

Maintenance of meiotic prophase arrest in vertebrate oocytes by a G_s protein-mediated pathway[☆]

Rebecca R. Kalinowski,^a Catherine H. Berlot,^b Teresa L.Z. Jones,^c Lavinia F. Ross,^a
Laurinda A. Jaffe,^{a,*} and Lisa M. Mehlmann^{a,*}

^aDepartment of Cell Biology, University of Connecticut Health Center, Farmington, CT 06032, USA

^bWeis Center for Research, Geisinger Clinic, Danville, PA 17822, USA

^cMetabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract

Maintenance of meiotic prophase arrest in fully grown vertebrate oocytes depends on an elevated level of cAMP in the oocyte. To investigate how the cAMP level is regulated, we examined whether the activity of an oocyte G protein of the family that stimulates adenylyl cyclase, G_s , is required to maintain meiotic arrest. Microinjection of a dominant negative form of G_s into *Xenopus* and mouse oocytes, or microinjection of an antibody that inhibits the G_s G protein into zebrafish oocytes, caused meiosis to resume. Together with previous studies, these results support the conclusion that G_s -regulated generation of cAMP by the oocyte is a common mechanism for maintaining meiotic prophase arrest in vertebrate oocytes.

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Introduction

Fully grown oocytes of mammals, frogs and fish remain arrested in meiotic prophase within the ovarian follicle until luteinizing hormone (LH) acts on the follicular cells to cause meiosis to resume (see Masui and Clarke, 1979). In mammals, maintenance of the prophase arrest depends on the presence of ovarian follicle cells, but in frogs, oocytes remain arrested even in the absence of follicle cells. Nevertheless, evidence indicates that in both of these vertebrate groups, the activity of a G_s G protein is required to maintain the arrest. This has been determined by injecting oocytes with an inhibitory antibody made against the 10 C-terminal amino acids of the α subunit of G_s (Gallo et al., 1995;

Mehlmann et al., 2002). Because G_s stimulates adenylyl cyclase, it acts to elevate cAMP. Thus, a requirement for G_s in maintaining meiotic arrest fits well with other evidence indicating a requirement for cAMP and adenylyl cyclase in the oocyte to maintain arrest (Eppig, 1991; Eppig et al., 2004; Horner et al., 2003; Maller and Krebs, 1977). The α subunit and/or $\beta\gamma$ subunit complex of G_s could be the activator of adenylyl cyclase (Hanoune and Defer, 2001; Simonds, 1999; Sheng et al., 2001). It is not fully understood how cAMP controls the activation state of the cyclin-dependent kinase/cyclin B complex (CDK1/CYB) that determines whether the cell progresses from prophase to metaphase; however, recent work has identified the CDC25 phosphatase, which directly regulates the activity of CDK1, as at least one substrate of the cAMP-dependent kinase, protein kinase A (Ferrell, 1999; Duckworth et al., 2002; Lincoln et al., 2002; Kishimoto, 2003).

A role for G_s activity in maintaining meiotic arrest is consistent with several other previous studies. Under some experimental conditions, the G_s activator cholera toxin can have an inhibitory effect on spontaneous nuclear envelope or “germinal vesicle” breakdown (GVBD) in isolated mouse oocytes (Downs et al., 1992; Vivarelli et al., 1983).

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* Corresponding authors. Department of Cell Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06032. Fax: +1-860-679-1661.

E-mail addresses: bkalinowski@neuron.uhc.edu (R.R. Kalinowski), chberlot@geisinger.edu (C.H. Berlot), tlzj@helix.nih.gov (T.L.Z. Jones), lross@neuron.uhc.edu (L.F. Ross), ljaffe@neuron.uhc.edu (L.A. Jaffe), lmehlman@neuron.uhc.edu (L.M. Mehlmann).

Conversely, general inhibition of G protein function by sequestration of G protein $\beta\gamma$ subunits has been reported to cause GVBD in *Xenopus* oocytes (Sheng et al., 2001; see Discussion), as does general inhibition of G protein-coupled receptors (GPCRs) by G protein receptor kinases or β -arrestin (Wang and Liu, 2003). However, G protein receptor kinases can phosphorylate proteins other than GPCRs (see Wang and Liu, 2003) and β -arrestin also interacts with proteins other than GPCRs (Chen et al., 2003; Pierce and Lefkowitz, 2001). In addition, injection of *Xenopus* oocytes with G_s antisense oligonucleotides has been reported to cause an increase in MAPK activity like that seen in response to maturation-inducing steroids, although the effect of the antisense oligonucleotides on meiotic resumption was not examined (Romo et al., 2002).

All of the G protein modifiers used to date could have nonspecific targets; even the antibody made against G_s , which is quite specific in its binding to the G_s protein in lysates of mouse and *Xenopus* oocytes (Mehlmann et al., 2002, supplementary material), could in principle interact with other proteins within the cytoplasm of a living oocyte. Since the concept that oocyte cAMP is generated by G_s activity and adenylyl cyclase activity in the oocyte itself differs from the long standing paradigm that meiotic arrest is maintained in mammalian oocytes due to transfer of cAMP from follicle cells via gap junctions (see Anderson and Albertini, 1976; Eppig et al., 2004; Webb et al., 2002), we felt that it was important to test the G_s requirement for maintaining meiotic arrest using an independent method. We also wished to examine the possible role of other heterotrimeric G proteins in maintaining meiotic arrest, particularly for frog, since a previous study suggested this possibility (Sheng et al., 2001).

To inhibit G_s function, we used a dominant negative form of G_s , in which the sequence of the α subunit of rat G_s was point-mutated at multiple positions, resulting in a protein that blocks signaling by G_s -linked receptors ($\alpha_s(\alpha3\beta5/G226A/A366S)$; Berlot, 2002). The effectiveness of this dominant negative G_s , which we refer to as G_s DN, was demonstrated by transfecting it into tissue culture cells that also expressed the LH receptor. The cAMP response to agonist addition in the G_s DN-transfected cells was reduced by 97% relative to the response in control cells without G_s DN (Berlot, 2002). The mutations in this dominant negative α_s mutant increase receptor affinity and decrease receptor-mediated activation (substitutions in the $\alpha3\beta5$ loop region), prevent an activating conformational change required for dissociation of α from $\beta\gamma$ (G226A), and decrease affinity for GDP (A366S). *Xenopus* and rat $G\alpha_s$ subunits are 92% identical in amino acid sequence, and although they may not be functionally identical in all respects (see Antonelli et al., 1994), their amino acid sequences are 100% identical in the positions that were modified in G_s DN. Therefore, it is likely that G_s DN should behave as a G_s dominant negative in *Xenopus* oocytes as it does in mammalian cells. We injected RNA encoding G_s DN into *Xen-*

opus and mouse oocytes to determine if it caused meiosis to resume. To examine the generality of the G_s requirement for maintaining meiotic arrest in vertebrate oocytes, we also investigated the effect of G_s inhibition on meiotic arrest in zebrafish oocytes.

Materials and methods

G_s constructs, in vitro transcription

The G_s dominant negative DNA (G_s DN) consisted of the α subunit of rat G_s with point mutations at seven positions: G226A, N271K, K274D, R280K, T284D, I285T, A366S; this construct was originally named $\alpha_s(\alpha3\beta5/G226A/A366S)$ (Berlot, 2002). G_s DN and the control constructs, R280K (Berlot, 2002) and G226A (Iiri et al., 1999), were in the vector pcDNA1/Amp (Invitrogen, Carlsbad, CA). The plasmids were linearized using *Xba*I and RNA was transcribed in vitro using T7 polymerase.

Antibodies and other reagents

Affinity-purified antibodies against the G_s α -subunit (RM) and against the G_q α -subunit (QL) were provided by Allen Spiegel (NIH, Bethesda, MD). These antibodies were made against 10-amino-acid peptides corresponding to the C-termini of *Xenopus* and mouse $G\alpha_s$ and $G\alpha_q$ (Gallo et al., 1996; Shenker et al., 1991; Simonds et al., 1989). Non-immune rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies were concentrated in PBS as described in Gallo et al. (1995). DHP (Steraloids, Newport, RI) was dissolved in EtOH (10 mg/ml) before diluting in the frog or fish oocyte culture medium (see below). Hypoxanthine (Sigma) was made as a 200-mM stock in 1 N NaOH, diluted to 4 mM in the mouse oocyte culture medium, MEM (see below), and the pH was adjusted to 7.2 with HCl. Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA) and activated by incubation in the presence of 10 mM DTT and 0.1 mM ATP at 37°C for 15 min before microinjection.

Culture and microinjection of follicle-free Xenopus oocytes

Frogs (*Xenopus laevis*) were purchased from Nasco (Fort Atkinson, WI) and were used without gonadotropin priming. Stage VI follicle-free oocytes (approximately 1200–1300 μ m diameter) were obtained by treating pieces of ovary with collagenase (Duesbery and Masui, 1993; Gallo et al., 1995). The oocytes were cultured at 18–20°C in 50% Leibovitz's L-15 medium, 15 mM HEPES, pH 7.8, 100 μ g/ml gentamicin (all components from Invitrogen) on agarose-coated dishes (2% Sigma type V, high gelling temperature agarose in modified Ringer's). Oocytes were cultured for 15–18 h after isolation before use; this culture

period allows the oocytes to recover their protein synthesis capability after collagenase treatment (Smith et al., 1991). Oocytes were injected with 50 nl of RNA, using a Picospritzer (General Valve Corporation, Fairfield, NJ), or 50 nl of an antibody solution, using a syringe-controlled injection system and pipets backfilled with mercury (see Runft et al., 1999). The volume of a *Xenopus* oocyte is approximately 1000 nl.

DHP was used as the maturation-inducing steroid for *Xenopus* oocytes because we found that it usually caused GVBD at a somewhat lower concentration than was seen with progesterone. GVBD was scored by observation of a white spot at the animal pole using a stereoscope, and confirmed by fixing for 5 min in 4% trichloroacetic acid and halving the oocytes with a scalpel, to determine whether the GV was present (Gallo et al., 1995). Oocytes were photographed using a stereoscope (Wild M3C, Leica Inc., Rockleigh, NJ) and a DC4800 digital camera (Eastman Kodak, Rochester, NY). Chromosomes and polar bodies were fluorescently labeled by incubating live defolliculated oocytes in 10 µg/ml H33258 Hoechst stain (Gallo et al., 1995). Chromosomes were photographed using a Zeiss Axioskop with a 10×, 0.3 NA neofluar objective (Carl Zeiss, Inc., Thornwood, NY) and a Kodak DC4800 digital camera. These and other data figures were assembled using Adobe Photoshop 6.0.

Culture and microinjection of follicle-enclosed and isolated mouse oocytes

NSA (CF1) mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN). For experiments with follicle-enclosed oocytes, antral follicles were dissected from the ovaries of 22- to 25-day-old, unprimed mice using fine forceps and 30-gauge needles (Mehlmann et al., 2002). Approximately 10–20 follicles were obtained per mouse, ranging in size from approximately 260 to 470 µm in diameter, and were cultured in 200-µl drops of medium under light mineral oil (Fisher Scientific, Pittsburgh, PA) on a tray maintained at 37°C. The medium used was MEM with Earle's salts, L-glutamine, nonessential amino acids, 120 U/ml penicillin G (potassium salt), 50 µg/ml streptomycin sulfate, 0.24 mM sodium pyruvate, 0.1% polyvinyl alcohol, and 20 mM HEPES, pH 7.2, and was supplemented with 1 mg/ml BSA (Fraction V, Calbiochem; other reagents from Sigma).

Follicle-enclosed mouse oocytes were microinjected in a chamber constructed of two pieces of coverglass that were held apart by a 300-µm spacer composed of three layers of double-stick tape (see Mehlmann et al., 2002). The tape held a small piece of coverslip hanging from the top coverslip, forming a 1-mm wide ledge in which the follicles were placed using a mouth-controlled pipet. This assembly was mounted on a U-shaped plastic slide over a reservoir of medium. The chamber was observed using an upright microscope (Zeiss Axioskop) with a 20× lens (0.5 or 0.75

N.A.). A micropipet was used to roll the follicle in order to position the oocyte near the upper surface for optimized viewing of the oocyte. Only oocytes in which a nucleolus could be seen were injected and used for these experiments. Two follicles were put in the injection chamber at a time and were kept in the chamber at 18–22°C for 10–30 min; after injection, they were transferred back to a culture dish at 37°C (1–10 follicles per 200-µl drop).

Oocytes were injected using a syringe-controlled injection system and pipets backfilled with mercury (see Mehlmann et al., 2002; Jaffe and Terasaki, in press). Injection volumes (14 pl) were calibrated by drawing up a comparable length of oil into the injection pipet, then expelling the oil and measuring the diameter of the drop. (The volume of a mouse oocyte is approximately 200 pl.) The success of the injection was confirmed by including calcium green 10-kDa dextran (Molecular Probes) in the RNA solution (final concentration in the oocyte = 10 µM) and checking the oocytes for fluorescence (see Mehlmann et al., 2002).

For these experiments, we divided the follicles obtained from a single mouse into two dishes, choosing follicles of equivalent size and appearance for each dish. One dish was used for injection and the other was set aside as a control. Injections were performed within approximately 1–2 h after the dissection. Six hours after injection, follicles were opened using 30-gauge needles to determine if the oocyte had undergone GVBD. After opening the injected follicles, the follicles in the control dish were also opened. If GVBD in the control dish exceeded 25%, the results from injections into follicles from that mouse were disregarded; 10% of the mice we tested were unacceptable by this criterion. Discounting these, the rate of spontaneous GVBD in control, uninjected oocytes was 7%.

For experiments with isolated oocytes, 4- to 12-week-old mice were used. Fully grown (approximately 70–75 µm diameter) immature oocytes were collected, pipetted to removed cumulus cells, and injected as previously described (Mehlmann and Kline, 1994). The medium used was MEM as described above, supplemented with 250 µM dbcAMP during dissection and injection. Oocytes were subsequently transferred to MEM with 4 mM hypoxanthine.

Culture and microinjection of follicle-enclosed zebrafish oocytes

Zebrafish (*Danio rerio*, wild type) were kindly provided by Dr. Stephen DeVoto (Wesleyan University, Middletown, CT) or purchased from Carolina Biological (Burlington, NC). Males and females were maintained together in a filtered, aerated 30-gal aquarium containing deionized water and 1.5 g/gal Tropic Marin sea salts (Marinus Inc., Long Beach, CA). The temperature was 25–28°C, and the light cycle was 14 h light and 10 h dark. Fish were fed twice a day with dry fish food (TetraMin) supplemented two times per week with live brine shrimp or frozen *Drosophila*. Fish were killed between 1 and 3 h after the light turned on by

incubation in an ice bath for 30 s followed by decapitation. Ovaries were placed into 3 ml of the same medium described above for *Xenopus* oocytes, but at pH 7.2, on 2% agarose-coated dishes. Follicle-enclosed oocytes were isolated using fine forceps under a dissecting microscope; the follicle was pinched from the ovary at the stalk, resulting in an oocyte surrounded by a three-layer follicular epithelium approximately 10 μm in thickness (Selman et al., 1993, 1994), which we were unable to remove without damaging the oocytes. Before use, the follicle-enclosed oocytes were cultured for 2–3 h to identify and remove any that had been damaged by the isolation procedure. Approximately 15–130 follicles were obtained per fish; follicles from 1 to 4 fish were pooled for each experiment. No more than 20 follicles were cultured in a 35-mm dish.

Follicles of 650–700 μm in diameter were used for the experiments. All oocytes in this size range underwent GVBD in response to 30 nM DHP and none underwent spontaneous GVBD in the absence of DHP. For microinjection, follicles were placed in a chamber constructed from a plastic slide supporting two parallel coverslips, with the follicles resting on the bottom coverslip. The slide had a rectangular cut out 15 mm long, 5 mm deep, and 1.5 mm thick, with the coverslips attached above and below with silicon grease. The slide was held on the stage of an upright microscope, and viewed with a 10 \times objective, 0.3 N.A. Injections were made using pipets backfilled with mercury (see Jaffe and Terasaki, in press). Injection volumes were calibrated by measuring the decrease in the length of the column of solution in a loading capillary (0.5 mm inner diameter, Drummond Scientific Company, Broomall, PA) that was held on the injection slide. Injection volumes were 5–10 nl, corresponding to approximately 3–5% of the oocyte volume (180 nl). After injection, the oocytes were removed from the chamber and incubated in 4 ml of medium in a 35-mm culture dish, at 18–20°C, or at 25°C. Follicles were photographed using a stereoscope (Wild M3C) and a Kodak DC4800 digital camera. Indirect fiber optic lighting was used to visualize the GV and oocyte clearing (see Fig. 4B).

Immunoblotting

For immunoblotting, *Xenopus* oocyte membranes (Gallo et al., 1996; “type 2” membranes) and a homogenate of whole mouse brain (Mehlmann et al., 2001) were prepared as previously described. Mouse oocytes were prepared by freezing oocytes with liquid N_2 , then just before use, solubilizing them in SDS sample buffer (Mehlmann et al., 1998). Zebrafish samples were prepared by lysing approximately 20 oocytes in a glass homogenizer in 20 mM HEPES, pH 7.0, 1 mM EDTA, 2 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 mM Pefabloc, and 10 $\mu\text{g}/\text{ml}$ leupeptin, followed by centrifugation at 1000 $\times g$ for 5 min at 4°C. The supernatants were used for gel samples. The protein content of mouse oocytes was estimated as 25 ng per oocyte (Schultz and Wassarman,

1977). A BCA protein assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard was performed to determine the protein concentration for samples from frog and fish oocytes. Proteins were separated by SDS-PAGE and blots were incubated with the $\text{G}\alpha_s$ antibody (1.7 $\mu\text{g}/\text{ml}$) or the $\text{G}\alpha_q$ antibody (1.0 $\mu\text{g}/\text{ml}$), and developed with ECL Plus reagents (Amersham Life Science, Inc., Arlington Heights, IL). Immunodensities were compared using scanned images of the films and NIH Image (available at <http://rsb.info.nih.gov/nih-image/>).

Online supplemental material

Video1.mov. Microinjection of a follicle-enclosed mouse oocyte. The micropipet was advanced towards the follicle from the left, pushed through the mural granulosa cell layers, pulled back, then pushed forward again into the oocyte. A drop of silicon oil was introduced into the oocyte cytoplasm as a consequence of the injection. After the pipet was withdrawn from the follicle, the success of the injection was confirmed by the presence of a fluorescent marker (calcium green dextran) in the oocyte.

The movie was made by imaging the follicle using a Zeiss Axioskop with a 20 \times /0.75 N.A. fluar lens, and recording the video signal from a Kodak DC 4800 camera using a Sony PC5 camcorder. The movie was downloaded by firewire into a Macintosh computer using iMovie and then cropped using Adobe Premiere 6.0. The images were collected at 30 frames per second; every other frame was subsequently deleted, to reduce the file size, so the Quick-time movie is 15 frames per second.

Results

*Injection of a dominant negative form of G_s causes resumption of meiosis in *Xenopus* oocytes*

To examine whether inhibiting $\text{G}\alpha_s$ with a dominant negative form of the $\text{G}\alpha_s$ protein (G_sDN) would cause *Xenopus* oocytes to resume meiosis, we injected oocytes with G_sDN RNA. Oocytes injected with ≥ 1 ng of this RNA underwent GVBD, as indicated by the formation of a white spot at the animal pole (Fig. 1A) and confirmed by fixing oocytes in 4% trichloroacetic acid, halving them with a scalpel and observing the absence of the germinal vesicle using a stereoscope.

The time course of GVBD in response to injection of G_sDN RNA was similar to that seen in uninjected oocytes incubated with 10 μM of a maturation-inducing progesterone derivative, 4-pregnen-17 α 20 β -diol-3-one (DHP) (Fig. 2A); this indicated that the translation of the injected RNA to make protein occurs rapidly. Other studies have shown that protein production in *Xenopus* oocytes can be detected as early as 1 h after RNA injection (e.g., Martínez-Torres and Miledi, 2001), so the lack of an obvious delay in GVBD

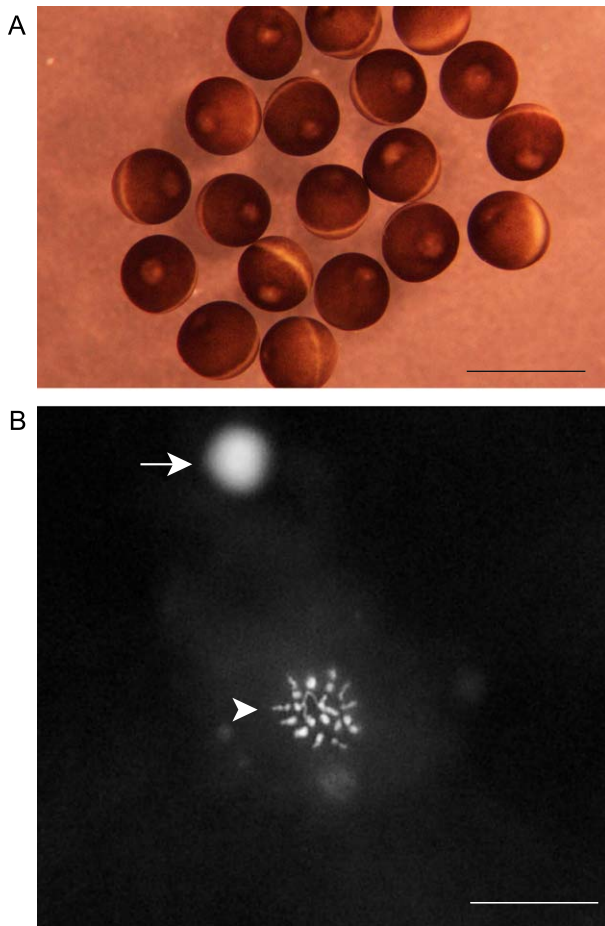


Fig. 1. Injection of G_s dominant negative RNA causes meiotic maturation of *Xenopus* oocytes. (A) White spot formation indicative of GVBD. Oocytes were injected with 10 ng of RNA and photographed 18 h later. Scale bar = 2.0 mm. (B) DNA staining showing the first polar body (arrow) (5/6 oocytes) and second metaphase chromosomes (arrowhead) (6/6 oocytes). Oocytes were injected with 3 ng of RNA; 24 h later, they were stained with 10 μ g/ml Hoechst 33258 and visualized by fluorescence microscopy. Scale bar = 50 μ m.

to account for the time required for protein synthesis is not surprising. Analyses of cellular levels of G_s and another G_s -linked receptor (β -adrenergic) have shown that G_s is in approximately 200 \times molar excess (Post et al., 1995), making it reasonable that even a small amount of a mutant G_s protein with high receptor affinity (Berlot, 2002) could inhibit receptor- G_s coupling.

To examine whether G_s DN-injected oocytes proceeded through meiosis and arrested normally at metaphase II, we stained the injected oocytes with a DNA specific dye (H33258), 24 h after injection, and examined them by fluorescence microscopy. Observation of the animal pole of the live oocytes showed condensed chromosomes and a polar body (Fig. 1B). This pattern of condensed chromosomes and a polar body was similar to that seen after injection of a G_s inhibitory antibody or exposure to steroid (Gallo et al., 1995) and indicated that the G_s DN-injected oocytes had progressed to metaphase II.

Specificity controls

As controls, we injected oocytes with two different RNAs, each of which encode an α_s subunit with a single amino acid substitution (R280K or G226A). These two substitutions are also present in G_s DN. When transfected

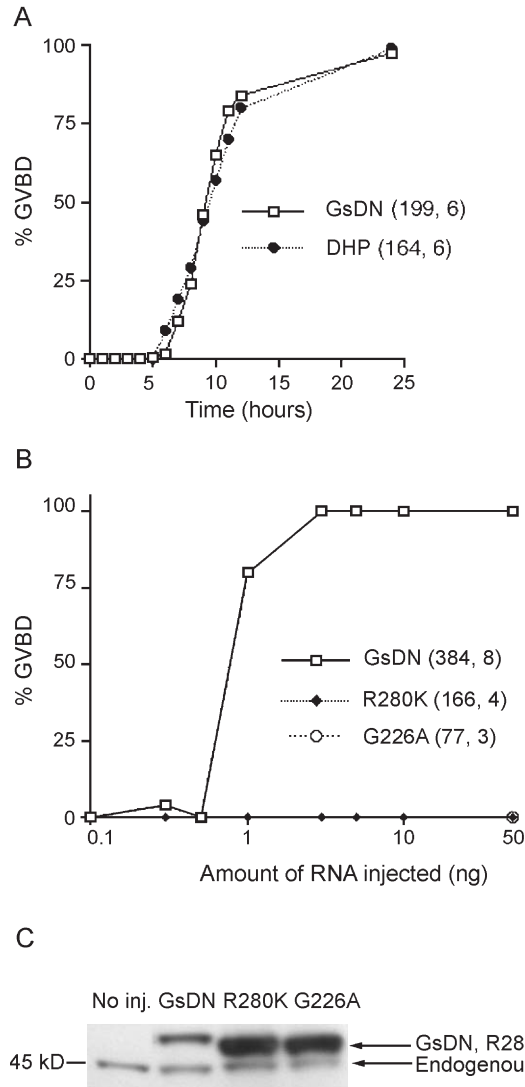


Fig. 2. Kinetics and concentration dependence of GVBD in response to injection of G_s dominant negative (G_s DN) RNA into *Xenopus* oocytes. (A) Oocytes were injected with 1–50 ng of G_s DN RNA or exposed to 10 μ M DHP, and scored for GVBD at various times after injection or DHP application. The numbers in parentheses indicate the number of oocytes and number of animals tested. (B) Oocytes were injected with various amounts of G_s DN RNA or a control RNA (R280K or G226A) and scored for GVBD 24 h later. (C) Immunoblot of *Xenopus* oocyte membranes showing expression of G_s DN, R280K, and G226A proteins after injection of 1 ng of G_s DN RNA, or 50 ng of R280K or G226A RNA. Membranes were prepared 24 h after RNA injection and 10 μ g of protein was loaded in each lane of the gel. The blot was probed with an antibody against the α subunit of G_s (RM). The lower bands show endogenous G_s and the upper bands show the exogenously expressed proteins. G_s DN migrates slightly more slowly than R280K and G226A (see Berlot, 2002). The densities of the R280K and G226A bands were 2.5 \times the density of the G_s DN band.

into tissue culture cells, the R280K and G226A mutated forms of α_s exhibit little or no dominant negative activity and also have little or no ability to activate adenylyl cyclase in response to receptor stimulation (Iiri et al., 1999; Berlot, 2002). G226A is of particular interest because this mutation increases the $\beta\gamma$ affinity of α_s (Miller et al., 1988; Lee et al., 1992), and thus it provides a test of whether the stimulation of GVBD by G_s DN could be due to its increased affinity for $\beta\gamma$ rather than its interference with receptor-mediated activation of α_s .

Injection of ≥ 1 ng of G_s DN RNA induced GVBD, but injection of 50 ng of either R280K or G226A RNA did not (Fig. 2B). To confirm that the control proteins were effectively expressed, we analyzed the membranes of injected oocytes by immunoblotting (Fig. 2C). Oocytes produced protein from all of the injected RNAs, and the amount of R280K and G226A protein expressed after injection of 50 ng of RNA was greater than the amount of G_s DN protein expressed after injection of 1 ng of RNA. Thus, although more control protein was produced, it did not cause GVBD.

We also considered whether the stimulation of oocyte maturation by G_s DN might result from inhibition of other heterotrimeric G proteins since this dominant negative α_s mutant can block signaling from the calcitonin receptor to both G_s and G_q (Berlot, 2002). For this reason, we examined the possible role of other G proteins in maintaining meiotic arrest. Stimulation of oocyte maturation by G_s DN is unlikely to result from inhibition of G_i since inactivation of 90% of the G_i in *Xenopus* oocytes by pertussis toxin did not cause meiotic resumption (Kline et al., 1991), and a role for the PTX-insensitive G_i family G protein, G_z , is unlikely since G_z protein is not present at detectable levels in *Xenopus* oocytes (Kalinowski et al., 2003). Furthermore, injection of an inhibitory antibody (EC; Wilson et al., 1993) against the only detectable G_i family G protein in *Xenopus* oocytes, $G\alpha_{i3}$, did not cause GVBD (Gallo et al., 1995). (α_{i1} , α_{i2} , α_t , and α_o proteins were not detectable by immunoblotting; Gallo et al., 1995.)

The G_q family of heterotrimeric G proteins are also expressed, at low levels, in *Xenopus* oocytes (Gallo et al., 1996). To examine if this G protein family could function in maintaining meiotic arrest, we injected an inhibitory antibody against $G_q/G_{11}/G_{14}$ (QL), which recognizes G proteins of this family in *Xenopus* oocytes (Gallo et al., 1996). At a concentration of 1 mg/ml (7 μ M), which has been previously demonstrated to eliminate the function of G_q family G proteins in *Xenopus* eggs (Runft et al., 1999), the QL antibody did not cause GVBD ($n = 20$ oocytes). Another G_q family member, $G_{15/16}$, is not present in *Xenopus* eggs, at least in a functional form (Runft et al., 1999). Therefore, neither G_i nor G_q family G proteins appear to have a function in maintaining meiotic arrest. The mouse and human genomes contain one other less well-characterized G protein α subunit family, $G_{12/13}$ (Wilkie et al., 1992), for which a *Xenopus* homolog could exist. With this possible caveat, we concluded that the stimulation of meiotic re-

sumption by injection of the G_s dominant negative RNA is very likely due to inhibition of G_s .

Injection of a dominant negative form of G_s causes resumption of meiosis in follicle-enclosed and isolated mouse oocytes

We next examined whether expression of the dominant negative G_s would also cause GVBD in mouse oocytes. In the first series of experiments, we dissected antral follicles and injected the oocytes within the follicles; a video of the injection process is shown in the Online supplemental material (Video1.mov). We injected the follicle-enclosed mouse oocytes with 42 pg of G_s DN RNA, cultured the follicles for 6 h after the injection to allow time for the oocytes to express the protein, removed the oocytes from the follicles, and scored them for GVBD. We injected 60 pg of the R280K RNA as a control.

Injection of G_s DN RNA caused GVBD in 88% of follicle-enclosed oocytes (Fig. 3A) and these oocytes subsequently formed first polar bodies. The R280K mutant did not cause GVBD (Fig. 3A). All oocytes injected with R280K, as well as uninjected oocytes, underwent spontaneous GVBD after they were removed from the follicles, and almost all were subsequently observed to have formed first polar bodies (73% and 83%, respectively). This demonstrates that the control oocytes were healthy and that the RNA did not have nonspecific or toxic effects.

Injection of G_s DN RNA also caused GVBD in isolated oocytes that were incubated in the presence of hypoxanthine to maintain meiotic arrest. Eighty percent of the oocytes underwent GVBD by 3.5–4.5 h after injection (Fig. 3B). The R280K and G226A control RNAs did not cause GVBD (Fig. 3B). Oocytes injected with the G_s DN, R280K, and G226A RNAs produced similar amounts of protein, as determined by immunoblotting (Fig. 3C). Additional control experiments showed that injection of pertussis toxin (9 μ g/ml, $n = 11$ oocytes), or the G_q -family inhibitory antibody QL (1.0 mg/ml=7 μ M, $n = 7$ oocytes), which recognizes a $G\alpha_q$ family protein in mouse oocytes (Fig. 3D), did not cause GVBD in isolated mouse oocytes in hypoxanthine-containing medium. These results provide further evidence that G_s activity in mouse oocytes is required to maintain meiotic prophase arrest.

Injection of a G_s inhibitory antibody causes resumption of meiosis in zebrafish oocytes

To examine if G_s activity might be a widespread mechanism for maintaining meiotic arrest in vertebrate oocytes, we investigated whether G_s activity was also required in zebrafish oocytes. We tried to express the G_s DN protein in zebrafish oocytes, but injection of the G_s DN RNA resulted in little if any protein synthesis (Fig. 4A). We do not have an explanation for this poor translation of the injected RNA, but we have also noted that RNA encoding GFP is not

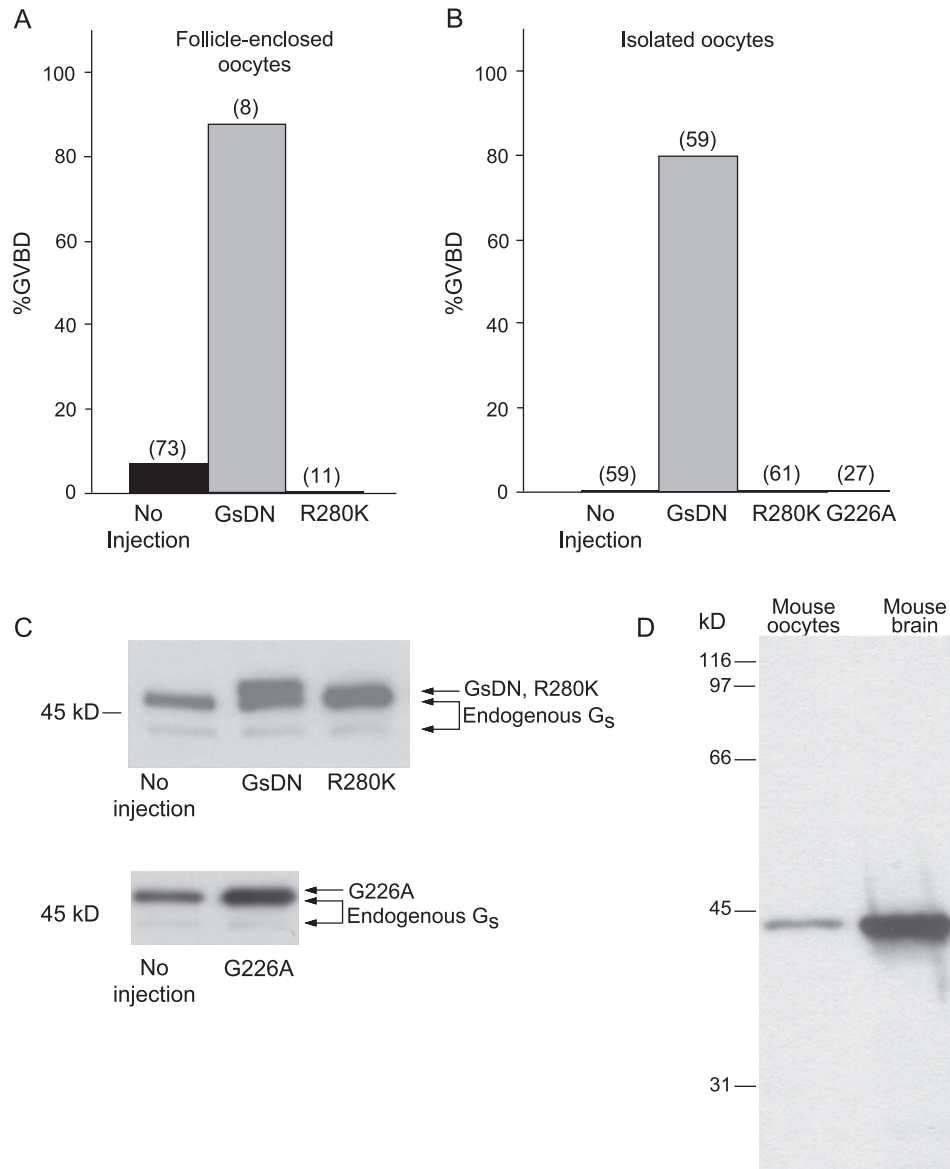


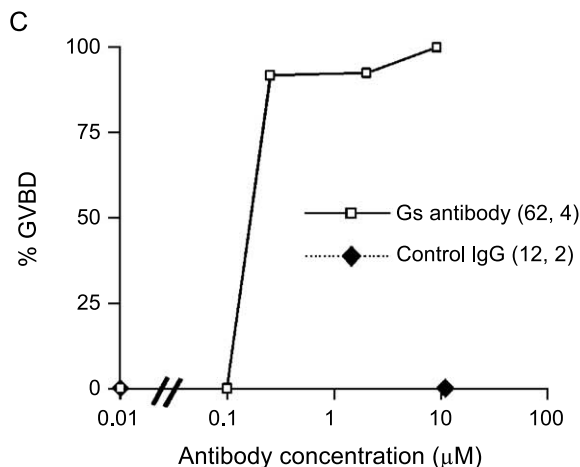
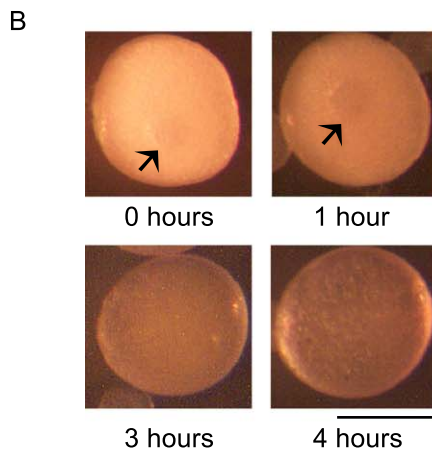
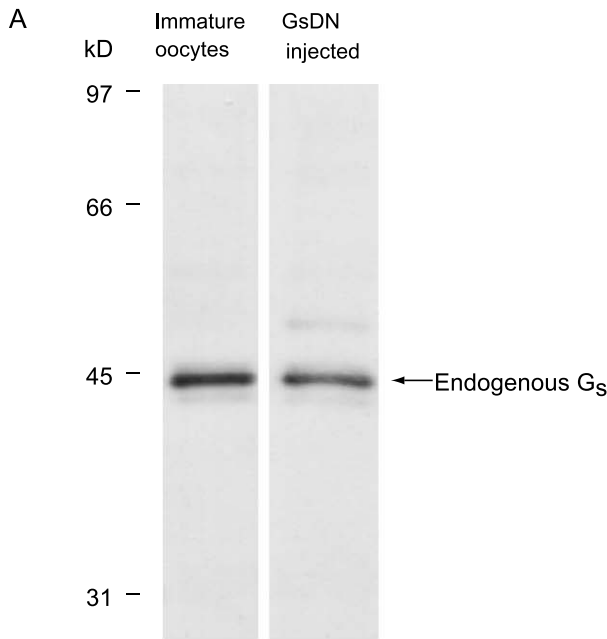
Fig. 3. Injection of G_s dominant negative RNA causes meiotic maturation of follicle-enclosed and isolated mouse oocytes. (A) Follicle-enclosed oocytes were injected with RNA encoding G_s DN (42 pg) or R280K (60 pg), or not injected; 6 h after injection, the oocytes were removed from their follicles and scored for GVBD. (B) Isolated oocytes, maintained in 4 mM hypoxanthine, were injected with RNA encoding G_s DN (26 pg), R280K (30 pg), or G226A (24 pg), or not injected; they were scored for GVBD at 3.5–4.5 h after injection. For A and B, the numbers in parentheses indicate the number of oocytes per group. (C) At 4–5 h after injection, sets of isolated oocytes were prepared for immunoblotting. The blots were probed with an antibody against the α subunit of G_s (RM). The upper blot shows oocytes that were injected with G_s DN or R280K RNA, or not injected; 59 oocytes = 1.5 μ g protein per lane. The lower blot shows oocytes that were injected with G226A RNA, or not injected; 27 oocytes = 0.7 μ g protein per lane. The G_s DN protein ran slightly more slowly on the gel than the R280K or G226A proteins, and also more slowly than the two alternative splice variants of the G_s α subunit that are present endogenously in mouse oocytes (see Mehlmann et al., 2002). Because the exogenously expressed protein bands were not separable from the upper band of endogenous G_s , densitometric measurements were performed on the combination of the upper G_s band plus the exogenously expressed protein band. Relative to the density of the upper G_s band from the uninjected eggs on the same blot, the densities for G_s DN, R280K, and G226A were 1.8, 1.7, and 1.8, respectively. (D) Immunoblot showing specific recognition of a G_{α_q} family protein in mouse oocytes by the QL antibody (100 oocytes = 2.5 μ g protein); brain homogenate (5 μ g) was run for comparison.

translated to a detectable level in zebrafish oocytes (R.R. Kalinowski, unpublished results). Consistent with the lack of significant expression of G_s DN protein, G_s DN RNA injection did not cause GVBD.

As an alternative approach, we injected zebrafish oocytes with the G_s inhibitory antibody (RM) that was previously

shown to cause GVBD when injected into frog and mouse oocytes (Gallo et al., 1995; Mehlmann et al., 2002). This antibody, which was made against the common C-terminal 10 amino acids of the α subunit of mammalian and frog G_s (see Materials and methods), specifically recognized a band at approximately 45 kDa in zebrafish oocytes (Fig. 4A) and

caused GVBD when injected into these oocytes (Fig. 4B). At 25°C, GVBD occurred at approximately 2–3 h after antibody injection, similar to the time required for GVBD following addition of the maturation-inducing hormone,



DHP. At about the same time that GVBD occurred, the opaque oocyte began to clear; this is thought to be due to a change in the structure of the yolk (Selman et al., 1994). GVBD and cytoplasmic clearing were also observed in G_s antibody-injected or DHP-treated oocytes that were cultured at 18°C; although the time course was slower (approximately 6–8 h to GVBD), it was similar for both antibody injection and hormone treatment.

Injection of a final cytoplasmic concentration of ≥ 0.037 mg/ml (0.25 µM) of the G_s antibody caused GVBD in 94% of zebrafish oocytes (Fig. 4C); 1.6 mg/ml (11 µM), injected as a control, did not cause GVBD (Fig. 4C). We concluded that as in frog and mouse oocytes, meiotic arrest in zebrafish oocytes is maintained by the activity of a G_s G protein.

Discussion

Accumulating evidence indicates that fully grown vertebrate oocytes remain arrested at meiotic prophase due to the activity of a G_s protein within the oocyte, which activates adenylyl cyclase, elevating oocyte cAMP (see Introduction). In particular, injection of a G_s inhibitory antibody into oocytes of frog (Gallo et al., 1995), mouse (Mehlmann et al., 2002), and fish (present results) causes meiosis to resume. The present results add to this evidence by demonstrating that a dominant negative form of $G\alpha_s$ (Berlot, 2002) also causes meiosis to resume in both frog and mouse oocytes, and that other heterotrimeric G proteins are not required to maintain prophase arrest. In the case of mouse oocytes, the somatic cells of the follicle have also been considered as a possible source of cAMP in the oocyte (Anderson and Albertini, 1976; Eppig et al., 2004; Webb et al., 2002). Although our results do not directly address this question, they do indicate that cAMP from a source outside of the oocyte is not sufficient to maintain meiotic arrest.

Fig. 4. An antibody against the α subunit of G_s recognizes G_s in zebrafish oocytes and causes GVBD when injected. (A) Immunoblot of proteins from zebrafish follicle-enclosed oocytes, stained with the RM antibody against α_s ; 10 µg of protein per lane. Lane 1, immature oocytes, showing the endogenous G_s protein. Lane 2, oocytes that were injected 20 h earlier with 30 ng of G_s DN RNA, showing little or no expression of G_s DN protein; the faint band above the endogenous G_s may represent G_s DN. (B) Photographs of zebrafish follicles after injection of 0.037 mg/ml (0.25 µM) of the G_s antibody (final cytoplasmic concentration) and incubated at 25°C. Arrows indicate the germinal vesicle. By 3 h after injection, the germinal vesicle was no longer visible and the opaque cytoplasm had begun to clear. By 4 h, the oocyte reached maximum clarity. Scale bar = 500 µm. (C) Zebrafish oocytes were injected with various amounts of G_s antibody or control IgG, incubated at 18°C, and scored for GVBD 16–24 h later. The numbers in parentheses indicate the number of oocytes tested and the number of separate experiments. Uninjected oocytes that were incubated for 24 h did not undergo GVBD (0/57), while oocytes that were incubated in 30 nM DHP did (49/49).

Contributions of G_s α and $\beta\gamma$ subunits in maintaining meiotic arrest

Among the approximately nine known mammalian adenylyl cyclase isoforms, all are activated by α_s , but some can also be activated by G protein $\beta\gamma$ subunits (Hanoune and Defer, 2001). This raises the question of whether the $\beta\gamma$ subunits as well as the α subunit of G_s contribute to maintaining meiotic arrest in oocytes. Based on the presence of multiple adenylyl cyclase RNAs, several isoforms of this protein are probably present in mouse oocytes (Horner et al., 2003). Among these, adenylyl cyclase 3 (AC3) appears to be essential for maintaining cAMP in the oocyte since approximately 50% of the fully grown oocytes in AC3 knockout mice undergo spontaneous maturation within the follicle (Horner et al., 2003). AC3 is activated by the α subunit of G_s rather than its $\beta\gamma$ subunits (Tang and Gilman, 1991), but $\beta\gamma$ -sensitive adenylyl cyclase isoforms may also be present. In frog oocytes, injection of RNA encoding $\beta\gamma$ subunits elevates cAMP, indicating that these oocytes contain a $\beta\gamma$ -stimulated adenylyl cyclase (Sheng et al., 2001). RNA encoding a novel adenylyl cyclase that differs in its sequence from known mammalian isoforms has been identified in *Xenopus* oocytes (Torrejón et al., 1997), but its $\beta\gamma$ sensitivity has not been examined.

The possible role of $\beta\gamma$ subunits in maintaining meiotic arrest in *Xenopus* oocytes has been investigated by injecting RNAs encoding proteins that can sequester $\beta\gamma$ subunits. Injection of large amounts (10–40 ng) of RNA encoding G protein α subunits (α_{i2} , α_t , α_o , α_q) in some cases causes GVBD, but variable results have been reported (Guttridge et al., 1995; Lutz et al., 2000; Sheng et al., 2001). GVBD can also be induced by injection of *Xenopus* oocytes with RNA encoding the noncatalytic C-terminal region of β -adrenergic receptor kinase if the protein is targeted to the membrane by addition of a geranylgeranylation site (Sheng et al., 2001). Because both the G protein α subunits and the C-terminal region of β -adrenergic receptor kinase can bind G protein $\beta\gamma$ subunits, these findings have been interpreted to indicate that sequestering $\beta\gamma$ subunits can cause GVBD. Sequestering $\beta\gamma$ subunits may turn off G protein signaling by α -subunits as well since the $\alpha\beta\gamma$ complex is required for activation of G proteins by receptors (Iiri et al., 1999; Sheng et al., 2001).

Based on this previous work, we considered the possibility that the GVBD we observed in response to injection of G_s DN RNA was due in part to sequestration of $\beta\gamma$ subunits since one of the mutations in G_s DN (G226A) increases the $\beta\gamma$ affinity of α_s (Miller et al., 1988; Lee et al., 1992). However, our control experiments showed that a form of the G_s α subunit with only the G226A mutation did not cause GVBD, indicating that sequestration of a receptor, rather than sequestration of $\beta\gamma$, is the predominant mechanism by which G_s DN causes GVBD. Taken together, the previous observations of GVBD in response to agents that sequester $\beta\gamma$, and our results of GVBD in response to G_s DN and to an

antibody against α_s , suggest that both α_s and $\beta\gamma$ derived from G_s function in maintaining meiotic arrest.

Receptor activation of G_s and implications for possible mechanisms of hormonal stimulation of meiotic resumption

The G_s G protein, by itself, is not constitutively active (Iiri et al., 1994), and for this reason, it is likely that a receptor activates G_s in oocytes. A key difference between mammalian and frog oocytes is that while mammalian oocytes resume meiosis spontaneously when removed from their follicles, frog oocytes do not (see Masui and Clarke, 1979). Fish oocytes from different species appear to differ in their ability to maintain meiotic arrest in the absence of follicle cells (Bhattacharyya et al., 2000; Greeley et al., 1987). One possible explanation of these species variations is that in mammalian and some fish oocytes, the receptor that maintains G_s in its active state might require a stimulus from the follicle to be activated to a level that maintains meiotic arrest (Figs. 5A, B). In contrast, in frog and some other fish oocytes, the receptor that activates G_s might have sufficient constitutive activity to maintain arrest, even in the absence of a stimulus from the follicle (Fig. 5D). Various G_s -linked receptors have varying degrees of constitutive activity, with agonists acting to further increase this activity, and inverse agonists acting to decrease the activity (Chidiac et al., 1994; de Ligt et al., 2000; Eggerickx et al., 1995; Seifert and Wenzel-Seifert, 2002). Another possible explanation of the species variation as to whether follicle cells are required to maintain prophase arrest could be differences in the level of cAMP phosphodiesterase activity in the oocyte.

In the model of a mouse follicle shown in Fig. 5A, the stimulus from the follicle is depicted as a ligand associated with the membrane of the cumulus cells. We favor this alternative because evidence indicates that soluble factors in the follicular fluid are not sufficient to maintain meiotic arrest (Racowsky and Baldwin, 1989). Cumulus cell contact with the oocyte is also not sufficient, even in the presence of follicular fluid; contact between the cumulus cell mass and the mural granulosa cells is also essential (Eppig et al., 2004; Racowsky and Baldwin, 1989). Therefore, the cumulus cell ligand depicted in Fig. 5A could only be active if the cumulus/granulosa cell bridge was intact. The nature of the signal that crosses this bridge is unknown. Our drawing shows the cumulus cell ligand associated with a G_s -linked receptor in the oocyte, but this is not the only possible way in which the presence of the follicle cells could keep oocyte cAMP elevated. It is also possible that the G_s -linked receptor in the oocyte has the same level of activity regardless of the presence of follicle cells. Other proposed mechanisms by which the follicle cells might maintain arrest are by reducing the level of cAMP phosphodiesterase activity in the oocyte or by supplying cAMP to the oocyte through gap junctions (Eppig et al., 1985, 2004; Webb et al., 2002).

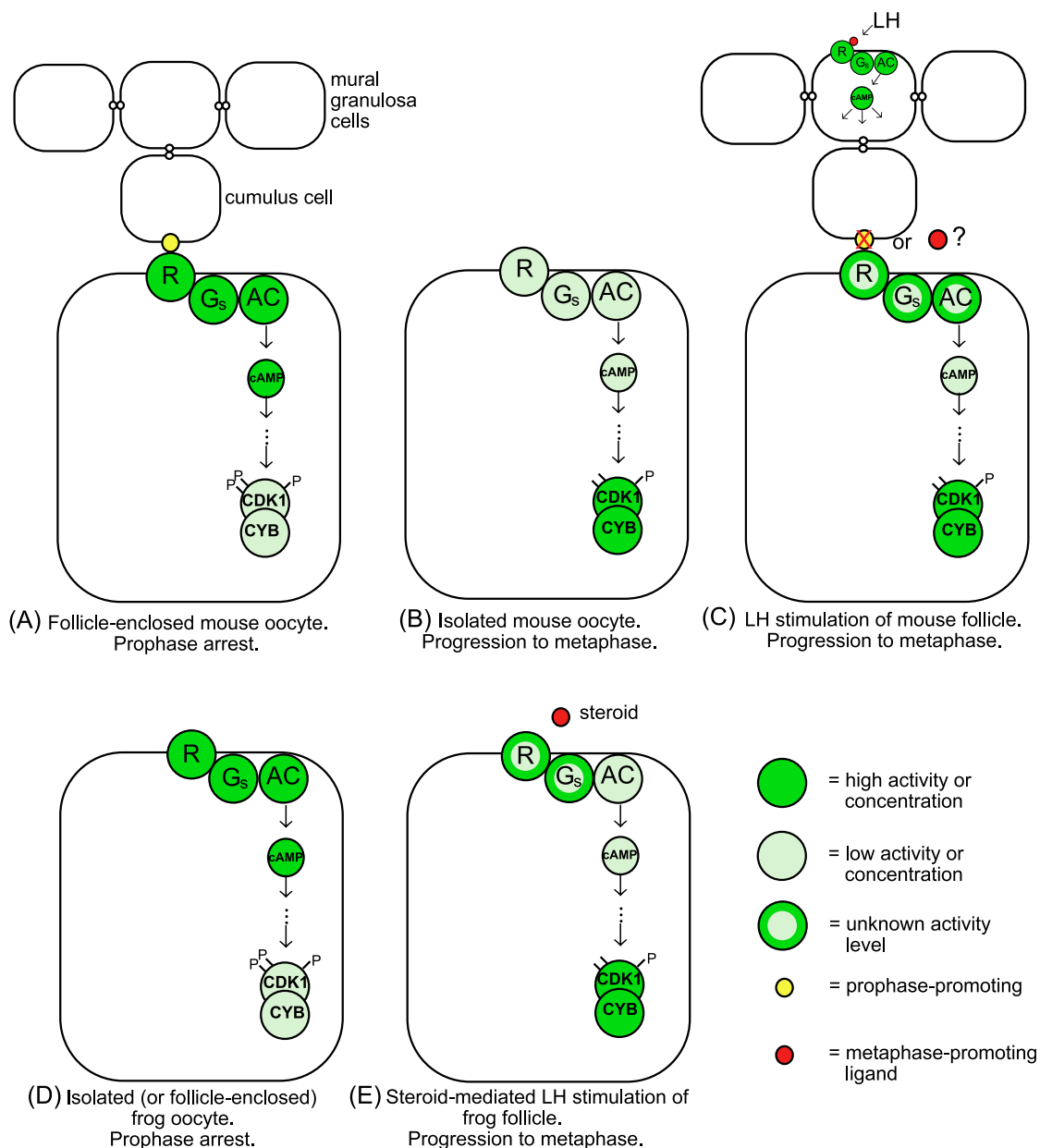


Fig. 5. A working hypothesis for the function of an oocyte G_s -linked receptor in the maintenance and release of prophase arrest in mouse and frog oocytes. (A) In the fully grown mouse oocyte that is enclosed in its follicle and arrested in prophase, the receptor (R) is hypothesized to be maintained in an active state by cell–cell interactions with somatic cells. A ligand associated with the cumulus cells may keep the oocyte receptor active, activating G_s and in turn adenylyl cyclase (AC), thus maintaining a high concentration of cAMP in the oocyte. This would result, indirectly, in keeping the cyclin-dependent kinase/cyclin B complex (CDK1/CYB) in its phosphorylated and inactive state, keeping the oocyte in prophase. See main text for further discussion of this and alternative models. (B) A possible explanation of the spontaneous progression to metaphase in mouse oocytes that are isolated from their follicles is that in the absence of a signal from the follicle, the oocyte receptor, and hence G_s and AC, are not sufficiently activated to maintain cAMP at a level that can keep CDK1 inactivated. The receptor is hypothesized, however, to have some level of agonist-independent activity, such that in the presence of a cAMP phosphodiesterase inhibitor, cAMP in the isolated oocyte would be sufficiently elevated to maintain prophase arrest. (C) In response to luteinizing hormone (LH), which acts on receptors on the mural granulosa cells, the mouse oocyte resumes meiosis. Activated LH receptors are linked through G_s and AC to cAMP production in the granulosa cells, leading to transcription of multiple genes, and resulting in meiotic resumption and other responses (Conti, 2002). As a consequence of LH action, the cAMP concentration in the oocyte decreases; the communication pathways between the somatic cells and the oocyte cAMP decrease are not well understood. The somatic cells could send a signal to the oocyte by inactivating the prophase-promoting ligand (red X) and/or by generating a metaphase-promoting ligand (red dot) (Downs et al., 1988; Rackowsky et al., 1989; Sato et al., 1993; Su et al., 2003); bidirectional signaling between the oocyte and somatic cells may also be essential (Su et al., 2003). Based on the model shown here, possible molecular targets at the oocyte level include the G_s -linked receptor, G_s , AC (perhaps by way of another receptor linked to G_i), or cAMP itself (by way of a phosphodiesterase; see Richard et al., 2001). (D) In the frog oocyte, the G_s -linked receptor may have enough agonist-independent activity to maintain prophase arrest even in the absence of the somatic cells. (E) LH action on the frog follicle cells causes the release of meiotic arrest by way of the production of a steroid. The steroid acts on the oocyte to decrease AC activity, which causes a decrease in the cAMP concentration. This occurs without a change in cAMP phosphodiesterase activity (Sadler and Maller, 1987) and does not involve a G_i -linked receptor (see Kalinowski et al., 2003). How the steroid acts is unknown, but the G_s -linked receptor, G_s , and AC are possible targets.

Identification of G_s -linked receptors in oocytes, their possible ligands from the follicle cells, and their relationship to other receptors that have been proposed to function in mediating steroid-induced oocyte maturation (Bayaa et al., 2000; Maller, 2001; Tian et al., 2000; Zhu et al., 2003) will be essential next steps towards understanding the regulation of meiotic prophase arrest. In particular, it has been proposed that activation of a G_i -linked progesterin receptor in fish oocytes could mediate GVBD in response to steroid, since the level of GVBD in response to steroid was decreased in oocytes that were injected with antisense oligonucleotides targeting this receptor (Zhu et al., 2003). However, when expressed in tissue culture cells, this receptor is activated by progesterone and 17,20 β , 21-trihydroxy-4-pregnen-3-one with similar concentration dependence, while the stimulation of GVBD in the fish oocyte requires a much lower concentration of 17,20 β , 21-trihydroxy-4-pregnen-3-one than progesterone (Thomas and Trant, 1989; Thomas and Das, 1997). This observation raises the question of whether the G_i -linked receptor that has been isolated from a fish ovary cDNA library is necessarily the same steroid receptor that induces GVBD. Another unresolved issue is whether G_i is required for steroid-induced GVBD in fish (Yoshikuni and Nagahama, 1994; Thomas et al., 2002); if so, this would indicate a fundamentally different mechanism than for steroid-induced GVBD in frogs, where G_i appears to be neither necessary nor sufficient for GVBD (see Kalinowski et al., 2003, and references therein). In frogs, and perhaps in fish and mammals as well, an alternative possibility is that the meiosis-inducing hormone might decrease adenylyl cyclase activity in the oocyte by decreasing the activity of G_s or a G_s -linked receptor (Sadler and Maller, 1983) (see Figs. 5C, E).

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