Decay-accelerating factor in the periovulatory rat ovary

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Abstract

One of the most prominent inflammatory reactions is the activation of the complement system. The activated complement system does not distinguish between pathogens and the host cell. In order to prevent autologous complement-mediated attack, host cells express a variety of both membrane-bound and fluid-phase complement regulatory proteins which control activity of the complement cascade by acting on convertase enzymes or the membrane-attack complex. Although the process of ovulation is facilitated by the inflammatory reaction, this reaction has the potential to cause serious damage to growing follicles, ovulated follicles, and other important ovarian tissues. This study was undertaken to characterize the expression and regulation of decay-accelerating factor (DAF), a complement regulator, as a potential mediator of ovarian tissue protection from ovulatory inflammation. DNA microarray and Northern blot analyses showed that an ovulatory gonadotropin stimulus dramatically yet

transiently induced DAF mRNA expression in the immature rat ovary. Northern blot and PCR analyses revealed that of the three known DAF isoforms, glycosylphosphatidylinositol (GPI)-, soluble-, and transmembrane-(TM) DAF, GPI-DAF was the predominant form. *In situ* hybridization localized GPI-DAF mRNA expression in the theca-interstitial cells of the periovulatory ovary. Neither the anti-progestin RU486 nor the cyclooxygenase inhibitor indomethacin significantly inhibited human chorionic gonadotropin (hCG)-induced GPI-DAF mRNA expression *in vivo*. *In vitro* theca cell culture studies indicated that hCG induces GPI-DAF mRNA expression through the protein kinase A pathway. This study suggests that gonadotropin-induced GPI-DAF may be involved in the protection of ovarian tissues from the potential attack by the complement system activated by the inflammatory response associated with ovulation.

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Introduction

Complement, a central element of innate immunity, initiates and coordinates immediate immune reactions which protect the body from microbes, foreign particles, and altered self cells. It consists of a series of plasma proteins that when activated directly or indirectly destroy invading organisms and mediate humoral and cellular interactions of the immune response, including chemotaxis, phagocytosis, cell adhesion, and B cell differentiation (Walport 2001). The complement system can be activated via three major pathways: the classical pathway, the alternative pathway, and the lectin pathway. The classical pathway is stimulated by antibody–antigen complexes, the alternative pathway by spontaneous hydrolysis of native complement factor C3, and the lectin pathway by mannose-binding lectins which recognize microbial saccharides (Nauta *et al.* 2004). Although the preliminary steps of each pathway differ, all three generate a C3 convertase enzyme, which

cleaves C3 into C3a and C3b. The C3b fragment generates the C5 convertase enzyme, allowing subsequent formation of the membrane-attack complex (MAC, C5b-9), which inserts itself into cell membranes causing damage by cytolytic mechanisms (Cole & Morgan 2003). Products produced during the complement cascade, such as the anaphylatoxins C5a and C3a, are capable of binding to receptors on mast cells, inducing them to release histamines and prostaglandins (PGs), and can serve as chemoattractants for neutrophils, eosinophils, and leukocytes (Kuby 1997, Nauta *et al.* 2004). Besides causing cell lysis, C5b-9 can promote production and release of cytokines, PGs, leukotrienes, and reactive oxygen species (Nauta *et al.* 2004).

As the complement system is non-selective, unable to distinguish between pathogen and host cell, host tissues have constructed a defense mechanism consisting of both membrane-bound and fluid-phase complement regulatory proteins, which check the activity of the complement

cascade at the level of convertase enzymes and MAC (Liszewski *et al.* 1996, Hourcade *et al.* 2000). Complement regulators include membrane cofactor protein (CD46), membrane inhibitor of reactive lysis (CD59), and decay-accelerating factor (DAF, CD55). DAF and CD59 are detected in various tissues, while CD46 is detected predominantly in testes (Kumar *et al.* 1993, Mead *et al.* 1999). In the human ovary, CD46 and DAF protein are found in granulosa cells of primordial follicles, and in both granulosa and theca cells of developing follicles, while CD59 is only localized to granulosa and theca cells of developing follicles (Oglesby *et al.* 1996). In the rat, DAF protein was reported to be present in the endothelium of the ovary (Spiller *et al.* 1999). DAF provides the first line of defense against complement-mediated damage to host cells by accelerating the decay of both the classical and alternative pathway C3 and C5 convertases. In primates and rodents, several protein isoforms of DAF have been identified. The predominant form of the protein is the glycosylphosphatidylinositol-anchored form (GPI-DAF), but alternative splicing produces a transmembrane-domain containing form (TM-DAF) and a secreted form (soluble-DAF) (Hinchliffe *et al.* 1998, Miwa *et al.* 2000). When the complement attack overcomes this DAF-mediated defense, it is vital that cells possess a second line of defense in order to minimize the damaging effects of the MAC. CD59, another GPI-anchored protein, prevents MAC assembly by binding to component C8 and/or C9, preventing lytic pore formation (Miwa & Song 2001).

Inflammation has been postulated to be a key component of ovulation (Espey 1980, Ness *et al.* 2000), whereby the oocyte is released from the interior of the follicle (Brannstrom & Enskog 2002). For example, there is a rapid increase in the leukocyte population (Brannstrom & Enskog 2002), upregulation of pro-inflammatory gene expression (Richards *et al.* 2002), an increase in inflammatory cytokines (Norman & Brannstrom 1996), and increased proteolytic activities in the periovulatory ovary (Curry & Osteen 2003), all of which have been regarded as an indication of the existence of an acute inflammatory reaction at the time of ovulation (Espey 1980). In addition, numerous reports have shown that treatment with anti-inflammatory agents decreases the ovulation rate (Brannstrom & Enskog 2002), further supporting the idea that inflammation is important for successful ovulation. Complement components have been documented in the reproductive tract during the menstrual cycle. Indeed, there is evidence that active complement is necessary for ovulation to proceed normally. Females suffering from the autosomal dominant disorder hereditary angioedema, in which complement function deteriorates, often display polycystic or multifollicular ovaries (Perricone *et al.* 1992). While the inflammation associated with ovulation facilitates follicular rupture, it could also damage nearby ovarian tissue by its own proteolytic and cytolytic nature. Thus, we hypothesized that complement regulators should be

upregulated during the periovulatory period in order to protect the ovarian tissues from attack of activated complement at the time of ovulation. In this study, we have characterized the ovarian expression of a critical component of this protective system, namely DAF, in association with factors involved in ovulation.

Materials and Methods

Materials

Pregnant mare's serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), RU486, and indomethacin were purchased from Sigma. Cell strainers of 40 and 70 µm were supplied by Becton Dickinson Falcon (Billerica, MA, USA). Restriction enzymes, RNaseOUT, and MLV reverse transcriptase were purchased from Invitrogen. [α -³⁵S]UTP was provided by Milan Panic Biomedicals (Irvine, CA, USA). [α -³²P]UTP was purchased from Amersham. Media for cell culture was obtained from Gibco.

Animals and treatments

Animal procedures were carried out in accordance with the University of Kentucky Animal Care and Use Committee. Twenty-day-old female Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Harlan, IN, USA). The animals were kept in a 14 h light:10 h darkness cycle and given water and rat chow. Using the gonadotropin-primed superovulation model, 21-day-old rats were injected with 10 IU PMSG, then 48 h later, injected with 10 IU hCG. For DNA microarray analysis in construction of the Rat Ovarian Gene Expression Database (rOGED, see below), tissue samples were collected at PMSG 0, 12 and 48 h and hCG 6 and 12 h; five animals were used per time point. For total RNA extraction, one ovary from each of four animals was collected at each time point. The remaining six ovaries were used for granulosa cell isolation and residual ovarian cell collection (Ko *et al.* 1999). As it is nearly impossible to isolate exclusively pure populations of a specific ovarian cell type in a short amount of time, there may exist minor crossover of cell types in the granulosa or residual ovarian cell samples. Uteri and oviducts from both ovaries of each animal were also collected and used for microarray analysis. For *in situ* hybridization, ovaries with intact oviducts were harvested at PMSG 48 h and hCG 6, 12 and 24 h, then stored at -80 °C for later sectioning. In order to study the effect of progesterone (P4) and PGs on GPI-DAF expression, animals were primed with PMSG, followed by injection of RU486 (2 mg/kg body weight) at 47 h post-PMSG and injection of hCG at 48 h post-PMSG. Indomethacin (1 mg/kg body weight) was administered 3 h after hCG injection. Rats were killed at PMSG

48 h, hCG 6 and 24 h. Oviducts of rats killed at hCG 24 h were examined and the number of oocytes counted to validate efficacy of treatments. One animal was used for each time point ($n=3$). One ovary from hCG 6 h, hCG 6 h+RU486 and hCG 6 h+indomethacin was also frozen at -80°C and later used for *in situ* hybridization.

rOGED

The expression profile of GPI-DAF was identified using rOGED (<http://web5.mccs.uky.edu/kolab/rogedendo.aspx>), which was previously developed in our laboratory. The procedures used are described in detail elsewhere (Jo *et al.* 2004). The database provides the gene expression profiles of all genes and expressed sequence tags included in the Affymetrix Rat Expression 230A and 230B Gene-Chips. The microarray data is displayed in graphical format, showing the expression pattern over a time course of PMSG 0, 12 and 48 h and hCG 6 and 12 h for whole ovary, granulosa cells, and residual (non-granulosa) cells.

Northern analysis

Northern analysis was performed to determine the temporal expression of the GPI-DAF transcript, the isoform-specific expression of DAF mRNA, and the possible regulation of GPI-DAF by the P4 and PG pathway. Total RNA was extracted from the whole ovary, granulosa cells, or residual tissue using Trizol reagent according to the manufacturer's protocol, then quantified by spectrophotometry. Five to ten micrograms of total RNA were separated on a 1% MOPS-formaldehyde agarose gel. The RNA was transferred to a nylon membrane (Schleicher & Schuell Inc., Keene, NH, USA) and fixed to the membrane by UV cross-linking (UV crosslinker, UVP CL-1000; VWR, West Chester, PA, USA). The blot was briefly washed in water, then air dried to remove residual $20\times$ SSC. Plasmids containing partial cDNA for the gene of interest were linearized with appropriate restriction enzymes. Antisense RNA probe was made by *in vitro* transcription using SP6 or T7 polymerase (Invitrogen) and [α - ^{32}P]UTP (10 mCi/ml). The probe was purified with mini Quick Spin RNA columns (Roche). The membrane was prehybridized in UltraHyb hybridization buffer (Ambion Inc., Austin, TX, USA) for 30 min at 68°C , then probe was added to a concentration of 10^6 c.p.m./ml and hybridized at 68°C for 12–16 h. Blots were washed twice (5 min each) with shaking in $2\times$ SSC, 0.1% SDS at room temperature, then washed at high stringency ($0.1\times$ SSC, 0.1% SDS) during two 15 min washes at 68°C . The membrane was exposed to film (Kodak Biomax XAR) for 48 h at -80°C .

Theca cell isolation and culture

Ovaries were harvested from immature rats primed with PMSG for 48 h. Granulosa cells were removed from the

ovaries as described previously (Ko *et al.* 1999). Briefly, the ovaries were incubated in preincubation media for 20 min at 37°C to allow dissociation of granulosa cells, washed with cold serum-free 4F media (15 mM Hepes (pH 7.4), 50% DMEM and 50% Ham's F12 with bovine transferrin (5 $\mu\text{g}/\text{ml}$), human insulin (2 mg/ml), hydrocortisone (40 ng/ml) and antibiotics) three times, and granulosa cells were removed by follicular puncture. Theca cells were then isolated from the residual ovarian tissue by the discontinuous density gradient centrifugation method as described by Magoffin & Erickson (1988). Cells were plated at a concentration of 200 000/well in McCoy's media containing $1\times$ penicillin–streptomycin and $1\times$ L-glutamine and incubated in a humidified atmosphere of 5% CO_2 at 37°C . Cells were treated immediately after plating to achieve optimal luteinizing hormone (LH) receptor (LH-R) responsiveness. Treatments were as follows: hCG (0.1 IU/ml), cycloheximide (CHX, 10 $\mu\text{g}/\text{ml}$), forskolin (protein kinase A (PKA) pathway activator, 10 μM) and phorbol 12-myristate 13-acetate (PMA, PKC pathway activator, 20 nM). Cells were incubated for 6 h after treatment, then harvested and RNA isolated using Trizol reagent. The effectiveness of 10 $\mu\text{g}/\text{ml}$ CHX in blocking protein translation was demonstrated with a [^{35}S]methionine incorporation assay. Briefly, cells were treated with CHX or vehicle (ethanol), then cultured with media containing [^{35}S]methionine for 6 h. Cells were washed with PBS, precipitated with trichloroacetic acid, and activity measured in a scintillation counter.

RT-PCR

For the study of regulatory factors in GPI-DAF expression, RT-PCR was performed on total RNA extracted from theca cell cultures by the Trizol method. Total RNA (0.5–1 μg) was reverse transcribed using 250 ng random hexamer and 200 U MLV reverse transcriptase as routinely performed in our laboratory (Ko *et al.* 2003). PCR amplification was done at 20 and 25 cycles on an Eppendorf Mastercycler. The PCR products were separated on a 1.5% agarose gel, stained with SYBR Green I (Sigma) for 20 min, and scanned on a phosphorimager (FujiFilm FLA-5000). The identity of the RT-PCR bands was confirmed by Southern blotting using [α - ^{32}P]UTP-labeled GPI-DAF riboprobe. The detailed procedure is described elsewhere (Ko & Park-Sarge 2000). Primers for GPI-DAF were 5'-cta atg cca cgc caa act cg-3' and 5'-cag ctt gta ccc ttt gtc gc-3', numbered as 131–150 and 572–591 in the sequence AB026903; for TM-DAF were 5'-gcc tta ggg act act ata gg-3' and 5'-gcc gtc atc taa ttc aca gg-3', numbered as 2557–2576 and 2830–2849 in the sequence AB032395; for soluble-DAF were 5'-gcc aat cag tca ggt agc ac-3' and 5'-ccc tta cca ttt cgt tca gg-3', numbered as 1350–1369 and 1711–1730 in the sequence AF039584; and for ribosomal protein L19 were 5'-ggc

tac aga aga ggc ttg cc-3' and 5'-cat atg cct gcc ctt ccg-3'. Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA).

In situ hybridization

Frozen ovaries were sectioned at 10 µm on a Bright OTF cryostat and mounted onto Superfrost/Plus Microscope slides (VWR, West Chester, PA, USA). Sections were fixed, pretreated, and hybridized with antisense RNA probe as described previously (Ko *et al.* 2003). Plasmids containing cDNA for the gene of interest in the pCRII-TOPO vector (Invitrogen) were linearized with the appropriate restriction enzyme, then [α -³⁵S]UTP-labeled RNA probes were synthesized using T7 or Sp6 polymerase. RNA probe (2×10^7 c.p.m./ml) in hybridization buffer was applied to sections and incubated at 42 °C in a humidity chamber for 15–18 h. Slides were washed and dehydrated as previously described (Ko *et al.* 2003). The slides were exposed to Kodak Biomax XAR film for 4 days and dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA) for autoradiography. The slides were exposed for 4–6 weeks at 4 °C. After developing with Kodak D19, slides were stained with Gill's No. 1 hematoxylin (Electron Microscopy Sciences, Fort Washington, PA, USA) and 1 mg/ml eosin (Sigma). The signal was visualized on an Olympus CKX41 microscope.

Statistics

For the *in vitro* theca cell culture experiment, the effect of treatments on GPI-DAF expression was analyzed by one-way ANOVA or Student's *t*-test. GPI-DAF expression in non-treated, hCG- and hCG+CHX-treated cells or non-treated, forskolin- and forskolin+CHX-treated cells was assessed by one-way ANOVA using the University of Kentucky StatServer (<http://sta.kbrin.uky.edu>). The Student's *t*-test was performed for the time course GPI-DAF expression using Microsoft Excel. *P* values of less than 0.05 were regarded as significant.

Results

Identification of gonadotropin-stimulated DAF mRNA expression in the periovulatory ovary

Recently, we have generated rOGED, which provides genome-wide temporal mRNA expression profiles in the intact ovary, granulosa cell, and residual ovarian tissue (ovarian cell types excluding granulosa cell) simultaneously (Jo *et al.* 2004) (<http://web5.mccs.uky.edu/kolab/rogedendo.aspx>). The rOGED profile revealed that hCG dramatically, yet transiently, induced GPI-DAF mRNA expression in the ovary (Fig. 1A). GPI-DAF mRNA expression was increased 6-fold by 6 h post-hCG

administration in the residual tissue, while the expression was barely detectable in the granulosa cells. Northern blot analysis showed a spatio-temporal GPI-DAF mRNA expression pattern identical to that of the rOGED profile (Fig. 1B).

Isoforms of DAF mRNA

The Northern analysis detected 4.3, 2.9, and 1.6 kb transcript bands (Fig. 1B). To determine whether each of the three transcripts represents a different isoform of DAF mRNAs, three identical RNA blots were hybridized with GPI-, TM-, and soluble-form specific probes respectively. The GPI-form specific probe detected all three of the bands, while no band was detected by the TM- or soluble-form specific probe (Fig. 2). This result was confirmed by a subsequent RT-PCR assay, in which simultaneous amplification of GPI-DAF and TM-DAF or GPI-DAF and soluble-DAF in the same PCR tube resulted in predominant amplification of GPI-DAF over TM-DAF (Fig. 3) and soluble-DAF (not shown).

Spatio-temporal DAF mRNA expression in the ovary and oviduct

To localize the *in vivo* DAF mRNA expression, *in situ* hybridization was performed using tissue sections of gonadotropin-primed immature rat ovaries with attached oviducts. The strongest GPI-DAF mRNA signal was detected in the theca-interstitial cells of ovaries collected 6 h after hCG (Fig. 4). As was predicted from the Northern blot analysis, mRNA signal was detected only by the GPI-DAF probe (Fig. 4); no detected signal was observed in the tissue sections hybridized with TM- and soluble-DAF probes (not shown). The expression decreased to basal level by 24 h after hCG (Fig. 4A). No prominent GPI-DAF mRNA signal was seen in the granulosa cell layer (Fig. 4B); however, strong GPI-DAF mRNA signal was detected in the luminal epithelium of the oviduct (Fig. 4C).

P4 and PG do not induce DAF expression

PG and P4 are critically involved in ovulation (Richards *et al.* 1998). To test whether these hormones have a regulatory role in GPI-DAF expression in the ovary, we measured GPI-DAF mRNA expression levels in the RU486- or indomethacin-treated immature rat ovary. Ovaries were collected at 6 and 24 h post-hCG administration and were examined for GPI-DAF mRNA expression (Fig. 5). The effect of RU486 and indomethacin treatment on the ovulatory process was validated by checking for the presence of oocytes in the oviduct of 24 h post-hCG animals. Twenty-five to thirty oocytes were retrieved from each oviduct of the control group (hCG only), while approximately half that number were seen in

A. DAF rOGED profile **B. Northern blot**

Figure 1 mRNA expression profile of GPI-DAF. (A) Expression of GPI-DAF was determined using the Rat Ovarian Gene Expression Database (rOGED), which contains DNA microarray analysis from the Affymetrix Rat Expression 230A and 230B gene chips. Immature rats were treated with 10 IU PMSG, followed by 10 IU hCG 48 h later. Tissue samples were collected at PMSG 0, 12 and 48 h and hCG 6 and 12 h. Five animals were used per time point; four ovaries (from each of four individual animals) were used for total RNA extraction, while the remaining six ovaries were used for granulosa cell isolation and residual ovarian cell collection. The experiment was repeated for whole ovary and granulosa cells. s.d. error bars are shown for whole ovary. (B) Autoradiograph for Northern blot analysis shows GPI-DAF mRNA expression increases at hCG 6 and 12 h, specifically in residual tissue. RNA from whole ovary, granulosa cells and residual tissue was collected at PMSG 0, 12 and 48 h and hCG 6 and 12 h as above. Five micrograms of RNA were separated by electrophoresis, then blots were prepared and probed with antisense GPI-DAF probe. The 28S RNA band demonstrates equal loading of lanes.

Figure 2 Characterization of isoform expression for DAF. Northern analysis revealed the existence of three transcripts for GPI-DAF. Three isoforms of DAF exist in the rat: a glycosylphosphatidylinositol (GPI), transmembrane (TM) and soluble (Sol) form. In order to determine whether the TM or soluble form are also expressed in the periovulatory ovary, Northern analysis was performed using the GPI-DAF probe, and probes specific for TM-DAF and soluble-DAF respectively. No signal was detected for TM-DAF or soluble-DAF in the ovary of PMSG 48 h, hCG 6 or 24 h primed rats by Northern blot analysis. Reverse agarose gel image of 28S RNA band shows equal amounts of RNA for each sample.

oviducts of indomethacin-treated rats, and no oocytes were seen in those of RU486-treated animals. Northern blot (Fig. 5B) and *in situ* hybridization (Fig. 5C) analyses showed that there was no marked reduction of GPI-DAF mRNA expression in either RU486- or indomethacin-treated animals.

Regulatory factors on DAF expression

To determine which signaling pathways are involved in the hCG-induced GPI-DAF mRNA expression in theca cells, we analyzed thecal cell responsiveness to hCG treatment *in vitro* using cells isolated from PMSG 48 h primed immature rats. First, we measured the GPI-DAF mRNA transcript in theca cells which had not been treated over a time period of 72 h. Immediately after isolation, the theca cells displayed a basal level of GPI-DAF mRNA expression. Interestingly, however, significant induction of GPI-DAF mRNA was detected within 6 h of culture, after which it decreased considerably but increased again by 24 h (Fig. 6A). Due to the lengthy cell isolation procedure (4 h), and the fact that the LH-R expression peaks at PMSG 48 h, cells were treated immediately after plating to avoid losing LH-R activity. Although the expression level was slightly increased by the hCG treatment (Fig. 6B), the level of increase was substantially lower than was observed *in vivo*. When cultured in the presence of the PKA pathway activator forskolin or the PKC activator PMA for 6 h, no significant increase was observed (Fig. 6B). To ascertain whether or not intermediary protein synthesis was necessary for hCG or the PKA pathway to upregulate DAF expression, cells were treated with hCG or forskolin in the presence

Figure 3 Expression of GPI-DAF vs TM-DAF in PMSG 48 h, and hCG 6 h ovary. (A) RT-PCR comparing relative expression levels of GPI-DAF and TM-DAF in the PMSG 48 h and hCG 6 h ovary. Primers for GPI-DAF or TM-DAF were used to amplify cDNA for 20, 25, 30 and 35 cycles. In the hCG 6 h ovary, TM-DAF PCR product is not detected until 30 cycles, while GPI-DAF is detectable after only 20 cycles. Thus TM-DAF transcript is present, albeit at very low levels. GPI-DAF and TM-DAF plasmids were used as template for control. (B) Graph depicting relative signal intensity of PCR products. In the hCG 6 h ovary, GPI-DAF PCR product shows approximately five times greater signal intensity than the TM-DAF PCR product.

or absence of CHX, a protein translation inhibitor. Surprisingly, addition of CHX to either forskolin or hCG treatment significantly increased the GPI-DAF mRNA

expression, while treatment with hCG or forskolin alone did not show a significant increase (Fig. 6B).

CD59 is expressed in the periovulatory ovary

We wondered if other complement regulators showed the same spatio-temporal expression as GPI-DAF. In the ovary, the complement regulator CD59 shows a different expression profile compared with GPI-DAF; while highly expressed in residual tissues from PMMSG 0 h to hCG 12 h, it sharply increases in granulosa cells after hCG 6 h (Fig. 7).

Discussion

In this study, we found that the mRNA transcript for GPI-DAF increases dramatically by 6 h following hCG treatment, and this increase in expression occurs specifically in the theca-interstitial layer of the rat ovary. The upregulation of this complement regulator may be significant as a variety of inflammatory mediators and complement factors have been detected in the periovulatory ovary, many specifically in the theca layer. In the rat, neutrophils increase 8-fold in the theca layer of ovulating follicles, while macrophages increase 5-fold (Brannstrom & Enskog 2002). The expression of several chemokines and cytokines increases in the rat ovary at hCG 6 h, including monocyte chemotactic protein-1 and -3, macrophage inflammatory proteins-1 α , -1 β , and -1 γ (Wong *et al.* 2002), and the interleukin-8 (IL-8)-like neutrophil chemoattractant CINC/gro (Ushigoe *et al.* 2000). Protein expression of both CINC/gro and its human counterpart IL-8 was localized to the theca layer (Runesson *et al.* 2000). Functionally active complement is present in follicular fluid during the preovulatory period, and it has been hypothesized that it plays a significant role in release of the oocyte. High concentrations of the anaphylatoxins C3a, C4a and C5a were found in human follicular fluid, the presence of these by-products indicating that complement in follicular fluid is partially activated (Perricone *et al.* 1990). In addition, *r*OGEED shows the

Figure 4 Confirmation of GPI-DAF mRNA expression profiles by *in situ* hybridization. Immature 21-day-old rats were primed with PMMSG (10 IU), followed by hCG (10 IU) 48 h after PMMSG injection. Ovaries with intact oviducts were collected at PMMSG 48 h and hCG 6, 12 and 24 h, sectioned, and hybridized with antisense probe for GPI-DAF. (A) GPI-DAF mRNA transcript is localized to the theca-interstitial cells (TIC), while no signal is visible in granulosa cells (GC). Expression peaks at hCG 6 h. (B) Whole ovary at hCG 6 h shows expression of GPI-DAF throughout the theca cell layer. (C) GPI-DAF in oviduct shows strong signal in the luminal epithelium (LE), but not in the smooth muscle layer (SM). Original magnification of slides $\times 10$ for (A) and (C), $\times 2$ for (B). POF, pre-ovulatory follicle.

Figure 5 GPI-DAF mRNA expression is not directly controlled by P4 or PG pathways. (A) Immature rats (21 days old) were treated with 10 IU PMSG, followed by 10 IU hCG at PMSG 48 h. One group of animals was treated with RU486 (2 mg/kg body weight) at PMSG 47 h, while another group was treated with indomethacin (1 mg/rat) at PMSG 51 h. Ovaries were harvested at both 6 and 24 h post-hCG for both RU486- and indomethacin-treated animals. Control animals were killed at PMSG 48 h, hCG 6 and 24 h. One animal was used for each time point/treatment; the experiment was performed three times. (B) Ten micrograms of total RNA were separated by electrophoresis, and blots were probed with GPI-DAF antisense probe. (C) *In situ* hybridization of sections from hCG 6 h, hCG 6 h+RU486, and hCG 6 h+indomethacin with GPI-DAF antisense probe. No marked decrease in mRNA transcript is seen between hCG-treated and RU486- or indomethacin-treated groups.

mRNA expression of several complement components, including C1qb, C3, C4, C5r1, factor h, and C1 qbp, in the ovary of the superovulated immature rat (Jo *et al.* 2004) (<http://web5.mcscs.uky.edu/kolab/rogedendo.aspx>). Thus, it is clear that many immune factors, including complement, are expressed in the periovulatory ovary, strongly indicating that the expression of GPI-DAF

Figure 6 Forskolin induces GPI-DAF mRNA expression *in vitro*. (A) Theca cells were isolated from PMSG 48 h primed 23-day-old immature rats by the discontinuous density gradient centrifugation method and cultured up to 72 h without treatment, and the basal level of GPI-DAF expression examined ($n=2$). Without treatment, GPI-DAF expression increases significantly after 6 h ($P=0.018$). 0 vs 12 h, $P=0.074$; 0 vs 18 h, $P=0.048$; 0 vs 24 h, $P=0.054$; 0 vs 72 h, $P=0.015$. The LH-R mRNA was present in theca cells cultured for 6 h, but not in cells cultured for 18 h (not shown). For subsequent experiments, cells were treated immediately after plating, despite the rise in basal level of GPI-DAF, to have optimal LH-R responsiveness. (B) Theca cells were isolated from PMSG 48 h primed 23-day-old immature rats and treated immediately with 0.1 IU/ml hCG, 10 μ M CHX, 10 μ M forskolin (PKA pathway activator), 20 nM PMA (PKC pathway activator) or hCG+CHX and forskolin+CHX. After 6 h, cells were harvested, RNA extracted and GPI-DAF mRNA transcript detected by semi-quantitative RT-PCR. Treatment with hCG+CHX or forskolin+CHX compared with non-treated cells was found to be significantly different from zero by one-way ANOVA analysis. L19 was used as an internal control. Relative expression is shown as the ratio of GPI-DAF to L19. The experiment was performed five times. NT, no treatment; FSK, forskolin. s.d. error bars are shown. Asterisks indicate a significant increase.

CD59 rOGED profile

Figure 7 mRNA expression profile of CD59 in ovary. CD59, another complement regulator, was also found to be expressed in the periovulatory ovary using the rOGED database (see Fig. 1 legend). Expression is consistently high in residual tissue, and increases dramatically in granulosa cells by hCG 12 h. S.D. error bars are shown for ovary.

and CD59 at this time may be serving to keep these complement components in check.

Of the three isoforms of DAF which exist in the rat, GPI-DAF was found to be the predominant form expressed in the ovary. GPI-DAF is the major form expressed in most tissues of the rat, including lung, spleen and kidney (Miwa *et al.* 2000). The only tissue found thus far to express high levels of TM-DAF mRNA is the testis (Miwa *et al.* 2000, 2001). It has been speculated that since mature sperm cannot synthesize new protein, the more stable TM protein may be needed to provide protection (Miwa *et al.* 2001). The ovary, like most tissues, being able to make new proteins, would not need to express TM-DAF preferentially over GPI-DAF.

We sought to discover which factors are involved in the regulation of GPI-DAF mRNA expression in the periovulatory rat ovary. Since it was apparent that hCG induces GPI-DAF mRNA expression *in vivo*, we first investigated pathways stimulated by hCG. Perhaps the most well-known function of hCG is to activate the P4 and PG pathways, which are critical for successful ovulation (Richards *et al.* 1998). The anti-P4 compound RU486 and PG synthesis inhibitor indomethacin both exert anti-ovulatory effects (Richards *et al.* 1998), and null mutants for P4 receptor (PR) or the cyclooxygenase-2 gene are infertile due to anovulatory syndromes (Richards *et al.* 2002). PR is transcribed in granulosa cells of pre-ovulatory follicles in response to LH; as a nuclear receptor transcription factor, it mediates the action of P4 by controlling the transcription of specific genes (Robker *et al.* 2000). PG may also activate genes critical to

ovulation by binding to its membrane receptor (Richards *et al.* 1998). The administration of RU486 and indomethacin *in vivo* did not block GPI-DAF mRNA expression, indicating that GPI-DAF is not one of the genes under regulation of PR or PGs. Therefore, GPI-DAF may be activated directly by hCG. Interestingly, while we have seen only a minor increase of GPI-DAF mRNA expression by PMSG (Fig. 1), GPI-DAF transcript showed an 11-fold increase in immortalized rat granulosa cells (Sasson *et al.* 2003). This dramatic increase may be in part due to the artificial state of the cell line, which expresses follicle-stimulating hormone receptors at 20-fold higher rates than primary cells.

To see whether or not GPI-DAF is under the direct regulation of a protein kinase cascade, we studied the effect of several factors on GPI-DAF mRNA expression in theca cells *in vitro*. We demonstrated that the expression of GPI-DAF in theca cells *in vitro* is induced by forskolin, which activates the PKA pathway (Fig. 6B). Interestingly, cotreatment of cells with either CHX+hCG or CHX+forskolin induced an even more dramatic increase in GPI-DAF than hCG or forskolin alone. This increased expression could be due to increased mRNA stability sometimes afforded by CHX or may indicate that synthesis of a new protein actually serves to inhibit induction of GPI-DAF. It was also noticeable that a significant increase in GPI-DAF expression was detected in theca cell culture without any treatment in the 6 h after isolation (Fig. 6A). Whether this is due to the stress of the cell isolation procedure or removal from its *in vivo* conditions, this rise may mask effects of treatment when assayed at 6 h. This result, along with the fact that inhibitors of P4 and PG did not suppress GPI-DAF induction, would indicate that hCG effects transcription of GPI-DAF via activation of the PKA cascade. This does not rule out activation of protein kinase by other receptors, such as cytokine receptors. In murine endothelial cells, DAF is induced by tumor necrosis factor- α , and this induction is PKC-dependent (Ahmad *et al.* 2003). However, we did not observe induction of GPI-DAF after treatment with several cytokines, nor with a PKC agonist (data not shown). The LH surge *in vivo* and the subsequent changes in the physical and biochemical environment surrounding the theca cells, such as the rapid increase in follicular size, angiogenesis, influx of leukocytes, and loosening of the extracellular structure by proteinases, may contribute to the fine regulation of GPI-DAF expression *in vivo*.

Coupled with previous reports that complement regulators are detected in the ovary of human and other species, our findings strongly indicate that DAF may play a crucial role in preventing damage that would otherwise be induced by activation of the complement cascade, supporting the reports of a correlation between deteriorating complement function and cystic or multifollicular ovaries in women with hereditary angioedema (Perricone *et al.* 2000). We hypothesize that a lack of functional DAF or

other complement regulators could contribute to inappropriate damage to ovarian tissue. After hundreds of ovulatory cycles, a lack of protection could lead to accumulated damage in the older female, which might cause destruction of small follicles, a decrease in follicular reserve, and possibly premature menopause. Two independent groups have generated a DAF knockout mouse (Sun *et al.* 1999, Lin *et al.* 2001); however, the phenotype of the ovary has not been reported. The DAF knockout mice are fertile (Sun *et al.* 1999), yet to the best of our knowledge, no long-term fertility assays have been performed. We plan to perform a long-term fertility assay to test our hypothesis that complement regulators prevent accumulated damage in aging females. We will take into account the fact that mice, unlike any other mammal, have two genes for DAF, and the DAF knockout mice are null only for *Daf1*. Most of the GPI-DAF product is produced from *Daf1*, but *Daf2* is also capable of producing GPI-DAF (Harris *et al.* 1999).

The presence of complement in the ovary during the periovulatory period requires a delicate balance. Complement may contribute and even be necessary for successful ovulation, yet at the same time the amplifying nature of the cascade must be controlled to prevent excess inflammation and subsequent tissue damage. We believe that the presence of active inflammatory and complement factors before ovulation requires a regulatory system to protect the healthy tissue.

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