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Oncogenic Ras-induced germinal vesicle breakdown is independent of phosphatidylinositol 3-kinase in *Xenopus* oocytes

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Abstract A number of reports have identified phosphatidylinositol 3-kinase as a downstream effector of Ras in various cellular settings, in contrast to others supporting the notion that phosphatidylinositol 3-kinase acts upstream of Ras. Here, we used *Xenopus* oocytes, a model of Ras-mediated cell cycle progression (G2/M transition) to analyze the contribution of phosphatidylinositol 3-kinase to insulin/Ras-dependent signaling pathways leading to germinal vesicle breakdown and to ascertain whether phosphatidylinositol 3-kinase acts upstream or downstream of Ras in those signaling pathways. We analyzed the process of meiotic maturation induced by progesterone, insulin or micro-injected oncogenic Ras (Lys12) proteins in the presence and absence of specific inhibitors of phosphatidylinositol 3-kinase activity. As expected, the progesterone-induced maturation was independent of phosphatidylinositol 3-kinase since similar rates of germinal vesicle breakdown were produced by the hormone in the presence and absence of wortmannin and LY294002. In contrast, insulin-induced germinal vesicle breakdown was completely blocked by pre-incubation with the inhibitors prior to insulin treatment. Interestingly, similar rates of germinal vesicle breakdown were obtained in Ras (Lys12)-injected oocytes, independently of whether or not they had been pre-treated with phosphatidylinositol 3-kinase inhibitors. The effect of wortmannin or LY294002 on MAPK and Akt activation by progesterone, insulin or Ras was also analyzed. Whereas insulin activated those kinases in a phosphatidylinositol 3-kinase-dependent manner, progesterone and Ras were able to activate those kinases in the absence of phosphatidylinositol 3-kinase activity. Since Ras is a necessary and sufficient downstream component of insulin signaling pathways leading to germinal vesicle breakdown, these observations demonstrate that phosphatidylinositol 3-kinase is not a downstream effector of Ras in insulin/Ras-dependent signaling pathways leading to entry into the M phase in *Xenopus* oocytes.

Key words: Ras; Phosphatidylinositol 3-kinase; Germinal vesicle breakdown; *Xenopus* oocyte; Insulin; Signal transduction

1. Introduction

Fully grown (stage VI) *Xenopus* oocytes are physiologically arrested at the G_2 stage of the first meiotic prophase. Physiological doses of progesterone and pharmacological doses of insulin are able to break the blockade and induce entry into the M phase triggering germinal vesicle breakdown (GVBD) through activation of a variety of signaling pathways (reviewed in [1–3]). Thus, progesterone leads to a decreased adenylate cyclase activity, with a resulting drop in overall cAMP levels and the subsequent cascade of protein kinase A (PKA)-dependent phosphorylations. In contrast, insulin or IGF-1 triggers a cascade of phosphorylations initiated by the specific receptor tyrosine kinase (RTK). Both progesterone and insulin-initiated pathways eventually converge into the final activation of the MPF kinase complex, a universal activator of M phase transition in eukaryotic cells and an absolute requirement for GVBD [4,5]. MAPK activation appears to be a necessary and sufficient step in the process of GVBD induction [6–10].

Ras and other oncogenic proteins have also been shown to induce meiotic maturation when micro-injected into *Xenopus* oocytes [11–17]. Several lines of evidence indicate that Ras proteins are essential components in pathways of insulin-induced maturation, but not in progesterone-induced GVBD [18–20].

Because the progesterone (PKA-dependent) and insulin/Ras (RTK-dependent) signaling cascades are already partially characterized, injection of putative signaling intermediates into *Xenopus* oocytes is a valuable tool to analyze their potential contribution in signaling pathways controlling cell proliferation. Thus, the *Xenopus* system has been succesfully used to analyze the functional contribution of a variety of isolated, purified modular domains present in proteins involved in the regulation of RTK/Ras signaling pathways and/or cell cycle progression [21–28]. These reports demonstrate that *Xenopus* oocytes constitute a useful biological model system to functionally analyze components of RTK/Ras signaling pathways.

Phosphatidylinositol 3-kinases (PI3K) are a family of evolutionarily conserved enzymes capable of phosphorylating phosphoinositides at the 3′ position of the inositol ring. The common heterodimeric PI3K isoforms are composed of a catalytic subunit (p110) and a regulatory subunit (p85). Activation of the p85-p110 PI3K complex, through a variety of molecular mechanisms, is known to play essential roles in signal transduction processes related to a variety of cellular activities such as cytoskeletal re-arrangement, cellular migration, differentiation, protection from apoptosis and mitogenesis (reviewed in [29–32]).

PI3Ks are recognized as essential components of receptor tyrosine kinase-activated signaling pathways, such as those initiated by PDGF and insulin, in mammalian cells [33–35]. However, analysis of the participation of PI3K in RTK/Ras signaling pathways has yielded apparently conflicting reports. Thus, whereas some reports have identified Ras as a downstream target in various cellular settings [36–38], others clearly place PI3K upstream of Ras and/or MAPK [39–42].

A number of reports have described the participation of PI3K [21,27,43–45], its distal kinase Akt [46] and the signaling inositol phosphatase SIP/SHIP [47] in the process of insulin-

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induced GVBD in oocytes. PI3K has also been reported to participate in the regulation of glucose transport in the oocytes [48,49]. In view of the conflicting reports in mammalian cells, we decided to utilize the model system of cell cycle progression in *Xenopus* oocytes to ask whether Ras participates upstream or downstream of PI3K in the signaling pathways leading to cell cycle progression into the M phase. Our experimental approach involved micro-injection of activated Ras oncoproteins and subsequent analysis of the resulting process of meiotic maturation under basal, native conditions or under conditions where the endogenous PI3K activity was blocked by specific inhibitors.

2. Materials and methods

2.1. p21Ras protein purification

Transforming H-Ras (Lys12) protein was expressed in *Escherichia coli* and purified from the insoluble fraction by extraction with urea, as previously described [17,26,27].

2.2. Oocyte preparation, micro-injection and maturation

Adult female *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI, USA), Xenopus I (Ann Arbor, MI, USA) and Carolina Biological Supply (Burlington, NC, USA) and stimulated to ovulate by injecting 50 U of pregnant mare serum gonadotropin (Calbiochem, San Diego, CA, USA, 367222) several days prior to oocyte extraction. Ovarian fragments were surgically removed from frogs anesthetized by hypothermia. Fully grown stage VI oocytes were manually dissected into ND-96 medium (5 mM HEPES, 96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 1.8 mM $CaCl_2$, pH 7.8 and 10 μg/ml each of penicillin and streptomycin sulfate). The oocytes were allowed to recover overnight in the same buffer before further treatment and were always maintained at 20°C. For induction of meiotic maturation, groups of 10–30 oocytes were incubated in ND-96 without KCl in the presence of insulin (7.5 μM, Sigma), progesterone (15 μM, Sigma) or were micro-injected into the cytoplasm with 5 ng p21Ras (Lys12) (60 nl, in 20 mM Tris, pH 7.5). Controls were micro-injected with buffer alone. When needed, wortmannin (100 nM, Sigma) [50], LY294002 (30 μM, Calbiochem) [51], PD98059 (50 μM, New England Biolabs) [52] or BAPTA-AM (20 μM, Molecular Probes) [53] were added to the medium at least 1 h before micro-injection of p21Ras or exposure to hormones. Wortmannin or BAPTA-AM were added to the medium and washed away after 30 min. LY294002 or PD98059 were maintained in the medium during the course of the experiment.

Meiotic maturation was assayed by scoring the disappearance of the nucleus (GVBD) in oocytes fixed with 10% trichloroacetic acid. In most cases, the absence of the nucleus correlated with the appearance of a white spot in the animal pole.

2.3. Protein kinase assays

Clarified extracts were utilized for the different kinase assays. For preparation of the extracts, specific groups of oocytes were homogenized in ice-cold buffer (20 μl per oocyte) containing 50 mM Tris pH 7.5, 50 mM β-glycerophosphate, 50 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 7 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride and 10 μg/ml each of aprotinin and leupeptin. Assays of kinase activity on specific substrates (myelin basic protein (MBP), histone H2) were performed directly in cytosolic extracts or in immunocomplexes, as described below. To ensure specificity, protein kinase A inhibitor was always present in the reaction mixtures.

For MAPK activity determination, MBP kinase assays were performed as described [17] using 1.5 mg/ml of MBP (Sigma) as substrate in a buffer containing 20 mM Tris pH 7.5, 100 mM β-glycerophosphate, 15 mM $MgCl_2$, 10 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 20 μM cold ATP, 1 μCi [γ-^{32}P]ATP, with protein kinase A inhibitor peptide (7 μM, Gibco BRL). 5 μl of oocyte extracts was incubated with 25 μl of the reaction buffer at 30°C for 20 min. Radioactivity in the MBP band was visualized by autoradiography after SDS-PAGE on 10–20% polyacrylamide gradient minigels.

For Akt activity determination, histone H2 kinase assays [54] were performed on immune complexes resulting from 90 μl of clarified extracts incubated with 400 ng anti-Akt antibodies (Santa Cruz SC-1618) and protein G agarose beads for 2–3 h at 4°C. Immunoprecipitates were washed twice with lysis buffer, twice with a buffer containing 0.5 M LiCl, 100 mM Tris-HCl, 1 mM EDTA, pH 8.0, and once with kinase buffer. The kinase reaction was carried out with 25 μl of the kinase buffer containing 2.5 μg H2B, 5 μM cold ATP, 7 μM protein kinase A inhibitor and 10 μCi [γ-^{32}P]ATP. Radioactivity in the histone H2B band was visualized by autoradiography after SDS-PAGE on 10–20% polyacrylamide gradient minigels.

3. Results

3.1. The effect of PI3K inhibitors on induction of GBVD by progesterone, insulin and micro-injected Ras (Lys12) protein

We first analyzed the kinetics of GVBD induced by progesterone, insulin or micro-injected Ras, in the presence and absence of wortmannin and LY294002, two specific inhibitors of PI3K activity [50,51]. Separate groups of oocytes were incubated with the PI3K inhibitors (or DMSO in the case of untreated, control oocytes) and then treated with either progesterone or insulin or micro-injected into the cytoplasm with purified, bacterially expressed p21Ras (Lys12) [17,26,27]. As additional controls, separate sets of insulin-induced or Ras-injected oocytes were treated with the MEK inhibitor

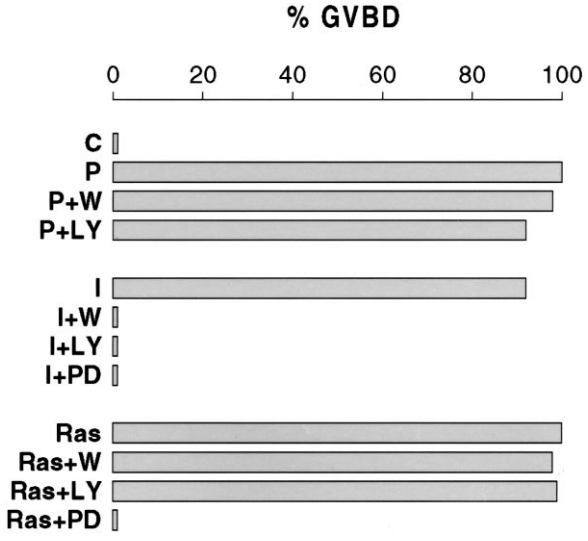

Fig. 1. The effect of PI3K inhibitors on *Xenopus* oocyte GVBD induced by insulin, progesterone or micro-injected, oncogenic Ras (Lys12) proteins. Separate groups of oocytes, maintained in ND-96 medium, were incubated in ND-96 medium after addition of hormones or micro-injection of Ras proteins, as indicated. When needed, wortmannin, LY294002 and PD98059 were added to the medium 1 h prior to incubation with hormones or micro-injection. Bars represent the percentage of GVBD measured after 16 h under the different conditions. Similar results were obtained in three separate experiments. C, untreated oocytes. P, oocytes treated with 15 μM progesterone. P+W, oocytes pretreatment with wortmannin and incubation in the presence of progesterone. P+LY, oocytes pretreated with LY294002 and incubated with progesterone. I, incubation in the presence of 7.5 μM insulin. I+W, oocytes pretreatment with wortmannin and incubation in the presence of insulin. I+LY, oocytes pre-treated with LY294002 and incubated with insulin. I+PD, pretreatment with PD98059 and incubation with insulin. Ras, oocytes injected with 5 ng each of purified Ras (Lys12) protein and incubated in regular medium. Ras+W, pretreatment with wortmannin prior to Ras injection. Ras+LY, oocytes pre-treated with LY294002 prior to Ras injection. Ras+PD, pretreatment with PD98059 prior to Ras injection.

PD98059 [52] to demonstrate the dependence of GVBD on MAPK activation (Fig. 1).

We observed that progesterone-induced maturation was independent of PI3K activity, since similar rates of GVBD were produced by the hormone in the presence and absence of wortmannin or LY294002 (Fig. 1). In contrast, and consistent with a previous report [44], insulin-induced GVBD was completely blocked by pre-incubation with the PI3K inhibitors prior to insulin treatment. Interestingly, similar rates of GVBD were obtained in Ras (Lys12)-injected oocytes, independently of whether or not they were pre-treated with wortmannin or LY294002 (Fig. 1).

Ras proteins have been shown to be essential signaling intermediates in insulin signaling pathways leading to GVBD, but not in progesterone-induced GVBD [18–20]. Therefore, our observations are not consistent with PI3K acting as a downstream effector of Ras in the oocytes, but rather suggest that PI3K acts upstream of Ras or, in a parallel signaling pathway, in the process of insulin/Ras-induced GVBD.

3.2. The effect of PI3K inhibitors on MAPK activation

Previous studies have indicated that MAPK activation is a necessary and sufficient step in the processes of GVBD induction by the hormones progesterone and insulin and by micro-injected Ras proteins [6–10,17].

Here, we measured MAP kinase activity (using MBP as a substrate) in total cell lysates of oocytes after stimulation with the hormones or micro-injection with Ras proteins, in the presence and absence of wortmannin or LY294002 (Fig. 2). In agreement with previous reports [7,8,17], autoradiographic estimation of the MBP kinase activity in comparison to untreated controls confirmed that the MAPK activity was significantly activated in oocytes shortly after treatment with either progesterone or insulin or micro-injection with the activated Ras (Lys12) oncoprotein. This activation was specifically blocked by the MEK inhibitor PD98059 (Fig. 2). In contrast, pretreatment of the oocytes with PI3K inhibitors prior to hormone treatment or Ras micro-injection showed a different pattern of responses in MAPK activation. Thus, whereas insulin-induced activation of MAPK was completely blocked by pretreatment with wortmannin or LY294002, the activation of MAPK by progesterone or micro-injected Ras was completely unaffected by the same treatment with the PI3K inhibiting drugs (Fig. 2). These observations with MAPK activation completely parallel the behavior observed in the measurements of the kinetics of GVBD under similar experimental conditions (Fig. 1).

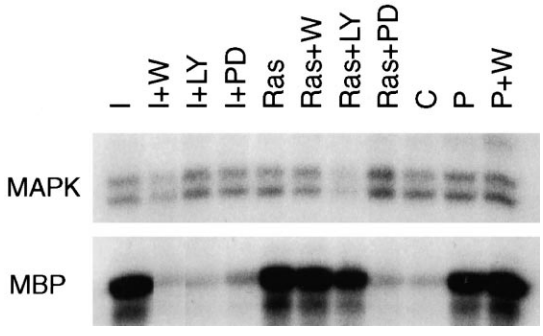

Fig. 2. The effect of PI3K inhibitors on MAPK activation by insulin, progesterone or micro-injected, oncogenic Ras (Lys12) proteins in *Xenopus* oocytes. MAPK activity was estimated in the oocytes by performing in vitro MBP kinase assays in cleared lysates (5 μl) from oocytes treated under the same conditions as in Fig. 1. Radioactivity incorporated in the MBP band was visualized by autoradiography. The upper panel shows similar amounts of MAPK protein in all samples assayed. I, treatment with 7.5 μM insulin for 16 h. I+W, pretreatment with wortmannin prior to insulin treatment. I+LY, pretreatment with LY294002 prior to insulin treatment. I+PD, pretreatment with PD98059 prior to insulin treatment. Ras, injection of 5 ng Ras (Lys12). Ras+W, pretreament with wortmannin prior to Ras injection. Ras+LY, pretreatment with LY294002 prior to Ras injection. Ras+PD, pretreatment with PD98059 prior to Ras injection. C, control oocytes. P, 15 μM progesterone. P+W, pretreatment with wortmannin prior to progesterone treatment.

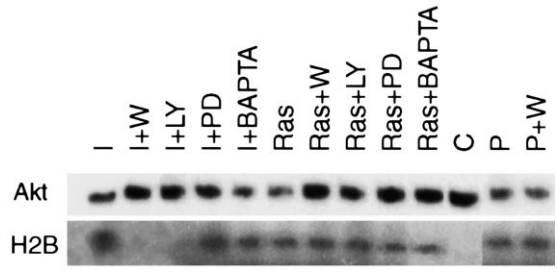

Fig. 3. The effect of PI3K inhibitors on Akt activation by insulin, progesterone or micro-injected, oncogenic Ras (Lys12) proteins in *Xenopus* oocytes. Akt activity was estimated by performing in vitro histone 2B kinase assays in specific anti-Akt immunoprecipitates from oocyte-cleared lysates. Radioactivity incorporated into histone 2B (H2B) was visualized by autoradiography. The upper panel shows the presence of equal amounts of Akt protein in all samples assayed. I, treatment with 7.5 μM insulin for 16 h. I+W, pretreatment with wortmannin prior to insulin treatment. I+LY, pretreatment with LY294002 prior to insulin treatment. I+PD, pretreatment with PD98059 prior to insulin treatment. I+BAPTA, pretreatment with BAPTA-AM prior to insulin treatment. Ras, injection of 5 ng Ras (Lys12). Ras+W, pretreament with wortmannin prior to Ras injection. Ras+LY, pretreatment with LY294002 prior to Ras injection. Ras+PD, pretreatment with PD98059 prior to Ras injection. Ras+BAPTA, pretreatment with BAPTA-AM prior to Ras injection. C, control oocytes. P, 15 μM progesterone. P+W, pretreatment with wortmannin prior to progesterone treatment.

MAPK activation appears to be a prerequisite for GVBD and MPF activation induced by hormones or micro-injected Ras proteins [6–10,17]. Our present observations clearly indicate that only insulin requires contribution of PI3K for activation of MAPK, whereas progesterone and oncogenic Ras were able to activate MAPK without the contribution of the activity of PI3K. Since both Ras [18–20] and PI3K ([44], our observations) have been shown to be downstream signaling elements of insulin-initiated signaling pathways leading to GVBD, it follows that PI3K cannot be a downstream effector of Ras in the signaling pathways leading to cell cycle progression and entry into the M phase in *Xenopus* oocytes.

3.3. The effect of PI3K inhibitors on Akt kinase activity

Akt, or protein kinase B, has been identified as a serine/threonine kinase acting distally to PI3K in pathways regulating mammalian cell differentiation and survival (reviewed in [30]). A recent report has shown that Akt overexpression in *Xenopus* oocytes resulted in GVBD through a process involving downstream activation of type 3 phosphodiesterase PDE3 [46].

In order to further characterize the role of PI3K signaling in Ras-induced GVBD, we measured the Akt activity present in different sets of wortmannin- or LY294002-treated and un-

treated oocytes that were also treated with progesterone or insulin or micro-injected with oncogenic Ras. In vitro Akt kinase assays were performed, using histone 2B as a substrate [54], on immunoprecipitates of oocyte lysates obtained using specific anti-Akt antibodies (Santa Cruz). Fig. 3 shows the pattern of Akt activity detected under various sets of conditions in the same groups of oocytes assayed for MAPK activity in the previous figure. Insulin treatment resulted in a significant activation of Akt activity in the oocytes and this activation was totally dependent on PI3K since pre-incubation with wortmannin or LY294002 completely blocked Akt activation by insulin. Progesterone or micro-injected Ras also induced activation of Akt in the oocytes (Fig. 3). However, in those cases, prior inactivation of PI3K by pre-incubation with PI3K inhibitors did not affect significantly the level of Akt activation achieved (Fig. 3). The process of Akt activation by insulin or micro-injected Ras was further probed in the presence of the specific MEK inhibitor PD98059 or the Ca^{2+} chelator BAPTA-AM. Neither of these reagents altered the pattern of Akt activation observed with insulin or Ras oncogenes alone (Fig. 3).

These observations further support the notion that Ras proteins are not upstream activators of PI3K in the process of GVBD in oocytes. In addition, they also indicate that Ras proteins are able to induce activation of Akt without the contribution of PI3K or the MEK/MAPK cascade. In summary, our findings underscore the notion that Akt may be activated in oocytes through PI3K-dependent and -independent pathways, as has also been reported in mammalian cells [32,55,56].

4. Discussion

Xenopus oocytes provide a valuable system for dissecting insulin-induced signaling pathways and ascertaining the contribution of Ras and PI3K to those pathways. Ras proteins have been previously shown to be necessary and sufficient for insulin-induced GVBD [11,18–20], with the MAPK cascade lying downstream of Ras in the signaling pathway leading to activation of MPF, the p34/cdc2 kinase complex whose activation is an essential requirement for entry into the M phase [4–8,10,17]. PI3K has been shown to be an essential component of insulin-induced GVBD signaling since treatment with wortmannin or micro-injection of isolated SH2 domains blocked the process [21,27,43,44]. However, the functional relationship between Ras and PI3K in oocytes has not been fully resolved yet. Questions remain about the potential participation of PI3K and Ras in common or separate signaling cascades initiated by insulin. More importantly, when PI3K and Ras act in the same pathway, it is important to determine whether PI3K functions as a downstream effector of Ras or as an upstream element of their shared signaling pathway.

In this report, we showed that, in contrast to insulin, micro-injected Ras (Lys12) proteins and progesterone were able to induce GVBD in oocytes under conditions where the endogenous PI3K activity was completely inhibited by pretreatment with specific inhibitors [50,51]. In further contrast with insulin, micro-injected Ras proteins and progesterone were able to activate downstream kinases such as MAPK and Akt during the process of GVBD induction in the presence of wortmannin or LY294002. Since PI3K and Ras proteins are clearly necessary and sufficient downstream elements of insulin-initiated signaling pathways [18–21,27,43,44], our observations demonstrate that PI3K cannot be a downstream effector of Ras in the insulin signaling pathway. Our observations also strongly suggest that the observed activation of Akt by Ras and progesterone in wortmannin- or LY294002-treated oocytes is PI3K-independent. Since wortmannin is an irreversible inhibitor of PI3K [50] and no new protein synthesis occurs during oocyte GVBD after 2 h of hormonal stimulation [57,58], it is highly unlikely that late PI3K-generated lipids are responsible for the observed activation of Akt.

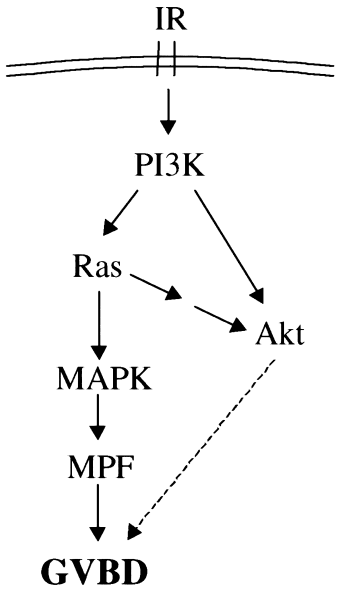

Fig. 4. Differential contribution of PI3K and Ras to insulin-induced GVBD. Schematic model depicting functional interactions among PI3K, Ras, MAPK and Akt in insulin-induced signaling pathways initiated by its specific surface receptor (IR) and leading to GVBD in oocytes. MAPK activation occurs downstream of Ras and is an essential requirement for MPF activation and GVBD [6–10,17]. Injection of an activated Akt has been reported to induce GVBD in oocytes [46]. The observations presented in this report, together with previously published reports [18–20,39,44], indicate that PI3K acts upstream of the endogenous, normal Ras in insulin induction of GVBD. Because PI3K is not a downstream effector of Ras in oocytes, exogenously injected, oncogenic Ras (Lys12) proteins are able to induce activation of MAPK and MPF in the absence of PI3K activation by insulin. The results also indicate that Akt can be activated in a PI3K-dependent and -independent fashion.

Our results cannot formally discern whether PI3K acts upstream of Ras or in a separate, parallel pathway. However, the observation that oocyte maturation induced by a constitutively active mutant of PI3K is inhibited by dominant negative Ras [39] strongly indicates that PI3K and Ras act in the same signaling cascade. We conclude (Fig. 4) that PI3K acts upstream of Ras in the insulin-induced signaling cascade triggering GVBD and entry into the M phase in oocytes. Our observations also indicate that Ras can activate Akt in a MEK/MAPK- and PI3K-independent fashion.

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