# Stimulation of *Xenopus* Oocyte Maturation by Inhibition of the G-Protein $\alpha_S$ Subunit, a Component of the Plasma Membrane and Yolk Platelet Membranes

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***Abstract.*** Oocytes of *Xenopus laevis* undergo maturation when injected with an affinity-purified antibody against the COOH-terminal decapeptide of the α subunit of the G-protein $G_s$, an antibody that inhibits $G_s$ activity. Germinal vesicle breakdown, chromosome condensation, and polar body formation occur, with a time course similar to that for oocytes treated with progesterone. The $\alpha_s$ antibody-injected oocytes also acquire the ability to be activated by sperm. Coinjection of the catalytic subunit of cAMP-dependent protein kinase, or incubation with cycloheximide, inhibits maturation in response to injection of the $\alpha_s$ antibody; these experiments show that the $\alpha_s$ antibody acts at an early point in the pathway leading to oocyte maturation, before formation of maturation promoting factor, and like progesterone, its action requires protein synthesis. Immunogold electron microscopy shows that $\alpha_s$ is present in the yolk platelet membranes as well as the plasma membrane. These results support the hypothesis that progesterone acts by inhibiting $\alpha_s$, and suggest that the target of progesterone could include yolk platelet membranes as well as the plasma membrane.

DESPITE the recent flood of information about proteins that regulate the cell cycle (Masui, 1992; Murray and Hunt, 1993), the mechanisms by which hormones reinitiate the meiotic cell cycle in oocytes have remained elusive. In particular, receptors for the hormones have not been identified (see Liu and Patino, 1993, for a description of progress). However, a role for a G-protein in the hormonal stimulation of oocyte maturation has recently been demonstrated for the stimulation of starfish oocyte maturation by 1-methyladenine (Shilling et al., 1989; Tadenuma et al., 1991, 1992; Chiba et al., 1992, 1993; Jaffe et al., 1993). 1-Methyladenine is produced by the follicle cells surrounding the oocyte and acts on the oocyte's external surface (Kanatani, 1985; Yoshikuni et al., 1988). Exposure to the hormone activates $G_i$, and the dissociated βγ subunit of $G_i$ activates the subsequent events of oocyte maturation. The effector protein for βγ is unknown.

Whether a G-protein functions in vertebrate oocyte maturation is less clear, although studies of frogs provide some indications of this. Frog oocyte maturation is initiated by progesterone produced by the follicle cells (see Masui and Clarke, 1979; Schuetz and Glad, 1985). The oocyte's response to progesterone does not require transcription, and some experiments indicate an action at the plasma membrane level (see Masui and Clarke, 1979). Some if not all of the hormone's action appears to be mediated by a decrease in cAMP, based on the following evidence: (*a*) cAMP levels in the oocyte decrease by 20–40%, within 1–2 min after applying the hormone (see Maller et al., 1979; Cicirelli and Smith, 1985; Cork et al., 1990), and adenylyl cyclase activity also decreases (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981). (*b*) Injection of the cAMP-dependent protein kinase catalytic subunit inhibits progesterone-induced oocyte maturation (Maller and Krebs, 1977; Daar et al., 1993). Likewise, conditions that raise cAMP (cholera toxin, GTP-γ-S injection) inhibit progesterone-induced maturation (Maller et al., 1979; Cork et al., 1990). (*c*) Injection of the cAMP-dependent protein kinase type II regulatory subunit, or an inhibitor of cAMP-dependent protein kinase, causes oocyte maturation (Maller and Krebs, 1977; Huchon et al., 1981; Daar et al., 1993).

Because of these experiments implicating cAMP in the stimulation of oocyte maturation by progesterone, and because of the regulation of adenylyl cyclase by G-proteins (see Hepler and Gilman, 1992), the role of a G-protein in transducing the progesterone signal has been examined. In support of the involvement of a G-protein, progesterone inhibits adenylyl cyclase activity in oocyte membranes that are stimulated by the G-protein activators GppNHp or cholera toxin, but not adenylyl cyclase activity in oocyte membranes that are stimulated by forskolin or manganese, agents that act directly on adenylyl cyclase (Sadler and

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Maller, 1981, 1983; Jordana et al., 1984). A pathway involving hormonal stimulation of $G_i$ was suggested by the finding that in some conditions pertussis toxin, which inhibits receptor-mediated activation of the $G_i$ and $G_o$ classes of G-proteins, inhibits or slows oocyte maturation in response to progesterone (Sadler et al., 1984; Pellaz and Schorderet-Slatkine, 1989; but see Goodhardt et al., 1984; Mulner et al., 1985). However, pertussis toxin does not affect adenylyl cyclase inhibition by progesterone in isolated oocyte membranes (Goodhardt et al., 1984; Olate et al., 1984; Sadler et al., 1984). Also, unlike typical G-protein–mediated processes, in which the rate of GTP exchange onto α subunits increases with receptor stimulation, applying progesterone to isolated oocyte membranes decreases the rate of GTP exchange with the membranes (Sadler and Maller, 1983). These unusual properties led to the suggestion that progesterone might act by inhibition of $G_s$ (Sadler and Maller, 1983, 1985; Jordana et al., 1984; Allende, 1988).

In the present paper, we investigate the role of G-proteins in frog oocyte maturation by injecting oocytes with affinity-purified antibodies against the COOH-terminal decapeptides of G-protein α subunits, which inhibit the activity of these G-proteins in mammalian cells (Simonds et al., 1989*b*; Nair et al., 1990; Meinkoth et al., 1992; Aridor et al., 1993; Wilson et al., 1993). We show that an antibody against $\alpha_s$, which inhibits $\alpha_s$ activity in frog oocyte membranes, stimulates maturation when injected into frog oocytes. The $\alpha_s$ antibody acts at an early point in the pathway leading to oocyte maturation, before formation of maturation promoting factor (MPF)[1], and its action requires protein synthesis. We investigate the intracellular localization of $\alpha_s$ in the oocyte and find that it is present on membranes of the yolk platelets as well as the plasma membrane. These results support the previously proposed hypothesis that progesterone could act by inhibiting $\alpha_s$, and raise the possibility that the target of the hormone is not limited to the plasma membrane.

## Materials and Methods

### Oocytes

Pieces of ovary were removed from hypothermically anesthetized *Xenopus laevis* (Nasco, Fort Atkinson, WI). Stage 6 oocytes were either manually dissected from their follicles, or were isolated by treatment with 2% collagenase; the collagenase-treated oocytes were washed in 100 mM K phosphate, pH 6.5, and 0.1% BSA, and sorted using a stereoscope (Duesbery and Masui, 1993). Staining of live oocytes with 10 μg/ml bisbenzimide H33342 fluorochrome (Calbiochem, La Jolla, CA) to visualize DNA showed that some follicle cells remained on manually dissected but not on collagenase-isolated oocytes. Oocytes were kept at 18–20°C on agarose-lined dishes in a medium composed of 50% Leibovitz's L-15 medium, 10 mM Hepes, pH 7.2, 50 μg/ml gentamycin (all components from GIBCO BRL, Gaithersburg, MD). Oocytes were used within 1 d after isolation. Progesterone (Sigma Chemical Co., St. Louis, MO) was dissolved in EtOH (10 mg/ml) before dilution in the above medium.

Formation of a white spot at the animal pole, an indicator of germinal vesicle breakdown (GVBD), was observed and photographed using a Wild M5A stereoscope (Kramer Scientific, Yonkers, NY). GVBD was confirmed by fixing oocytes in 4% TCA, and then cutting them with a scalpel and examining them with a stereoscope. Chromosomes and polar bodies were detected by incubating live collagenase-defolliculated oocytes in 10 μg/ml bisbenzimide H33342 fluorochrome in modified Ringers (100 mM NaCl, 1.8 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 5.0 mM Hepes, pH 7.8) for 30 min. Oocytes in the dye solution were mounted animal pole up between a slide and a coverslip separated by a .03″ thick silicon rubber spacer ("calendared sheet," North American Reiss Corp., Blackstone, VA) cut to make a central well and secured with silicon grease. The preparations were observed and photographed with a Zeiss Axioskop, using a 40×, 0.75 NA neofluar objective (Carl Zeiss, Inc., Thornwood, NY). Photographs were made using T-MAX 400 film (Eastman Kodak Corp., Rochester, NY). Fertilization of in vitro matured oocytes was done as described by Kline et al. (1991). In brief, oocytes at 3 h after white spot formation were treated with pepsin and cysteine to loosen the vitelline envelope, which was then removed with forceps. The vitelline-free oocytes were inseminated with sperm in an extract of egg jelly.

Oocytes from starfish (*Asterina miniata*) were collected as described by Jaffe et al. (1993).

### G-Protein Specific Antibodies and Other Biochemicals

G-protein α-subunit specific antibodies were produced by immunizing rabbits with peptides corresponding to mammalian G-protein α subunits (see Table I). The RM, EC, GO, GC, and AS antibodies, as well as the RM peptide antigen, were provided by Allen Spiegel (National Institutes of Health, Bethesda, MD). The KQ antibody was provided by Keith Mostov (University of California, San Francisco, CA). The sc-387 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The sequences of the peptides used to produce the antibodies, and the similarity of the corresponding sequences of *Xenopus* G-protein α subunits are summarized in Table I. Antibodies were affinity purified with a column of the corresponding peptide (Simonds et al., 1989*b*). For microinjection, the antibodies were spin-dialyzed and concentrated into 100 mM K aspartate, 10 mM Hepes, pH 7.0, using 30 kD Millipore Ultrafree-MC filter units (Millipore Corp., Bedford, MA). Protein concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard. Rabbit IgG (Sigma) was used for nonimmune control injections.

Cycloheximide was obtained from Sigma Chem. Co. The catalytic subunit of cAMP-dependent protein kinase was obtained from Promega Corp. (Madison, WI). Bovine transducin βγ subunits were provided by Yee-Kin Ho (University of Illinois, Chicago, IL) and were spin-dialyzed into the same buffer as the antibodies.

### Microinjection

For microinjection, oocytes were supported on a microscope slide in a pool of modified Ringers retained by a U-shaped plastic frame. Microinjections were made using either an oil-filled constriction pipette (Hiramoto, 1974) or a "Nanoject" automatic injector (Drummond Scientific Co., Broomall, PA). Injection volumes for the constriction pipette were calibrated by measuring the decrease in the length of the column of solution in the capillary from which the pipette was loaded (30–50 nl). Injection volumes for the "Nanoject" injector were calibrated by measuring the diameter of a sample of injectate expelled into air (50 nl). For calculation of protein concentrations in the cytoplasm, the volume of the stage 6 oocyte was taken to be 1.0 μl; antibody concentrations were calculated based on the molecular weight of IgG (150 kD).

### Preparation of Cortices, Membranes, and Yolk Platelets

Cortices were isolated from manually or collagenase-defolliculated oocytes as described by Elinson et al. (1993). In brief, oocytes were cut in half with a scalpel in a buffer containing 100 mM Pipes, pH 6.9, 10 mM EGTA, 1 mM $MgSO_4$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml $\alpha_2$-macroglobulin, 100 μg/ml soybean trypsin inhibitor. Four animal halves and four vegetal halves were placed membrane side down on a 8-mm square piece of nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The half oocytes were flattened by pressing down a 12-mm square coverslip (Bradford Scientific, Inc., Epping, NH) until pigment granules of the cortex began to disperse as viewed with a stereoscope. The filter was separated from the coverslip and rinsed twice in the above buffer. Each filter contained ~50 μg protein. Electron microscopy of these filters showed a morphology similar to that obtained by Elinson et al. (1993); see also Fig. 6.

A membrane fraction to be used for immunoblotting was prepared by a

1. *Abbreviations used in this paper*: GVBD, germinal vesicle breakdown; MPF, maturation promoting factor.

*Table I. G-Protein α-Subunit Peptides Used to Produce Antibodies*

| Antibody | G-protein subunit | Antigen sequence* | *Xenopus* sequence similarity‡ |
|---|---|---|---|
| RM | $\alpha_s$ | RMHLRQYELL | 10/10 |
| KQ | $\alpha_s$ | KQLQKDKQVYRA | 12/12 |
| EC§ | $\alpha_{i3}$ | KNNLKECGLY | 8/10 |
| GO§ | $\alpha_o$ | ANNLRGCGLY | 9/10 |
| GC§ | $\alpha_o$ | GCTLSAEERAALERSK | 16/16 |
| sc-387§ | $\alpha_o$ | KMVCDVVSRMEDTEPFSAEL | 18/20 |
| AS | $\alpha_t$, $\alpha_{i1}$, $\alpha_{i2}$ | KENLKDCGLF | 9/10 |

*For RM, EC, GO, and AS antibodies, the antigens corresponded to the COOH-terminal decapeptides of the indicated mammalian G-protein α subunits (Simonds et al., 1989*a*). For KQ, the antigen corresponded to amino acids 28–39 (Pimplikar and Simons, 1993). For GC, the antigen corresponded to a peptide near the $NH_2$-terminal (amino acids 2–17) (Thambi et al., 1989). For sc-387, the antigen corresponded to amino acids 105–124 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All antibodies except KQ and GC were affinity purified.

‡Sequences for *Xenopus* $\alpha_s$, $\alpha_{i3}$, $\alpha_o$, and $\alpha_{i1}$ are described by Olate et al. (1990).

§The EC and GO antibodies react most strongly with $\alpha_{i3}$ and $\alpha_o$, respectively, but there is some cross-reactivity (Simonds et al., 1989*a*). The GC antibody is specific for $\alpha_o$; only 6/16 of the amino acids of the antigen are identical with those of *Xenopus* $\alpha_{i3}$. The sc-387 antibody is also specific for $\alpha_o$; only 2/20 of the amino acids of the antigen are identical with those of *Xenopus* $\alpha_{i3}$.

procedure modified from that of Tilley et al. (1988). Approximately 1,000 collagenase-defolliculated oocytes were suspended in 10 ml of 100 mM $K^+$ glutamate, 10 mM EGTA, 20 mM Hepes, pH 7.2, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml soybean trypsin inhibitor, and 100 μg/ml $\alpha_2$-macroglobulin, in a 15-ml Dounce homogenizer (Wheaton, Millville, NJ) on ice. The oocytes were lysed with 6–8 strokes using a loose-fitting pestle (pestle B). The lysate was diluted with 8 ml of the above buffer, layered onto a 0.5-vol sucrose cushion (1.5 M sucrose in the same buffer) in a 32-ml tube, and centrifuged at 4°C in a SW-27 rotor at 16,000 *g* ($r_{av}$) for 10 min. The interface was removed, diluted fivefold, and further homogenized with four strokes of pestle B. The centrifugation step was repeated and the interface was removed and diluted as described above. This diluted sample was centrifuged in a microfuge (4°C) for 10 min at 16,000 *g* to pellet the membranes. The supernatant was removed, and the pellet was resuspended in the above buffer, aliquoted, frozen in liquid $N_2$, and stored at −70°C. About 5 μg of membrane protein was obtained per oocyte.

A less purified membrane fraction, to be used for measurement of adenylyl cyclase activity, was prepared by the procedure of Finidori-Lepicard et al. (1981). This membrane preparation was chosen because of previous work (Finidori-Lepicard et al., 1981) demonstrating that it preserved progesterone-responsive adenylyl cyclase activity. Approximately 1,000 collagenase-defolliculated oocytes were suspended in 5 ml of 1 mM $NaHCO_3$, 3 mM $Na_2EDTA$, in a 15-ml Dounce homogenizer on ice. The oocytes were lysed with 10 strokes using a tight-fitting pestle (pestle A). The lysate was centrifuged at 4°C in a microfuge at 1,000 *g* for 15 min; this step pelleted yolk and pigment granules. The supernatant was centrifuged at 4°C in a microfuge at 12,000 *g* for 15 min; this step pelleted a crude membrane fraction. The pellet was resuspended in the above buffer, aliquoted, frozen in liquid $N_2$, and stored at −70°C. About 6 μg of protein was obtained per oocyte.

A fraction enriched in yolk platelets and lacking plasma membrane was prepared by collecting cytoplasm from individual collagenase-defolliculated oocytes. Oocytes were cut open with a scalpel in the same buffer as used for the cortices. A core of cytoplasm was collected from each oocyte, using a mouth-controlled suction pipette (~0.4 mm diameter). Cytoplasm from 40 oocytes was pooled in a microfuge tube and spun at 1,000 *g* for 1 min, to pellet the yolk. About 15 μg of protein was obtained per oocyte.

### *Immunoblotting*

Gel electrophoresis and immunoblotting were done as described by Jaffe et al. (1993). For samples of cortices prepared on nitrocellulose filters, the filters were loaded directly into dry sample wells, followed by addition of sample buffer. Affinity-purified antibodies were used at a concentration of 1.6–3.8 μg/ml. The KQ antiserum was used at a dilution of 1:2,000. The GC antiserum was used at a dilution of 1:200. As a positive control, we made immunoblots from *Xenopus* brain membranes, prepared by homogenizing a brain and collecting a 12,000-*g* pellet.

### *Adenylyl Cyclase Assays*

Adenylyl cyclase activity was assayed at 37°C for 90 min using a 60-μl incubation volume that contained 50 mM Tris-HCl, pH 7.5, 1 mM cAMP, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM 3-isobutyl-1-methylxanthine, 2 mM β-mercaptoethanol, 100 μM [α-$^{32}$P]ATP (300–500 cpm/pmol), 10 mM creatine phosphate, 24 μg creatine kinase, 25 μM GTPγS, and 200 μg of membrane protein (see above). The reaction was stopped by the addition of 100 μl of 25 mM Tris-HCl, pH 7.5, 1.3 mM ATP, 2% (wt/vol) SDS, 0.2 mM [$^3$H]cAMP (1,000 cpm/nmol), and heating 5 min at 95°C. The [$^{32}$P]cAMP was isolated by the method of Salomon et al. (1974).

### *Immunogold Electron Microscopy of Isolated Cortices*

Cortices prepared on nitrocellulose filters as described above were fixed for 1 h in 2% paraformaldehyde in 100 mM Pipes, pH 6.9, 10 mM EGTA, 1 mM $MgSO_4$ (P10EM buffer) and washed with P10EM. After blocking with 0.1 M glycine and then with 1% BSA in P10EM, the cortices were incubated for 4 h in 10 μg/ml of primary antibody in P10EM + 1% BSA, washed, blocked with 1% BSA, and incubated overnight in a 1:20 dilution of 5 nm gold-labeled goat anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) in P10EM + 1% BSA. The cortices were then washed, fixed for 30 min in 2.5% glutaraldehyde in cacodylate buffer, washed, fixed for 30 min in 1% $OsO_4$ and 0.8% ferricyanide, washed, incubated in 0.5% uranyl acetate for 30 min, washed, dehydrated, embedded, sectioned, and observed with a Philips CM-10 electron microscope.

For quantitation of the immunogold labeling, micrographs of 18 adjacent but nonoverlapping areas of the plasma membrane and adjacent cortical cytoplasm were taken at an initial magnification of 21,000 and enlarged to 57,000. Gold particles over the total area of cortical cytoplasm, associated with the cytoplasmic face of the plasma membrane, and associated with the cytoplasmic surface of the yolk platelet membranes were analyzed. Counting, as well as area and linear measurements, were done on a digitizing tablet using SigmaScan software (Jandel, San Rafael, CA).

## *Results*

### *G-Protein $\alpha_s$ and $\alpha_{i3}$ Subunits Were Present in Frog Oocyte Membrane Fractions*

To identify the G-proteins present in frog oocyte membranes, we made immunoblots of two oocyte fractions, "cortices," which consisted of the plasma membrane with the associated vitelline layer, as well as some adhering cytoplasmic components (Elinson et al., 1993), and a membrane fraction prepared by homogenization followed by centrifugation on a sucrose cushion (Tilley et al., 1988) (see Materials and Methods). Both were prepared from fully grown (stage 6) oocytes that had been defolliculated with collagenase, in order to avoid possible follicle cell contamination. The antibodies were made against peptides of mammalian G-protein α subunits; the peptide sequences were identical or almost identical to the corresponding sequences of *Xenopus* oocyte α subunits (Table I).

Using these antibodies, $\alpha_s$ and $\alpha_{i3}$ were identified in frog oocyte membranes. The RM antibody against the COOH-terminal decapeptide of mammalian/*Xenopus* $\alpha_s$ showed labeling of a 43-kD band in both cortices and the membrane fraction (Fig. 1). In addition, the RM antibody detected a band at 38 kD in the membrane fraction. The 43-kD band, but not the 38-kD band, was also recognized by an antibody against amino acids 28–39 of $\alpha_s$ (KQ, see Table I). Based on this, the 38-kD polypeptide could be either $\alpha_s$ lacking ~5 kD at its $NH_2$ terminus, or a different protein containing a sequence similar to the COOH-terminal decapeptide of $\alpha_s$. The EC antibody against the COOH-terminal decapeptide of mammalian $\alpha_{i3}$ showed labeling of a 39-kD band in both cortices and the membrane fraction (Fig. 1), as well as a 50-kD band in the membrane fraction.

Although RNA for $\alpha_o$ and $\alpha_{i1}$ is present in stage 6 *Xenopus* oocytes (Onate et al., 1992), neither $\alpha_o$ nor $\alpha_{i1}$ protein was detected by immunoblotting of the oocyte membranes. The GO antibody against the COOH-terminal decapeptide of $\alpha_o$ detected labeling of a band at 39 kD in both cortices and the membrane fraction, but the GO antibody recognizes both $\alpha_o$ and $\alpha_{i3}$ (Simonds et al., 1989*a*). Therefore, blots were also probed with the GC antibody, which is specific for $\alpha_o$ (see Table I). The GC antibody did not detect a band in either oocyte fraction, but it was strongly reactive with a 39-kD band of *Xenopus* brain membranes. These results indicated that detectable levels of $\alpha_o$ were not present in these oocyte fractions, and that the GO signal represented cross-reactivity with $\alpha_{i3}$. This conclusion was confirmed by blotting with another antibody specific for $\alpha_o$ (sc-387; see Table I); this antibody did not recognize a specific band in either oocyte fraction, but did recognize a specific band in *Xenopus* brain membranes. Likewise, although the AS antibody against $\alpha_{i1/2}$ recognized a specific band in *Xenopus* brain membranes, it did not detect a specific band in either oocyte cortices or the oocyte membrane fraction. The absence of detectable $\alpha_o$ and $\alpha_{i1/2}$ protein in the oocyte suggests that, although injection of brain $\alpha_o$ can stimulate GVBD (Kroll et al., 1991), neither $\alpha_o$ nor $\alpha_{i1/2}$ participates in the natural process of oocyte maturation.

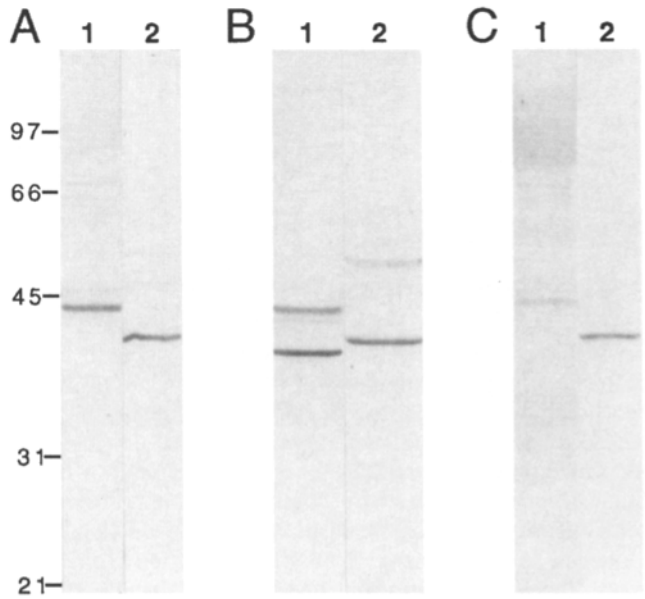


*Figure 1.* Immunoblots of oocyte cortices (*A*), membranes (*B*), and yolk platelets (*C*) with antibodies against G-protein α subunits. For each: (lane *1*) RM antibody against $\alpha_s$, (lane *2*) EC antibody against $\alpha_{i3}$. The amount of protein loaded per gel lane: cortices, 50 μg; membranes, 70 μg; and yolk platelets, 100 μg.

### *Oocytes Injected with an Antibody That Inhibited $\alpha_s$ Underwent Germinal Vesicle Breakdown with a Time Course Similar to That with Progesterone*

To examine the possible function of the $\alpha_s$ and $\alpha_{i3}$ subunits in the stimulation of oocyte maturation, we injected oocytes with affinity-purified antibodies against their COOH-terminal decapeptides. Fully grown (stage 6) oocytes were isolated from ovaries of nonhormonally primed frogs, either by manual dissection or by treatment with collagenase (see Materials and Methods). Injection of the RM antibody at concentrations of ≥0.2 μM caused GVBD, as indicated by formation of a white spot at the animal pole (Figs. 2 and 3). The time course for stimulation of white spot formation by $\alpha_s$ antibody (0.3–2.0 μM) was similar to that seen for oocytes treated with progesterone (3 μM) (Fig. 4). We confirmed that the white spot was indicative of GVBD by cutting open fixed oocytes; germinal vesicles were not present. Injections of <0.1 μM of the $\alpha_s$ antibody did not cause GVBD (Fig. 3). Likewise, injections of 2.2 μM of the EC antibody (against $\alpha_{i3}$), or 3.3 μM of nonimmune IgG, did not cause GVBD (Fig. 3). As additional controls, oocytes were injected with the filtrate collected during spin dialysis and concentration of the $\alpha_s$ antibody (20 oocytes, 4 animals), or with RM antibody preincubated with the RM peptide (10 oocytes, 1 animal; Fig. 3); these solutions did not cause GVBD.

The RM antibody has been shown to inhibit $\alpha_s$ activity in membranes from mammalian cells (Simonds et al., 1989*b*; Nair et al., 1990). To determine if this antibody inhibited $\alpha_s$ in frog oocyte membranes, we examined its effect on GTP-γ-S–stimulated adenylyl cyclase activity in isolated membranes (Table II). Like progesterone (10 μM), the RM antibody (2 μM) caused a decrease in adenylyl cyclase activity, indicating an inhibition of $\alpha_s$. As a control, we used the EC antibody, which did not affect

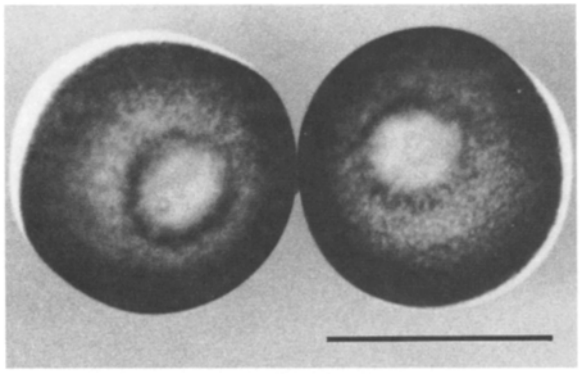

*Figure 2.* White spot formation in oocytes injected with $\alpha_s$ antibody. Oocytes were injected with 50 nl of 1 mg/ml antibody solution (0.3 μM cytoplasmic concentration). White spots formed at 11.5 h after injection; the photograph was taken at 2.5 h after white spot formation. Bar, 1.0 mm.

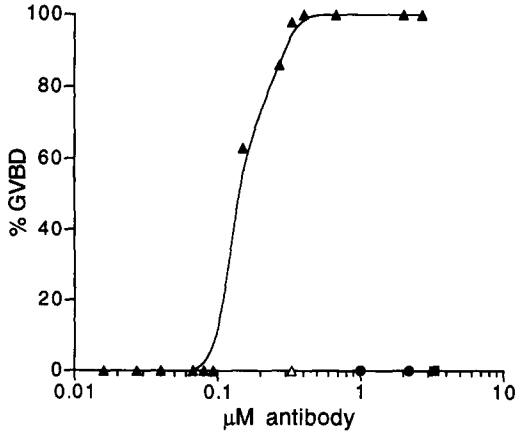


*Figure 3.* Concentration dependence of $\alpha_s$ antibody stimulation of GVBD. The x-axis indicates the antibody concentrations in the cytoplasm. The y-axis indicates the % of oocytes undergoing GVBD, as indicated by formation of a white spot at the animal pole or by the absence of a germinal vesicle in TCA-fixed oocytes. Oocytes were scored for GVBD at 24 h after injection. ▲, RM antibody against $\alpha_s$ ($n$ = 273 oocytes, 21 animals); △, RM antibody against $\alpha_s$ preincubated with an equal amount (wt/wt) of the RM peptide ($n$ = 10 oocytes, 1 animal). ●, EC antibody against $\alpha_{i3}$ ($n$ = 38 oocytes, 4 animals); ■, nonimmune IgG ($n$ = 7 oocytes, 2 animals).

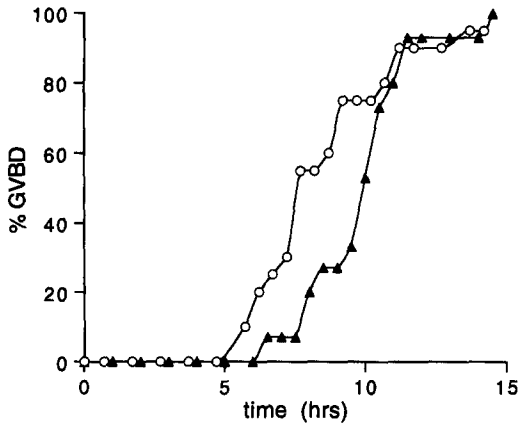


*Figure 4.* Time course of GVBD in oocytes injected with $\alpha_s$ antibody (RM) or exposed to progesterone. Oocytes were injected with 0.3 μM RM antibody (▲) ($n$ = 15 oocytes), or exposed to 3 μM progesterone (○) ($n$ = 20 oocytes). The time of GVBD was counted as the time of appearance of the white spot at the animal pole. These results are typical of seven similar experiments, using RM antibody concentrations ranging from 0.3 to 2.0 μM.

adenylyl cyclase activity (Table II). These results support the conclusion that injection into oocytes of the RM antibody against $\alpha_s$ causes maturation by inhibiting $\alpha_s$.

### *$\alpha_s$ Antibody-injected Oocytes Formed Condensed Chromosomes and a Polar Body at the Animal Pole*

To examine whether meiotic maturation in $\alpha_s$ antibody–injected oocytes proceeded beyond germinal vesicle breakdown, live collagenase-defolliculated oocytes were stained with a DNA-specific dye (H33342) and examined by fluorescence microscopy. Control oocytes treated with 3 μM progesterone and examined 9–18 h later (3–8 h after formation of a white spot) showed a radial array of condensed chromosomes at the animal pole (38/38 oocytes from four animals) (Fig. 5 *A*). The morphology of the chromosome array was typical of metaphase (see Gard, 1992) and was ~20–40 μm in diameter. 30/38 oocytes also showed a polar body containing a mass of condensed chromatin, located near the chromosomes (Fig. 5 *A'*).

Like the progesterone-treated oocytes, oocytes injected with the $\alpha_s$ antibody (0.3–2.0 μM final concentration in the cytoplasm) and examined 8–19 h later (3–7 h after formation of a white spot) showed an array of condensed chromosomes at the animal pole (35/35 oocytes, 4 animals) (Fig. 5 *B*). The array of chromosomes usually appeared quite similar to that seen in progesterone-treated oocytes. 16/35 oocytes also showed a polar body associated with the chromosomes (Fig. 5 *B'*).

### *$\alpha_s$ Antibody-matured Oocytes Activated in Response to Sperm*

To examine whether oocytes that underwent meiotic maturation in response to injection of the $\alpha_s$ antibody also acquired the ability to be activated by sperm, we removed the vitelline envelope from these oocytes at 3 h after white spot formation, and exposed them to a suspension of sperm in egg jelly extract (see Materials and Methods). Such oocytes responded to sperm like oocytes matured by exposure to progesterone. A few minutes after sperm addition, a dark spot appeared at one position on the animal half of the oocyte, and from this point, a wave of contraction passed over the oocyte surface (8 oocytes, 2 animals). These results indicated that the $\alpha_s$ antibody-matured oocytes could be activated by sperm.

### *$\alpha_s$ Antibody Stimulated Oocyte Maturation at a Step before MPF Formation and Required Protein Synthesis*

Both the protein synthesis inhibitor cycloheximide (Wasser-

*Table II. Inhibition of Adenylyl Cyclase in Oocyte Membranes by an Antibody against $\alpha_s$*

| Sample | Adenylyl cyclase activity (pmol cAMP/mg/min)* | Percent of GTP-γ-Sample |
|---|---|---|
| GTP-γ-S (25 μM) | 2.13 ± 0.13 | |
| GTP-γ-S (25 μM) + progesterone (10 μM) | 0.98 ± 0.08‡ | 46 |
| GTP-γ-S (25 μM) + RM antibody (2 μM) | 1.51 ± 0.18§ | 71 |
| GTP-γ-S (25 μM) + EC antibody (2 μM) | 2.17 ± 0.16 | 102 |

* Mean ± standard error of the mean, for three experiments performed in triplicate.
‡Significantly different from the GTP-γ-S value ($p < 0.01$, analyzed with Instat for MacIntosh, GraphPad, San Diego, CA).
§ Significantly different from the GTP-γ-S value ($p < 0.05$).

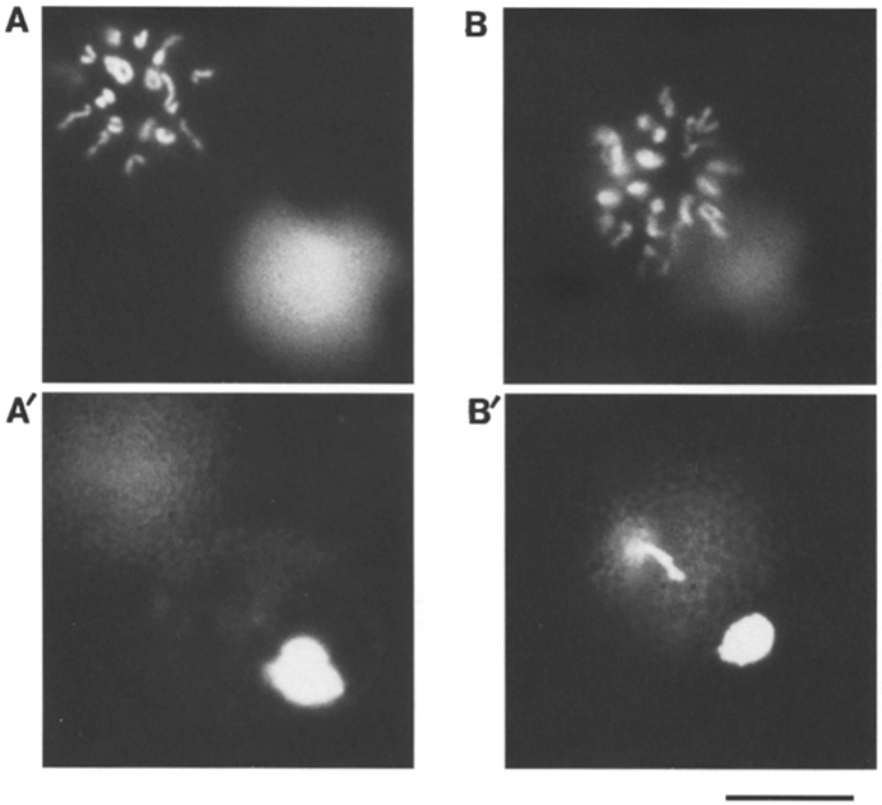


*Figure 5.* DNA staining of live oocytes that had been exposed to progesterone (3 μM) (*A* and *A′*), or injected with the RM antibody against $\alpha_s$ (0.3 μM) (*B* and *B′*). In *A* and *B*, the microscope was focused at the level of the second metaphase chromosomes. In *A′* and *B′*, the microscope was focused at the level of the tightly condensed chromatin in the polar body (same oocytes as in *A* and *B*). Bar, 50 μm.

man and Masui, 1975) and the catalytic subunit of cAMP-dependent kinase (Maller and Krebs, 1977; Daar et al., 1993) inhibit oocyte maturation in response to progesterone, but not in response to injection of MPF. We used these inhibitors to determine the point in the pathway at which the $\alpha_s$ antibody stimulated oocyte maturation. Oocytes injected with the $\alpha_s$ antibody at a concentration of 0.7 μM, and then incubated in the presence of cycloheximide (10 μg/ml), did not undergo GVBD (Table III). Likewise, oocytes coinjected with the $\alpha_s$ antibody (0.7 μM) and the catalytic subunit of cAMP-dependent kinase (0.6 μM) did not undergo GVBD (Table III). These results indicated that the $\alpha_s$ antibody acts at an early point in the pathway leading to oocyte maturation, before MPF formation, and its action requires protein synthesis.

*Table III. Effect of Inhibitors of Progesterone-induced GVBD on $\alpha_s$ Antibody-induced GVBD*

| Injection | Percent GVBD§ |
|---|---|
| RM + cycloheximide* | 0 (20, 2) |
| RM* | 100 (20, 2) |
| RM + $PKA_{cat}$‡ | 0 (24, 3) |
| RM + $PKA_{cat}$ buffer‡ | 100 (24, 3) |

*Oocytes were injected with the RM antibody against $\alpha_s$ (0.7 μM) and then incubated with or without cycloheximide (10 μg/ml). GVBD was scored at 24 h after injection.
‡Oocytes were injected with a mixture of the RM antibody against $\alpha_s$ (0.7 μM) and the catalytic subunit of cAMP-dependent protein kinase ($PKA_{cat}$) (0.6 μM); control oocytes were injected with the RM antibody (0.7 μM) mixed with the phosphate buffer in which the $PKA_{cat}$ was supplied. GVBD was scored at 24 h after injection. In two of the three experiments, the oocyte pigment became somewhat spotty after injection of the solutions containing $PKA_{cat}$. This was not seen in the buffer-injected control oocytes.
§Numbers in parentheses indicate the number of oocytes and the number of animals.

### *$\alpha_s$ Was Localized on the Oocyte Plasma Membrane and Yolk Platelet Membranes*

To determine the localization of $\alpha_s$, we used a gold-labeled secondary antibody to visualize the binding of the $\alpha_s$ antibody to cortices prepared as described in the Materials and Methods. Gold particles were associated both with the cytoplasmic face of the plasma membrane and with the cytoplasmic face of yolk platelet membranes (Fig. 6, *A* and *B*; Table IV). There were clumps of gold particles on the membranes, suggesting that the G-proteins were present in patches. Although these were the main sites of labeling, occasional gold particles were present on endoplasmic reticulum membranes (Table IV, see * footnote). Gold particles were not present on mitochondrial membranes.

Cortices incubated with nonimmune rabbit IgG showed far fewer gold particles than cortices incubated with the $\alpha_s$ antibody (Fig. 6 *C*, Table IV). Cortices incubated with the antibody against $\alpha_{i3}$ showed labeling similar to that seen with the $\alpha_s$ antibody, with gold particles on both the plasma membrane and yolk platelet membranes (Table IV). In addition, the $\alpha_{i3}$ antibody labeled endoplasmic reticulum membranes (Table IV, see * footnote).

To confirm the presence of $\alpha_s$ and $\alpha_{i3}$ in the yolk platelets, we prepared a yolk platelet fraction of the oocyte cytoplasm (see Materials and Methods). Electron microscopic examination of the pellet showed that >90% of the material in this fraction was yolk platelets, and that membranes were present around the yolk platelets. Immunoblots of this fraction with the $\alpha_s$ and $\alpha_{i3}$ antibodies showed

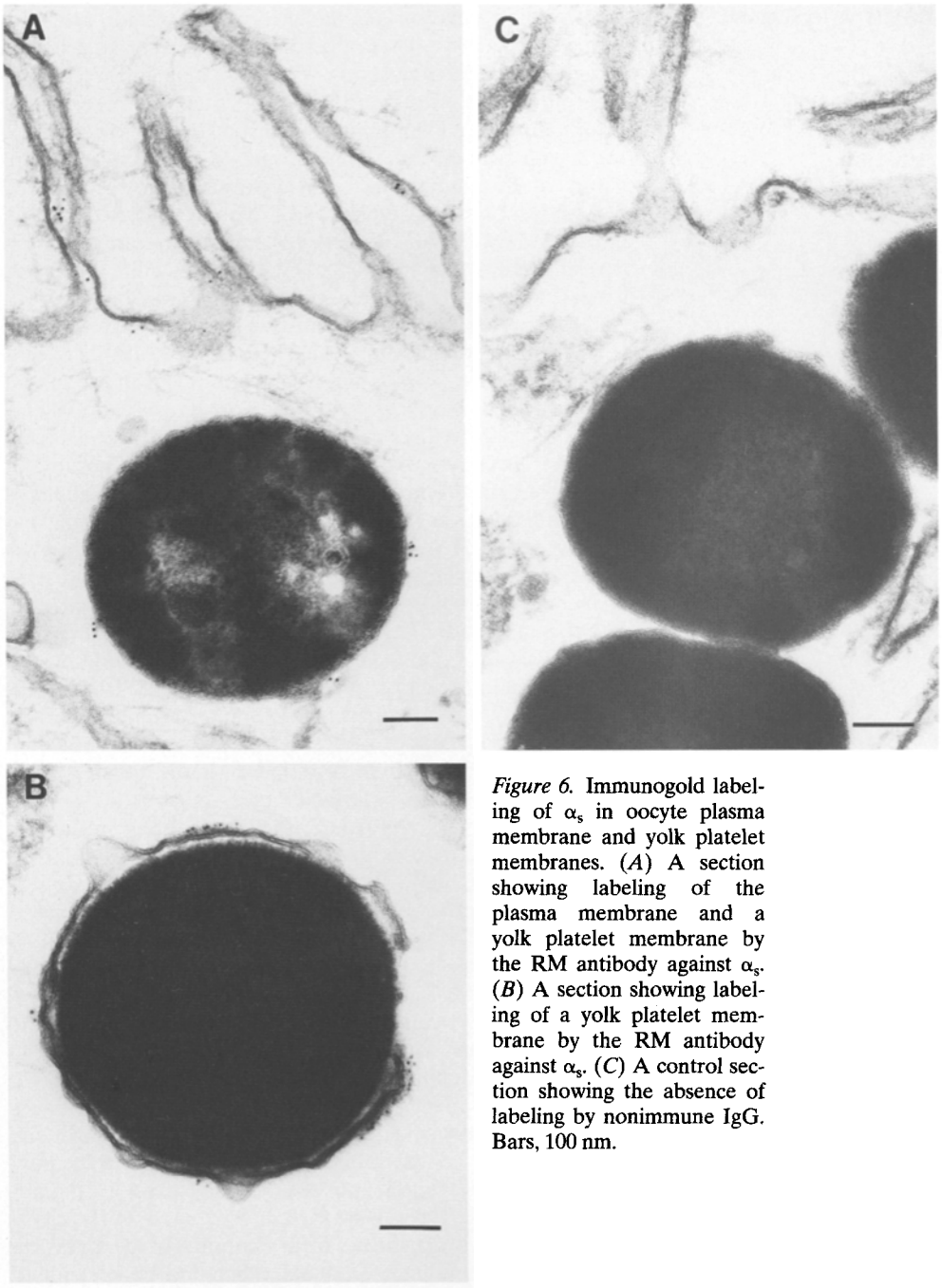


*Figure 6.* Immunogold labeling of $\alpha_s$ in oocyte plasma membrane and yolk platelet membranes. (*A*) A section showing labeling of the plasma membrane and a yolk platelet membrane by the RM antibody against $\alpha_s$. (*B*) A section showing labeling of a yolk platelet membrane by the RM antibody against $\alpha_s$. (*C*) A control section showing the absence of labeling by nonimmune IgG. Bars, 100 nm.

specific labeling of 43- and 39-kD bands, respectively, as was seen in the cortex and membrane fractions (Fig. 1).

### *G-Protein Effects on Oocyte Maturation Differed in Frog and Starfish*

Because βγ subunits cause maturation of starfish oocytes (Jaffe et al., 1993; Chiba et al., 1993), we examined their effect on frog oocytes. Injection of 3–10 μM of bovine transducin βγ subunits did not cause GVBD (12 oocytes, 2 animals); the same preparation of βγ subunits, at a concentration of ≥1.8 μM, caused GVBD in 100% of starfish oocytes (Jaffe et al., 1993). Injection of transducin βγ subunits into frog oocytes also had no inhibitory effect on the response to 0.1 μM progesterone. GVBD occurred in 11/19 progesterone-treated oocytes preinjected with 10 μM βγ subunits, compared with 15/20 control oocytes preinjected with BSA or nonimmune IgG (results from two experiments with oocytes from two different animals, exposed to progesterone at 3–5 h after injection, and scored for GVBD at 20 h after applying progesterone).

To examine whether the RM antibody against $\alpha_s$ stimulated maturation in starfish oocytes as in frog oocytes, we injected it into oocytes of *Asterina miniata*. The RM antibody recognized a 44-kD protein in starfish oocytes, based on immunoblotting (Jaffe et al., 1993), but at a concentration of 8 μM (40 times the effective concentration in frog oocytes), it neither stimulated GVBD, nor inhibited GVBD in response to 1-methyladenine ($n$ = 5 oocytes). These results indicated that although G-proteins can function in

*Table IV. G-Protein α-Subunit Localization in Xenopus Oocytes*

| Antibody | Particles per μm² of thin section* | Particles per μm of plasma membrane‡ | Particles per μm of yolk platelet membrane§ |
|---|---|---|---|
| RM ($\alpha_s$) | 4.4 ± 0.8 | 1.5 ± 0.4 | 6.2 ± 1.5 |
| EC ($\alpha_{i3}$) | 8.8 ± 0.8 | 2.2 ± 0.4 | 3.4 ± 0.9 |
| control IgG | 1.0 ± 0.2 | 0.3 ± 0.2 | 0.8 ± 0.1 |

*Determined by counting the total number of gold particles in 18 areas of electron micrographs of thin sections of oocyte cortices, each with an area of about 5 μm². Areas for counting were selected to include plasma membrane and associated cortical cytoplasm. Mean ± standard error of the mean. Of the total number of particles counted, 5% of the RM particles and 19% of the EC particles were associated with membranes identified as endoplasmic reticulum by the presence of ribosomes.

‡Determined by counting the number of gold particles in the segments of plasma membrane in the micrographs described in * footnote. The length of plasma membrane in each micrograph was ~3.5 μm. Microvillar membranes were not included because the membrane was not clearly defined in all planes of section. Mean ± standard error of the mean.

§Determined by counting the number of gold particles in the yolk platelet membranes in the micrographs described in * footnote. The length of yolk platelet membrane in each micrograph was ~2 μm. Mean ± standard error of the mean.

the regulation of oocyte maturation in both starfish and frog, the G-protein types involved differ for the two species.

## Discussion

To investigate the possible role of G-proteins in oocyte maturation in vertebrates, we injected frog oocytes with antibodies that inhibit G-protein function. An antibody that inhibits $\alpha_s$ (RM) initiates both nuclear and cytoplasmic maturation; that is, it causes oocytes to undergo germinal vesicle breakdown and progress through the meiotic cell cycle, and to acquire the ability to be activated by sperm. The concentration of the $\alpha_s$ antibody required to stimulate oocyte maturation is ~0.2 μM, which is similar to the concentration of this same antibody that is needed to inhibit $\alpha_s$ when it is injected into mammalian cells (Meinkoth et al., 1992). Like the effect of progesterone, the stimulatory effect of the $\alpha_s$ antibody can be blocked by the protein synthesis inhibitor cycloheximide, or by the catalytic subunit of cAMP-dependent protein kinase. These results indicate that the antibody acts at an early point in the pathway leading to oocyte maturation, since these agents do not block GVBD in response to injection of MPF. Our findings do not establish that inhibition of $\alpha_s$ occurs during the initiation of meiotic maturation by progesterone. They do, however, support earlier findings that suggested this hypothesis (see Introduction). Interestingly, $\alpha_s$ activity is a factor that can either prevent or stimulate the cell cycle in somatic cells, although these cells are arrested at a different point in the cell cycle, before DNA synthesis (Meinkoth et al., 1992; Chen and Iyengar, 1994).

### *Possible Mechanisms of $\alpha_s$ Activation and Inhibition in the Oocyte*

The effect of the RM antibody indicates that $\alpha_s$ is activated in the immature *Xenopus* oocyte. The activation of $\alpha_s$ could be due to a constitutively active membrane receptor of the seven transmembrane family that couples to G-proteins (see Baldwin, 1994), or from the presence of a very high concentration of receptor in which a small fraction of the receptor is in an active conformation (see Milano et al., 1994). These possibilities are suggested by the fact that the RM antibody was made against the carboxy terminus of $\alpha_s$, a region that is important for receptor interactions (see Conklin and Bourne, 1993). However, the presence in the oocyte membrane of endogenous receptors of this family has not been definitively established (see Dascal and Cohen, 1987; Miledi and Woodward, 1989). If a receptor of the seven transmembrane family is responsible for activating oocyte $G_s$, it would seemingly need to be active in the absence of agonist, since there is no obvious source of an agonist which could act on an oocyte without follicle cells, and the presence of follicle cells is not required to maintain *Xenopus* oocytes in the immature state.

Alternatively $\alpha_s$ could be activated by a protein from within the oocyte. An antibody made against the same $\alpha_s$ sequence as RM inhibits GppNHp stimulation of adenylyl cyclase, suggesting that the antibody can inhibit the exchange of GDP for GTP, independent of its effect on the coupling of seven transmembrane receptors to G-proteins (Nair et al., 1990). An example of a cytoplasmic protein that can activate G-proteins, including $G_s$, is tubulin; activation occurs when GTP is tranferred from tubulin to the G-protein α subunit (Roychowdhury et al., 1993; Roychowdhury and Rasenick, 1994; Mark Rasenick, University of Illinois College of Medicine, Chicago, IL, personal communication). Tubulin is abundant in the oocyte (Gard, 1991), and so could be a factor that activates $\alpha_s$. Yet another possibility is that some fraction of the $\alpha_s$ in the *Xenopus* oocyte is constitutively active.

*Xenopus* $\alpha_s$ may have differences in nucleotide exchange and activation compared to mammalian $\alpha_s$, based on sequence differences in the region of residues 70–140 (Antonelli et al., 1994). While mammalian and *Xenopus* $\alpha_s$ are 92% identical, *Xenopus* $\alpha_s$ does not activate mammalian adenylyl cyclase in an in vitro expression system, though chimeras which replace the *Xenopus* region 70–140 with the corresponding mammalian region are active (Antonelli et al., 1994). The region homologous to residues 70–140 in the crystal structure of the α subunit, transducin, is part of an α-helical domain which forms a lid to the guanine nucleotide–binding pocket, and may affect GTP binding (Noel et al., 1993).

Since *Xenopus* $\alpha_s$ differs from mammalian $\alpha_s$, it may be regulated by unique mechanisms. It could be speculated that progesterone inhibits $\alpha_s$, either by activating a receptor that inhibits $\alpha_s$ (see a related example described by Negishi et al., 1993), or by inhibiting coupling of a constitutively active receptor with $\alpha_s$.

### *Possible Functions of G-Proteins in Yolk Platelet Membranes*

Although G-proteins were initially thought to be exclusively plasma membrane enzymes, functions of G-proteins on intracellular membranes have recently been identified in a variety of processes (Bomsel and Mostov, 1992; Jones, 1994). The presence of $\alpha_s$ on yolk platelet membranes suggests that these membranes as well as the plasma membrane could be sites of progesterone action. Progesterone has been thought to act exclusively on the plasma mem-

brane based on experiments in which steroids coupled to agarose beads or polyethylene oxide (molecular weight 20,000) were shown to induce maturation in *Xenopus* oocytes (Ishikawa et al., 1977; Godeau et al., 1978). However, an action mediated by steroid dissociated from the polymer cannot be eliminated (see Bronson and Stumpf, 1991). Furthermore, although injection of progesterone in an aqueous solution is ineffective, injection of progesterone in paraffin oil results in oocyte maturation (Tso et al., 1982).

Localization of $\alpha_s$ and $\alpha_{i3}$ on the yolk platelet membranes may be significant in other aspects of oocyte physiology. Heterotrimeric G-proteins have recently been implicated as regulatory factors in endocytosis (Haraguchi and Rodbell, 1990), endosome fusion (Colombo et al., 1992, 1994), and polarized vesicular transport (Bomsel and Mostov, 1993; Pimplikar and Simons, 1993). Thus the presence of G-proteins on yolk platelet membranes could be related to the formation of the yolk platelets by endocytosis, endosome fusion, and polarized vesicular transport (see Danilchik and Gerhart, 1987) during oogenesis. These G-proteins could also function in the regulation of ion transport across yolk platelet membranes (Fagotto and Maxfield, 1994), or in proteolysis of yolk glycoproteins during embryogenesis (Mallya et al., 1992).

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