Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the G_s G protein

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The arrest of meiotic prophase in mouse oocytes within antral follicles requires the G protein G_s and an orphan member of the G protein–coupled receptor family, GPR3. To determine whether GPR3 activates G_s, the localization of $G\alpha_s$ in follicle-enclosed oocytes from *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ mice was compared by using immunofluorescence and $G\alpha_s$GFP. GPR3 decreased the ratio of $G\alpha_s$ in the oocyte plasma membrane versus the cytoplasm and also decreased the amount of $G\alpha_s$ in the oocyte. Both of these properties indicate that GPR3 activates G_s. The follicle cells around the oocyte are also necessary to keep the oocyte in prophase, suggesting that they might activate GPR3. However, GPR3-dependent G_s activity was similar in follicle-enclosed and follicle-free oocytes. Thus, the maintenance of prophase arrest depends on the constitutive activity of GPR3 in the oocyte, and the follicle cell signal acts by a means other than increasing GPR3 activity.

Introduction

Meiosis begins early in oogenesis but does not proceed beyond prophase until luteinizing hormone from the pituitary acts on the somatic cells of the follicle surrounding the oocyte (Eppig et al., 2004). For the prolonged period of storage before oocyte growth and during the growth period, meiotic arrest is maintained by inherent factors in the oocyte (Eppig et al., 2004). However, once the oocyte has grown to its full size and an antral space has begun to form between the somatic cells of the follicle, a different mechanism for maintaining meiotic arrest comes into play. After this point, the oocyte will resume meiosis spontaneously if isolated from the follicle, leading to the conclusion that maintenance of meiotic arrest requires an inhibitory signal from the follicle cells (Pincus and Enzmann, 1935; Erickson and Sorensen, 1974).

The inhibitory signal has been proposed to be cAMP that enters the oocyte from the somatic cells (Anderson and Albertini, 1976; Piontkewitz and Dekel, 1993; Webb et al., 2002; Eppig et al., 2004). However, this long-standing hypothesis has recently been challenged by evidence that the regulation of meiotic arrest requires a G protein, a receptor, and adenylyl cyclase in the oocyte (Mehlmann et al., 2002, 2004; Horner et al., 2003; Kalinowski et al., 2004; Ledent et al., 2005). This has led to the concept that follicle cells control the oocyte cell cycle by regulating rates of cAMP production or degradation within the oocyte, but the communication pathway between follicle cells and the oocyte has not been determined.

The requirement for elevated cAMP in the oocyte to maintain prophase arrest was established by several findings (Conti et al., 2002; Eppig et al., 2004; Mehlmann, 2005). (1) After removal from the antral follicle, the oocyte's cAMP content decreases in parallel with meiotic resumption (Tornell et al., 1990). (2) Isolated oocytes can be maintained in prophase arrest by elevating cAMP (for example, with membrane permeant cAMP analogues or cAMP phosphodiesterase [PDE] inhibitors; Cho et al., 1974; Schultz et al., 1983; Eppig et al., 1985). (3) Oocytes within complexes of follicular somatic cells, which maintain meiotic arrest in vitro, resume meiosis in response to a permeant cAMP antagonist (Eppig, 1991). Elevated cAMP maintains meiotic arrest by activating protein kinase A, which phosphorylates and, thus, inhibits the phos-

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Abbreviations used in this paper: eCG, equine chorionic gonadotropin; PDE, phosphodiesterase.

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phatase CDC25B; this prevents CDC25B from dephosphorylating and activating CDK1 and, therefore, prevents the prophase-to-metaphase transition (Duckworth et al., 2002; Lincoln et al., 2002).

The cAMP that maintains meiotic arrest is produced in the oocyte by a pathway requiring the activity of the heterotrimeric G protein G_s (Mehlmann et al., 2002; Kalinowski et al., 2004) and type 3 adenylyl cyclase (Horner et al., 2003). Supporting these conclusions, the injection of follicle-enclosed oocytes with a $G\alpha_s$ inhibitory antibody or a dominant-negative form of $G\alpha_s$ causes meiosis to resume (Mehlmann et al., 2002; Kalinowski et al., 2004), as does the deletion of the gene for type 3 adenylyl cyclase, which is expressed in the oocyte (Horner et al., 2003).

In addition to G_s, the maintenance of meiotic prophase arrest requires an orphan member of the G protein–coupled receptor family, GPR3 (Mehlmann et al., 2004; Ledent et al., 2005). GPR3 was identified in a mouse oocyte cDNA library by a search for seven-transmembrane proteins (Mehlmann et al., 2004) and was of particular interest because the overexpression of GPR3 in various cell lines had been shown to elevate cAMP (Eggerickx et al., 1995; Uhlenbrock et al., 2002; Bresnick et al., 2003). *Gpr3* RNA is highly localized in the oocyte versus the surrounding somatic cells, and in *Gpr3* knockout mice, the majority of oocytes in antral follicles resume meiosis spontaneously (Mehlmann et al., 2004; Ledent et al., 2005). Oocytes in $Gpr3^{-/-}$ preantral follicles remain arrested in prophase, but as antral spaces begin to form between the somatic cells (early antral stage), the oocytes resume meiosis independently of an increase in luteinizing hormone. As a consequence of this disruption of the normal coordination of meiotic progression and fertilization, *Gpr3* knockout females are subfertile (Ledent et al., 2005). The spontaneous progression of meiosis that occurs in *Gpr3* knockout oocytes can be reversed by an injection of *Gpr3* RNA into preantral follicle-enclosed oocytes followed by culture to the early antral/antral stage (Mehlmann et al., 2004). It is thought that GPR3 in the oocyte maintains meiotic arrest by activating G_s; however, this has not been directly tested.

We investigated this hypothesis by taking advantage of two properties of G_s that have been established in somatic cells: the activation of G_s causes its α subunit to move from the plasma membrane to the cytoplasm (Rasenick et al., 1984; Ransnas et al., 1989; Levis and Bourne, 1992; Wedegaertner et al., 1996; Iiri et al., 1997; Thiyagarajan et al., 2002; Yu and Rasenick, 2002; Hynes et al., 2004; Allen et al., 2005) and also to be degraded at an increased rate (see Discussion). We compared the localization of $G\alpha_s$ in follicle-enclosed oocytes from $Gpr3^{+/+}$ and $Gpr3^{-/-}$ mice by using immunofluorescence and $G\alpha_s$GFP and found that GPR3 causes the ratio of $G\alpha_s$ in the oocyte plasma membrane versus the cytoplasm to decrease. GPR3 also causes the total amount of $G\alpha_s$ protein in the oocyte to decrease. Both of these properties indicate that GPR3 activates G_s in the oocyte. We then used the $G\alpha_s$ distribution to determine whether the presence of follicle cells is necessary to keep GPR3 active in the oocyte.

Results

The localization of $G\alpha_s$ in *Gpr3* knockout and wild-type oocytes indicates that GPR3 activates G_s in the oocyte

To investigate whether GPR3 activates G_s in the mouse oocyte, we compared the plasma membrane-to-cytoplasm distribution of $G\alpha_s$ in $Gpr3^{+/+}$ and $Gpr3^{-/-}$ oocytes. To avoid the problem that $Gpr3^{-/-}$ oocytes within early antral and antral follicles spontaneously resume meiosis, which would complicate a direct comparison with $Gpr3^{+/+}$ oocytes, we performed these experiments using preantral follicles (120–190-μm diam). These preantral follicles contained oocytes with ~70-μm diam that were not yet competent to resume meiosis when isolated (Erickson and Sorensen, 1974). At this stage, GPR3 is not required to maintain meiotic arrest (Mehlmann et al., 2004). However, we predicted that to ensure a continuously effective block to the progression of meiosis, the GPR3–G_s regulatory system would need to be in place before the oocyte acquires the inherent factors that allow meiotic resumption, and results that are described below support this prediction.

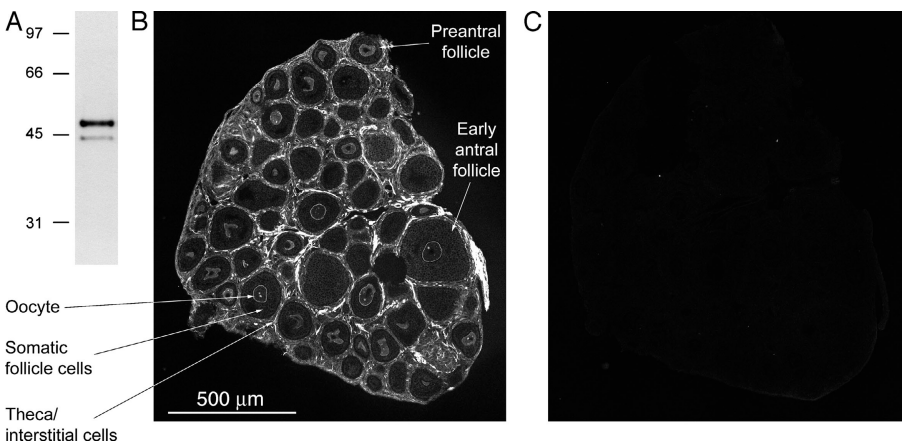

Figure 1. **Immunofluorescence localization of $G\alpha_s$ in a mouse ovary.** (A) Immunoblot demonstrating $G\alpha_s$ antibody specificity. 1 μg of ovary lysate ($Gpr3^{+/+}$) was loaded on the gel; both the 52- and 45-kD splice variants of $G\alpha_s$ (Robishaw et al., 1986) were seen. (B) A section of a $Gpr3^{+/+}$ ovary labeled with an antibody against $G\alpha_s$. This ovary, which was obtained from a mouse that had not been injected with eCG to stimulate antral follicle formation, contained mostly preantral follicles and some early antral follicles. Follicles with 120–190-μm diam were classified as preantral, whereas those with ≥200-μm diam were classified as early antral (with multiple small antral spaces) or antral (with a single large antral space; Fig. 3 A). These categories were not completely distinct because some follicles with <200-μm diam showed a formation of antral spaces. (C) A control section labeled with nonimmune IgG. Confocal microscope settings and bars were the same for B and C.

By using an antibody against the COOH-terminal decapeptide of $G\alpha_s$ (Simonds et al., 1989), which specifically recognized $G\alpha_s$ in mouse ovary (Fig. 1 A), we examined the distribution of $G\alpha_s$ immunofluorescence in preantral follicle-enclosed oocytes in frozen sections of ovaries (Fig. 1, B and C) from *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ mice. These sections showed that $G\alpha_s$ was present at a high level in theca/interstitial cells that surround each follicle and in the oocyte. $G\alpha_s$ was also present at lower levels in the follicular somatic cells.

In *Gpr3*$^{+/+}$ oocytes, $G\alpha_s$ fluorescence was present in both the plasma membrane and in the cytoplasm; within the cytoplasm, irregularly shaped bright spots up to several micrometers in diameter as well as diffuse fluorescence were visible in most sections (Fig. 2, A and C). In *Gpr3*$^{-/-}$ oocytes, $G\alpha_s$ was more strongly localized in the plasma membrane (Fig. 2, B and C).

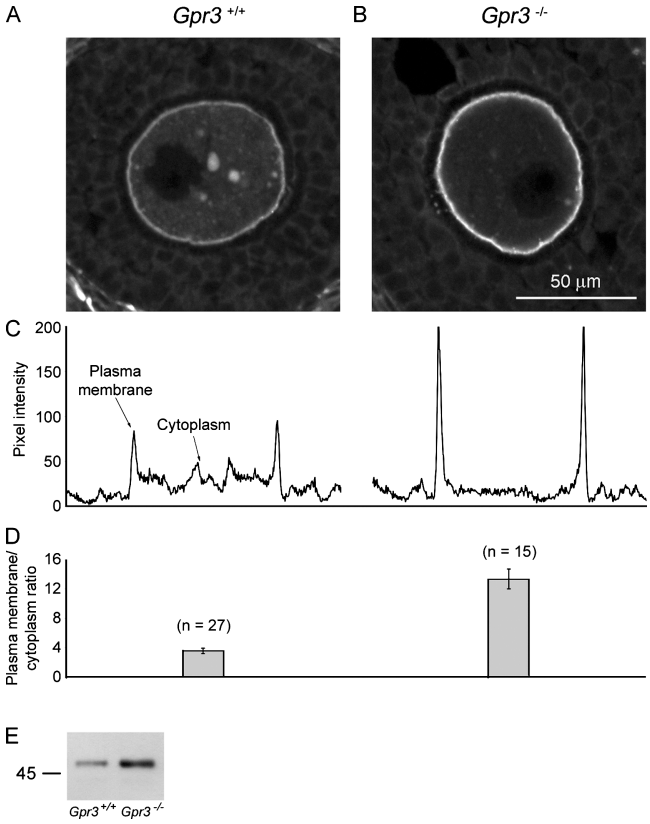

Figure 2. **Immunofluorescence localization and immunoblotting of $G\alpha_s$ in oocytes in *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ ovaries.** (A and B) $G\alpha_s$ in *Gpr3*$^{+/+}$ (A) and *Gpr3*$^{-/-}$ (B) oocytes in a preantral follicle. The confocal microscope settings and bars were the same for A and B; the dark region in each oocyte is the nucleus. (C) Pixel intensity on a scale of 0–255 along a line drawn through each oocyte image, choosing a line that avoided the nucleus. (D) Plasma membrane-to-cytoplasm $G\alpha_s$ fluorescence ratios for *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes, which were determined as described in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1). Mean ± SEM (error bars; *n* = number of oocytes). The fluorescence ratios for *Gpr3*$^{+/+}$ oocytes (3.6 ± 0.3) and *Gpr3*$^{-/-}$ oocytes (13.4 ± 1.3) were significantly different (*t* test, P = 0.0001). Data are from two *Gpr3*$^{+/+}$ and two *Gpr3*$^{-/-}$ mice; ovaries from mice of the two genotypes were processed in parallel. (E) Immunoblot comparing the amount of $G\alpha_s$ protein in *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes that were isolated from preantral follicles. 15 oocytes per lane. The 52-kD splice variant of $G\alpha_s$ is predominant in mouse oocytes, although a small amount of the 45-kD form is present (Fig. 4 B; Mehlmann et al., 2002).

Intensity measurements (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1) showed that in *Gpr3*$^{-/-}$ oocytes, the $G\alpha_s$ fluorescence was higher in the plasma membrane and lower in the cytoplasm than in *Gpr3*$^{+/+}$ oocytes (Table I). For each oocyte, we determined the plasma membrane-to-cytoplasm fluorescence intensity ratio and found that the mean ratio was significantly smaller for *Gpr3*$^{+/+}$ oocytes than for *Gpr3*$^{-/-}$ oocytes (Fig. 2 D). These results show that GPR3 causes $G\alpha_s$ to move from the oocyte plasma membrane to the cytoplasm; the simplest interpretation of this observation is that GPR3 activates G_s.

The localization of $G\alpha_s$ in oocytes from preantral and antral follicles indicates that GPR3 in the oocyte is already fully active at the preantral stage

To test whether the $G\alpha_s$ distribution in oocytes in *Gpr3*$^{+/+}$ preantral follicles was similar to that in oocytes in *Gpr3*$^{+/+}$ early antral and antral follicles, we compared the oocyte plasma membrane-to-cytoplasm $G\alpha_s$ fluorescence ratio for follicles in the 120–190-μm diam range (preantral) with that for follicles in the 200–475-μm diam range (early antral and antral; Fig. 3, A–D). The ratios were the same for oocytes within follicles of the two size ranges, indicating that the level of G_s activation in preantral follicle-enclosed oocytes is the same as in early antral and antral follicle-enclosed oocytes. These results indicated that the GPR3 in the oocyte is already fully active at the preantral stage. Consistent with this conclusion, oocytes from preantral versus early antral and antral follicles contained almost the same amounts of *Gpr3* RNA, with slightly more *Gpr3* RNA in the oocytes from preantral follicles (Fig. 3 E).

Additional evidence that GPR3 activates G_s in the oocyte

As another test of whether GPR3 activates G_s in the oocyte, we compared the amount of $G\alpha_s$ protein in *Gpr3*$^{-/-}$ with *Gpr3*$^{+/+}$ oocytes because the activation of G_s increases the rate of degradation of its α subunit (see Discussion). Oocytes were dissected from preantral follicles, and the amount of $G\alpha_s$ protein in each sample was then analyzed by immunoblotting (Fig. 2 E). The amount of $G\alpha_s$ was found to be 2.2–3.6 times greater in the

Table I. **$G\alpha_s$ immunofluorescence intensities for the plasma membrane and cytoplasm of *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes**

Region of intensity measurement	*Gpr3*$^{+/+}$	*Gpr3*$^{-/-}$
Plasma membrane intensity	91 ± 4 (*n* = 27)[a]	191 ± 13 (*n* = 15)[a]
Cytoplasm intensity	29 ± 2 (*n* = 27)[b]	16 ± 2 (*n* = 15)[b]

$G\alpha_s$ immunofluorescence intensities in the plasma membrane and cytoplasm of *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes were measured as described in Fig. S1. Except for the detector gain, the confocal microscope settings were the same for all measurements; differences in gain were adjusted for by multiplying by constant conversion factors. Intensities are expressed as equivalent values at detector gain 800 on a scale of 0–255. Mean ± SEM (*n* = number of oocytes). Data are from two *Gpr3*$^{+/+}$ and two *Gpr3*$^{-/-}$ mice. Ovaries from mice of the two genotypes were processed in parallel.
[a]These two values are significantly different (*t* test, P < 0.0001).
[b]These two values are significantly different (*t* test, P = 0.0001).

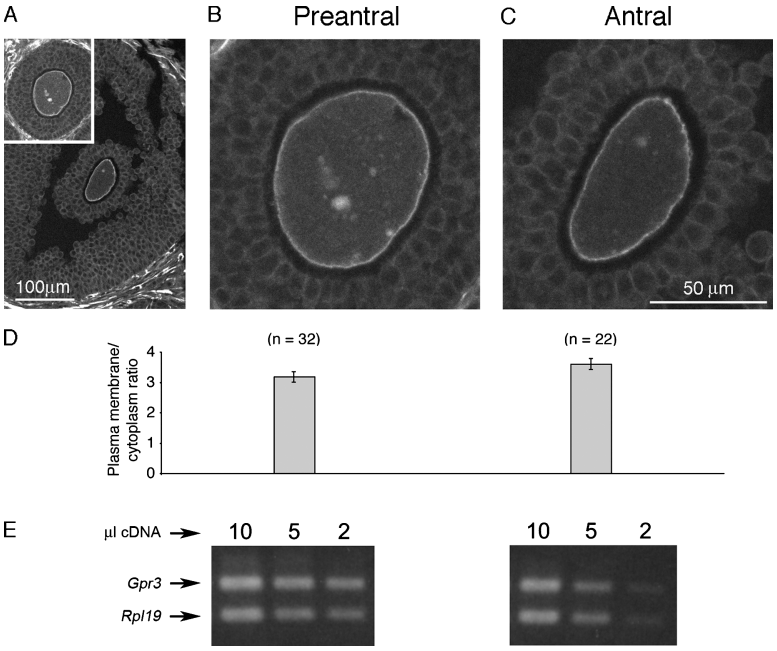

Figure 3. **Immunofluorescence localization of $G\alpha_s$ in oocytes within preantral and early antral/antral follicles of *Gpr3*$^{+/+}$ ovaries.** (A) Low magnification view of a section of an antral follicle labeled with the $G\alpha_s$ antibody. Inset shows a preantral follicle. The detector gain was the same for both images. (B and C) High magnification view of the oocyte in the preantral (B) and antral (C) follicle shown in A. The confocal microscope settings and bars were the same for B and C. (D) Plasma membrane-to-cytoplasm fluorescence ratios for oocytes in preantral follicles (3.2 ± 0.2) or early antral/antral follicles (3.6 ± 0.2). Mean ± SEM (error bars; *n* = number of oocytes). The ratios were not significantly different (*t* test, P = 0.09). Data are from two *Gpr3*$^{+/+}$ mice, one of which was injected with eCG 43 h before ovary collection to increase the number of antral follicles. (E) RT-PCR to compare the amount of *Gpr3* RNA in oocytes from preantral and early antral/antral follicles. The indicated numbers of microliters of cDNA were used for each RT-PCR; 1 μl cDNA was derived from 0.08 oocytes (supplemental Materials and methods). A mixture of primers for *Gpr3* and *Rpl19* was included in each reaction. The ratios of band intensities for *Gpr3* and *Rpl19* PCR products in each lane of the gel were used to obtain a relative measure of the amount of *Gpr3* RNA per oocyte. The relative amount of *Gpr3* RNA in oocytes from preantral follicles compared with oocytes from early antral/antral follicles was 1.3–1.6 (range for this and another similar experiment).

Gpr3$^{-/-}$ oocytes versus the *Gpr3*$^{+/+}$ oocytes (range for two independent experiments, each with two to three sets of 15 oocytes of each genotype).

Consistent with the conclusion that GPR3 activates G_s in the oocyte, the injection of *Gpr3* RNA increased the amount of cAMP in isolated *Gpr3*$^{+/+}$ oocytes (~1.7 times under the conditions of these measurements; supplemental Materials and methods, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1). However, the process of oocyte isolation, which is necessary for such assays, changes the amount of cAMP in the oocyte (Tornell et al., 1990); this limits the usefulness of biochemical measurements of cAMP to detect G_s activation by GPR3 in this system. Instead, we developed an optical method to monitor GPR3 activation in live oocytes.

$G\alpha_s$GFP as a monitor of GPR3 activation in live oocytes

To confirm our immunofluorescence results and to establish a method for investigating GPR3 activation in live oocytes, we examined the GPR3 dependence of $G\alpha_s$ distribution by using a GFP fusion protein. To validate the use of $G\alpha_s$GFP as a monitor of receptor activation, we showed that in oocytes expressing an exogenous β-adrenergic receptor and exposed to isoproterenol, $G\alpha_s$GFP moved from the plasma membrane to the cytoplasm, as previously described for somatic cells (supplemental Results and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1).

Preantral follicle-enclosed oocytes were injected with RNA encoding the α subunit of G_s fused with GFP ($G\alpha_s$GFP; Hynes et al., 2004), were cultured overnight to allow protein expression, and were imaged with a confocal microscope while the oocytes were still within their follicles (Fig. 4 A). Under the conditions of these experiments, $G\alpha_s$GFP was expressed at a level only slightly greater than that of endogenous $G\alpha_s$ (Fig. 4 B). This level of $G\alpha_s$GFP expression did not disrupt the normal physiology of the oocyte, as indicated by the resumption of meiosis in $G\alpha_s$GFP-expressing oocytes that were isolated from antral follicles (supplemental Results).

In *Gpr3*$^{+/+}$ oocytes, $G\alpha_s$GFP was present in both the plasma membrane and the cytoplasm; the cytoplasmic fluorescence included both diffuse and particulate components (Fig. 4 C). In contrast, in *Gpr3*$^{-/-}$ oocytes, $G\alpha_s$GFP was highly localized in the plasma membrane (Fig. 4 D). This difference in $G\alpha_s$GFP fluorescence distribution in the oocyte could not be attributed to an optical difference in the surrounding follicle cells because the follicles used were similar in size and optical density for the two genotypes. Quantitation of $G\alpha_s$GFP fluorescence (Fig. S1) showed that the plasma membrane-to-cytoplasm fluorescence ratio was smaller for *Gpr3*$^{+/+}$ oocytes than for *Gpr3*$^{-/-}$ oocytes (Fig. 4 E), as was also observed with $G\alpha_s$ immunofluorescence (Fig. 2 D). Although the ratios that were obtained by the two methods were close, they were not identical; factors such as fixation or overexpression artifacts could possibly account for this lack of exact correspondence.

To determine whether GPR3 in the oocyte could account for the difference in $G\alpha_s$GFP localization in oocytes from *Gpr3*$^{-/-}$ and *Gpr3*$^{+/+}$ mice, we injected *Gpr3*$^{-/-}$ oocytes within preantral follicles with a mixture of *Gpr3* RNA and $G\alpha_s$GFP RNA, cultured the follicles overnight, and observed them with confocal microscopy. If GPR3 in the oocyte is responsible for causing the $G\alpha_s$GFP plasma membrane-to-cytoplasm ratio to decrease, we would expect that the injection of *Gpr3*$^{-/-}$ oocytes with *Gpr3* RNA would decrease the relative amount of $G\alpha_s$GFP in the plasma membrane versus the cytoplasm.

In *Gpr3*$^{-/-}$ oocytes that had been injected with 7 pg of a control RNA (*β-globin*), $G\alpha_s$GFP was highly localized in the plasma membrane (Fig. 5, A and D), as in *Gpr3*$^{-/-}$ oocytes that were injected with $G\alpha_s$GFP RNA alone (Fig. 4, D and E). In contrast, in *Gpr3*$^{-/-}$ oocytes that had been injected with 7 pg of *Gpr3* RNA, $G\alpha_s$GFP fluorescence was present in the cytoplasm

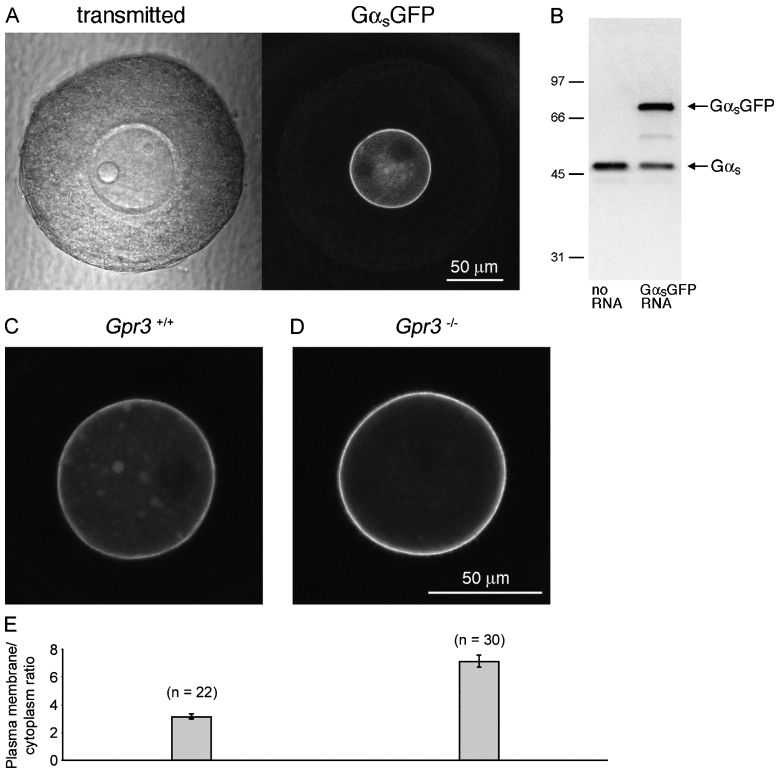

Figure 4. **$G\alpha_s$GFP fluorescence in *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ preantral follicle-enclosed oocytes.** (A) Scanning transmission and fluorescence images of a *Gpr3*$^{+/+}$ preantral follicle in which the oocyte had been injected with $G\alpha_s$GFP RNA 20 h before. In the transmitted light image of the oocyte, the bright sphere on the left is an oil drop that was introduced by microinjection; the nucleus and nucleolus are visible on the top right. In the fluorescence image, the oil drop and nucleus appear as dark areas where $G\alpha_s$GFP is excluded. (B) Immunoblot comparing the amount of $G\alpha_s$GFP and endogenous $G\alpha_s$ in $G\alpha_s$GFP RNA-injected and noninjected oocytes from a *Gpr3*$^{+/+}$ mouse. Oocytes were injected with the RNA while inside their preantral follicles; after culturing the follicles overnight, the oocytes were isolated, and gel samples were prepared (14 oocytes per lane). The blot was probed with an antibody against $G\alpha_s$ (RM). The ratio of $G\alpha_s$GFP protein in the injected oocytes to $G\alpha_s$ in uninjected control oocytes was 1.1–1.5 (range for two experiments). The expression of $G\alpha_s$GFP caused some reduction of endogenous $G\alpha_s$ protein. (C and D) $G\alpha_s$GFP fluorescence in *Gpr3*$^{+/+}$ (C) and *Gpr3*$^{-/-}$ (D) oocytes. (C) Fluorescence is present in the plasma membrane, in spots in the cytoplasm, and diffusely within the cytoplasm. (D) Fluorescence is present primarily in the plasma membrane. The confocal microscope settings and bars were the same for C and D. (E) Plasma membrane-to-cytoplasm fluorescence ratios for *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes. Mean ± SEM (error bars; *n* = number of oocytes). Ratios for *Gpr3*$^{+/+}$ oocytes (3.1 ± 0.2) and *Gpr3*$^{-/-}$ oocytes (7.1 ± 0.4) were significantly different (*t* test, $P < 0.0001$). Data are from three *Gpr3*$^{+/+}$ and three *Gpr3*$^{-/-}$ mice.

and was not localized in the plasma membrane (Fig. 5 B). As the amount of *Gpr3* RNA was reduced while keeping the amount of $G\alpha_s$GFP RNA constant, the fraction of $G\alpha_s$GFP in the plasma membrane increased. The injection of 7 fg *Gpr3* RNA resulted in a plasma membrane-to-cytoplasm fluorescence ratio that was comparable with that in *Gpr3*$^{+/+}$ oocytes (compare Fig. 5, C and D with Fig. 4, C and E). Similar results were obtained by using a GPR3-RFP construct, which allowed us to monitor the level of GPR3 expression as a function of the amount of RNA injected and to show that the amount of GPR3 protein correlates with the amount of $G\alpha_s$GFP internalization (supplemental Results and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1). Because an increase in the relative amount of $G\alpha_s$ in the cytoplasm versus the plasma membrane is an indicator of G_s activation, these findings confirm that within the oocyte, GPR3 activates G_s.

GPR3 constitutively activates G_s in the oocyte independently of follicle cells

One way in which the somatic cells of antral and early antral follicles could maintain prophase arrest in the oocyte is by producing a ligand that increases the activity of GPR3. Alternatively, the follicle cells could inhibit meiotic resumption by another pathway such as activating another G_s-linked receptor

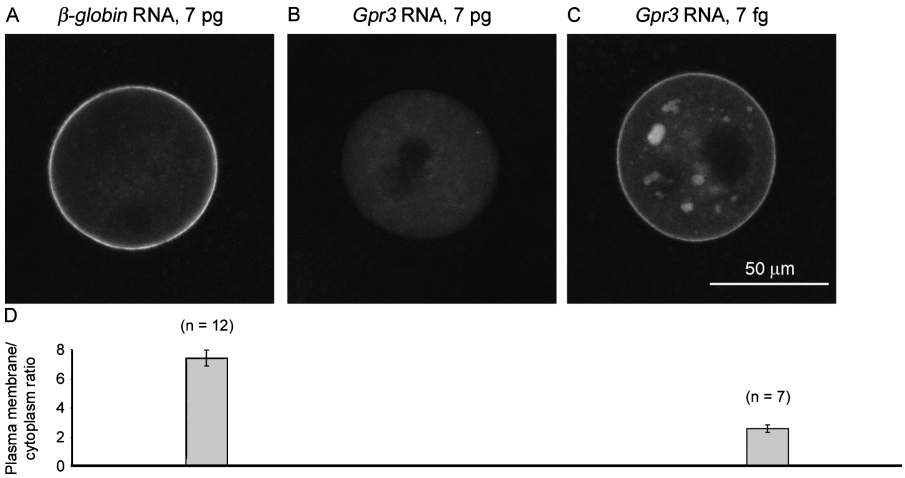

Figure 5. **$G\alpha_s$GFP fluorescence in *Gpr3*$^{-/-}$ preantral follicle-enclosed oocytes that had been coinjected with $G\alpha_s$GFP RNA and *Gpr3* RNA.** Oocytes were injected with 15 pg $G\alpha_s$GFP RNA and the indicated amounts of *Gpr3* or a control RNA encoding β-globin, and the follicles were cultured overnight before imaging. (A–C) 7 pg *β-globin* (A), *Gpr3* (B), and 7 fg *Gpr3* (C) RNA. The confocal microscope settings and bars were the same for all images. (D) Plasma membrane-to-cytoplasm $G\alpha_s$GFP fluorescence ratios for *Gpr3*$^{-/-}$ oocytes that were injected with the indicated RNAs. Mean ± SEM (error bars; *n* = number of oocytes). Data are from three mice. For the images of oocytes that had been injected with 7 pg *Gpr3* RNA (*n* = 10), no plasma membrane fluorescence could be identified, so no ratio was calculated. Ratios for oocytes that were injected with 7 pg *β-globin* RNA (7.4 ± 0.5) or 7 fg *Gpr3* RNA (2.6 ± 0.2) were significantly different (*t* test, $P < 0.0001$). The ratio for *Gpr3*$^{-/-}$ oocytes + 7 fg *Gpr3* RNA was not different from that for *Gpr3*$^{+/+}$ oocytes (Fig. 4 E; $P = 0.14$), and the ratio for *Gpr3*$^{-/-}$ oocytes + 7 pg *β-globin* RNA was not different from that for *Gpr3*$^{-/-}$ oocytes (Fig. 4 E; $P = 0.69$).

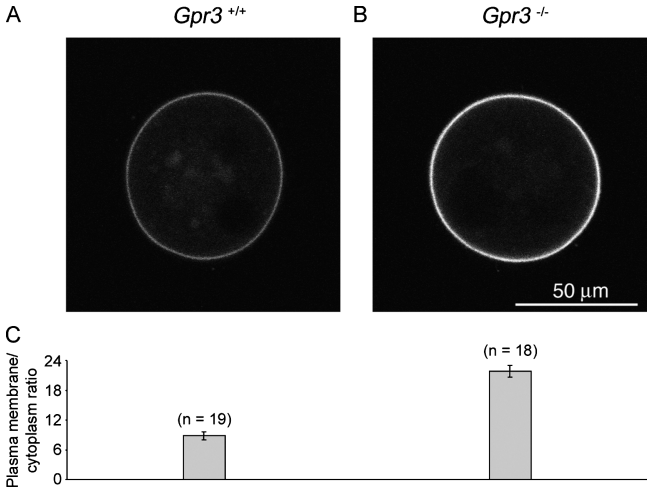

Figure 6. **$G\alpha_s$GFP fluorescence in *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes that were isolated from preantral follicles and injected with $G\alpha_s$GFP RNA.** (A and B) *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes. The confocal microscope settings and bars were the same for A and B. (C) Plasma membrane-to-cytoplasm fluorescence ratios for *Gpr3*$^{+/+}$ (8.8 ± 0.8) and *Gpr3*$^{-/-}$ oocytes (21.8 ± 1.2). Mean ± SEM (error bars; *n* = number of oocytes). The ratios for *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes were significantly different (*t* test, $P < 0.0001$). Data are from two *Gpr3*$^{+/+}$ and two *Gpr3*$^{-/-}$ mice.

Table II. **The GPR3-dependent activation of G_s in the oocyte is independent of follicle cells**

Specimen	PM/CYTO (*Gpr3*$^{+/+}$)	PM/CYTO (*Gpr3*$^{-/-}$)	PM/CYTO (*Gpr3*$^{-/-}$) / PM/CYTO (*Gpr3*$^{+/+}$)
Follicle-enclosed oocytes[d]	3.1 ± 0.2 (*n* = 22)[a]	7.1 ± 0.4 (*n* = 30)[b]	2.3 ± 0.2[c]
Isolated oocytes[e]	8.8 ± 0.8 (*n* = 19)	21.8 ± 1.2 (*n* = 18)	2.5 ± 0.3[c]
Isolated oocytes (with optical correction)	4.1 ± 0.5 (*n* = 19)[a]	10.1 ± 1.0 (*n* = 18)[b]	–

Second and third columns indicate the ratios of plasma membrane $G\alpha_s$GFP fluorescence (PM) to cytoplasm $G\alpha_s$GFP fluorescence (CYTO) in the oocyte. Mean ± SEM (*n* = number of oocytes). Data for follicle-enclosed oocytes are from three mice of each genotype, and data for isolated oocytes are from two mice of each genotype. For each row, the last column indicates the value listed in the third column divided by the value listed in the second column. See the last section of Results and Fig. 7 for an explanation of the optical correction.
[a]These two values are not significantly different (*t* test, P = 0.08).
[b]These two values are significantly different (*t* test, P = 0.01).
[c]These two values are not significantly different (*t* test, P = 0.6).
[d]Corresponds to Fig. 4 E.
[e]Corresponds to Fig. 6 C.

in the oocyte, inhibiting the breakdown of cAMP by a PDE in the oocyte, or supplying cAMP to the oocyte by way of gap junctions (see Discussion). To test the first of these possibilities, we examined whether the GPR3 stimulation of $G\alpha_s$ requires the presence of follicle cells.

Because the immunofluorescence results described in Fig. 3 indicated that the GPR3–G_s complex is equally active in oocytes in preantral and in antral and early antral stage follicles, we performed these experiments using oocytes from preantral follicles, which have the important technical advantage of not resuming meiosis when isolated. This allowed us to measure the $G\alpha_s$GFP distribution that was established after the injection of $G\alpha_s$GFP RNA into isolated oocytes.

Oocytes were isolated from *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ preantral follicles, injected with $G\alpha_s$GFP RNA, cultured overnight, and imaged by confocal microscopy. The difference in $G\alpha_s$GFP localization between *Gpr3*$^{+/+}$ and *Gpr3*$^{-/-}$ oocytes was still evident, indicating that GPR3 activates G_s even in the absence of follicle cells (Fig. 6).

A comparison of plasma membrane-to-cytoplasm fluorescence ratios from follicle-enclosed and isolated oocytes is complicated by optical factors (see next paragraph). However, by dividing the plasma membrane-to-cytoplasm fluorescence ratio for *Gpr3*$^{-/-}$ oocytes by the plasma membrane-to-cytoplasm fluorescence ratio for *Gpr3*$^{+/+}$ oocytes, we obtained a measure of GPR3-dependent G_s activation that could be directly compared for follicle-enclosed and isolated oocytes (Table II, right column). This analysis indicated that the follicle cells do not increase the activation of G_s by GPR3.

The measurements in Table II also showed that the absolute values for plasma membrane-to-cytoplasm ratios of $G\alpha_s$-GFP fluorescence were greater in the isolated oocytes versus the follicle-enclosed oocytes regardless of the presence of GPR3. We suspected that this difference resulted, at least in part, because confocal imaging of an isolated oocyte involves light traveling through ~35 μm of optically clear medium to reach the plasma membrane versus ~35 μm of optically dense cytoplasm to reach the cytoplasmic imaging plane in the center of the oocyte. As a consequence, the plasma membrane of isolated oocytes appears artificially brighter (Fig. 7 A). A follicle-enclosed oocyte is free of this artifact because the paths to the cytoplasm and membrane both involve light travelling through cytoplasm (either follicle cell cytoplasm or oocyte cytoplasm; Fig. 7 A).

To determine the magnitude of this optical effect, we injected preantral follicle-enclosed oocytes with RNA-encoding YFP attached to the palmitoylation domain of neuromodulin (YFP-Mem) in order to introduce a fluorescent marker of the plasma membrane that should be independent of signals from the follicle cells. We also injected these oocytes with YFP protein, which stays in the cytoplasm, in an amount that resulted in a fluorescence ratio for the plasma membrane (YPF-Mem) to cytoplasm (YFP) that was similar to that of oocytes expressing $G\alpha_s$GFP. We then imaged their fluorescence before and after removal from the follicle (Fig. 7, B and C). These measurements indicated that to directly compare $G\alpha_s$GFP ratios in isolated oocytes with those in follicle-enclosed oocytes, we need to use a correction factor of 2.2 times (Fig. 7). This value fits well with another measurement of the absorbance of light by the mouse oocyte cytoplasm (Terasaki, 2005).

After correction for this optical factor, the plasma membrane-to-cytoplasm $G\alpha_s$GFP fluorescence ratios for follicle-enclosed and isolated oocytes were much closer (Table II, second and third columns). For the *Gpr3*$^{-/-}$ oocytes, a small but statistically significant difference remained, which could indicate some effect of the follicle cells on G_s activity. However, with regard to the *Gpr3*-dependent activation of G_s (Table II, third column), the presence of follicle cells had no detectable stimulatory effect. Therefore, although GPR3 and G_s are required

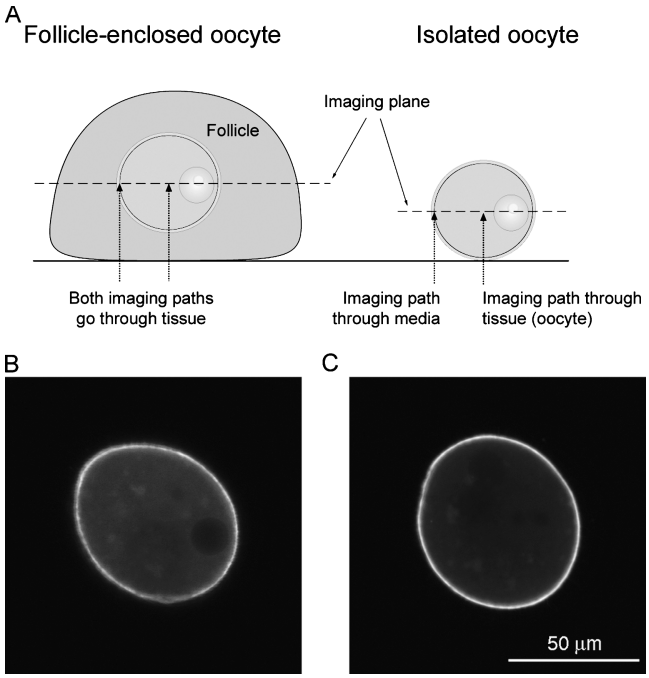

Figure 7. **Determination of the optical correction factor for comparing the plasma membrane-to-cytoplasm fluorescence ratios in preantral follicle-enclosed and isolated oocytes.** (A) Diagram of the optical difference in comparing follicle-enclosed with isolated oocytes. (B) YFP-Mem and YFP fluorescence from an oocyte in a preantral follicle. (C) YFP-Mem and YFP fluorescence from the same oocyte as shown in B after isolation from its follicle. The detector gain for B was 3.2 times that for C, reflecting the fact that the fluorescence intensity collected from an oocyte inside a preantral follicle is less than that collected from an isolated oocyte. Other confocal microscope settings and bars were the same for B and C. The plasma membrane-to-cytoplasm fluorescence ratio that was measured for B was 3.5 and that for C was 8.2. Based on measurements of 14 such oocytes, removal from the follicle resulted in an increase in the apparent plasma membrane-to-cytoplasm fluorescence ratio of 2.2 ± 0.2 (mean ± SEM); data from two mice.

to maintain meiotic arrest in a follicle-enclosed oocyte (Mehlmann et al., 2002, 2004; Kalinowski et al., 2004; Ledent et al., 2005), the signal from follicle cells that maintains meiotic arrest acts by a mechanism other than providing a ligand that activates GPR3.

Discussion

Optical methods have provided important insights about the function of G proteins and their receptors in intact cells; in this study, we applied such methods to study receptor–G protein interactions in a cell (the oocyte) within an intact tissue (the ovarian follicle). Our results showed that GPR3, which is a member of the G protein–coupled receptor family that is required to maintain meiotic arrest, constitutively activates the G_s G protein in the mouse oocyte.

GPR3 activates G_s in the mouse oocyte

To determine whether GPR3 activates G_s in the oocyte, we examined the distribution of $G\alpha_s$ in *Gpr3* knockout and wild-type oocytes within their follicles. The activation of G_s causes its α subunit to dissociate from the plasma membrane, thus providing a way to examine the activation of G_s in single cells within tissues. Translocation of the activated α subunit of G_s into the cytoplasm has been demonstrated in somatic cells by fractionation of soluble and particulate components (Rasenick et al., 1984; Ransnas et al., 1989; Levis and Bourne, 1992; Wedegaertner et al., 1996; Jones et al., 1997; Huang et al., 1999; Thiyagarajan et al., 2002; Yu and Rasenick, 2002), by immunofluorescence microscopy (Wedegaertner et al., 1996; Iiri et al., 1997; Thiyagarajan et al., 2002), and, most recently, by GFP fusion protein microscopy (Yu and Rasenick, 2002; Hynes et al., 2004; Allen et al., 2005).

In particular, these microscopic methods have shown a consistent correlation between G_s activation and translocation of its α subunit from the plasma membrane into the cytoplasm whether G_s is activated by a receptor, by cholera toxin, or by point mutation. In addition, $G\alpha_s$ is not internalized in response to receptor stimulation if the α subunit is point mutated such that its activation is impaired (Iiri et al., 1997). We validated the use of $G\alpha_s$ localization as an indicator of its activation in oocytes by showing that $G\alpha_s$GFP moves from the oocyte plasma membrane to the cytoplasm when an exogenously expressed β-adrenergic receptor is stimulated with isoproterenol.

Incorporation of GFP into an internal sequence of $G\alpha_s$ does not prevent receptor-mediated activation of GTP-binding and adenylyl cyclase (Yu and Rasenick, 2002; Hynes et al., 2004). The $G\alpha_s$GFP fusion protein that we used, which includes a linker sequence on either side of the inserted GFP, is almost identical to $G\alpha_s$ in terms of the receptor-mediated activation of adenylyl cyclase (Hynes et al., 2004). We used $G\alpha_s$GFP as well as immunofluorescence to visualize the $G\alpha_s$ distribution within follicle-enclosed oocytes. Both localization methods showed that the ratio of $G\alpha_s$ in the plasma membrane versus cytoplasm is smaller in $Gpr3^{+/+}$ oocytes than in $Gpr3^{-/-}$ oocytes, indicating that GPR3 activates G_s. The injection of *Gpr3* RNA into $Gpr3^{-/-}$ oocytes restored the $G\alpha_s$GFP distribution that is characteristic of $Gpr3^{+/+}$ oocytes, showing that GPR3 in the oocyte is sufficient to maintain the activity of G_s in the oocyte.

The mechanism of $G\alpha_s$ internalization is incompletely understood (Hynes et al., 2004; Allen et al., 2005), but depalmitoylation of the activated α subunit is likely to be an important factor (Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996; Thiyagarajan et al., 2002). $G\alpha_s$ is then repalmitoylated (Jones et al., 1997) and, as a consequence, is thought to associate with intracellular membranes for transport back to the plasma membrane (Wedegaertner et al., 1996; Hynes et al., 2004). Dissociation of α from βγ subunits could also contribute to $G\alpha_s$ internalization, but whether such dissociation occurs during G_s activation is unknown (Frank et al., 2005).

Bright spots of $G\alpha_s$ fluorescence within the cytoplasm of $Gpr3^{+/+}$ oocytes are suggestive of an association of $G\alpha_s$ with clusters of intracellular membranes. Punctate localization of $G\alpha_s$ has been seen in somatic cells after receptor activation (Wedegaertner et al., 1996; Yu and Rasenick, 2002; Hynes et al., 2004; Allen et al., 2005), and, in these cells, colocalization of $G\alpha_s$ with the monomeric G protein RAB11 supports the idea that $G\alpha_s$ associates with vesicles for recycling to the plasma membrane (Hynes et al., 2004). Because the $G\alpha_s$ accumulations in oocytes

are up to several micrometers in diameter and are irregular in shape, and because the fluorescence is present throughout the spots rather than at their peripheries, these spots are unlikely to be single vesicles; whether they function in the recycling of $G\alpha_s$ to the oocyte plasma membrane remains to be determined.

As another test of whether GPR3 activates G_s in the oocyte, we compared the amount of $G\alpha_s$ protein in oocytes that had been isolated from *Gpr3*$^{+/+}$ or *Gpr3*$^{-/-}$ follicles. The activation of $G\alpha_s$ by cholera toxin, point mutation, or receptor activation increases its rate of degradation (Levis and Bourne, 1992; Moravcová et al., 2004). Thus, a decrease in $G\alpha_s$ protein is an indicator of G_s activation. We found that the amount of $G\alpha_s$ protein in *Gpr3*$^{-/-}$ oocytes was approximately three times that in *Gpr3*$^{+/+}$ oocytes, indicating that the presence of GPR3 reduces the amount of $G\alpha_s$ protein. This finding, as well as our finding that GPR3 overexpression elevates cAMP in the oocyte, are additional evidence that GPR3 activates G_s in the mouse oocyte.

For somatic cells, it has been speculated that one function of the internalization and degradation of activated $G\alpha_s$ may be signal attenuation; internalization of $G\alpha_s$ potentially separates it from the receptor that activates it and from adenylyl cyclase, both of which are localized in the plasma membrane (Wedegaertner and Bourne, 1994). However, for the maintenance of meiotic arrest in oocytes, it is not clear why attenuation of the G_s signal would be useful. Another proposed function of internalization of $G\alpha_s$ is to allow it to interact with molecules in the cytoplasm (Wedegaertner and Bourne, 1994). In the oocyte, much of the adenylyl cyclase 3 protein is found in the cytoplasm (Horner et al., 2003), so the internalization of $G\alpha_s$ could allow it to activate this pool of adenylyl cyclase as well as regulate other cytoplasmic proteins such as tubulin (Roychowdhury et al., 1999).

These results tie together previous findings that both GPR3 (Mehlmann et al., 2004; Ledent et al., 2005) and G_s (Mehlmann et al., 2002; Kalinowski et al., 2004) are required to maintain meiotic arrest and support the conclusion that GPR3–G_s interaction is responsible for the activation of adenylyl cyclase that maintains elevated cAMP in the oocyte, thus maintaining meiotic prophase arrest.

GPR3 activity in the oocyte is not increased by the presence of follicle cells

In somatic cells, GPR3 expression elevates cAMP apparently independently of an extracellular ligand (Eggerickx et al., 1995; Uhlenbrock et al., 2002; Bresnick et al., 2003). Likewise, our measurements of $G\alpha_s$GFP distribution indicate that even without the follicle cells, GPR3 activates G_s in the oocyte. Previous studies have also shown that G_s is active in follicle-free oocytes because the injection of a $G\alpha_s$ inhibitory antibody or a dominant-negative form of $G\alpha_s$ overcomes the inhibition of spontaneous meiotic resumption by a cAMP PDE inhibitor (Mehlmann et al., 2002; Kalinowski et al., 2004). However, this previous work did not quantitatively compare G_s activity in oocytes with and without the follicle cells. Based on the $G\alpha_s$GFP distribution, we now conclude that the follicle cells have no detectable effect on GPR3-dependent G_s activity in the oocyte. Instead, GPR3 appears to be either inherently active or activated by a molecule within the oocyte cytoplasm or plasma membrane.

How follicle cells signal to the oocyte to maintain meiotic arrest is unknown. Our results indicate that the target of the follicle cell signal is not GPR3 but do not exclude the possibility that the follicle cell signal activates a different, unidentified G_s-linked receptor that could be present in the oocyte membrane. The small but statistically significant difference in the plasma membrane-to-cytoplasm ratio of $G\alpha_s$GFP, comparing follicle-enclosed with isolated oocytes from *Gpr3*$^{-/-}$ mice, suggests a role for a second G_s-linked receptor. Another possible explanation of the meiosis-inhibiting effect of follicle cells is that additional cAMP enters the oocyte from follicle cells by way of gap junctions (Anderson and Albertini, 1976; Piontkewitz and Dekel, 1993; Webb et al., 2002; Eppig et al., 2004). Although this has been a long-standing hypothesis, it has not been definitively tested.

Alternatively, follicle cells could reduce cAMP PDE activity in the oocyte. Consistent with a role for a PDE inhibitor in maintaining meiotic arrest, oocytes from mice lacking the PDE3A isoform remain in prophase when isolated (Masciarelli et al., 2004). In addition, cilostamide-sensitive cAMP PDE activity in cumulus cell–oocyte complexes increases with time after isolation from the follicle; because cilostamide is an inhibitor of the oocyte-specific PDE3A isoform, this finding indicates that the presence of follicle cells may suppress oocyte PDE activity (Richard et al., 2001). Mouse follicular fluid contains the PDE inhibitor hypoxanthine, which is an effective inhibitor of meiotic resumption in isolated oocytes (Eppig et al., 1985). However, because the concentration of hypoxanthine in follicular fluid is not precisely known, its physiological significance is uncertain (Eppig et al., 1985; Eppig, 1991).

Although follicular fluid alone has some inhibitory effect on meiotic resumption in isolated oocytes, the inhibition is stronger when there is contact between the somatic cells and the oocyte (Tsafriri and Channing, 1975; Racowsky and Baldwin, 1989). This suggests that somatic cell molecules that act on the oocyte are, at least in part, membrane associated rather than free in the follicular fluid. The somatic cells extend processes that contact the oocyte; these contacts may be the sites where the meiosis-inhibiting signal is transmitted to the oocyte (Anderson and Albertini, 1976).

Although our findings argue against stimulation of GPR3 activity in the oocyte by an agonist from the follicle cells, GPR3 activity could be inhibited by an inverse agonist when luteinizing hormone acts on the somatic cells to cause meiosis to resume in the oocyte (Eppig et al., 2004; Park et al., 2004; Mehlmann, 2005). GPR3 is related to the melanocortin receptors (Joost and Methner, 2002), and the melanocortin-4 receptor shows constitutive activity that is inhibited by an endogenous inverse agonist, agouti-related protein (Nijenhuis et al., 2001).

Two sequential but overlapping mechanisms for maintaining meiotic prophase arrest

The GPR3–G_s mechanism for maintaining meiotic prophase arrest becomes necessary only at the early antral stage; at the preantral stage, meiotic arrest does not require GPR3 (Mehlmann et al., 2004) or the presence of follicle cells (Erickson and Sorensen, 1974). Why fully grown oocytes within preantral folli-

cles are incompetent to resume meiosis is unknown. Cell cycle regulatory protein levels have not been compared in oocytes from preantral with early antral follicles, but a comparison of ~55-μm diam oocytes from 12-d-old mice with ~80-μm diam oocytes from antral follicles of adult mice has shown that the small oocytes contain lower concentrations of cyclin B and CDK1 (Kanatsu-Shinohara et al., 2000). It is possible that the concentrations of these proteins, which are essential for meiotic resumption, stay low until the early antral stage.

Although the GPR3–G_s system is not required at the preantral stage, GPR3 is already present and activating G_s. Thus, as seems logical, the oocyte establishes the GPR3–G_s regulatory system before the oocyte becomes competent to resume meiosis, therefore ensuring that progression to metaphase does not precede the luteinizing hormone signal.

Materials and methods

Mice

Gpr3 knockout mice were obtained from Deltagen, Inc. and were genotyped as previously described (Mehlmann et al., 2004). Ovaries were collected from 20–25-d-old mice. For Fig. 3, one mouse was injected interperitoneally with 5 IU equine chorionic gonadotropin (eCG; provided by A.F. Parlow, National Hormone and Peptide Program, Torrance, CA). All animal studies were approved by the University of Connecticut Health Center Animal Care Committee.

Isolation and culture of follicles and oocytes

Preantral follicles (120–190-μm diam) and early antral/antral follicles (250–430-μm diam) were dissected from ovaries by using Dumont mini forceps (11200-14; Fine Science Tools). For some experiments, oocytes were isolated from the dissected follicles by using 30-gauge needles; residual follicle cells were removed by mouthpipetting through a micropipette that had been cut with a diamond knife (10100-00; Fine Science Tools) such that the inner diameter at the end was ~60 μm. All manipulations were performed in MEMα medium (12000-022; Invitrogen) supplemented with 25 mM $NaHCO_3$, 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 5% FBS (16000-044; Invitrogen).

For overnight culture of preantral follicles, the medium also contained 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (all from Sigma-Aldrich), and 10 ng/ml ovine follicle stimulating hormone (National Hormone and Peptide Program). 4–10 follicles were placed on Millicell culture plate inserts (PICMORG50; Millipore) inside coverslip-bottomed dishes (P35G-0-20-C-INV; MatTek Corp.) containing 1.6 ml of medium. The dishes were thoroughly rinsed with MEMα before use. The cultures were maintained at 37°C with 5% CO_2 in humidified air for 20–34 h before imaging. Isolated oocytes were cultured in 200 μl MEMα drops containing 5% FBS under light mineral oil (Fisher Scientific) for 21–28 h before imaging.

Immunoblotting

An ovary was homogenized in 100 μl PBS with protease inhibitors by using a 0.1-ml Dounce homogenizer. Oocytes were isolated from preantral follicles as described above. An affinity-purified antibody against the COOH-terminal decapeptide of $G\alpha_s$ (RM) was provided by A. Spiegel (National Institutes of Health, Bethesda, MD) and used at 1.7 μg/ml; blots were developed by using ECL Plus reagents (GE Healthcare).

Immunofluorescence microscopy

Ovaries were fixed in 4% PFA (Electron Microscopy Sciences) in PBS for 3 h at 4°C, rinsed in PBS, incubated in 30% sucrose for 1–2 h, and transferred to freezing medium (H-TFM; Triangle Biomedical Sciences, Inc.) in 6-mm–diam gelatin capsules (5214; Ernest F. Fullam, Inc.). The samples were frozen in isopentane that was cooled with dry ice, and 8–10-μm sections were cut using a cryostat. The sections were blocked with 1% BSA and 5% normal goat serum in PBS, stained using 3 μg/ml $G\alpha_s$ antibody or 3.6 μg/ml nonimmune rabbit IgG (MP Biomedicals) and 20 μg/ml of a fluorescein-labeled goat anti–rabbit secondary antibody (MP Biomedicals), and mounted with Vectashield mounting medium (Vector Laboratories). The sections were imaged by using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.) with the pinhole set for an optical section thickness of 3 μm. Fluorescence was excited with the 488-nm line of an argon laser and was detected by using a 500–550-nm emission filter. Tile scans as shown in Fig. 1 (B and C) were collected by using a 20× NA 0.75 Fluar objective (Carl Zeiss MicroImaging, Inc.) and were used to identify follicles for imaging with a 40× NA 1.2 water immersion objective (C-Apoachromat; Carl Zeiss MicroImaging, Inc.). For these and all other images, the photomultiplier amplifier offset was adjusted so that the baseline was above zero, and the gain was adjusted so that peak intensities were not saturated.

Plasma membrane and cytoplasmic fluorescence intensities were measured by using MetaMorph software (Molecular Devices) as described in Fig. S1 and in supplemental Materials and methods. For the analysis of $G\alpha_s$ immunofluorescence images, we subtracted a background value that was determined by measuring the fluorescence intensity of oocytes in sections that were processed in parallel but incubated with nonimmune IgG instead of the anti-$G\alpha_s$ primary antibody. These background intensity values, which, for different sets of sections, ranged from three to seven on a scale of 0–255, have been subtracted from the $G\alpha_s$ fluorescence intensity values shown in Table I, but the figures are shown without background subtraction.

Microinjection

Preantral follicle-enclosed oocytes and isolated oocytes were injected while held between coverslips that were separated by a piece of double stick tape that formed a 100-μm spacer (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1; Jaffe and Terasaki, 2004; Mehlmann et al., 2004). The preantral follicles (120–190-μm diam) were partially pulled into the coverslip space by applying suction with a mouth-controlled pipette and were pushed in further using the microinjection pipette. This flattened the follicles such that the oocytes could be seen clearly for injection. After injection, the follicles were swept out of the coverslip space by using the microinjection pipette such that they fell into the reservoir of medium that was formed by the injection slide. All injection volumes were 15 pl and were calibrated as previously described (Jaffe and Terasaki, 2004). For experiments in which follicle-enclosed oocytes were injected with RNA-encoding YFP-Mem, a second injection of YFP protein was performed after overnight culture; this was accomplished by putting the follicle-enclosed oocyte back into the coverslip space on an injection slide.

RNAs and protein used for injection

RNA-encoding $G\alpha_s$GFP was transcribed from a cDNA that was identical to α_s-CFP, as described previously by Hynes et al. (2004), except that ECFP was replaced by EGFP. This construct, which was in the vector pcDNAI/Amp (Invitrogen), was linearized with XbaI, and RNA was transcribed using T7 polymerase (mMessage mMachine; Ambion). The cDNA for mouse *Gpr3* (D21062) in pHGCX was as described previously by Saeki et al. (1993) and Mehlmann et al. (2004). The cDNA for mouse *Gpr3-RFP* consisted of the monomeric form of RFP (*mRFP1*, AF506027.1; Campbell et al., 2002) fused to the COOH terminus of mouse *Gpr3* and inserted into the vector pHGCY. *mRFP1* cDNA was provided by R.Y. Tsien (University of California, San Diego, San Diego, CA). *Gpr3* and *Gpr3-RFP* cDNAs were linearized with PacI, and RNA was transcribed using T7 polymerase. pSP64-Xβm, which is a plasmid containing *Xenopus laevis β-globin* cDNA, was obtained from D.A. Melton (Harvard University, Cambridge, MA) and linearized with PstI; RNA was transcribed using SP6 polymerase. The cDNA for the rat β_2 adrenergic receptor (*Adrb2*) in pBluescript SK+ was obtained from N. Ancellin (GlaxoSmithKline, Les Ulis, France) and linearized with Not1; RNA was transcribed using T7 polymerase. The YFP membrane marker pEYFP-Mem (BD Biosciences) was subcloned into pBluescript II SK+ and linearized with SapI; RNA was transcribed using T7 polymerase.

Except where indicated, an ~200-bp poly(A) tail was added to RNAs by incubation with *Escherichia coli* poly(A) polymerase I (Ambion; Aida et al., 2001) followed by LiCl precipitation and resuspension in nuclease-free water. RNA concentrations were estimated by a comparison with RNA standards (Ambion) that were run on an agarose gel. Except where indicated, the $G\alpha_s$GFP RNA concentration was adjusted to ~1 μg/μl such that a 15-pl injection introduced 15 pg/oocyte. YFP-Mem RNA was used at ~1 pg/oocyte. Amounts of other RNAs are indicated in the text. YFP protein was made in bacteria by using EYFP (BD Biosciences) subcloned into pRSETA (Invitrogen), and the protein was purified on a Ni–nitrilotriacetic acid column (QIAGEN) and a HiTrap Q HP column (GE Healthcare), concentrated using a 10,0000 MWCO centrifugal filter device (Amicon Ultra-4; Millipore), and used at a concentration of 4 μM in the oocyte.

Imaging of live follicles and oocytes

Follicles were imaged while held on a Millicell membrane over a coverslip-bottomed dish (see Isolation and culture...oocytes). Isolated oocytes were imaged while held in 5-μl droplets under light mineral oil on a coverslip-bottomed dish. The dishes were placed within a covered microscope stage (Carl Zeiss MicroImaging, Inc.) maintained at 37°C; water-saturated 5% CO_2 in air was passed through the chamber. The follicles and oocytes were imaged by using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.), with the pinhole set for an optical section thickness of 5 μm, and a 40× NA 0.8 water immersion objective (Achroplan; Carl Zeiss MicroImaging, Inc.). GFP fluorescence was excited with the 488-nm line of an argon laser and was detected by using a 500–550-nm emission filter. YFP fluorescence was excited with the 514-nm line of an argon laser and was detected by using a longpass 530-nm emission filter. RFP fluorescence was excited with the 543-nm line of a HeNe laser and was detected by using a longpass 560-nm emission filter.

Plasma membrane and cytoplasm intensities were measured by using MetaMorph software (Fig. S1 and supplemental Materials and methods). For both GFP and YFP images, controls using uninjected oocytes within preantral follicles showed no detectable difference in intensity when comparing the oocyte and surrounding follicle cells. Therefore, intensity measurements in follicle-enclosed oocytes expressing GFP or YFP were referenced with respect to the baseline fluorescence intensity in the surrounding follicle cells. Controls using uninjected oocytes that had been isolated from their follicles sometimes showed slightly higher autofluorescence than the surrounding medium (depending on the detector gain, a mean of zero to two units on a scale of 0–255). For those cases in which the oocyte autofluorescence was greater than one unit, it was subtracted from the fluorescence intensity values before calculating the plasma membrane-to-cytoplasm fluorescence ratio; figures are shown without background subtraction.

Statistics

Tests of statistical significance were performed by using Instat (GraphPad Software). For Table II, the SEM resulting from the division of one mean ± SEM divided by another mean ± SEM was calculated by using an equation for error propagation (http://www.rit.edu/~uphysics/uncertainties/Uncertaintiespart2.html).

Online supplemental material

Video 1 shows the microinjection of a preantral follicle-enclosed mouse oocyte. The supplemental Materials and methods section describes the use of MetaMorph software to measure plasma membrane and cytoplasm intensities (Fig. S1) as well as methods for RT-PCR and cAMP measurements. The supplemental Results section shows that $G\alpha_s$GFP expression does not prevent the spontaneous resumption of meiosis in oocytes that were isolated from antral follicles, that $G\alpha_s$GFP moves from the oocyte plasma membrane to the cytoplasm when an exogenously expressed β-adrenergic receptor is stimulated with isoproterenol (Fig. S2), and that the amount of GPR3 protein expression correlates with the amount of $G\alpha_s$GFP internalization (Fig. S3). Fig. S1 shows the quantitation of plasma membrane-to-cytoplasm fluorescence ratios. Fig. S2 shows the distribution of $G\alpha_s$GFP in an oocyte coexpressing the β_2-adrenergic receptor before and 30 min after exposure to 10 μM isoproterenol. Fig. S3 shows images of preantral follicle-enclosed *Gpr3*$^{-/-}$ oocytes coexpressing GPR3-RFP and $G\alpha_s$GFP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506194/DC1.

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