Protein kinase B/Akt is essential for the insulin- but not progesterone-stimulated resumption of meiosis in *Xenopus* oocytes

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In the present study, we have characterized the *Xenopus* Akt expressed in oocytes from the African clawed frog *Xenopus laevis* and tested whether its activity is required for the insulin- and progesterone-stimulated resumption of meiosis. A cDNA encoding the *Xenopus* Akt was isolated and sequenced, and its expression in the *Xenopus* oocyte was confirmed by reverse transcription PCR and Northern blotting. Using phosphospecific antibodies and enzyme assays, a large and rapid activation of the *Xenopus* Akt was observed upon insulin stimulation of the oocytes. In contrast, progesterone caused a modest activation of this kinase with a slower time course. To test whether the activation of Akt was required in the stimulation of the resumption of meiosis, we have utilized two independent ap-

proaches: a functional dominant negative Akt mutant and an inhibitory monoclonal antibody. Both the mutant Akt, as well as the inhibitory monoclonal antibody, completely blocked the insulin-stimulated resumption of meiosis. In contrast, both treatments only partially inhibited (by approx. 30%) the progesterone-stimulated resumption of meiosis when submaximal doses of this hormone were utilized. These data demonstrate a crucial role for Akt in the insulin-stimulated cell cycle progression of *Xenopus* oocytes, whereas Akt may have an ancillary function in progesterone signalling.

Key words: cell cycle, insulin action, phosphatase, progesterone signalling.

INTRODUCTION

Stage VI *Xenopus laevis* oocytes are arrested in a G2-like state of meiosis. Upon exposure to either the polypeptide growth factor insulin-like growth factor I (IGF-I) and insulin, or the steroid hormone progesterone, the oocyte re-enters the cell cycle [1,2]. The transition is accompanied by an increase in maturation promoting factor (MPF or Cdc2/cyclin B) which precedes germinal vesicle breakdown [3]. Most reports point towards the Mos-MEK1-ERK2 pathway [where ERK is an extracellular signal-related protein kinase, MEK is a MAPK/ERK kinase and Mos is a p42(MAPK) activator] and the polo-like kinase/CDC25 pathway as responsible for the activation of MPF in meiosis, most likely triggered by a decrease in cAMP (reviewed in [4]). In most cells insulin stimulates the class IA phosphatidylinositol 3-kinase (PI 3-kinase) by inducing the tyrosine phosphorylation of proteins which bind the p85/p55 regulatory subunit of this enzyme [5]. This binding causes a dramatic increase in the intracellular levels of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. In recent years a number of downstream targets of these lipids have been identified, including several Ser/Thr kinases such as Akt [also called protein kinase B (PKB)], its activating kinase PDK-1, several isoforms of protein kinase C and even ERK in some systems [6,7]. In addition, a number of other high affinity PtdIns(3,4,5)P₃ binding proteins have been identified in cells such as GRP-1 which may link to other signalling cascades [8,9].

The *Xenopus* oocyte has been extensively utilized to study insulin signalling since its large size facilitates microinjection, thereby allowing for the expression of exogenous proteins by

injection of mRNAs, or the modulation of endogenous proteins by injection of inhibitory molecules [10–15]. Moreover, the *Xenopus* oocyte IGF-I receptor and one of its substrates, xIRS-1, have been identified [16,17]. The tyrosine phosphorylation of this substrate presumably leads to the activation of the *Xenopus* PI 3-kinase and production of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ [17]. A variety of approaches have implicated the activation of the PI 3-kinase in the ability of insulin/IGF-1 to stimulate the resumption of meiosis [15,17–19]. More recently it was demonstrated that insulin/IGF-I or injection of PI 3-kinase stimulated Akt activity in the *Xenopus* oocyte [19,20] and that the expression of a constitutively active form of the mammalian Ser/Thr kinase Akt in oocytes could stimulate resumption of meiosis [21]. These results indicated that activated Akt was sufficient to induce the resumption of meiosis. However, these studies did not address the question of whether activation of endogenous Akt was necessary for the ability of insulin/IGF-I to induce meiosis. Indeed, in some studies it has been argued that although exogenous Akt is capable of mimicking a particular response to insulin, activation of endogenous Akt was not required for insulin to elicit these responses [22–24].

Recently, a classical *Xenopus* progesterone receptor has been identified in *Xenopus* oocytes and it has been argued that this receptor acts through a non-genomic signalling pathway to induce resumption of meiosis [25,26]. One possibility is that this receptor also couples to the PI 3-kinase pathway since, in mammalian cells, several reports have indicated that other members of the steroid receptor family can directly link to the PI 3-kinase [27,28]. The role of the PI 3-kinase pathway in the

Abbreviations used: DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; ERK-2/MAPK, extracellular signal-related kinase-2 mitogen-activated protein kinase; GSK, glycogen synthase kinase; GST, glutathione S-transferase; GVBD, germinal vesicle break down; HA, haemagglutinin; IGF-I, insulin-like growth factor 1; MEK, MAPK/ERK kinase; MPF, maturation promoting factor; myr, myristoylated; PDE, phosphodiesterase; PDK-1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; RT-PCR, reverse transcription PCR; xAkt, *Xenopus* Akt.

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progesterone-induced resumption of meiosis in the *Xenopus* oocytes has been controversial, with some studies indicating that activation of PI 3-kinase is required for the effects of progesterone, whereas other studies have indicated that it is not required [15,17,19,29].

In the present study we report the cloning and characterization of the Akt expressed in the *Xenopus* oocyte (hereafter described as xAkt). We show that insulin and progesterone, albeit to a much lower extent and with a slower time course, induce the activation of xAkt. Finally, using a functional dominant negative Akt mutant as well as an inhibitory monoclonal antibody, we demonstrate that xAkt is essential for insulin-stimulated resumption of meiosis whereas it has only an ancillary role in the progesterone stimulation.

EXPERIMENTAL

Materials

[γ - 32 P]ATP (37.2 Ci/mmol) and [α - 32 P]dCTP were obtained from NEN Life Science Products (Boston, MA, U.S.A.). If not otherwise listed, chemicals were obtained from Sigma. The pGAD10 *Xenopus* ovary library was a gift of Dr Peter Jackson, (Stanford University, CA, U.S.A.).

Cloning of xAkt

Total and poly(A)-rich RNAs from *Xenopus* stage VI oocytes (30 oocytes per preparation) were extracted and purified using the Trizol Reagent Kit according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD, U.S.A.). First strand cDNAs were generated using the First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) with random hexanucleotides as primer. Bulk First-Strand cDNA Reaction Mix™ (containing reverse transcriptase) was replaced by RNase-free water in some reactions to monitor genomic or cDNA contamination.

PCR were performed directly on 2 μ l of the first-strand cDNA using primers corresponding to sequences conserved in Akts from different species (primers Akt3 and Akt5, listed in Table 1). PCR incubation mixtures contained 50 mM KCl, 20 mM Tris/HCl, pH 8.4, 1.5 mM MgCl₂, 200 μ M of deoxynucleoside triphosphate, 0.5 μ M of each primer, and 2.5 units of *Taq* DNA Polymerase (Gibco BRL). The PCR conditions were as follows: denaturation at 94 °C for 5 min; followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min,

and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min.

The 3' and 5' portions of the cDNA were obtained by PCR on 50 ng of the pGAD10 *Xenopus* ovary library (ClonTech, Palo Alto, CA, U.S.A.) performed under the following conditions: denaturation at 95 °C for 2.5 min; followed by 6 cycles of denaturation at 95 °C for 45 s; annealing at 68 °C for 45 s, decreasing 0.4 °C per cycle; extension at 72 °C for 2 min; 22 cycles of denaturation at 95 °C for 45 s; annealing at 65 °C for 45 s; extension at 72 °C for 2 min; and final extension at 72 °C for 10 min. The PCR products were analysed on 1 % agarose gels stained with ethidium bromide and visualized with UV light. Genuine Akt products were identified by a second PCR, using internal primers Akt6 and Akt8, subcloned and sequenced. All fragments were subcloned into pCDNA3.1-HIS/V5-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced using vector-specific primers (5' SP6 and 3' BGH reverse) or Akt6 or Akt8. The sequence was used to design additional primers for full-length cloning and sequencing. Owing to difficulties in amplifying the 5' portion of the xAkt cDNA from the library, a primer containing the start codon and 21 additional nucleotides was designed based on the chicken Akt (Akt9). This primer was used to PCR the 5' portion of the xAkt cDNA. The sequence of the xAkt was confirmed by sequencing both strands using the Cyclist ExpoPfu DNA Sequencing kit (Stratagene, La Jolla, CA, U.S.A.).

Northern blotting

Total *Xenopus* oocyte RNA (30 μ g) was subjected to gel electrophoresis and transferred onto Biotrans nylon membranes (Pall Biosupport Corporation, NY, U.S.A.). The full length xAkt cDNA was digested by *Eco*RI, and a 712 bp fragment was recovered (nt 61–1323). This fragment was labelled to a specific activity of 2×10^8 cpm/ μ g of DNA using [α - 32 P]dCTP and the Random Primer DNA labelling system (Gibco BRL). Blots were hybridized with this probe using ExpressHyb™ Hybridization Solution (Clontech) according to the manufacturer's procedure. Membranes were washed several times for 10 min at 22 °C in $2 \times$ SSC (0.15 M NaCl/0.015 M sodium citrate), 0.005 % SDS and three times for 10 min at 68 °C in $0.1 \times$ SSC, 0.1 % SDS. Autoradiographs were obtained after 72 h of exposure at –80 °C.

Expression of xAkt in Cos7 cells

Cos7 cells were grown to 50–80 % confluence in 100 mm dishes and transfected with 5 μ g/plate of pcDNA3.1 or pcDNA3.1-xAkt using LIPOFECTAMINE™ reagent (Gibco BRL) according to the manufacturer's protocol. Cells were lysed in Nonidet P40 lysis buffer containing 1 % Nonidet P40, 10 % glycerol, 137 mM NaCl, 20 mM NaF, 1 mM NaPPi, 20 mM Tris/HCl, pH 7.4, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM PMSF. Lysates were subjected to 10000 *g* centrifugation for 15 min, and the supernatants were analysed by SDS/PAGE and Western blotting.

Generation of monoclonal antibody 1C8

An Akt3-GST (glutathione S-transferase) fusion protein [30] was purified and injected (approximately 50 μ g per injection) into the hind footpads of BALB/c mice. After two injections, the lymphocytes from the draining lymph nodes were fused to SP2/O myeloma cells as described previously [31]. The resulting hybridomas were screened for the generation of anti-

Table 1 Primers used for xAkt cloning

The name, nucleotide sequence and location of each of the primers used for cloning is listed.

Primer	Sequence (5'–3')	nt*
Akt3	ATCCGCTGCCTGCAGTGGACC	220–241
Akt5	TTGTCCAGCATGAGGTTCTCC	855–834
Akt6	TTCATGTGGATTCTCCAGAGG	263–284
Akt8	CCTCCATTGGCATACTCC	683–701
Akt6R	CCTCTGGAGAATCCACATG	284–265
Akt9	ATGAATGAAGTAGCGATAGTGAAGG	1–24
Akt13	CTAAGCATTTCCTAGCAG	1446–1436
BK81	GAAATTGAGATGGTGACGATGCAGG	pGAD10
BK82	CGAGGATCCGAATTGCGGCGCGTCGAC	pGAD10
pGAD5'	TTGATGATGAAGATACCCACCA	pGAD10
pGAD3'	GTGAACATGCGGGTTTTTCAGTATCTACGAT	pGAD10

* nucleotide sequence according to GenBank® accession number AF317656.

bodies which could react with Akt3 expressed in mammalian cells.

Construction of mRNA expression vectors and *in vitro* mRNA synthesis

The constructs encoding the haemagglutinin (HA)-tagged Akt, myristoylated (Myr) Akt, K179M-Akt, and A2myr-Akt in pSP64(polyA) have been described previously [21]. The construct encoding the HA-tagged A2myr K179M-Akt in pSP64(polyA) was made by swapping the wild-type kinase domain with the K179M kinase domain. A construct encoding myc-tagged phosphoinositide-dependent kinase-1 (PDK-1) ([32] a gift from Dr D. R. Alessi, University of Dundee, U.K.) and the HA-tagged phosphodiesterase, PDE3A [33] were subcloned into pSP64 (polyA). The pSP64 (polyA) constructs were transcribed using the SP6 polymerase according to the procedure supplied by the manufacturer (Message Machine Kit, Promega). The transcribed mRNA was purified by phenol/chloroform extraction, precipitated at -20°C with 1 vol. isopropanol and subsequently resuspended in diethyl pyrocarbonate (DEPC)-treated water. The mRNA was diluted to a concentration of 1 mg/ml in DEPC water and stored at -70°C .

Injection of *Xenopus* oocytes

Ovary fragments were surgically removed from *Xenopus*, which were primed with pregnant mare serum gonadotropin, and defolliculated oocytes were isolated after treatment with collagenase (2.5 mg/ml) in MBS buffer (88.0 mM NaCl, 1.0 mM KCl, 0.82 mM MgSO_4 , 2.4 mM Na_2HCO_3 , 10.0 mM Hepes, pH 7.4) for 1–1.5 h. Dumont Stage VI oocytes were selected for all the experiments. Oocyte storage and experiments were carried out in OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl_2 , 1.0 mM MgCl_2 , 1.0 mM Na_2HPO_4 , 5.0 mM Hepes, pH 7.8) supplemented with 0.1% BSA. Oocytes were routinely tested for their insulin responsiveness by incubation of 10 oocytes in 1 μM insulin in OR2 solution overnight at 22°C . Oocytes which exhibited an insulin-stimulated germinal vesicle break down (GVBD) of 80–100% were utilized for experiments. The mRNAs or H_2O (vehicle) were injected using a micro-manipulator (Drummond Scientific Company, Broomall, PA, U.S.A.). Resumption of meiosis was scored by the appearance of a white spot on the animal pole of the oocyte (GVBD) and by the activation of *Xenopus* ERK2 (see below).

Western-blot analyses

Expression of the HA-tagged Akt constructs was analysed after lysing injected oocytes in 10 μl /oocyte of lysis buffer (250 mM sucrose, 1 mM KCl, 1 mM MgCl_2 , 0.2 mM PMSF). Oocyte extracts were first pre-cleared by centrifugation at 10000 g in a Beckman model B centrifuge for 4 min at 4°C . Oocyte extracts were analysed by SDS/PAGE (8% gel) and transferred to nitrocellulose membranes (Immobilon, Millipore, Bedford, MA, U.S.A.) which were blocked overnight at 4°C in TBS with 5% BSA/0.2% Tween 20. The HA-tagged Akt was detected with the HA.11 polyclonal antibody (BabCO, Richmond, CA, U.S.A.). Phosphorylation of Akt was detected with either the Akt P-Thr³⁰⁸ or P-Ser⁴⁷³ antibodies (New England Biolabs, Beverly, MA, U.S.A.). Endogenous xAkt was detected with a panspecific Akt antibody [22]. ERK2 phosphorylation was detected as described previously [34]. Binding of the primary antibodies was assessed by the appropriate secondary antibody and either ECL[®]

(Amersham Biosciences, Piscataway, NJ, U.S.A.) or the Immun-Star[®] substrate (Bio-Rad Laboratories, Richmond, VA, U.S.A.).

Akt enzymic assays

Oocytes were lysed in 10 μl /oocyte of lysis buffer [50 mM Tris/HCl, pH 7.6, 50 mM β -glycerophosphate, 50 mM NaCl, 15 mM MgCl_2 , 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, 7 mM NaF, 1 mM dithiothreitol (DTT), 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ of aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin]. Lysates were pre-cleared as described previously and 40 μl of the extracts were subjected to immunoprecipitation with the indicated antibodies. Immunoprecipitates were washed twice in washing buffer (25 mM Hepes, pH 7.88, 0.1% BSA, 10% glycerol, 1% Triton X-100, 1M NaCl) and twice in kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl_2 , 1 mM DTT). In some experiments, either 1C8 or a control IgG was added and incubated with immunoprecipitates for 1 h at 4°C before the assay was performed. In these experiments, the antibody to Akt used was coupled to agarose so as to avoid the use of protein A-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Assays for Akt enzymic activity were performed at 30°C in kinase buffer after addition of 0.2 μCi [γ -³²P]ATP (NEN), 5 μM ATP and 0.15 mg/ml of the glycogen synthase kinase (GSK) 3 peptide (Crosstide) [35]. Phosphorylation of the peptide Crosstide was assessed after

Figure 1 PCR cloning and expression of *Xenopus* Akt

(A) Schematic presentation of the different PCR amplifications used to clone the full-length xAkt. The PCR primers are numbered and listed in Table 1. PCR(3–5) and PCR(6–8) indicate PCR products isolated after RT-PCR on total RNA and mRNA isolated from stage VI oocytes. The full-length xAkt cDNA was obtained by performing PCR (FLPCR) on 50 ng DNA from the pGAD10 *Xenopus* ovary library using sequence- and vector-specific primers. (B) Agarose gel electrophoresis of RT-PCR reaction products from total RNA isolated from stage VI *Xenopus* oocytes. The following samples were analysed: the PCR(3–5) products from oligo-dT (dT) or N6 primed (N6) RT reactions (performed on two different RNA batches), and a PCR control (B) in which the cDNA was left out of the reaction. The left-hand lane contains 1 kb markers (M). (C) Northern blot of *Xenopus* oocyte mRNA with xAkt probe. Total RNA (30 μg) from *Xenopus* oocytes were electrophoresed, transferred and hybridized to a probe containing 712 bp of the coding sequence. Size markers (kb) and the location of xAkt transcript are indicated.

Figure 2 Alignment and phylogenetic tree of Akt

(A) Alignment of the predicted amino acid sequence of xAkt with Akt1 from mouse (mAkt1) and chicken (ggAkt1, respectively) using ClustalW 1.8 and Boxshade3.21 (http://www.ch.embnet.org/software/BOX_form.html). Since the primer for the 5' end of the full-length xAkt clone was based on the chicken Akt1 sequence (see Figure 1), the first eight amino acids of the Akt sequence shown are actually those of the chicken Akt. Identical amino acids are shaded black, predicted PH and kinase domains are boxed, PDK1 and PDK2 phosphorylation sites are boxed and indicated with an asterisk. (B) Schematic model of the xAkt protein with the predicted domains and their locations indicated. Domains were identified by the use of the SMART program [50]. (C) Homology of xAkt with Akt from different species. Identities and positives were calculated by the use of the BLAST program. Positives indicate residues that are identical (%) or have similar functional characteristics. (D) Phylogenetic tree of xAkt. The phylogenetic tree was compiled by the use of the Phylip phylogeny algorithm {Phylip and Retree [51] at BioNavigator by eBioinformatics Pty Ltd (<http://www.eBioinformatics.com>)}. gg, *Gallus gallus*; m, mouse; r, rat; h, human; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans*; dd, *Dictyostelium discoideum*.

Figure 3 Use of antibodies to mammalian Akt to detect the xAkt protein

(A) Extracts from *Xenopus* interphase eggs were immunoprecipitated (IP) with a monoclonal antibody (AB) to mammalian Akt (1C8). Controls containing either no 1C8 antibody or no extract were also performed as indicated. Immunoprecipitates (IP) or supernatants of precipitates (S) were subjected to SDS/PAGE and Western blotting using a pan-specific rabbit polyclonal anti-Akt antibody (diluted 1:1000). Total lysates were also run as control (Lys). (B) Extracts from stage VI oocytes were immunoprecipitated with either control IgG, the monoclonal antibody to Akt (1C8) or a polyclonal antibody to Akt1 (Akt1) (Santa Cruz, SC-1618). The precipitates were analysed by SDS/PAGE and Western blotting using the pan-specific rabbit polyclonal anti-Akt antibody. (C) Comparison of recombinant xAkt expressed in Cos7 cells and endogenous xAkt. Cos7 cells were transfected with empty pcDNA3.1 vector (Emp) or pcDNA3.1 containing the insert encoding the complete xAkt cDNA (xAkt). Lysates of these transfected cells and *Xenopus* oocytes (Extract) were analysed by SDS/PAGE and Western blotting using the pan-specific antibody to Akt.

electrophoresis of the reaction products on a 40% urea polyacrylamide gel.

RESULTS

Cloning of Akt by reverse transcription (RT)-PCR

The cDNA coding for xAkt was cloned as follows (Figure 1A). RT-PCR with primers Akt3 and Akt5 (see Table 1 for a description of the primers used), designed from sequences with greatest homology among Akts from different species, amplified a fragment of the predicted size (634 bp) on total RNA or mRNA isolated from stage VI oocytes (Figure 1B). The sequencing of this fragment confirmed the amplification of an Akt-like cDNA. Two specific primers were designed from the sequence of this product (Akt6 and Akt8), and were found to amplify, by PCR, a 439 bp fragment from both *Xenopus* oocyte cDNA and from the 634 bp PCR product which contained sequence identical to the first PCR.

The 3' cDNA end of xAkt was obtained by PCR of a *Xenopus* ovary library (pGAD10) using an Akt-specific primer (Akt5 or 6) and vector-specific primers BK82 or pGAD10-5'. All fragments were cloned into TOPO-pCDNA3-HIS/V5 and sequenced using vector-specific primers or Akt-specific primers. The sequences generated were utilized to design additional primers for full length cloning and sequencing. Extension of the 5' cDNA proved difficult, and was isolated by PCR with a primer designed from the start codon of chicken Akt (Akt9) and Akt6R and subsequent

sequencing. A full-length clone was isolated and subcloned by using primers for Akt9 and Akt13 on the pGAD10 *Xenopus* ovary library.

To determine the expression of the xAkt mRNA, a 0.7 kb *EcoRI* insert of the xAkt cDNA was randomly labelled with [α - 32 P]dCTP and used as a probe in a Northern blot of 30 μ g of *Xenopus* oocyte RNA (Figure 1C). A major transcript of 2.3 kb was observed with this probe (Figure 1C).

The deduced amino acid sequence of the full-length xAkt had the highest homology to Akt1 from chicken and mouse (Figures 2A and 2C). The critical features of mammalian Akt [36] were all conserved in the *Xenopus* Akt sequence, including the N-terminal pleckstrin homology (PH) domain, a centrally positioned Ser/Thr kinase domain, and the two regulatory phosphorylation sites, the phosphoinositide-dependent kinase 1 (PDK1) phosphorylation site Thr³⁰⁹ in the 'T-loop' activation region of the kinase domain as well as the Ser⁴⁷⁴ in the hydrophobic motif C-terminal to the kinase domain (Figure 2B). The alignment of the protein sequences indicated that the xAkt belongs to a family of kinases highly conserved between species (95% similar to chicken Akt), and a Phylip phylogeny algorithm predicts that it is more closely related to the Akt1 family (Figures 2C and 2D) than to the other Akt members.

Expression and activation of Akt in the *Xenopus* oocyte

Xenopus oocytes or egg extracts were analysed for the presence of the Akt protein by immunoprecipitation with a monoclonal antibody (1C8) raised against mammalian Akt and blotting with a pan-specific anti-Akt polyclonal antibody (Figure 3A). A 60 kDa immunoreactive band was detected in total extracts and after immunoprecipitation. The observation of this band in the precipitates was dependent on the presence of both the antibody and extracts during the precipitation and was depleted from the supernatants of the precipitates (Figure 3A). In addition a distinct Akt antibody also immunoprecipitated an Akt immunoreactive polypeptide of the same size from oocyte extracts (Figure 3B). Finally, the full length cDNA of the xAkt was expressed in Cos7 cells and found to encode an immunoreactive polypeptide of a size identical to the immunoreactive polypeptide in *Xenopus* oocyte extracts (Figure 3C).

To test whether Akt enzymic activity was stimulated during resumption of meiosis, *Xenopus* oocytes were treated with either insulin or progesterone, lysed and the endogenous xAkt was precipitated and tested for enzymic activity. Insulin was found to induce a 7-fold activation of xAkt activity within 30 min of treatment (Figure 4A). In contrast, no significant stimulation of enzymic activity was observed after 30 min treatment with progesterone (Figure 4A). In agreement with the changes in enzymic activity, insulin induced a comparable 7-fold increase in the phosphorylation of Ser⁴⁷⁴ of the endogenous xAkt, whereas progesterone had little effect (Figure 4B). To clarify further whether Akt enzymic activity was stimulated by progesterone, a more complete time course was performed. A consistent but smaller increase in Akt enzymic activity was observed with progesterone treatment at later times, with a maximal effect of 2.5-fold after 4 h of treatment. For comparison, the percentage GVBD after different periods of time with progesterone (Figure 4C) is also shown. The maximal Akt activation by progesterone at 4 h corresponds to 40% GVBD.

Use of a localization deficient mutant of Akt to test the role of Akt in insulin and progesterone-induced resumption of meiosis

We have observed previously that injection of an mRNA encoding a constitutively active Akt into *Xenopus* oocytes induced

Figure 4 Effect of insulin and progesterone on xAkt activity in *Xenopus* oocytes

(A) Stage VI oocytes were incubated with either buffer (0), 5 μ M insulin (Ins) or 5 μ M progesterone (Prog) for 30 min. Extracts were prepared as described in the Experimental section. Aliquots of each extract (40 μ l) were immunoprecipitated for 2 h with either control IgG or 2 μ l of purified monoclonal anti-Akt1 antibody (Santa Cruz, SC-1618) pre-absorbed to protein G-Sepharose. The Akt activity of the precipitates was measured as described in the Experimental section and the autoradiogram of the phosphorylation reaction products is shown (middle panel), the amount of total xAkt detected by Western blotting in the precipitates is also shown (bottom panel). The phosphorylation of the peptide (32 P-pep) substrate was quantified and is plotted as fold increase over the non-hormone treated group after subtraction of the amount of phosphorylated peptide detected in the control Ig precipitates (top panel). (B) The same precipitates were analysed for the presence of phosphorylated Akt protein by immunoblotting with P-Ser⁴⁷⁴ and P-Thr³⁰⁹ (numbers correspond to the position in the *Xenopus* enzyme) phosphospecific antibodies as well as with the panspecific (xAkt) antibody (lower panels). The intensity of the reaction with the Akt P-Ser⁴⁷⁴ antibody was measured and is shown as fold over basal (top panel). (C) Time course of Akt activation (left-hand panel) and germinal vesicle break down (% GVBD, right-hand panel) by progesterone. Oocytes were incubated with 1 μ M progesterone for the indicated times, lysed and the extracts from 15 oocytes were precipitated and assayed for enzymic activity. The inset shows an autoradiograph of a representative experiment. The results of three experiments have been averaged and plotted as the means $\pm$ S.E. * P < 0.001 for the effect of progesterone at 4 h versus the unstimulated control.

resumption of meiosis [21]. This construct (called Myr-Akt) was active since it is constitutively directed to the membrane by its fusion to the *src* myristoylation sequence. This observation, together with the data above showing that the *Xenopus* Akt was activated by insulin, prompted us to investigate whether this activation was an absolute requirement for the insulin/IGF-1- and progesterone-induced resumption of meiosis. To this end, constructs encoding a kinase deficient Akt mutant (K179M), as well as a localization deficient mutant (A2myr-Akt), were injected into the oocytes prior to insulin stimulation. This latter mutant lacks the glycine which is myristoylated in the *src* myristoylation sequence. In addition, it lacks the PH domain which normally directs Akt to the membrane in the presence of PtdIns(3,4,5) P_3

Figure 5 Effect of A2myr-Akt on insulin and progesterone-stimulated meiotic resumption

(A) Expression of the Akt constructs (as described in the Experimental section). Oocytes (in groups of 20) were injected with mRNA encoding the indicated HA-epitope-tagged Akt (K179M, A2myr and Myr), or water as a control, and 6–8 h later they were treated with insulin. After scoring, the oocytes were lysed and the total lysates were analysed by SDS/PAGE and Western blotting using an antibody to the HA-epitope (HA.11). The position of full-length Akt, as well as the PH-domain-deleted Akts (Δ PH Akt) are indicated. (B) The oocytes were scored for induction of GVBD (resumption of meiosis) 15 h after insulin treatment (% GVBD). (C) The oocyte extracts were also analysed for ERK2 activation, by Western blotting, with an antibody to the phosphorylated ERK2. The phosphorylated enzyme is indicated (P-ERK2). (D) Kinase deficient A2myr (A2myr-K179M)-Akt does not inhibit insulin-stimulated meiosis. Oocytes were injected with mRNA encoding the indicated HA-epitope tagged Akt and then stimulated with insulin as in (A). Resumption of meiosis was scored and the levels of the expressed proteins were analysed as in (A). (E) Effect of A2myr-Akt on the progesterone-induced resumption of meiosis. Oocytes (in groups of 20) were injected with either mRNA encoding the A2myr-Akt, or water, and then treated with 0.1 μ M progesterone. Resumption of meiosis was scored (% GVBD) at 15 h after hormone stimulation. Results are means $\pm$ S.E. of at least three independent experiments. * P < 0.015 for the effect of A2myr-Akt versus water injection prior to progesterone stimulation.

Figure 6 A2myr-Akt injection does not inhibit insulin stimulation of xAkt

(A) Oocytes were injected with (A2Myr) or without (–) the mRNA of A2myr-Akt as indicated, and 8 h later treated with (Ins) or without (0) 1 μ M insulin for 0.5 h. Oocytes were lysed and the extracts incubated with either a monoclonal anti-Akt antibody (α Akt) bound to protein G–Sepharose or protein A–Sepharose previously incubated with a pre-immune serum (PI) or an antibody to the PH domain of Akt (α PH-Akt) as indicated. The absorbed xAkt was assayed for enzymic activity as described previously. Shown is an autoradiograph of a representative gel of the phosphorylated peptide substrate (32 P-pep) as well as the quantification of the amount of enzymic activity. (B) A2myr-Akt and kinase deficient A2myr-Akt (A2myr-K179M) injection does not block xAkt Ser⁴⁷⁴ phosphorylation after insulin treatment. Oocytes were injected with the indicated mRNAs and treated as in (A). Oocyte lysates were analysed for phosphorylated Akt protein (P-S474) by immunoblotting with anti-P-Ser⁴⁷³ Akt phosphospecific antibodies.

but it contains no mutations in either its catalytic domain or its phosphorylation sites [37]. Injection of the A2myr-Akt construct completely blocked insulin-stimulated resumption of meiosis and activation of ERK (Figures 5B and 5C). The lack of effect of the kinase deficient Akt (the K179M mutant) may be due to its lower level of expression (Figure 5A). To attempt to understand the mechanism whereby this construct inhibits the insulin-stimulated biological response, a kinase deficient version of the A2myr-Akt (A2myr-K179M-Akt) was constructed and tested. The A2myr-K179M-Akt did not inhibit the insulin-stimulated resumption of meiosis although it was expressed at levels comparable to the A2myr-Akt construct (Figure 5D).

To determine whether progesterone stimulated oocyte maturation via a similar mechanism as insulin, the A2myr-Akt was tested for its ability to inhibit this response. In contrast to the dramatic effect of the A2myr-Akt on the insulin-stimulated resumption of meiosis, it had little effect on the progesterone-stimulated resumption of meiosis when 1 μ M progesterone was used (results not shown). However, a 30% inhibition of GVBD was observed when A2myr-Akt was expressed in oocytes prior to

Figure 7 Ability of PDE3A or Myr-Akt expression to overcome inhibition by either the functional dominant negative Akt or the inhibitory monoclonal antibody

(A) Inhibition by A2myr-Akt. Oocytes (in groups of 20) were injected with (bottom panel) A2myr-Akt mRNA alone or in combination with the mRNA encoding PDK1 (PDK) or PDE3A (3A). Insulin (1 μ M) was added 8 h after the mRNA injections and % GVBD (top panel) was assessed 15 h after insulin stimulation (Ins). Western blots (central panels) show the amount of A2myr-Akt (A2myr) and PDE3A present in the extracts. (B) Inhibition by 1C8 monoclonal antibody. Oocytes were injected with 30 ng of 1C8 (0.2 μ M) alone or in combination with either buffer, the mRNA encoding PDE3A (3A) or Myr-Akt (Myr) as indicated (bottom panel). Insulin (1 μ M) was added 8 h after injection and % GVBD (top panel) was assessed 15 h after insulin stimulation (Ins). Western blots show the amount of Myr-Akt (Myr) and PDE3A present in the extracts. Results shown are representative of the three independent experiments performed.

stimulation with submaximal concentrations (0.1 μ M) of progesterone (Figure 5E).

To probe further the mechanism whereby the A2myr-Akt mutant blocks the insulin-stimulated resumption of meiosis, we examined its ability to block the activation of endogenous Akt in insulin-stimulated oocytes. Oocytes expressing A2myr-Akt were stimulated with insulin and the endogenous xAkt was tested for its enzymic activity after it was specifically precipitated with an antibody directed to the PH domain (since the A2myr-Akt lacks the PH domain, these antibodies would not recognize this

Figure 8 1C8 inhibits the enzymic activity of xAkt

Activated xAkt from extracts of insulin-treated oocytes was captured by anti-Akt antibodies coupled to agarose beads (Santa Cruz, SC-1618 AC). A control of lysates from non-insulin treated cells was also incubated with these beads. The bound xAkt was then incubated with either 2 μ M monoclonal anti-Akt antibody 1C8 or a control IgG in kinase assay buffer (bottom panel). After 1 h at 4 °C, [γ - 32 P]ATP and peptide substrate were added. The phosphorylation reaction and analysis of the product were performed in duplicate. Shown is the autoradiograph of the peptide gel (middle panel) and quantification of the amount of Akt activity expressed as fold over basal, where basal was the amount of activity measured in the reactions with the non-insulin treated lysates (top panel). The experiment shown is representative of the three performed.

protein). Expression of the A2myr-Akt did not block the insulin-stimulated increase in enzymic activity of the endogenous xAkt (Figure 6A). This result was further substantiated by the finding that expression of either A2myr-Akt or A2myr-K179M-Akt did not block the insulin-stimulated phosphorylation of endogenous Akt on Ser⁴⁷⁴, a phosphorylation which generally correlates with activation of the enzyme (Figure 6B).

To rule out the possibility that the A2myr-Akt mutant was acting via a non-specific effect on resumption of meiosis, we examined whether PDE3A, a downstream target of Akt in *Xenopus* oocytes [21], was capable of overcoming the inhibitory effects of this construct. Co-expression of PDE3A was found to overcome the blockage in resumption of meiosis caused by expression of A2myr-Akt (Figure 7A). This finding demonstrated that A2myr-Akt does not cause a non-specific blockage of oocyte maturation. Attempts to overcome the inhibitory effect of A2myr-Akt by overexpression of PDK1, however, were unsuccessful (Figure 7A), possibly due to the inability to obtain high enough expression of this enzyme.

Use of an inhibitory antibody to test the role of Akt in insulin- and progesterone-induced resumption of meiosis

Because of the undefined nature of the functional dominant negative mutant of Akt, a second independent strategy was used to determine the role of xAkt in the resumption of meiosis. A monoclonal antibody (1C8) was generated which inhibits the activity of mammalian Akt in a cell-free assay with an IC₅₀ of 25 nM (see Experimental section). This monoclonal antibody (1C8) was also capable of recognizing the xAkt in a precipitation reaction (Figure 3A). This antibody was therefore tested for its ability to inhibit the xAkt in an *in vitro* kinase reaction. Under these conditions, the antibody (at 2 μ M) inhibited by more than 70 % the enzymic activity of the xAkt (Figure 8).

Figure 9 Effect of Akt antibody 1C8 on the insulin- and progesterone-stimulated resumption of meiosis

(A) Effect of an inhibitory antibody (AB) on insulin and progesterone-stimulated oocyte GVBD. Oocytes (in groups of 20) were injected with 30 ng of either the monoclonal anti-Akt antibody 1C8, mouse IgG, an unrelated monoclonal antibody M3S1, or buffer, and stimulated with either 1 μ M insulin or 1 μ M progesterone 1 h after injection. Results shown are the percentage of oocytes exhibiting GVBD 16 h after hormone stimulation. (B) Effect of the inhibitory antibody on the ability of low doses of progesterone to stimulate GVBD. Oocytes (in groups of 20) were injected with either anti-Akt antibody 1C8 or control mouse IgG, and 1 h later, stimulated with the indicated concentration of progesterone. Results shown are the percentage of oocytes exhibiting GVBD 21 h after hormone stimulation and are means $\pm$ S.E. of three independent experiments. * P < 0.05.

The monoclonal antibody 1C8 was therefore tested for its effect on the insulin-stimulated resumption of meiosis by injection into oocytes prior to insulin stimulation. The 1C8 antibody at 200 nM completely inhibited oocyte maturation stimulated by 1 μ M insulin (Figure 9). Both control IgG and an unrelated monoclonal antibody (M3S1) at the same concentrations had no effect. The 1C8 inhibitory effects on insulin-stimulated resumption of meiosis were further assessed with graded concentrations of antibody (Figure 10). The 1C8 antibody blocked insulin-stimulated oocyte maturation with an IC₅₀ of approximately 50 nM, a value similar to that for Akt inhibition in a cell-free kinase assay (IC₅₀ of 25 nM). The antibody injection also blocked Mos accumulation and ERK2 activation with similar potencies (Figure 10).

In contrast to the effects on the insulin response, 1C8 (even at 200 nM) had no effect on the ability of 1 μ M progesterone to stimulate oocyte maturation (Figure 9A). To evaluate further the role of Akt in progesterone signalling, the anti-Akt 1C8 antibody was injected into oocytes prior to incubation with increasing concentrations of progesterone. Injection of this antibody

Figure 10 Dose dependence of Akt antibody 1C8 inhibition of insulin stimulated GVBD, Mos activation and ERK2 activation

Oocytes (in groups of 20) were injected to give the indicated final concentrations of the monoclonal Akt antibody 1C8, and then stimulated with 1 μ M insulin. The percentage of oocytes exhibiting GVBD 15 h after hormone stimulation is shown (upper panel). The oocytes were lysed and the extracts were analysed by SDS/PAGE and Western blotting using anti-Mos^{ae} antibodies (middle panel) or anti-ERK2 antibodies (lower panel). ERK2 phosphorylation (P-ERK2) is seen by the shift in molecular mass of this enzyme. The experiment shown is representative of the two performed.

blocked resumption of meiosis by approx. 30% when 0.1 μ M progesterone was used to stimulate the oocytes (Figure 9B).

To verify the specificity of the inhibitory effect of the monoclonal antibody, we have examined whether overexpression of a constitutively active Akt (Myr-Akt) or PDE3A, a downstream target of Akt in *Xenopus* oocytes, is capable of overcoming this block. Expression of either the constitutively active Akt or PDE3A overcame the blockage in resumption of meiosis caused by the monoclonal antibody 1C8 (Figure 7B). This finding demonstrated that the antibody did not cause a non-specific blockage of maturation.

DISCUSSION

The protein kinase Akt is one of the major downstream targets of the PI 3-kinase pathway, although its actual role in various insulin/IGF-I signalling pathways is still a matter of debate [7,22–24]. In the present study, we have isolated a cDNA which encodes for the *Xenopus* Akt. The deduced sequence of this enzyme is highly homologous to that of the mammalian enzyme, retaining all the essential domains of this molecule, including the N-terminal PH domain, a centrally positioned Ser/Thr kinase domain, and the two regulatory phosphorylation sites. Moreover, we show that both the Akt mRNA and protein are expressed in the oocytes and that insulin/IGF-I potently and rapidly stimulates the enzymic activity of the xAkt. Finally, we provide direct evidence that endogenous xAkt plays an essential role in the insulin/IGF-1 induced re-entry into the cell cycle in the *Xenopus* oocyte.

To test the role of the xAkt in the insulin-stimulated resumption of meiosis of the oocyte, we have utilized two distinct approaches. First we attempted to utilize various mutant Akt constructs to block the endogenous Akt. Many previous studies have utilized

such mutant enzymes as 'dominant' negatives. However, relatively few studies have identified the mechanism(s) whereby these mutants block subsequent biological responses. In the present work, we utilized a mutant Akt (called A2myr-Akt) which lacks the PH domain, but contains the 14 amino acid *src* myristoylation sequence without the glycine at the second amino acid position, which is normally myristoylated [37]. This mutant Akt has some basal enzymic activity but is incapable of stimulating GVBD [21]. In the present study, we found that expression of this mutated form of the enzyme potently blocked the insulin-induced stimulation of oocyte GVBD and the increase in ERK2 phosphorylation. Quite surprising, expression of this construct did not inhibit either the activation or the phosphorylation of the endogenous xAkt. Moreover, we found that a kinase deficient version of this molecule was not able to inhibit insulin-stimulated GVBD. Finally, the inhibitory effect of the construct was not due to a non-specific toxic effect, since expression of a downstream target of Akt, the phosphodiesterase 3A [21], was capable of overcoming this blockage and stimulated induction of meiosis. These results implicate the xAkt in the insulin-induced resumption of oocyte maturation.

The mechanism whereby various Akt mutants inhibit particular responses has been controversial [7]. In some studies, expression of various kinase inactive Akt mutants has been shown to block the stimulation of the enzymic activity of the endogenous Akt [22–24]. However, mutations which do not disrupt kinase activity have also been shown to act as functional dominant negatives. For example, an Akt construct with the membrane-targeting signal of Ki-Ras, the so-called CAA_X motif, was shown to act as a functional dominant negative [38]. Exactly how such constructs act is not clear. In the case of the CAA_X-Akt, it was proposed that this construct acts by binding to and sequestering downstream substrates of Akt like GSK-3. Alternatively, it is possible that these constructs work to phosphorylate a substrate and thereby induce a feedback inhibition. In agreement with this hypothesis is the finding that a kinase deficient version of the A2myr-Akt is not able to block meiosis, indicating that its basal activity is important for mediating the negative effects of A2myr-Akt. Such a mechanism is also consistent with a variety of data indicating that Akt can feedback to inhibit various insulin-stimulated responses [39–43].

In the present study therefore, we have utilized a second independent method to verify the conclusion that xAkt plays a critical role in the insulin-induced resumption of oocyte maturation. This alternate strategy made use of a monoclonal antibody, 1C8, which is a potent inhibitor of mammalian Akt. This antibody was also capable of recognizing the xAkt in precipitation reactions and was found to be capable of inhibiting the xAkt enzymic activity in an *in vitro* kinase assay. Injection of the 1C8 antibody into the *Xenopus* oocytes potently blocked the insulin-induced resumption of meiosis and activation of ERK2 with an IC₅₀ of 50 nM. This IC₅₀ is close to the potency of the antibody in inhibiting mammalian Akt kinase activity in a cell-free system (IC₅₀ of 25 nM). Moreover, the 1C8 blockade could be completely overcome by injection of either the mRNA encoding a constitutively active Akt, or PDE3A, a downstream target of Akt. These latter findings indicate that the inhibitory effect of 1C8 is not due to a nonspecific effect of the antibody.

The above findings demonstrate that xAkt plays an essential role in the IGF-1/insulin signalling pathway that controls meiotic resumption. These same tools, therefore, were utilized to explore the role of xAkt in the progesterone-stimulated resumption of meiosis. Several studies have implicated the PI 3-kinase pathway in progesterone-induced resumption of meiosis (overview on meiosis signalling in Scheme 1). Progesterone induced activation

Scheme 1 Schematic representation of the signalling pathways controlling resumption of meiosis in *Xenopus* oocytes

The Scheme illustrates the possible pathways whereby insulin/IGF-1 and progesterone can stimulate resumption of meiosis. Insulin has been shown to cause activation of PI 3-kinase, and inhibitors of the PI 3-kinase pathway, like wortmannin (Wort.), block the insulin-stimulated meiotic resumption. In the present study the critical role of the downstream kinase, Akt, in the insulin-stimulated meiosis is demonstrated by the use of two inhibitors of the Akt, a PH-domain-deleted A2myr-Akt (A2myr) and the inhibitory monoclonal antibody 1C8. PKA exerts its negative effect on meiosis progression at several steps in the pathways leading to activation of the G2-M transition [52]. Akt phosphorylates and activates PDE3, thereby decreasing cAMP and leading to activation of the G2-M transition [21]. The involvement of the PI 3-kinase/Akt pathway in the progesterone-stimulated resumption of meiosis is less clear. In addition to this pathway, the progesterone receptor has been reported to control the availability of $G\beta\gamma$, thereby regulating the activity of the adenylate cyclase.

of PI 3-kinase, and injection of a dominant negative PI 3-kinase, blocked oocyte maturation induced by progesterone [29]. However, pharmacological inhibition of PI 3-kinase by wortmannin has provided unclear results with, at best, only a delay in oocyte maturation [17,19,20,44]. That the PI 3-kinase pathway may be an important component of steroid hormone signalling has been suggested recently by the observation that in mammalian cells oestrogen receptors physically interact with PI 3-kinase, and estrogen agonists activate PI 3-kinase and PKB/Akt [27,28]. Moreover, Bagowski et al. [44] have recently found that the *Xenopus* progesterone receptor also associated with the PI 3-kinase and that progesterone stimulated its enzymic activity. The time course of activation of PI 3-kinase by progesterone in their study was consistent with the time course of activation of Akt by progesterone in this study. Also of potential relevance is the finding that the stimulation of maturation of oocytes from the starfish *Asterina pectinifera* by 1-methyladenine requires the activity of both PI 3-kinase and Akt. Most recently, the action of Akt in the starfish has been shown to be via the phosphorylation of Myt1, a cyclin-dependent kinase-inhibitory kinase of the Wee1 family [45]. However, the site in the starfish Myt1, which is phosphorylated by Akt, is not conserved in the *Xenopus* Myt1.

In the present study, we have found that both the A2myr-Akt mutant and the inhibitory antibody had a small (30%) but

reproducible effect on progesterone-induced oocyte maturation. This inhibition was only observed when using submaximal progesterone concentrations. These data indicate that, at low concentrations of progesterone, activation of Akt may play a role in the progesterone-induced oocyte maturation. Moreover, the finding that this inhibition is only observed at low progesterone concentrations may, in part, explain the divergence in the literature as to the role of the PI 3-kinase/Akt pathway in the progesterone response [15,17,19,29].

In summary we have demonstrated that the PI 3-kinase/Akt signalling pathway, via regulation of PDE3 activity and cAMP levels, plays an essential role in insulin/IGF-1-induced resumption of meiosis in *Xenopus* oocytes (Scheme 1). It is likely that this signalling cascade is also involved in the control of meiotic maturation in mammalian oocytes, since PDE3 inhibitors block both spontaneous and hormone-induced maturation in this system [46]. In contrast, although progesterone has some effects on the state of activation of Akt, this pathway is only partially required for signalling meiotic maturation. Progesterone may function to regulate cAMP levels by controlling free $G\beta\gamma$ availability, as recently suggested by studies using G proteins as scavenger [47,48]. The resulting decrease in PKA also leads to activation of the polo-like kinase cascade by multiple unknown steps. This kinase cascade pathway results in activation of

Cdc25C, the phosphatase that dephosphorylates and activates MPF (cyclin B/Cdc2) at the G2/M transition in meiosis I (Scheme 1) [49].

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