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# A novel role for glycogen synthase kinase-3 in *Xenopus* development: maintenance of oocyte cell cycle arrest by a β-catenin-independent mechanism

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## SUMMARY

**We have examined the expression of glycogen synthase kinase-3β in oocytes and early embryos of *Xenopus* and found that the protein is developmentally regulated. In resting oocytes, GSK-3β is active and it is inactivated on maturation in response to progesterone. GSK-3β inactivation is necessary and rate limiting for the cell cycle response to this hormone and the subsequent accumulation of β-catenin. Overexpression of a dominant negative form of the kinase accelerates maturation, as does inactivation by expression of *Xenopus* Dishevelled or microinjection of an inactivating antibody. Cell cycle inhibition by GSK-3β is not mediated by the level of β-catenin or by a direct effect on either the MAP kinase pathway or translation of mos and cyclin B1. These data indicate a novel role for GSK-3β in *Xenopus* development: in addition to controlling specification of the dorsoventral axis in embryos, it mediates cell cycle arrest in oocytes.**

Key words: Glycogen synthase kinase-3β, β-catenin, Cell cycle arrest, *Xenopus*, Oocyte maturation

## INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) is a widely conserved serine/threonine protein kinase, which is constitutively active in unstimulated cells and is inhibited by signal transduction pathways responding to various growth factors in different systems (see Cook et al., 1996 for review). Genetic analysis of *Drosophila* development has led to a description of a signalling pathway, apparently conserved in all metazoa, initiated by the wingless protein and resulting in inhibition of GSK-3 (Cadigan and Nusse, 1997). During *Xenopus* embryonic development, GSK-3 acts as a negative regulator of dorsal axis formation (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1996). Inhibition of GSK-3 activity leads to stabilization of the downstream effector β-catenin and expression of target dorsalizing genes (Wylie et al., 1996; Yost et al., 1996). GSK-3 promotes β-catenin phosphorylation, which stimulates its degradation by the ubiquitin-proteasome system (Orford et al., 1997; Aberle et al., 1997). Maternally expressed β-catenin is essential for dorsal determination (Heasman et al., 1994) and is localized to the future dorsal side of the embryo just after egg fertilization and cortical rotation (Rowning et al., 1997). Meiotic maturation is required for fertilization, and thus cortical rotation, of *Xenopus* oocytes (Masui and Clarke, 1979). Thus meiotic maturation can be considered as a preparation for asymmetric localization of β-catenin and dorsal axis formation.

Before meiotic maturation, oocytes are arrested just before the $G_2$-M transition of meiosis I. Physiologically, maturation occurs due to the activation of cdc2-cyclin B kinase, the maturation-promoting factor (see Dorée, 1990 for review) in response to the maturation hormone (progesterone in *Xenopus*). The combined activities of multiple signal transduction pathways are necessary for the activation of cdc2-cyclin B in *Xenopus* oocytes. Progesterone-mediated maturation requires synthesis of mos and activation of the MAP kinase (Sagata et al., 1988; Gotoh et al., 1995; Kosako et al., 1994) and the PI3 kinase (Muslin et al., 1993) pathways.

Both of these pathways result in activation of protein kinases which inhibit GSK-3 in vivo: $p90^{rsk}$ is activated by the MAP kinase pathway in *Xenopus* and inactivates GSK-3 in mammalian cells (Eldar-Finkelman et al., 1995; Saito et al., 1994) while PKB/akt is activated by the PI3 kinase pathway and inhibits GSK-3 in response to insulin in myotubes (Alessi et al., 1997; Cross et al., 1995). Insulin, as well as progesterone, can bring about meiotic maturation of *Xenopus* oocytes (El-Etr et al., 1979). Given that GSK-3 is active in non-proliferating cells, that GSK-3 inhibition appears to be a feature of various signalling pathways and that GSK-3 activity regulates β-catenin accumulation in early *Xenopus* embryos, we decided to investigate GSK-3 activity in *Xenopus* oocytes. We have found that GSK-3β is inactivated in response to progesterone, that GSK-3β can specifically block progesterone-mediated oocyte maturation, and that GSK-3β inhibition is a rate-limiting step in progesterone-mediated maturation.

## MATERIALS AND METHODS

### Recombinant mRNAs and proteins

Xdsh in pSP64R1, human GSK-3β WT and KM in pSP64T and the expression vectors for *Xenopus* β-catenin and Δβ-catenin, pXBC40, pXE28 and pXE48 were generous gifts of Sergei Sokol (Cambridge, USA), Igor Dawid (Bethesda) and David Kimelman and Randy Moon (Seattle), respectively. The pMal-mos expression plasmid was a gift of Dr Tim Hunt (London). The GST-Sea urchin cyclin B construct has been described previously (Abrieu et al., 1996). Capped mRNAs were transcribed by standard procedures (Promega).

### Immunological procedures

The activity-neutralizing *Xenopus* GSK-3β antibody used for western blotting, anti-raf-1, anti-mos and anti-ERK1 antibodies were purchased from Santa Cruz (sc-7291, sc-133, sc-94 and sc-086, respectively). The GSK-3β antibody used for immunoprecipitation and kinase assays GSK3L6 was raised in rabbits against a synthetic peptide corresponding to the 12 C-terminal residues of *Xenopus* GSK-3β, while the *Xenopus* β-catenin antibody was raised in rabbits against full-length recombinant myc-tagged β-catenin. For microinjection, 20 ng of neutralizing GSK-3β antibody were used per oocyte after concentration of the antibody by microcon (TM) and dialysis against PBS. Immunoprecipitations for kinase assays were performed in buffer A: 20 mM Tris pH 7.5, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM $Na_4P_2O_7$, 1 mM $Na_3VO_4$, 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, 1 μM microcystin. Protein-A sepharose-immune complexes were washed twice in the same buffer.

Western blots were probed with primary antibody at 50 ng/ml and the appropriate secondary antibody-HRP conjugate diluted according to recommendations (Sigma) and revealed by ECL.

### Kinase assays

The GSK-3 peptides were a generous gift of Chris Proud (Canterbury) and the assay procedure has been published previously (Welsh et al., 1997). Histone kinase assays and in-gel MBP kinase assays were performed as described (Labbé et al., 1991; Shibuya et al., 1992).

### *Xenopus* oocytes and embryos

Stage VI oocytes were selected after surgical removal of ovaries from mature female *Xenopus laevis* and treatment with collagenase (Sigma) at 1 mg/ml in OR-2 (5 mM Hepes pH 7.2, 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$). All further manipulations of oocytes were performed in modified Ringer's Solution (MMR: 5 mM Hepes pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 0.1 mM EDTA, 2 mM $CaCl_2$). Progesterone was used at 10 μg/ml. For oocyte extracts, oocytes were rinsed in buffer A, homogenised at 5 μl per oocyte in buffer A at 4°C, microcentrifugated at 13000 revs/minute for 1 minute at 4°C and the supernatant used for further analysis. In vitro fertilization and embryo cultivation was carried out as described (Newport and Kirschner, 1982).

## RESULTS

### GSK-3β in *Xenopus* oocytes and embryos

We performed western blots on oocyte extracts with a polyclonal antibody raised against recombinant *Xenopus* GSK-3β (Fig. 1A). A major band was observed at the expected size of 45 kDa, which comigrated with in vitro translated human GSK-3β (Fig. 1A, lane T) and overexpressed human GSK-3β, which is recognized by the anti-*Xenopus* antibody (lanes OP). Immunoprecipitation of $^{35}S$-labelled oocytes with this antibody resulted in three closely migrating bands at 45 kDa (not shown). No obvious difference in quantity or mobility was seen between immature stage VI oocytes arrested at prophase of meiosis I (lane S), oocytes matured with progesterone (lanes Pg) and arrested at metaphase of meiosis II, or fertilized eggs (lane F). Overexpressed GSK-3β from injection of mRNA is present at a level significantly higher than that of the endogenous protein, and diluting the mRNA ten-fold before injection still resulted in higher than endogenous levels of GSK-3β protein (lanes 0.1× and 1×).

GSK-3β controls β-catenin stability and nuclear accumulation during embryogenesis. We therefore analysed the expression of GSK-3β in early embryos. GSK-3β is present at a low level in the oocyte (Fig. 1A) and fertilized egg (Fig. 1A,B, lane F), and accumulates to a high level by 14 hours after fertilization (Fig. 1B, upper panel, lanes 45'-14h). The major change in accumulation occurs at around cell cycles 14-15 (7h30'). As well as the doublet at the expected size, a further, faster migrating protein band is seen to increase during early embryonic develoment, which might be a related kinase or a differentially spliced form of GSK-3β. Cdc2 protein kinase is shown as a loading control (Fig. 1B, lower panel).

### Endogenous GSK-3β is inactivated during oocyte maturation

GSK-3β can be assayed using a highly specific peptide model substrate (Welsh et al., 1997). In a preliminary experiment in total extracts, using the GSK-3β peptide, we could detect a GSK-3-like activity in stage VI oocytes, which decreased on maturation by progesterone (Fig. 2A). This activity was greatly increased in oocytes overexpressing GSK-3β (data not shown).

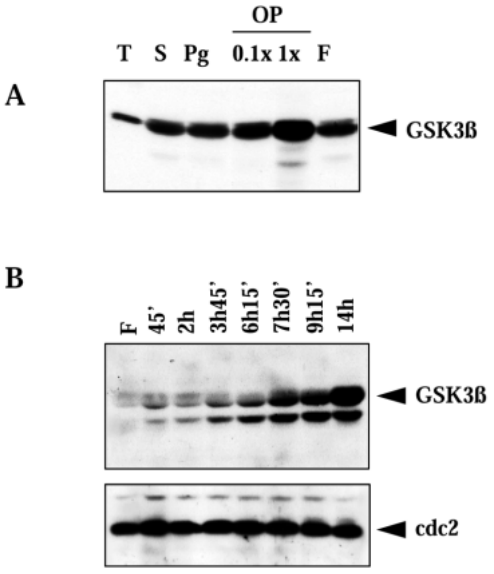


**Fig. 1.** Expression of GSK-3β in *Xenopus* oocytes and early embryos. (A) Western blot of GSK-3β in stage VI (S) and progesterone-matured (P) oocytes and fertilized eggs (F), GSK-3β translated in vitro (T) or overexpressed (OP) in stage VI oocytes at a low (0.1×) or high level (1×). The content of 4 oocytes was loaded in each lane. (B) Upper panel: western blot of GSK-3β in fertilized eggs (F) and embryos at the indicated times in hours (h) and minutes (') after fertilization. Lower panel: western blot of the same samples for cdc2, as a loading control. 50 μg of protein was loaded in each lane.

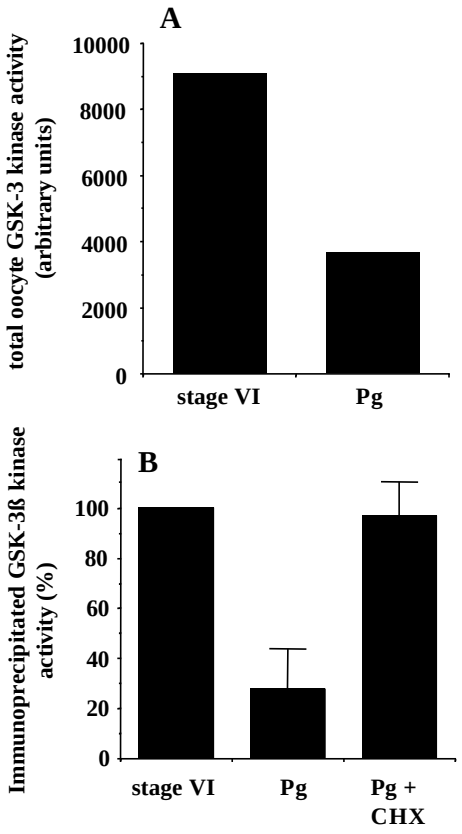


**Fig. 2.** GSK-3β is inactivated during meiotic maturation. (A) Kinase assay of GSK-3β in total extracts: 100 oocytes were homogenised and the extract of the equivalent of ten oocytes was used in a kinase assay in vitro, as described in Methods. Stage VI: extract from stage VI oocytes. Pg, extract from metaphase-arrested oocytes matured by progesterone. (B) Kinase assay of immunoprecipitated GSK-3β: as A, except that GSK-3β was immunoprecipitated from 30-100 oocytes using the GSKL6 antibody. The average values from eleven separate experiments (Pg) or four experiments (Pg + CHX) are presented and expressed in % with respect to activity of the stage VI samples. The error bars indicate the standard deviation.

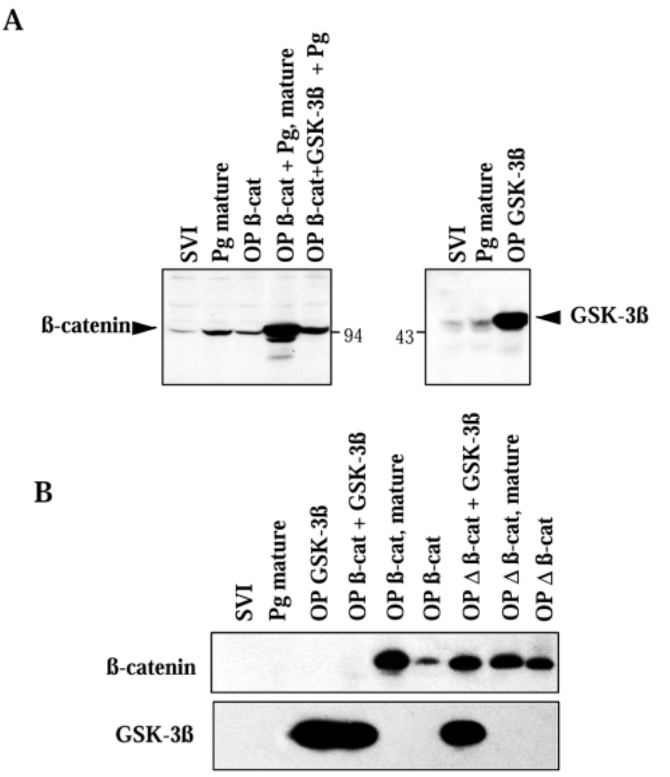


**Fig. 3.** β-catenin accumulation in *Xenopus* oocytes. (A) Left panel: western blot of *Xenopus* β-catenin in stage VI (SVI) and progesterone-matured (Pg mature) oocytes, stage VI (OP β-cat) and progesterone-matured (OP β-cat + Pg, mature) oocytes injected with β-catenin mRNA, and progesterone-treated oocytes injected with both β-catenin and GSK-3β mRNAs (OP β-cat + OP GSK3β + Pg), which do not mature. The equivalent of 4 oocytes was loaded in each lane. Right panel: western blot of GSK-3β showing the relative level of GSK-3β overexpression in this experiment. (B) Upper panel: western blot of *Xenopus* β-catenin in stage VI (SVI) and progesterone-matured (Pg mature) oocytes, stage VI (OP β-cat or Δβ-cat) and progesterone-matured (OP β-cat or Δβ-cat, mature) oocytes injected with β-catenin mRNA or Δβ-catenin, and progesterone-treated oocytes injected with both β-catenin and GSK-3β mRNAs (OP β-cat or Δβ-cat + GSK3β), which do not mature. The equivalent of 3 oocytes was loaded in each lane. Lower panel: western blot of GSK-3β showing the relative level of GSK-3β overexpression in this experiment.

However, we could not measure any GSK-3β kinase activity on immunoprecipitation of endogenous or overexpressed GSK-3β with the commercial *Xenopus* antibody, suggesting that this antibody may be inactivating. We therefore raised an antibody against a C-terminal peptide of *Xenopus* GSK-3β, since C-terminally tagged GSK-3β can be immunoprecipitated via the tag as an active kinase (e.g. He et al., 1995). This antibody recognized GSK-3β as a band at the expected size (not shown). Immunoprecipitation from 40-100 oocytes reproducibly showed that GSK-3β activity decreases between stage VI and metaphase (Fig. 2B) to about 27±16% (0-50% in eleven independent experiments). This decrease requires protein synthesis: it was abolished in progesterone-treated oocytes prevented from maturing by cycloheximide (CHX); concentrations of CHX from 2 μg/ml to 100 μg/ml all blocked oocyte maturation and the decrease in GSK-3β activity, in four independent experiments (Fig. 2B). In another experiment, we compared kinase activity in stage VI and mature oocytes with 1 hour postfertilization embryos and late blastula embryos: in this experiment, the kinase activity in stage VI oocytes was 6-fold that of M-phase oocytes, 2-fold that of 1 hour embryos and two thirds of that seen in late blastula embryos (data not shown), implying firstly that GSK-3β activity in oocytes is not insignificant, and secondly that GSK-3β begins to be reactivated early in the morula.

### Inactivation of GSK-3β allows β-catenin to accumulate in mature oocytes

One physiological consequence of inactivation of GSK-3β is the stabilization of β-catenin, a protein effector of the wingless pathway, which controls dorsal-ventral axis formation in the embryo. If GSK-3β kinase activity drops during oocyte maturation, this should result in stabilization of β-catenin. Indeed, Fig. 3A shows that β-catenin levels increase significantly during oocyte maturation (lanes SVI and Pg, mature). Microinjection of mRNA (about 50 ng/oocyte) for

*Xenopus* β-catenin leads to only a slight increase in its protein level in stage VI oocytes (lane OP β-cat) but, on maturation of injected oocytes with progesterone, β-catenin becomes highly overexpressed (lane OP β-cat + Pg, mature). Cooverexpression of GSK-3β, however, largely prevents this increase due to progesterone treatment and blocks maturation (lane OP β-cat + OP GSK3β + Pg). Injected β-catenin mRNA is under the same translational control as injected mRNA for GSK-3β, while GSK-3β protein does accumulate to high levels in stage VI oocytes (Fig. 3A, right panel), and efficiency of translation of exogenous mRNAs does not increase during meiotic maturation. This suggests that the lack of β-catenin protein accumulation in stage VI oocytes is a result of degradation and not a lack of synthesis.

To investigate this hypothesis and to see whether the block of maturation due to overexpression of GSK-3β is due to a lack of accumulation of β-catenin, we used a deletion (Δ) mutant version of β-catenin which cannot be phosphorylated by GSK-3β and is stabilized in *Xenopus* embryos (Yost et al., 1996), as well as the corresponding wild-type construct. We injected mRNA for wild type or Δβ-catenin (about 50 ng in each case) with or without mRNA for GSK-3β (30-50 ng/oocyte), or mRNA for GSK-3β alone and treated a subset of each population (40 oocytes) with progesterone. The results are shown in Fig. 3B. Again, wild-type β-catenin accumulated to only a low level in stage VI oocytes and not at all in oocytes coinjected with GSK-3β, but could accumulate to a high level in mature oocytes (Fig. 3B, upper panel, lanes 4-6). Δβ-catenin, however, could accumulate to a high level in stage VI oocytes and was unaffected by co-overexpression of GSK-3β (Fig. 3B, upper panel, lanes 7-9). Nevertheless, oocytes co-overexpressing GSK-3β and Δβ-catenin did not mature in response to progesterone and the expression of Δβ-catenin alone had no effect on the rate of oocyte maturation (data not shown). The level of overexpression of GSK-3β in these samples is shown in Fig. 3B, lower panel. In comparison, endogenous β-catenin and GSK-3β in either stage VI or mature oocytes are present at much lower levels and are not seen on these blots.

These results demonstrate several points: firstly that (1) inactivation of endogenous GSK-3β by progesterone-induced maturation correlates with an increase in endogenous β-catenin accumulation; (2) overexpressed β-catenin cannot accumulate in stage VI oocytes in which GSK-3β is active, but accumulates to high levels once it is inactive and (3) the lack of accumulation of β-catenin in stage VI oocytes is dependent on a GSK-3β phosphorylation site since the mutant version does accumulate. This demonstrates that GSK-3β is functionally active in stage VI oocytes and functionally inactive in mature oocytes. Secondly it shows that GSK-3β blocks oocyte maturation by a β-catenin-independent mechanism since Δβ-catenin cannot overcome the block due to maintenance of high GSK-3β.

### Oocyte maturation is specifically blocked by overproduction of wild-type GSK-3β and facilitated by the microinjection of a dominant negative GSK-3β

Since GSK-3β is inactivated during the response to hormone treatment, we wished to know whether this inactivation is necessary for activation of MPF and resumption of the meiotic cell cycle, or whether it is simply a consequence of MPF activation.

In fact, as mentioned above, microinjection of GSK-3β mRNA (approx 50 ng/oocyte) into stage VI oocytes prevented maturation induced by progesterone (Fig. 4A), an effect that was repeated in numerous independent experiments (not shown), as well as blocking maturation induced by microinjection of mos protein (data not shown). Because the microinjected mRNA is expressed in oocytes at a relatively high level (similar to that of 7-hour embryos: see Fig. 1) compared to the endogenous protein, we diluted the mRNA 10-fold and 100-fold before injection (Fig. 4A, 0.1× and 0.01× GSK3β); the inset (upper panel) shows the expression of GSK-3β. 10-fold diluted GSK-3β mRNA also abolished progesterone-induced maturation, an effect repeated in each of eight experiments (Fig. 4A and data not shown), while 100-fold less mRNA caused a severe delay in each of four experiments, but did not completely abolish maturation, showing that these oocytes are still viable (Fig. 4A). The histone kinase (cdc2) activities measured from these oocytes at 8 hours corroborate these results (Fig. 4A inset, lower panel) – histone kinase activity is low in untreated oocytes (lanes –) or progesterone-treated oocytes with moderate or high overexpression of GSK-3β (lanes 0.1×, 1×, +), high in oocytes treated with progesterone alone (lane +) or at a late stage with low levels of GSK-3β and progesterone (lane 0.01× +).

These results suggested that the block might be specific for GSK3. We therefore wanted to determine whether the level of GSK-3β kinase activity in a normal $G_2$-arrested oocyte is comparable to the level of kinase activity sufficient to block maturation by progesterone. If endogenous GSK-3β kinase activity is functionally relevant to oocyte cell cycle arrest, it should be comparable but lower, since it is not so high that it cannot be inactivated in response to progesterone. Fig. 4B demonstrates that this is so. Injection of undiluted GSK-3β mRNA (about 50 ng/oocyte) led to a GSK-3β kinase activity an average of six-fold that in an unstimulated oocyte, while injection of ten-fold diluted mRNA resulted in an average kinase activity 155% (±67%, $n$=4) of that of an untreated oocyte. Clearly either more protein is produced in the oocyte from the injected RNA than is activated, or the specific activity of endogenous GSK-3β is greater than the exogenous GSK-3β.

Although we confirmed that the overexpressed GSK-3β kinase was active and not acting as a dominant negative by peptide kinase assay, even moderate overexpression of GSK-3β might nonspecifically block by interacting with normal substrates of a physiologically relevant kinase active at lower levels than GSK-3β. We therefore injected high levels of mRNA (40-60 ng/oocyte) for a kinase-dead mutant of GSK-3β (GSK3β KD). Rather than blocking, this actually facilitated maturation induced by progesterone: oocytes started to mature about 1 hour earlier and maturation was much more synchronous – all oocytes had matured within 2 hours, as opposed to 5 hours with progesterone alone (Fig. 4C, Pg + GSK3β KD and Pg). As a parameter for comparison, we chose the time taken for 10% of the oocytes to undergo GVBD since this is the point at which a typical response-time curve gradient becomes maximal, and is also the time at which cyclin B-cdc2 kinase activity becomes detectable (Labbé et al., 1988) and after which cycloheximide addition will no longer block progesterone-induced GVBD (Wasserman and Masui, 1975).

This was advanced by between 15 and 20% by expression of GSK-3β KD (Fig. 4C) in each of three independent time courses. The expression of the kinase-dead (KD) and wild-type (WT) GSK-3β proteins in this experiment is shown in the inset panel, along with the endogenous levels in stage VI (S) and in progesterone-matured (P) oocytes.

GVBD is a late consequence of events initiated by progesterone in *Xenopus* oocytes. Earlier events include dephosphorylation of inhibitory residues on cdc2, phosphorylation of activating residues in the T-loop of MAP kinase and hyperphosphorylation of raf-1. All of these events were suppressed in oocytes expressing wild type GSK-3β but not KD GSK-3β, as shown in Fig. 4D (panels 1-4). These results suggest that GSK-3β blocks an event upstream of MAP kinase in the cascade of events that releases oocytes from $G_2$ arrest upon progesterone stimulation.

### Inhibition of GSK-3β facilitates oocyte maturation

Given that we could not measure any GSK-3β kinase activity using the commercial antibody although we could immunoprecipitate GSK-3β protein from $^{35}$S-labelled oocytes (data not shown) we surmised that this antibody may block kinase activity and thus GSK-3β function in oocytes. We therefore microinjected this antibody, and control

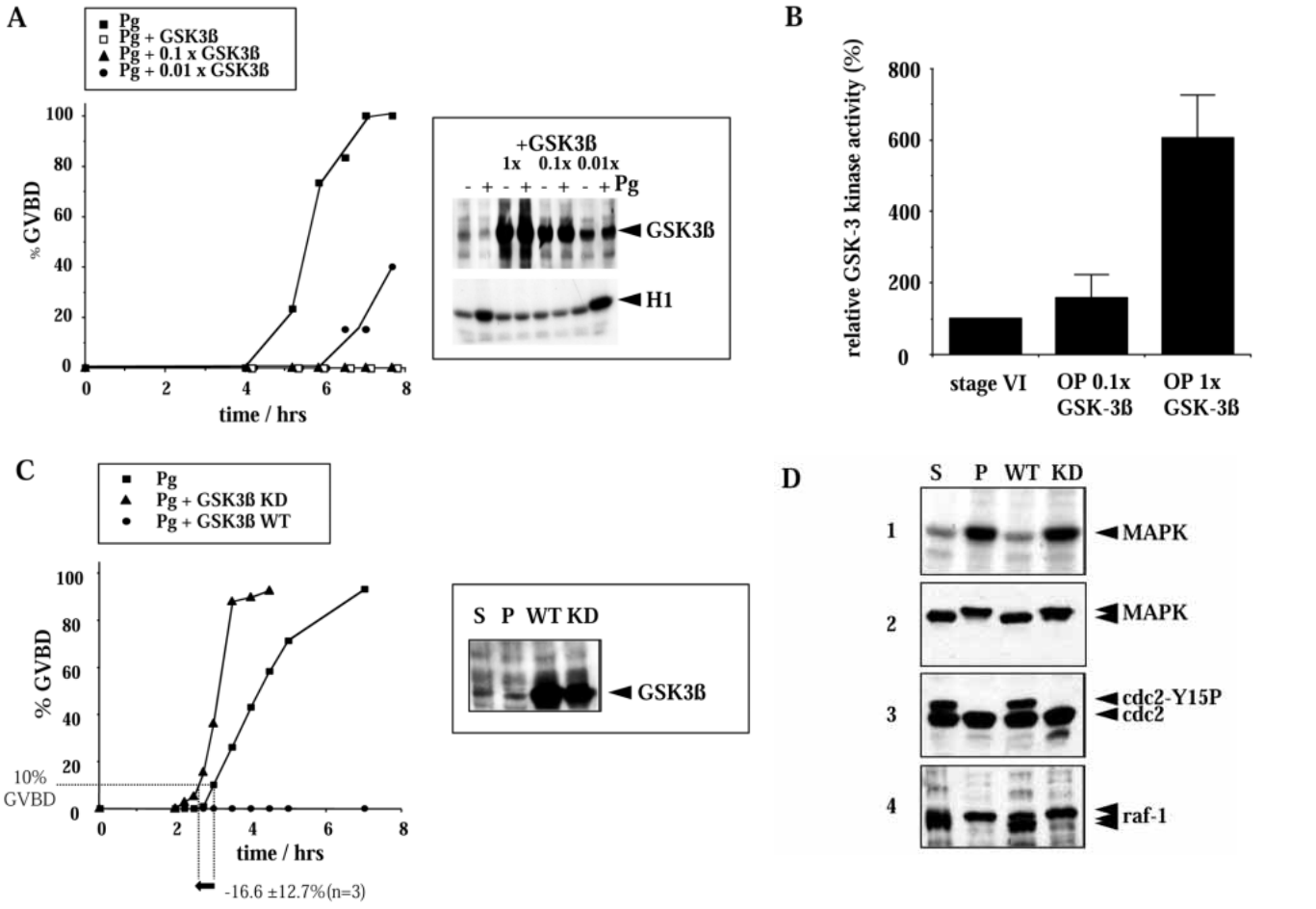


**Fig. 4.** Progesterone-induced maturation is blocked by expression of wild-type GSK-3β and facilitated by dominant-negative GSK-3β. (A) Oocytes were injected with 50 nl (about 50 ng) of mRNA for wild-type GSK-3β, or 50 nl of the same mRNA diluted 10-fold (0.1x GSK3β, filled triangles) or 100-fold (0.01× GSK3β, filled circles), or left uninjected (filled squares). The oocytes were then treated with progesterone (Pg) and GVBD was scored as a function of time. The inset panel shows a western blot for GSK-3β (upper panel) and histone H1 kinase assays (lower panel) of these samples before (–) and after (+) progesterone treatment. (B) GSK-3β was immunoprecipitated and assayed from control stage VI oocytes, or oocytes injected with either 10-fold dilted (OP 0.1×) or undiluted (OP 1×) mRNA for GSK-3β. The relative level of GSK-3β per oocyte is shown, with stage VI taken as 100%. Error bars show standard deviation from four experiments. In each experiment, we confirmed that the overproduced GSK-3β blocked the response to progesterone. (C) Oocytes were injected with 50 nl (about 50 ng) of mRNA for wild-type (WT) or dominant negative (kinase-dead, KD) GSK-3β, treated with progesterone, and scored for GVBD as in A. The inset panel shows the expression-level, by western blotting, of the GSK-3β WT and KD proteins in injected oocytes compared to uninjected control stage VI (S) and progesterone-matured (P) oocytes. 10% GVBD is indicated. The average ± standard deviation for the percentage of advancement of time taken to reach 10% GVBD in oocytes injected with GSK-3β KD mRNA, from three experiments, is shown. (D) Samples of oocytes as in B were analysed for MAP kinase activity, by in-gel MBP kinase assay (panel 1), MAP kinase kinase activity by phosphorylation-dependent SDS-PAGE mobility shift of MAP kinase (panel 2), tyrosine-phosphorylation status of cdc2 (panel 3) and activation-dependent mobility shift of raf-1 kinase (panel 4). Panels 2-4 are western blots.

immunoglobulins at the same concentration, and treated all oocytes with progesterone. As shown in Fig. 5, blocking GSK-3β function by antibody injection also facilitated the progesterone response, in each of three independent experiments. Interestingly, the effect was more marked than that of overexpression of kinase-dead GSK-3β, and the average advancement of 10% GVBD was 38 minutes, or 22±2.4%, while there was no difference (–0.2±1.6%) between progesterone treatment with or without injection of control immunoglobulins.

GSK-3β can be inactivated in vivo by the wingless pathway, which does not depend on either protein kinase B or MAP kinase pathways (Cook et al., 1996). The protein Dishevelled acts in this pathway and the *Xenopus* homologue acts as a negative regulator of GSK-3β during embryonic development (Sokol, 1996). This suggests that Dishevelled might be able to inactivate GSK-3β in oocytes. We therefore overexpressed *Xenopus* Dishevelled in stage VI oocytes by injecting mRNA (10-30 ng per oocyte) and measured the effect on endogenous GSK-3β kinase activity. Indeed, expression of Dishevelled caused a significant drop in GSK-3β activity, to 44±8% of control (*n*=3) (Fig. 6A) and enhanced the response of oocytes to progesterone in each of two independent time courses (Fig. 6B), with 10% GVBD occurring about 10% earlier.

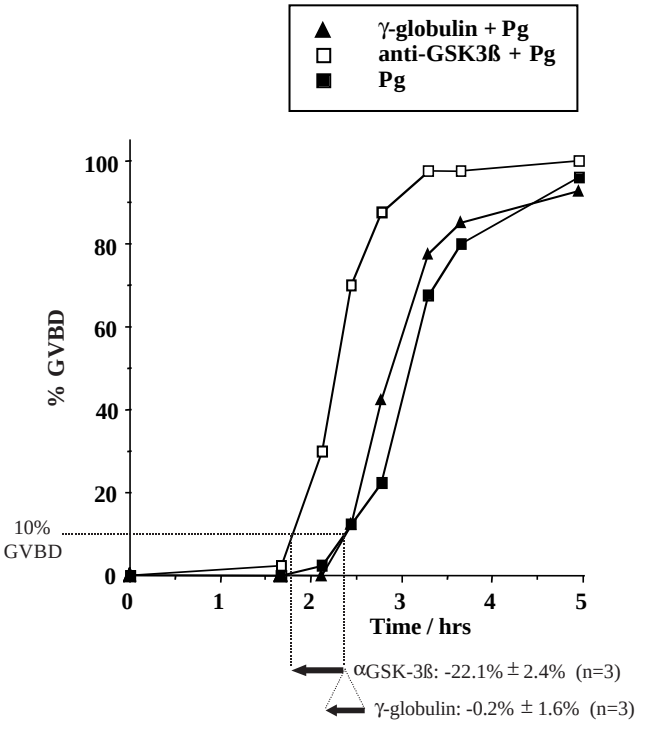


**Fig. 5.** Inactivation of endogenous GSK-3β by injection of an activity-neutralizing antibody facilitates progesterone-induced maturation. Oocytes were injected with GSK-3β activity-neutralizing antibody (anti-GSK3β, open squares) or the same concentration and quantity of rabbit γ-globulins (filled triangles) or left uninjected (filled squares). All oocytes were then treated with progesterone (Pg) and GVBD scored as a function of time. 10% GVBD is indicated. The average ± standard deviation for the percentage of advancement of time taken to reach 10% GVBD in oocytes injected with either an anti-GSK-3β antibody, or control immunoglobulins, from three experiments, is shown.

## Inactivation of GSK-3β in maturing oocytes occurs concurrently with MAP kinase and cdc2 kinase activation

So far no kinases modulated early in oocyte maturation upon hormone stimulation have been identified. The fact that keeping GSK-3β activity high blocks activation of the MAP kinase cascade and cdc2 kinase activation, and thereby blocks cell cycle progression, led us to ask whether GSK-3β inactivation might be an early event in the response to progesterone. However, one would also envisage that a rate-limiting step should be a relatively slow step. For these reasons,

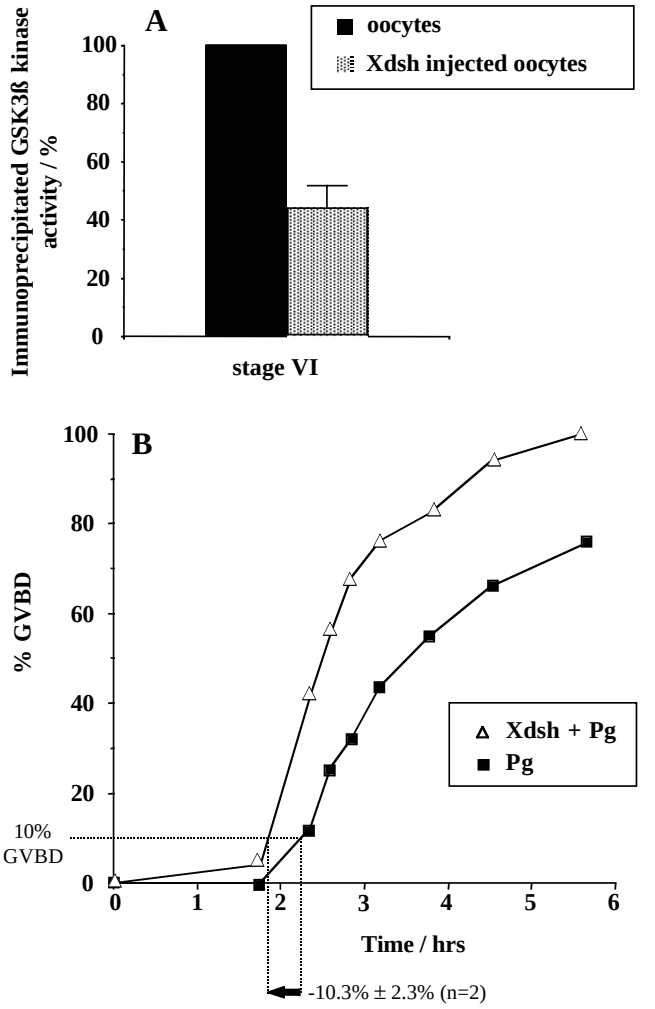


**Fig. 6.** Expression of Dishevelled causes GSK-3β inactivation in oocytes and facilitates progesterone-induced maturation. (A) GSK-3β was immunoprecipitated and its activity assayed from uninjected stage VI oocytes (black bar) and stage VI oocytes injected with *Xenopus* Dishevelled (Xdsh) mRNA (grey bar). Activity of GSK-3β from Xdsh-injected oocytes is expressed as a percentage of control activity. The average ± standard deviation of three independent experiments is shown. (B) Oocytes were injected with *Xenopus* Dishevelled (Xdsh) mRNA (open triangles) or left uninjected (filled squares), and then treated with progesterone (Pg) and GVBD scored as a function of time. 10% GVBD is indicated. The average ± standard deviation for the percentage of advancement of time taken to reach 10% GVBD in oocytes injected with Xdsh mRNA, from two experiments, is shown.

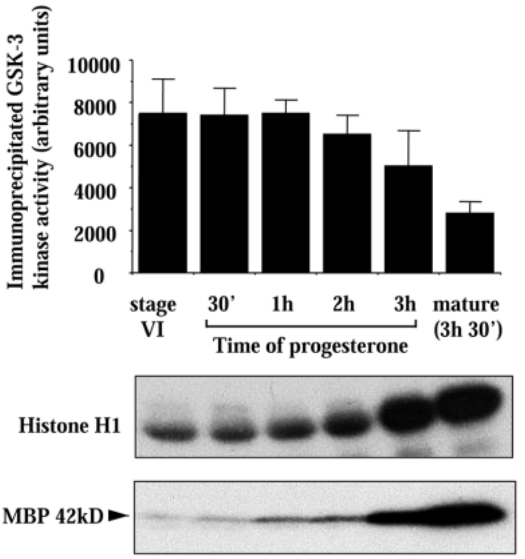


**Fig. 7.** GSK-3β inactivation during oocyte maturation occurs concurrently with MAP kinase and cdc2 kinase activation. Graph of immunoprecipitated GSK-3β kinase activity from extracts prepared from oocytes at 30 minutes, 1 hour, 2 hours and 3 hours after progesterone treatment, as well as mature oocytes. Three extracts of 30 oocytes were prepared at each timepoint and the immunoprecipitations and kinase assays were performed in triplicate. The average ± standard deviation for each point is shown. Middle panel: cdc2 (histone H1) kinase activity at each point. Bottom panel: MBP kinase activity in-gel of each sample, to assay MAP kinase, the position of which is indicated at 42 kD.

we undertook a time course of the inactivation of GSK-3β compared to the activation of kinases involved at a late stage, MAP kinase and cdc2. We found that in fact we could not temporally separate these processes (Fig. 7). We immunoprecipitated GSK-3β from extracts prepared in triplicate (from oocytes not yet having undergone GVBD, except for the final point) at different times after progesterone treatment, and compared immunoprecipitated GSK-3β kinase activity with cdc2 (H1 histone) kinase and MAP kinase (in-gel MBP) activity, in these extracts. Phosphorylated H1 and MBP were quantified by phosphor-imagery. GSK-3β activity started to decline (−14±12%) after 2 hours, at which point MAP kinase and cdc2 kinase activity were around 10% of their respective maxima; had lost 50±20% of the difference between mature and immature oocytes by 3 hours, at which point 10% GVBD had been reached and both MAP kinase and cdc2 kinase were at around 50% maximum, and was at a minimum only in mature oocytes. Similar results were obtained in four other experiments, although, in two cases, GSK-3β was at a minimum at around 10% GVBD, before MAP kinase and cdc2 kinase activation in these experiments (data not shown). In no case, however, was GSK-3β inactivated early in response to progesterone. While these experiments allow us to conclude that GSK-3β inactivation is not an early event, but do not allow sufficient temporal resolution to reproducibly distinguish the timing of GSK-3β inactivation from MAP kinase and cdc2 kinase activation, they are consistent with an inverse relationship between GSK-3β activity and the activity of MAP kinase and cdc2 kinase.

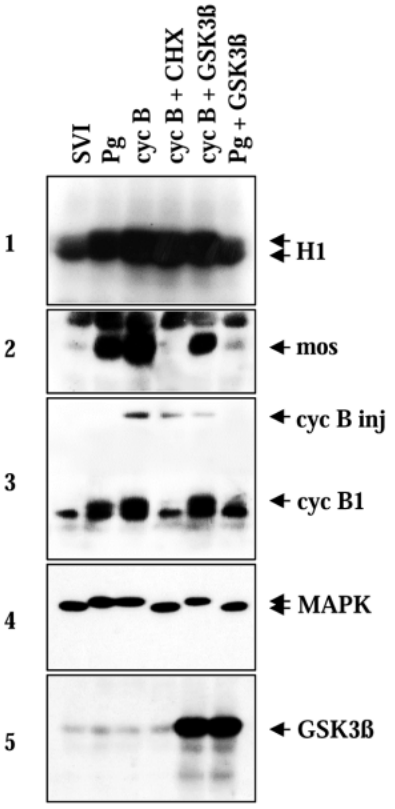


**Fig. 8.** GSK-3β overexpression does not block cdc2 kinase activation triggered by injection of recombinant cyclin B protein. Analysis of stage VI oocytes (SVI), oocytes matured by progesterone (Pg) or injection of GST-cyclin B protein in the absence (cyc B) and presence (cyc B + CHX) of cycloheximide and in oocytes previously injected and expressing GSK-3β mRNA (cyc B + GSK-3β), and of oocytes expressing GSK-3β mRNA and treated with progesterone (GSK3β + Pg). Histone H1 kinase assay (panel 1) and western blots for mos (panel 2), cyclin B1 (panel 3), MAP kinase (panel 4) and GSK-3β (panel 5) are shown.

## GSK-3β does not block oocyte maturation induced by cyclin B

How does an excess of GSK-3β block MPF activation? An overexpressed protein kinase could be generally toxic, although even relatively low levels of overexpression block maturation. Nevertheless, we investigated this possibility by injecting oocytes with cyclin B while overexpressing GSK-3β. If GSK-3β blocks activation of MPF specifically at a point upstream of MAP kinase activation (Fig. 4), while induction of GVBD by injection of cyclin B does not require MAP kinase activation or protein synthesis (D. L. F. et al., unpublished observations), GSK-3β overexpression might no longer block GVBD in this case. Our results are conclusive and agree with this reasoning (Table 1). All oocytes underwent GVBD when injected with GST-cyclin B alone (cyc B), or in the presence of cycloheximide (cyc B + CHX), as well as with GSK-3β overexpressed (cyc B + GSK3β). Half of the oocytes overexpressing GSK-3β were not injected with cyclin B but were treated with progesterone, as a control (Pg + GSK3β). None of these oocytes underwent GVBD. All oocytes treated with progesterone alone (Pg) matured. The histone kinase

**Table 1. GSK-3β overproduction does not block oocyte GVBD induced by cyclin B microinjection**

| Treatment | Oocyte GVBD/oocytes treated |
|---|---|
| Progesterone | 60/60 |
| injection GST-cyclin B | 30/30 |
| injection GST-cyclin B + CHX | 30/30 |
| injection GST-cyclin B + OP GSK-3β | 30/30 |
| Progesterone + OP GSK-3β | 0/20 |

Oocytes were microinjected with mRNA for GSK-3β and left for 12 hours. (OP GSK-3β), or left uninjected, and subsequently stimulated with progesterone or microinjected with GST-cyclin B protein, in the absence or presence (+ CHX) of 100 μg/ml cycloheximide. GVBD was assessed visually after several hours; oocytes were then frozen for further analysis (Fig. 8)

activities, levels of expression of mos and cyclin B1, and the mobilities of MAP kinase, are all shown in Fig. 8 (panels 1-4). Levels of GSK-3β expression are also shown (panel 5). These results demonstrate not only that GSK-3β overproduction does not block resumption of the cell cycle by general toxicity, but also that it does not directly block mos and cyclin B1 accumulation, nor prevent exogenously injected cyclin B from forming active histone kinase, nor block the feedback loop whereby the MAP kinase pathway is activated by active MPF. In this experiment, overexpressed GSK-3β was extremely active as a kinase as measured using the peptide substrate and, although the overexpressed kinase activity fell by 60% on maturation by injection of cyclin B, it was still active at well above endogenous levels (data not shown).

## DISCUSSION

The development of animal organs requires an extraordinary coordination of cell proliferation with morphogenesis. In *Drosophila*, the wingless signalling pathway controls the formation of the anteroposterior boundary of imaginal discs, and the homologous wnt pathways in vertebrates are essential for development of many organs (Cadigan and Nusse, 1997). These pathways also control cell proliferation in the target tissues. In *Drosophila*, the wingless pathway requires armadillo, a fly homologue of β-catenin, while the GSK-3 homologue shaggy negatively regulates the pathway and thus proliferation of the target cells. Ectopic expression of wingless leads not only to induction of ectopic structures but also general overproliferation of surrounding cells (Diaz-Benjumea and Cohen, 1995). The intersection of GSK-3 with cell cycle regulators has not yet been made, although the wingless pathway leads to repression of String, the cdc25C homologue (Johnston and Edgar, 1998).

With respect to cell proliferation, the prototype *wnt* was originally described as a site of integration for the mouse mammary tumour virus (Nusse and Varmus, 1982), and a family of *wnt* genes has since been discovered, with at least fifteen members being expressed in different tissues of *Xenopus* (Cadigan and Nusse, 1997), allowing for cell-type and temporal control of target gene expression and cell proliferation. Furthermore, wnt-1 and other wnt family members can prevent postconfluence proliferation inhibition in mouse fibroblasts (Bradbury et al., 1994). Moreover, GSK-3 is an active protein kinase in growth-arrested fibroblasts and is inhibited by a signal transduction pathway initiated by wingless protein as well as by mitogenic stimuli in several cell types (Cook et al., 1996; Eldar-Finkelman et al., 1995; Saito et al., 1994; Welsh et al., 1994). Perhaps significantly, the kinase that inhibits GSK-3β in response to insulin in myotubes, PKB, is also a cellular proto-oncogene, c-akt.

In the present work, we have investigated the possible involvement of GSK-3 in the block to proliferation that occurs early in development, where oocytes are arrested at the $G_2$/M phase boundary of the first meiotic cell cycle. We have found that GSK-3β is expressed and active in arrested oocytes, that release from the $G_2$ block, which occurs in the absence of transcription, is associated with inactivation of GSK-3β, and that the physiological effector mediating the release requires GSK-3β inactivation. Treatments that depress endogenous GSK-3β or which are expected to compete with it for binding to substrates or positive effectors accelerate the response to the maturation signal.

We have not investigated the mechanism of GSK-3β inhibition in response to progesterone, which may be complex given that multiple protein kinases that can phosphorylate GSK-3β are activated at M-phase. However, we have shown that, in oocytes, GSK-3β is responsive to overexpression of Dishevelled, an effector of the wnt pathway.

We have begun to analyse the mechanism by which GSK-3β contributes to the $G_2$ block in oocytes. Although maintaining GSK-3β at a high level prevents the synthesis of mos and the activity of the MAP kinase cascade in response to progesterone, it does not directly prevent these events since they occur in spite of a high level of GSK-3β if cyclin B injection is used to trigger maturation. This suggests that it acts upstream. Also, the accumulation of β-catenin, which is negatively regulated by GSK-3β in oocytes (this work) and embryos (eg Yost et al., 1996), is not the target of GSK-3β in the $G_2$ arrest since a mutant version of β-catenin, which cannot be phosphorylated by GSK-3β and which can therefore accumulate in the presence of high GSK-3β, does not overcome the cell cycle block.

GSK-3β is not the only kinase involved in $G_2$ arrest in oocytes. 20 years ago, it was shown that inactivation of PKA is both necessary and sufficient (if protein synthesis is allowed) to induce meiotic maturation of *Xenopus* oocytes (Maller and Krebs, 1977). In *Dictyostelium*, regulation of cell fate by PKA requires GSK-3 (Harwood et al., 1995) while, in *Drosophila*, both PKA and GSK-3 can lead to proteolysis of regulatory proteins (Jiang and Struhl, 1998). Both PKA and GSK-3 can also phosphorylate and regulate CREB (Bullock and Habener, 1998). One could envisage that PKA and GSK-3β may be involved synergistically in mediating $G_2$ arrest, by phosphorylating a common target inhibitory for cell cycle progression, and/or by preventing accumulation of an activator of cell cycle progression. Although the mechanism by which PKA exerts its inhibitory role is still not known, PKA has been proposed to have pleiotropic effects and to inhibit maturation at multiple points (Matten et al., 1994; Rime et al., 1994). For example, PKA has been reported to block maturation induced by the microinjection of recombinant cyclin B (Rime et al., 1992). In contrast, overproduction of active GSK-3β does not prevent microinjected cyclin B from forming an active complex with cdc2 and inducing GVBD. This shows that GSK-3β inhibition of cdc2 activation is due to prevention of an

upstream event in the cascade of events initiated by progesterone, and that its inhibitory effect can be bypassed by activating cdc2 kinase experimentally, by a mechanism that does not require the physiological signalling cascade.

Furthermore, the timing of GSK-3β inactivation does not correspond to the small but rapid change in cAMP that has been observed in response to progesterone (Cicirelli and Smith, 1985), while inhibition of endogenous protein kinase A has not yet been demonstrated in the case of oocyte maturation. A current problem in the elucidation of the mechanisms by which various effectors can bring about maturation of *Xenopus* oocytes is the identification of early events following stimulation. It is clear that MAP kinase is essential for activation of cdc2 kinase in *Xenopus* oocytes, although they are activated concurrently (Kosako et al., 1994; Shibuya et al., 1992). Similarly, activation of cdc2 kinase requires inhibition by phosphorylation of cdc2-inhibitory kinases such as Myt1, and the mechanism that has been proposed is via an enzyme downstream of MAP kinase, p90rsk (Palmer et al., 1998). These events, as well as GSK-3β inactivation (this work) seem to occur simultaneously, and this may be the basis for the high positive cooperativity of the 'cell-fate switch' recently demonstrated (Ferrell and Machleder, 1998), exemplifying the difficulty in delineating a temporal order of events. Tripping the switch might depend on, for example, progesterone-mediated stimulation of mos accumulation to a low but critical level set by another maturation-inhibiting kinase, casein kinase II (Chen and Cooper, 1997; Chen et al., 1997), although other scenarios are equally possible.

GSK-3 can both inhibit protein translation, by phosphorylating and inactivating the initiation factor eIF-2B (Welsh and Proud, 1993), and stimulate the ubiquitin-dependent degradation of at least β-catenin (Orford et al., 1997; Aberle et al., 1997). One potential target for GSK-3β regulation in the context of $G_2$ arrest could thus be the accumulation of a protein or proteins necessary for cell cycle resumption. This is interesting in the light of experiments reported by Furuno et al. (1994) and Nebreda et al. (1995) in which a catalytically inactive dominant-negative form of cdc2, which can still bind cyclins, blocks MAP kinase and MPF activation induced by progesterone, a phenotype identical to that of GSK-3β overexpression. These observations suggest that a newly accumulated protein, presumably a cyclin, must associate with free cdc2 to trigger activation of pre-MPF. Polyadenylation of an mRNA other than mos, and by implication accumulation of the protein, is necessary for *Xenopus* oocyte maturation (Barkoff et al., 1998). That cyclins are also degraded by the ubiquitin-proteasome system leads us to speculate that high GSK-3β may act in a similar way to dominant-negative cdc2, the former preventing the accumulation of a protein which is titrated by the latter. This would be consistent with the observation that GSK-3β-mediated arrest is bypassed by cyclin B microinjection.

As well as a role in $G_2$ arrest in oocytes, GSK-3 is clearly involved in dorsoventral development by maintaining the asymmetric distribution of β-catenin. β-catenin is specifically localized to the future dorsal side of 4-cell embryos and is regulated by GSK-3 activity (Larabell et al., 1997). Injected β-catenin mRNA is distributed throughout the embryo, suggesting that GSK-3 may be differentially active on the future dorsal/ventral sides very early in embryogenesis, promoting degradation of ventrally synthesized β-catenin. A subpopulation of GSK-3β may remain active, or GSK-3β may be reactivated soon after fertilization in a spatially restricted fashion. We have shown that accumulation of β-catenin oocurs during oocyte maturation, but cannot occur in oocytes in which GSK-3β is fully active, unless deleted for the GSK-3β phosphorylation site. Thus, the inhibition of the pool of GSK-3β involved in oocyte arrest is also absolutely required to allow β-catenin accumulation in the fertilized egg. GSK-3β expression is also developmentally regulated – only low levels of protein are present in the oocyte, while it accumulates during early embryogenesis. As such, GSK-3β is controlled at two levels: suppression of translation prevents high levels of GSK-3β protein accumulation, while signal transduction inhibits its kinase activity. A low level of GSK-3β protein in oocytes is necessary to allow release from $G_2$ arrest, since overexpression to levels seen several hours into embryogenesis blocks meiotic maturation.

The coordination of release of oocytes from $G_2$ arrest with the polarization of the early embryo thus seems to be controlled at a surprisingly sensitive level, in which GSK-3β regulation is critical. The nature of this regulation of GSK-3β and the mechanism by which GSK-3β inhibits cell proliferation is paramount to an understanding of embryonic development. Given the implications for cell proliferation, it is very important to know whether GSK-3 homologues exert similar roles in other animal systems.

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