3Ks [18], stimulated GVBD to an extent similar to progesterone or insulin [20]. Signalling inositol phosphatase/SH_2-containing inositol phosphatase (SIP/SHIP) was also implicated as a regulator of PI3K-induced *Xenopus* oocyte maturation [8]. Since SIP markedly reduced the level of PIP_3, it was concluded that the generation of PIP_3 is a necessary requirement for induction of GVBD in *Xenopus* oocytes. However, all previous work is with regard to the role of class-IA PI3K isoforms in the signalling pathway induced by insulin, although it is not clear yet if insulin plays a physiological role in *Xenopus* oocyte maturation *in vivo*.

Since an increase in MAPK activity, i.e. an increase in the level of phosphorylated MAPK, is required for *Xenopus* oocyte maturation [21], we investigated a possible involvement of the different kinase activities of PI3Kγ in cell-cycle progression in *Xenopus* oocytes. How PI3Kγ is translocated to the membrane *in vivo* is still under investigation. G$\beta\gamma$ subunits were proposed to activate PI3Kγ by allosteric interaction and/or by recruitment to the membrane [22], and association of PI3Kγ with p101 was shown to enhance the sensitivity to G$\beta\gamma$ [17]. It is not clear yet if *Xenopus* oocytes possess the regulatory subunit p101 and whether this protein is required for proper function and/or activation of PI3Kγ. Given these uncertainties regarding the activation mechanism, we used constitutive active PI3Kγ mutants (PI3Kγ-CAAX). The CAAX motif permanently attaches the enzyme to the membrane and thereby places it in close proximity to its lipid substrates. This approach circumvents co-expression of the regulatory subunit p101 and the use of agonists to stimulate G-protein-coupled pathways to activate PI3Kγ, which might trigger other signalling cascades and thus may interfere with PI3Kγ activation.

In the present study we demonstrate that expression of PI3Kγ-CAAX increased the levels of phosphorylated MAPK (P-MAPK) as well as phosphorylated Akt/PKB (P-Akt) and induced GVBD in *Xenopus* oocytes. In addition, we show that PI3Kγ-CAAX accelerated the effect of progesterone on *Xenopus* oocyte maturation, but had no effect on the insulin-induced response. Since various PI3Kγ mutants, with deficient lipid kinase activity, were not effective, we conclude that stimulation of oocyte maturation by PI3Kγ-CAAX is most likely mediated by an increase in the level of PIP_3.

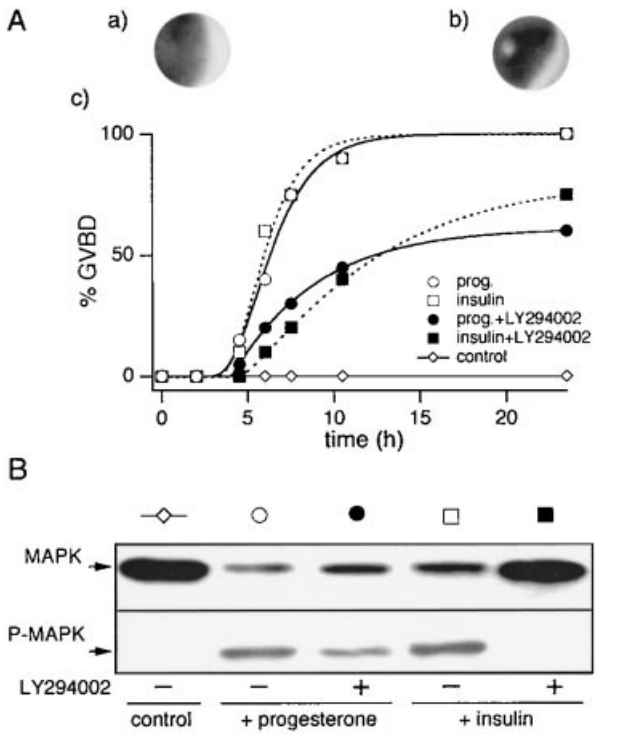

Figure 1 LY294002 inhibits GVBD induced by progesterone or insulin in *Xenopus* oocytes

(**A**) Panel a: immature stage-VI oocyte under control conditions. Panel b: mature oocyte after treatment with progesterone; the start of GVBD is indicated by the white spot on the dark animal pole. Panel c: time course of GVBD in different groups of uninjected oocytes treated with either progesterone (○, ●) or insulin (□, ■). Subgroups of oocytes were incubated with 20 μM LY294002 prior to and during stimulation (●, ■). The superimposed curves indicate fits according to eqn (1) to estimate the time of 50% GVBD (half-time), which in this case were 6.3 and 5.9 h in the presence of progesterone (solid line) and insulin (dashed line) respectively. Treatment with LY294002 increased the respective half-times to 12.4 and 12.6 h. (**B**) At the end of GVBD measurements (after 24 h), all oocytes in each group from (**A**) were collected, lysed and MAPK and P-MAPK assayed. No P-MAPK could be detected in untreated control oocytes (◇), although this group showed the highest level of MAPK. LY294002 caused a modest decrease in P-MAPK in the progesterone-stimulated group (●) and a complete disappearance of P-MAPK in the insulin-stimulated group (■). 20 oocytes were pooled for each group.

EXPERIMENTAL

Expression of PI3Kγ mutants

Engineering of chimaeric mutants of PI3Kγ (accession number X83368) with modified substrate specificity was described in detail previously [12]. In brief, a short region within the conserved catalytic domain of PI3Kγ was replaced by synthetic oligonucleotides corresponding to the sequences of PI3Ks of class II [cpk, which phosphorylate only phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate] and class-IV [FKBP12-rapamycin-associated protein (FRAP), lipid kinase-inactive member of the target-of-rapamycin family]. The protein kinase activity was unaffected in these two hybrid proteins. The point mutation K832R yielded a kinase-deficient PI3Kγ without protein or lipid kinase activities. For permanent membrane attachment, PI3Kγ was extended by a C-terminal isoprenylation signal of K-Ras (CAAX box). cDNAs coding for wild-type PI3Kγ, the membrane-localized PI3Kγ-CAAX, the substrate mutants PI3Kγ-cpk-CAAX and PI3Kγ-FRAP-CAAX and the kinase-deficient mutant PI3Kγ-K832R-CAAX were subcloned in the *Bam*HI/*Xba*I sites of the oocyte expression vector pGEM-HE (a kind gift from J. Tytgat, K. U. Leuven, Leuven, Belgium).

Oocyte preparation

Stage-V and -VI oocytes from *Xenopus laevis* (Figure 1A, panel a) were obtained surgically after anaesthetizing the animals with tricaine in ice-cold water. The follicular layer was removed manually after treatment with collagenase (3.1 mg/ml; Worthington Biochemical Corp.). Oocytes were kept in Barth medium containing 80 mM NaCl, 10 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris/HCl and 50 μg/ml penicillin/streptomycin, pH 7.4. In all experiments, equal amounts (50 nl) of either water or *in vitro*-transcribed mRNA (50–70 ng) were injected into each oocyte. The PI3K inhibitor LY294002 as well as the MEK (MAPK/extracellular-signal-regulated protein kinase kinase) inhibitor PD98059 (both from Calbiochem) were added immediately after the injection. For the experiment with the cdc2/cyclin B kinase inhibitor roscovitine (Sigma), oocytes were incubated overnight (12–15 h) before injection and during the entire experiment. Oocytes were placed in prepared 1%-agar-filled dishes with small holes in the agar to hold the oocytes in place. The cells were incubated at room temperature (20–25 °C) for the whole ex-

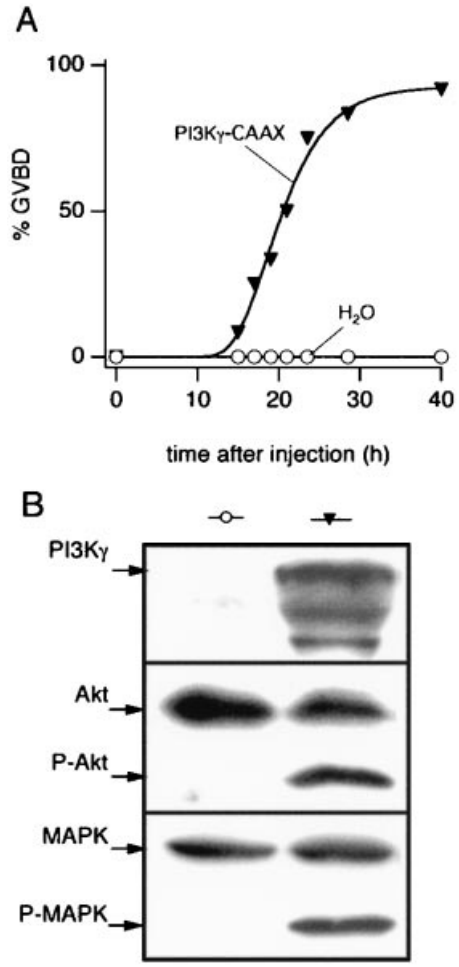

Figure 2 PI3Kγ-CAAX induces GVBD

(**A**) Time courses of GVBD in a sample batch of oocytes injected with either water (○) or mRNA coding for PI3Kγ-CAAX (▼). The estimated half-time of GVBD in this experiment was 20.5 h. (**B**) Immunoblots of expressed PI3Kγ (top panel), endogenous Akt and P-Akt (middle panel) and MAPK and P-MAPK (bottom panel) in the absence (○) or presence (▼) of PI3Kγ-CAAX in the oocytes from (**A**).

periment. To allow for protein synthesis, oocytes were kept for 12–22 h after mRNA injection before stimulation with either 15 μM progesterone or 8 μM insulin (Sigma) was performed.

Meiotic maturation

During maturation, the large oocyte nucleus (germinal vesicle), which resides in the darkly pigmented animal part of the oocyte, falls apart, a process called GVBD. Since GVBD correlates with the appearance of a white spot on the animal pole (Figure 1A, panel b), resumption of meiosis was measured by scoring the percentage of oocytes showing this spot at different time points after injection or stimulation. The total number of oocytes in each group (12–24) was considered as 100%. For statistical analysis of different batches of oocytes, we estimated the time required for the oocytes to undergo half-maximal GVBD by describing the time course of GVBD with the following equation:

$$\begin{aligned} \text{GVBD}\ (\%) &= 0 & |t \leqslant \Delta t \\ &= 100 \cdot \{1 - \exp[-(t-\Delta t)/\tau]\}^n & |t > \Delta t \end{aligned} \qquad (1)$$

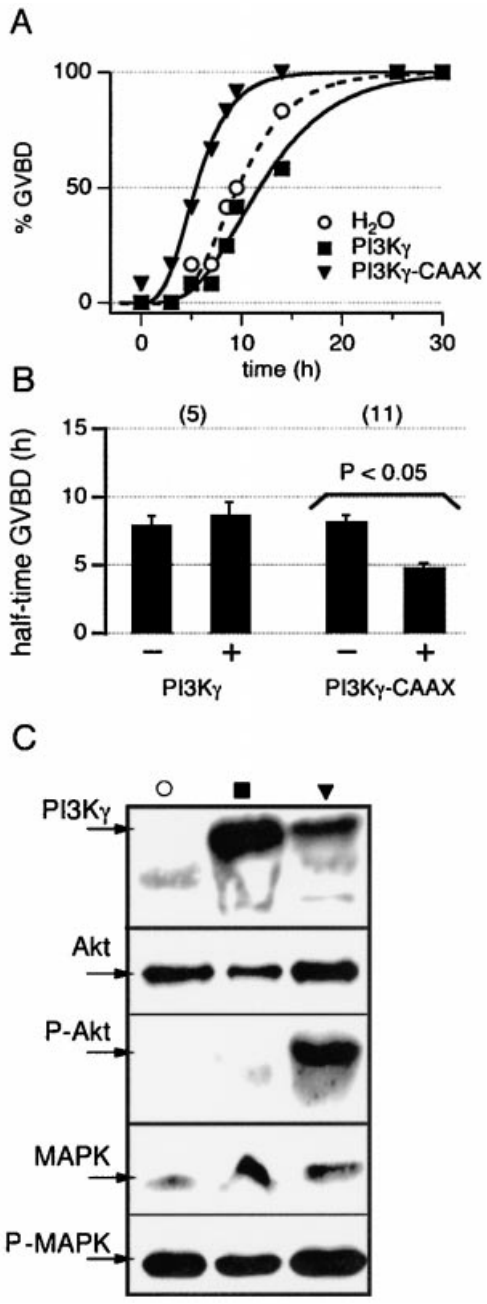

Figure 3 PI3Kγ-CAAX accelerates the progesterone-induced signalling pathway

(**A**) Time courses of GVBD in a sample oocyte batch. Groups of oocytes were injected with either water (○) or mRNA coding for PI3Kγ (■) or PI3Kγ-CAAX (▼) and stimulated with progesterone approx. 16 h after injection (time 0 h). Compared with the water-injected controls, PI3Kγ-CAAX reduced the half-time of GVBD after stimulation with progesterone from 9.5 to 5.4 h, whereas PI3Kγ slightly increased the half-time, to 11.8 h. (**B**) Mean estimated half-times of GVBD from fits to eqn (1) in independent batches of oocytes stimulated with progesterone in the absence (−) or presence (+) of the expression of PI3Kγ or PI3Kγ-CAAX. The half-times are expressed as the time delay after the start (time 0 h) of hormone stimulation. Data are represented as means ± S.E.M.; the numbers in parentheses above the bars indicate the total number of batches for each group. A significant difference between two groups is shown (two-tailed paired Student's t test). (**C**) Immunoblots of expressed PI3Kγ (top panel), endogenous Akt and P-Akt (middle panels) and MAPK and P-MAPK (bottom panels) for the three different groups of oocytes from (**A**).

with a time delay, Δt, a time constant, τ, and an exponent, n. The half-time of GVBD was determined as the time, t, at which eqn (1) reached 50%. Statistical significance was evaluated with a paired two-tailed Student's t test, and was set at $P < 0.05$.

Western-blot analysis of protein expression

In one set of experiments oocytes were collected immediately after the appearance of the white spot and kept at −80 °C until the end of the GVBD analysis (24–48 h), when all oocytes (independent of the appearance of the white spot) within each injection group and/or treatment were pooled together. For the experiments shown in Figure 1, all oocytes were collected 24 h after stimulation. For analysis of protein expression, equal numbers of oocytes for each injection (12–24) were lysed by vigorous pipetting in 10 μl of lysis buffer/oocyte and centrifuged for 15 min at 4 °C and 20000 ***g***. The lysis buffer contained 20 mM Tris/HCl, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 15 mM $MgCl_2$, 1 mM PMSF, 0.15 unit/ml aprotonin and 20 μM leupeptin [8]. The clear supernatant was transferred to Eppendorf tubes and 10 μl was used for each group. Expression of PI3Kγ was probed with a monoclonal antibody against human PI3Kγ (Alexis Corp., San Diego, CA, U.S.A.). The level of expression of all PI3Kγ-CAAX mutants was nearly identical, but in all batches it was lower than the amount detected for PI3Kγ without the CAAX box (see Figure 3B, below).

In a search for endogenous PI3Kγ we tested five different commercially available goat and rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) mapping different regions of PI3Kγ. P-MAPK was assayed with a polyclonal antibody against the two MAPK isoforms, P-Erk1 and P-Erk2 (also known as p44 and p42; New England Biolabs, Beverly, MA, U.S.A.). However, *Xenopus* oocytes express only Erk2 [5]. For measurement of the amount of P-Akt we used the monoclonal antibody Phosho-Akt (Ser-473) from New England Biolabs. Akt was assayed with a monoclonal antibody from BD PharMingen (San Diego, CA, U.S.A.) and MAPK was assayed with a monoclonal antibody from BD Transduction Laboratories (Lexington, KY, U.S.A.).

RESULTS

There are at least two different pathways leading to GVBD in *Xenopus* oocytes (for a review, see [2,5]). Progesterone, possibly acting on a recently cloned amphibian PR (xPR) [6,7], and insulin, stimulating the IR-1 receptor [3], cause the activation of MAPK and subsequent breakdown of the large oocyte nucleus (Figure 1A, panel b). In a first approach, we tested the possible involvement of endogenous PI3Ks in oocyte maturation by use of specific inhibitors.

Effect of PI3K inhibitors on GVBD induced by progesterone and insulin

Both LY294002 and wortmannin are selective inhibitors of PI3Ks and are used widely for the study of signal transduction in different systems. However, pretreatment of oocytes with wortmannin already induced GVBD by itself in the absence of either progesterone or insulin (results not shown). Similar results were reported recently by Carnero and Lacal [23]. Pre-incubation of oocytes with LY294002 had no effect on GVBD in the absence of stimulation, but impeded maturation induced by both progesterone and insulin (Figure 1A, panel c). For both agonists, the estimated half-times of GVBD were about 6 h in the absence and 12 h in the presence of 20 μM LY294002. In contrast to another report [10], the inhibitory effect of LY294002 was similar for the progesterone- and the insulin-induced GVBD (Figure 1A, panel c). At the end of the experiment (24 h), all oocytes for each group were collected, lysed and MAPK as well as P-MAPK assays performed (Figure 1B). Endogenous MAPK was present in all groups (Figure 1B, top panel), but only in the presence of progesterone or insulin could P-MAPK be detected (Figure 1B, bottom panel). LY294002 reduced the phosphorylation of MAPK for both stimulatory agents, but its effect was more pronounced in the oocytes stimulated with insulin, where no P-MAPK could be detected. In contrast, LY294002 caused only a moderate reduction in the level of P-MAPK after progesterone stimulation (compare LY294002 treatments in Figure 1B). Similar results were found in three independent batches of oocytes (not shown). On the average, incubation with LY294002 increased the half-times of GVBD 2.1-fold and 2.3-fold after treatment with progesterone and insulin, respectively.

PI3Kγ-CAAX stimulates *Xenopus* oocyte maturation

To test whether PI3Kγ is involved in oocyte maturation, we injected mRNA coding for PI3Kγ-CAAX and monitored the time course of GVBD. Expression of PI3Kγ-CAAX induced GVBD in the absence of hormone stimulation (Figure 2A). Figure 2(B) shows Western-blot analysis of the oocytes from Figure 2(A) using a monoclonal antibody raised against PI3Kγ. Expression of PI3Kγ could only be detected in oocytes injected with PI3Kγ mRNA (Figure 2B, top panel). We also tested five commercially available polyclonal antibodies directed against different regions of PI3Kγ, but could not detect any endogenous PI3Kγ in 10 independent batches of oocytes (results not shown). In contrast with the water-injected controls, expression of PI3Kγ-CAAX induced a substantial increase in the level of P-Akt, which itself becomes activated through a downstream product of PI3Ks (PIP_3) as well as the level of P-MAPK, a prerequisite for oocyte maturation (Figure 2B). The levels of endogenous Akt were similar for the two injections, as were those of endogenous MAPK (Figure 2B). Compared with the progesterone- and insulin-stimulated groups of oocytes (Figure 1), the time course of GVBD induced by PI3Kγ-CAAX alone was slower, but eventually reached 100% in 10 out of 13 batches tested. The estimated half-times of GVBD showed large variation between batches, ranging from 14.8 h to 42.1 h after injection. The mean half-time was 26.9 ± 2.2 h ($n = 13$). Expression of wild-type PI3Kγ without the membrane anchor induced some GVBD in only 2 out of 6 batches tested; the respective half-times were 47 h and 38 h, but maturation did not reach 100% even after an extended period of expression.

Acceleration of progesterone-induced maturation by PI3Kγ-CAAX

Since progesterone is the physiological trigger for maturation in *Xenopus* oocytes, we next addressed the question of whether this signalling pathway is influenced by the expression of PI3Kγ. To test this hypothesis, we injected oocytes with either water or mRNA coding for wild-type PI3Kγ or PI3Kγ-CAAX and monitored the time course of GVBD (Figure 3). About 16 h later, to allow for protein expression, injection was followed by treatment with progesterone. Expression of PI3Kγ-CAAX enhanced GVBD that was induced by progesterone compared with the water-injected controls (Figure 3A). The results from several independent batches of oocytes are summarized in Figure 3(B). Although the half-time values varied between 5.9 h and 11.2 h for control groups, expression of PI3Kγ-CAAX reduced the value in 10 out of 11 batches tested. The average shift in

the estimated half-times was 3.4 ± 0.6 h or, in other words, the presence of PI3Kγ-CAAX reduced the required time for 50% maturation to 60 ± 6% ($n = 11$). Expression of PI3Kγ had no significant effect on the time course of GVBD induced by progesterone. After injection of equal amounts of mRNA, PI3Kγ was expressed at a high level in both injected groups (Figure 3C, top panel), with the highest amount found in the oocytes injected with PI3Kγ. This rules out the possibility that the lack of effect of PI3Kγ is due to impaired expression of the protein. Compared with the water-injected control group, only expression of PI3Kγ-CAAX induced a substantial increase in the amount of P-Akt (Figure 3C, middle panel). Progesterone application strongly phosphorylated MAPK in all groups of oocytes (Figure 3C, bottom panel). Again, the absolute levels of endogenous Akt and MAPK were unaffected by the injections (Figure 3C).

Inhibition of PI3Kγ-CAAX-induced GVBD by PD98059 and roscovitine

The γ isoform of PI3K was shown to activate MAPK in COS-7 cells [22]. Activation of both MAPK and mitosis-promoting factor (MPF) are prerequisites for maturation in *Xenopus* oocytes. MPF is a protein kinase composed of the catalytic subunit cdc2 and the regulatory subunit cyclin B [5,21,24]. To clarify the involvement of these kinases in GVBD induced by PI3Kγ-CAAX and progesterone, we incubated different groups of injected oocytes with three different concentrations (10, 30 and 100 μM) of either PD98059, an inhibitor of the MAPK precursor MEK, or roscovitine, an inhibitor of cdc2 kinase. Oocytes were injected with water or mRNA coding for PI3Kγ-CAAX and incubated with control solution, PD98059 or roscovitine directly after injection. Both inhibitors were present during the entire course of the experiment. While 10 μM PD98059 only slightly delayed GVBD induced by expression of PI3Kγ-CAAX, 30 and 100 μM PD98059 reduced the total amount of GVBD to less than 50 and 20%, respectively (Figure 4A). Roscovitine was effective at the lowest concentration tested, 10 μM. Increasing concentrations did not induce a much stronger effect (Figure 4B). Neither inhibitor had any effect on the levels of endogenous P-Akt (Figure 4C, middle panel). However, P-MAPK levels were reduced at the lowest inhibitor concentration (10 μM), and P-MAPK was abolished completely in the presence of 30 and 100 μM PD98059 or roscovitine. Thus inhibition of MAPK phosphorylation by PD98059 correlates well with the inhibition of GVBD. The effect of roscovitine, however, is not necessarily directly linked to the block of cdc2 kinase, as it also potently inhibited phosphorylation of MAPK.

Mutations altering the substrate specificity of PI3Kγ

To further determine which kind of PI3Kγ activity (protein or lipid kinase activity) is necessary for the induction of GVBD, mutants of PI3Kγ with a PI3Kγ-CAAX background [12] were employed. For these experiments equal amounts of mRNA were always injected. Figures 5(A) and 5(B) summarize for all experiments the effect of such mutants on the rate of GVBD in the absence of hormone (Figure 5A) and following incubation with progesterone (Figure 5B). Only expression of PI3Kγ-CAAX induced GVBD in the absence of progesterone; 30 h after injection, more than 50% of the oocytes had started to mature (Figure 5A). PI3Kγ without the membrane anchor was much less effective. The PI3Kγ-FRAP-CAAX mutant, with protein but no lipid kinase activity, also had no significant effect on GVBD alone and did not alter the kinetics of progesterone-induced GVBD. Mutant PI3Kγ-K832R-CAAX, with no kinase activity at all, neither had an effect on GVBD alone nor altered the

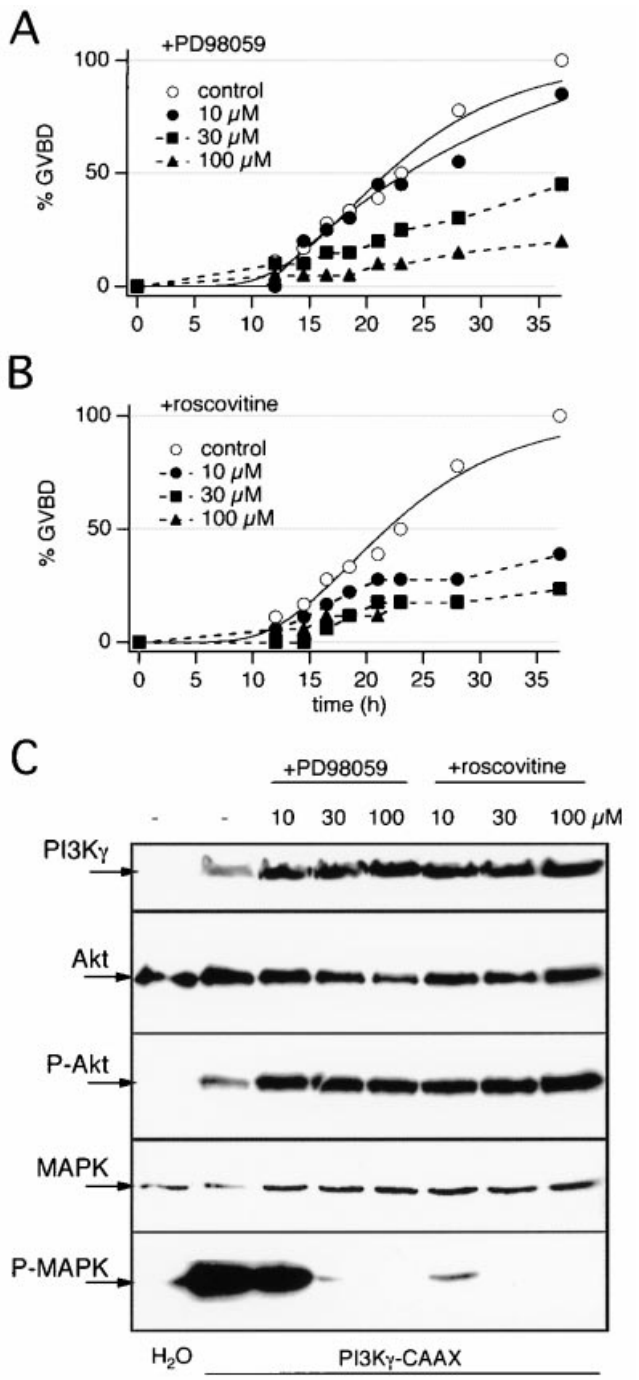

Figure 4 Effect of inhibitors of MAPK and cdc2 kinase on GVBD induced by PI3Kγ-CAAX

(**A**, **B**) Time courses of GVBD in a sample oocyte batch. Oocytes were injected with mRNA coding for PI3Kγ-CAAX and incubated with either control Barth medium (○) or the indicated concentrations of PD98059 (**A**) or roscovitine (**B**) directly after injection and during the entire experiment. The superimposed solid curves indicate fits according to eqn (1). (**A**) The estimated half-time of GVBD (time after injection) was 21.8 h for PI3Kγ-CAAX alone and 23.5 h in the presence of 10 μM PD98059. Incubation with either 30 or 100 μM reduced the total amount of GVBD to less than 50%. (**B**) Incubation of oocytes with 10, 30 or 100 μM roscovitine reduced the total amount of GVBD to less than 40%. Injection of water did not induce any GVBD (results not shown). For ease of comparison, the fitted curve for control-incubated PI3Kγ-CAAX is shown plotted again in (**B**). (**C**) Immunoblots of expressed PI3Kγ (top panel), endogenous Akt and P-Akt (middle panels) and MAPK and P-MAPK (bottom panels) for eight different groups of oocytes from (**A**) and (**B**). 18–20 oocytes were pooled for each group.

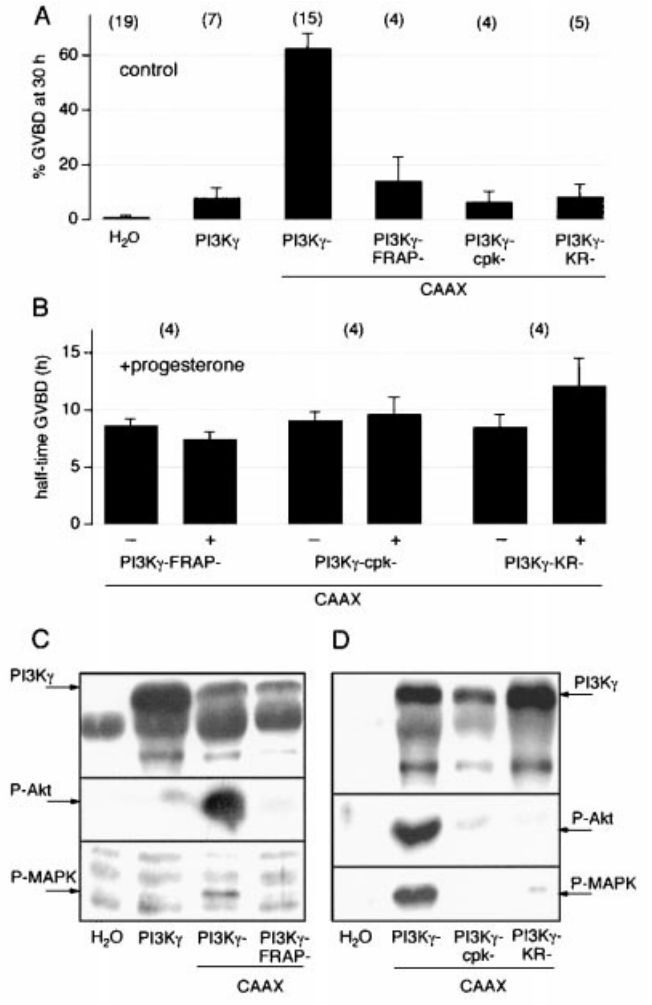

Figure 5 Effect of PI3Kγ-CAAX substrate mutants on GVBD with and without progesterone

(**A**, **B**) Summary of all groups injected with either water or mRNA coding for the PI3Kγ mutants, as indicated below the graphs. Total numbers of batches for each group are shown in parentheses. (**A**) Percentage of GVBD 30 h after injection in the absence of hormone. (**B**) Mean half-times for experiments on oocytes injected with the indicated PI3Kγ mutant mRNAs (+) or water (−) following stimulation with progesterone. (**C**, **D**) Immunoblots of PI3Kγ (top panels), P-Akt (middle panels) and P-MAPK (bottom panels) from two independent sample batches of oocytes, in the absence of progesterone, injected with water or PI3Kγ mutant mRNAs as indicated.

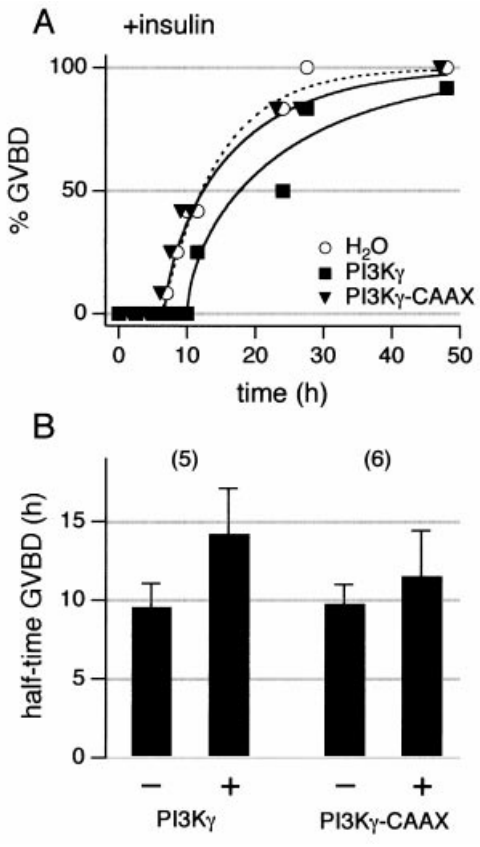

Figure 6 PI3Kγ-CAAX has no effect on GVBD induced by insulin

(**A**) Time courses of GVBD in a sample oocyte batch. Groups of oocytes were injected with either water (○), or mRNA coding for PI3Kγ (■) or PI3Kγ-CAAX (▼) and stimulated with insulin approx. 16 h after injection (time 0 h). The estimated half-times were 11.9 h for water injections and 17.5 and 11.4 h for PI3Kγ and PI3Kγ-CAAX injections, respectively. (**B**) Mean estimated half-times of GVBD from fits to eqn (1) in independent batches of oocytes following the expression of PI3Kγ or PI3Kγ-CAAX (+) or water injection (−) and stimulation with insulin. The half-times are expressed as the time delay after the start (time 0 h) of stimulation. Data are represented as means ± S.E.M.; the numbers in parentheses indicate the total number of batches for each group.

kinetics of progesterone-induced GVBD. This rules out the possibility that the enhancement of GVBD by PI3Kγ-CAAX is due to a CAAX-specific artifact, such as a change in the membrane-lipid environment following attachment of the protein to the membrane. Finally, we used mutant PI3Kγ-cpk-CAAX to test which of the phosphoinositides mediated the effect, since this mutant can phosphorylate only PI and phosphatidylinositol 4-phosphate (PIP), but not phosphatidylinositol 4,5-bisphosphate (PIP_2). In the absence of stimulation, PI3Kγ-cpk-CAAX did not lead to GVBD. In 2 out of 4 experiments it even delayed progesterone-induced GVBD. Western-blot analysis from two sample batches revealed that all PI3Kγ-CAAX mutants were expressed at a similar level (Figures 5C and 5D, top panels). None of the PI3Kγ-CAAX substrate mutants induced either P-Akt or P-MAPK (Figures 5C and 5D, middle and bottom panels).

Involvement of PI3Kγ in insulin-induced maturation

There is conflicting evidence with regard to the involvement of PI3Ks in progesterone- and insulin-induced *Xenopus* oocyte maturation [8,10,25]. We have already shown here that LY294002 inhibited both progesterone- and insulin-stimulated GVBD to a similar extent and inhibited phosphorylation of MAPK (Figure 1).

Expression of neither PI3Kγ nor PI3Kγ-CAAX had an effect on the time course of insulin-induced GVBD (Figure 6A). The results from several independent batches of oocytes are summarized in Figure 6(B). The mean half-time of maturation in the presence of insulin showed a large variation among the different batches; expression of both PI3Kγ and PI3Kγ-CAAX produced a slight increase, which was not significant. Furthermore, none of the substrate-specific mutants had any effect on insulin-induced GVBD (results not shown).

DISCUSSION

We have described the involvement of heterologously expressed PI3Kγ in the regulation of cell-cycle progression in *Xenopus*

oocytes. In particular we have shown that a constitutive active PI3Kγ-CAAX mutant induces GVBD by itself. Furthermore, we have presented evidence that this mutant accelerates specifically the response elicited by progesterone, the physiological trigger for maturation in oocytes, but does not affect the response to insulin.

Of the class-I PI3Ks, PI3Kγ is activated by G$\beta\gamma$ subunits [16] and assembles with a novel p101 subunit, which in turn enhances the response of PI3Kγ to G$\beta\gamma$ subunits [17,26]. Since PI3Kγ, but not PI3Kα, induces MAPK activation in COS-7 cells, this isoform was proposed to link G-protein-coupled receptors to MAPK activation [22].

Stimulation of *Xenopus* oocytes with either progesterone or insulin leads to GVBD and a substantial increase in the level of P-MAPK (Figure 1). There is conflicting evidence from the literature regarding the involvement of PI3Ks in the different pathways leading to *Xenopus* oocyte maturation. Early work by Muslin et al. [25] determined that the endogenous activity of p85, the regulatory subunit of class-IA PI3Ks, is stimulated after progesterone treatment. Moreover, inhibition of PI3K activation attenuates progesterone-induced maturation. In contrast, recent evidence indicates a possible role of PI3Ks only for insulin-induced oocyte maturation. The lipid phosphatase SIP/SHIP, which rapidly hydrolyses PIP_3, inhibits insulin-induced GVBD as well as GVBD induced by expression of a constitutive active form of class-IA PI3K [8]. Using the PI3K inhibitors wortmannin and LY294002, Lopez-Hernandez and Santos [10] showed that only insulin, not progesterone, required the contribution of PI3K for activation of MAPK and subsequent maturation [10]. However, the use of the irreversible inhibitor wortmannin should be considered with caution, because it was shown to induce GVBD by itself [23]. In our experiments, LY294002 inhibited progesterone- and insulin-induced GVBD to a similar extent, but exerted a much stronger inhibitory effect on the level of P-MAPK in the insulin-stimulated groups of oocytes (Figure 1). Since none of the inhibitors discriminate between the class-I PI3Ks, it is possible that different isoforms participate in different signalling pathways. From our data we suggest a participation of endogenous PI3Ks in both pathways leading to oocyte maturation.

Although we did not detect any endogenous PI3Kγ with the monoclonal and the different polyclonal antibodies directed against distinct regions of PI3Kγ (Figures 2 and 3), we cannot rule out the presence of PI3Kγ in *Xenopus* oocytes. Overexpression of PI3Kγ without membrane anchor had only a minor influence on GVBD and did not increase the level of either P-Akt or P-MAPK. There could be various reasons for this. PI3Kγ may have to be stimulated or require additional cofactors like the regulatory subunit p101 [26] to exert its effect on GVBD.

The use of constitutive active mutants, generated by fusion of the catalytic subunit with either a membrane-targeting signal or a regulatory subunit, has proved to be suitable in the identification of PI3K-regulated responses [9,19,27]. It circumvents the use of activators and/or co-expression of regulatory subunits. By means of a constitutive active PI3Kγ-CAAX mutant, we observed induction of GVBD in the absence of hormone stimulation, although with slower kinetics. Furthermore, expression of this mutant accelerated progesterone-induced maturation, although the insulin-induced pathway was unaffected. These results are in good agreement with previous studies showing that in order for insulin to induce maturation, the insulin receptor has to interact with the PI3K class-IA subunit p85 [28]. Since PI3Kγ does not assemble with p85, but instead with the regulatory subunit p101 [17,26], it is not expected to participate in the downstream effects of insulin.

The mechanism by which PI3Ks in general and PI3Kγ in particular could be activated in oocytes following progesterone stimulation is still unclear. A long-sought xPR was cloned recently and surprisingly, was found to be located in the cytosol and nucleus, but not attached to the plasma membrane [6,7]. Overexpression of xPR in *Xenopus* oocytes modestly accelerated progesterone-induced GVBD. These reports are in partial contrast with the current hypothesized model, where progesterone binds on the outside of the oocyte membrane to initiate the signalling cascade leading to maturation [5]. The question of how or whether this novel receptor is linked to the action of progesterone on the membrane-attached adenylate cyclase remains to be elucidated. Involvement of G_i-proteins was also proposed, possibly inducing the decrease in cAMP level following progesterone treatment. However, it is still under discussion as to whether PR binds directly to the G-protein α and/or $\beta\gamma$ subunits or to a molecule interfering with either subunit [5]. In starfish oocytes, MPF activation and maturation is induced by 1-methyladenine, acting on a not-yet-identified membrane receptor, linked to the $\beta\gamma$ subunit of a pertussis toxin-sensitive G-protein [29]. In this report, PI3K was found to be an essential component of MAPK activation, acting downstream of G$\beta\gamma$, and the authors hypothesized that the γ isoform mediates this effect. In contrast, two recent papers on *Xenopus* oocytes [30,31] present evidence that G-proteins might be responsible for the G_2 arrest of immature oocytes prior to progesterone stimulation. In particular it was shown that overexpression of G$\beta\gamma$ subunits inhibits progesterone-induced maturation and activation of MAPK. However, in COS-7 cells PI3Kγ was activated, but not inhibited, by G$\beta\gamma$ subunits liberated from G-proteins [22]. We have attempted to enhance the effect of the non-membrane-attached PI3Kγ on maturation by injecting mRNA coding for $G\beta_1$ and $G\gamma_2$ subunits alone and together with PI3Kγ mRNA, but could only detect a slowing of progesterone-stimulated maturation (results not shown); this is similar to the results described by Lutz et al. [30] and Sheng et al. [31]. This argues against a participation of at least these G$\beta\gamma$ subunits in PI3Kγ-induced maturation in *Xenopus* oocytes. Alternatively, co-expression of G$\beta\gamma$ might not have been sufficient to activate PI3Kγ or might require the expression of additional, as-yet-unknown, proteins to exert a positive effect on PI3Kγ.

The activation of both MPF and MAPK is a necessary requirement for mitotic progression and GVBD in *Xenopus* oocytes. Expression of PI3Kγ-CAAX leads to a substantial increase in the P-MAPK level (Figure 2). We hypothesize that this increase in P-MAPK is sufficient to induce GVBD, since incubation with the MEK inhibitor PD98059 suppressed P-MAPK and strongly reduced the amount of GVBD in the presence of PI3Kγ-CAAX (Figure 4). These observations are in good agreement with results by Fisher et al. [32], who showed that a low level of transiently activated MAPK is sufficient to induce GVBD. The influence of MPF (cdc2) as a downstream signalling factor could not be addressed unambiguously using the inhibitor roscovitine as even 10 μM affected P-MAPK levels. Thus in contrast with the recommended concentrations of 100 μM for *Xenopus* oocytes [33], we observed a presumably non-specific effect on the phosphorylation level of MAPK at a 10-fold lower concentration.

One downstream target of all PI3Ks is the protein Ser/Thr kinase Akt/PKB, which is activated via the phospholipid PIP_3 [34]. A constitutive active mutant of Akt/PKB was shown to induce GVBD and a 2-fold increase in the activity of endogenous phosphodiesterase in oocytes [20]. An increased activity of phosphodiesterase in turn is believed to cause the modest decrease in cAMP level observed within minutes of progesterone

stimulation [5]. These findings underscore the importance of PIP_3 as an early mediator of oocyte maturation upstream of MAPK activation.

A previous report by Bondeva et al. [12] using PI3Kγ mutants with varying substrate specificities in COS-7 cells revealed that Akt/PKB kinase was stimulated via the lipid kinase activity and MAPK via the protein kinase activity of PI3Kγ. Surprisingly, when expressed in *Xenopus* oocytes, mutant PI3Kγ-FRAP-CAAX, which harbours protein kinase but no lipid kinase activity, did not induce GVBD or increase the level of P-MAPK in the absence of hormone, nor did it significantly enhance the response to progesterone (Figure 5). Only expression of PI3Kγ-CAAX (Figures 2 and 3), not any of the other mutants tested (Figures 5C and 5D), increased the level of P-Akt. Thus we hypothesize that the production of lipids and subsequent activation of Akt/PKB kinase and not the protein kinase activity causes the effects on maturation. Mutant PI3Kγ-cpk-CAAX, which can only phosphorylate PI and PIP, but not PIP_2, also had no effect on the P-MAPK level and GVBD, further supporting the hypothesis that PIP_3 mediates the effect of PI3Kγ-CAAX on *Xenopus* oocyte maturation.

In conclusion, we have shown that a constitutive active PI3Kγ-CAAX mutant can activate MAPK and induce GVBD in *Xenopus* oocytes and that this effect is mediated through its lipid kinase activity and an activation of the protein kinase Akt/PKB.

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