0013-7227/01/$03.00/0
Printed in U.S.A.

Endocrinology 142(9):4076–4085

Characterization of Prohibitin in a Newly Established Rat Ovarian Granulosa Cell Line

WINSTON E. THOMPSON, ALICIA BRANCH, JOSEPH A. WHITTAKER, DEBORAH LYN, MOSHER ZILBERSTEIN, KELLY E. MAYO, AND KELWYN THOMAS

Department of Obstetrics and Gynecology (W.E.T., A.B.), Department of Anatomy and Neurobiology (J.A.W., K.T.), Department of Biochemistry (D.L.), Morehouse School of Medicine, Atlanta, Georgia 30310; Department of Obstetrics and Gynecology (M.Z.), Boston University School of Medicine, Boston, Massachusetts 02118; and Department of Biochemistry (K.E.M.), Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208

Prohibitin is an evolutionary conserved protein that is associated with cellular differentiation, atresia, and luteolysis in the rat ovary. However, the specific cellular location and function of prohibitin in ovarian cells has not been clearly elucidated. To characterize the expression of prohibitin during cell proliferation, differentiation, and cell death, we have successfully established a temperature-sensitive granulosa cell line, designated RGA-1. At a permissive temperature of 33 C, RGA-1 cells proliferate, but revert to a differentiated phenotype at a nonpermissive temperature of 39 C. Significant inductions of prohibitin mRNA and protein expression were observed in the differentiated phenotype when compared with proliferating cells. Differentiated RGA-1 cells were found to express inhibin α- and β-transcripts, as well as steroidogenic acute regulatory protein and peripheral-type benzodiazepine receptor proteins in a manner reminiscent of steroidogenic functional responses observed in primary differentiated granulosa cells. Prohibitin expression correlated well with the expression of these steroidogenic proteins. At 39 C, RGA-1 cells also displayed increases in p53 protein levels, indicative of growth arrest in the nonproliferating cells. Confocal and electron microscopic examinations revealed increased prohibitin localization to the mitochondria at 39 C, along with changes in mitochondrial size and shape. These changes were accompanied by marked reductions in cytochrome c oxidase subunit II levels and in unit mitochondrial transmembrane potential. In addition, cell fractionation studies demonstrated that the prohibitin protein was mainly localized to the mitochondrial membrane. Collectively, these findings suggest a role for prohibitin in mitochondrial structure and function during growth and differentiation in ovarian granulosa cells. Prohibitin expression may also be indicative of mitochondrial destabilization during apoptosis-related events. (*Endocrinology* 142: 4076–4085, 2001)

CELLULAR PROLIFERATION AND differentiation are controlled by extracellular signals that modulate gene expression patterns and subsequently affect regulation of the cell cycle. The ability of a cell to regulate this process of development is of vital importance to an organism. An imbalance between proliferation and differentiation can lead either to cell death or to uncontrolled cell growth resulting in the development of cancer. In the ovary, both hormonal and nonhormonal signals contribute to the development of individual follicles by triggering sequential, dynamic changes in granulosa cell proliferation, differentiation, and gene expression (1, 2). Ovarian granulosa cells play an important physiological role in supporting the development and selection of ovarian follicles by controlling oocyte maturation and by producing the steroid hormones, estradiol and progesterone, which are critical for maintenance of the ovarian cycle (3). For these reasons, analyses of the functional anatomy of granulosa cells, during growth and terminal differentiation, are pivotal to understanding how these cells contribute to the modulation of processes critical for oocyte development.

Abbreviations: COII, Cytochrome-c oxidase subunit II; JC-1, 5,5′, 6,6′-tetra-chloro-1,1′, 3,3′-tetra-ethyl-benz-imidazolo-carbo-cyanine iodide; PBR, peripheral-type benzodiazepine receptor; PCNA, proliferating cell nuclear antigen; RP, ribosomal protein; StAR, steroidogenic acute regulatory protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; tsA, temperature-sensitive mutant.

Recently, Thompson *et al.* (4) identified and characterized a mitochondria-associated protein, known as prohibitin, in rat granulosa cells isolated from preantral and early antral follicles. During the transitional stages of ovarian follicular differentiation in rats, increased prohibitin expression was observed at various developmental stages [namely, early antral follicles, preovulatory follicles, the corpus luteum, follicles undergoing atresia, and the ovarian germ cell (oocyte)]. Additional data, derived from studies involving corpus lutea-induced luteolysis, indicate that increases in prohibitin protein expression are correlated with initial events of apoptosis (5).

A member of a family of mitochondrial membrane proteins prohibitin (6–9) was cloned from cDNAs derived from transcripts that were more abundantly expressed in nondividing than regenerating rat liver cells (10), suggesting prohibitin may negatively regulate cellular proliferation. The antiproliferative activity of prohibitin was confirmed by microinjection of the mRNA into normal human diploid fibroblast-like cells and HeLa cells, where entry into the S phase of the cell cycle was inhibited (11). The antiproliferative activity was highest during G_0/G_1 and decreased as cells approached the S phase (12). In contrast, suppression of prohibitin expression using antisense oligonucleotide facilitated increased proliferation in these cells. Similarly, the yeast homologue of prohibitin Phb1p, has been implicated in reg-

ulating the replicative life span (7), mitochondrial morphology and inheritance (8), and the turnover of membrane proteins by the m-AAA protease, a conserved ATP-dependent protease localized in the inner membrane of yeast mitochondria (9). In mammalian cells, however, the exact function and mode of prohibitin regulation are currently unknown.

The inability of rat granulosa cells to divide, in culture, limits studies of proliferation and differentiation. In addition, cell death in granulosa cells occurs asynchronously (13). In an effort to deal with these issues, synchronized granulosa cell lines that are able to proliferate and retain differentiated, tissue-specific functions have been successfully established by SV40 transformation (14–18). We have successfully established a temperature-sensitive granulosa cell line, designated RGA-1, in an attempt to characterize the expression of prohibitin during cell proliferation, differentiation, and death. These cells retain cell-specific gene expression and exhibit temperature-sensitive characteristics in growth and morphology, on shifting to the nonpermissive temperature of 39 C. We have used this RGA-1 cell line as an *in vitro* model to study the role of prohibitin during growth, differentiation, and cell death and to determine whether increased prohibitin expression is associated with observed changes in mitochondrial morphology, function, cell differentiation, and apoptosis.

Materials and Methods

Reagents

RPMI-1640 medium, Trypsin/EDTA solution, and FCS were purchased from Atlanta Biological (Norcross, GA). McCoy 5A modified medium and gentamicin solutions were purchased from Life Technologies, Inc. (Grand Island, NY). Low- and high-density lipoprotein were purchased from Sigma (St. Louis, MO). Mitotracker Red CMXRos and 5,5′, 6,6′-tetra-chloro-1,1′, 3,3′-tetra-ethyl-benz-imidazolo-carbo- cyanine iodide (JC-1) were purchased from Molecular Probes, Inc. (Eugene, OR).

Antibodies

Drs. Douglas Stocco (Texas Tech University, Lubbock, TX) and Vassili Papadopoulos (Georgetown University, Washington, DC) generously provided polyclonal antibodies to the steroidogenic acute regulatory protein (StAR) and to the peripheral-type benzodiazepine receptor (PBR), respectively. All other antibodies used were obtained from commercial sources.

Transformation of rat granulosa cells by SV-40

Temperature-sensitive cells were isolated as previously described for temperature-sensitive cell line RGA-41S (16). Essentially, primary cultures of granulosa cells were isolated from ovaries of diethylstilbestrol-treated 27-d-old immature rats. Twenty hours after plating, the cells were infected with ts255 mutant SV40, at 33 C, in serum-free McCoy 5A modified medium, for 6 months, until the first colonies appeared. The designated cell line RGA-1 was derived from the first isolated colony. This colony was picked after short treatment with trypsin/EDTA solution. Cells from this colony were transferred into a 25-cm^2 plastic flask and maintained at the permissive temperature (33 C) until confluent. The cells were than passaged, and frozen stocks were kept in medium containing 10% dimethylsulfoxide and 15% FCS. Experiments for this report were initiated by thawing cells at the 8th passage.

Culture conditions for RGA-1 cells

Cells were cultured at the permissive (33 C) and nonpermissive (39 C) temperatures, to determine whether RGA-1 cells were temperature sensitive and expressed gene products associated with granulosa cells. Cells were analyzed for their growth potential, granulosa cell specific genes, steroidogenic capacity, and expression of prohibitin protein and RNA levels during growth, differentiation, and death. RGA-1 cells were cultured in the incubation medium (RPMI-1640 containing 10 μg/ml low-density lipoprotein, 30 μg/ml high-density lipoprotein, 50 μg/ml gentamicin, and 2.5% FBS), at both temperatures, under an atmosphere consisting of 5% CO_2-95% air. To evaluate the expression of prohibitin during differentiation or death, RGA-1 cells were first cultured at 33 C until 50% confluent and then shifted to 39 C.

Western blot analysis

Fifty micrograms of RGA-1 cell protein extracts from different conditions were subjected to one- and two-dimensional gel electrophoresis. The procedures used for one- and two-dimensional gel electrophoresis have been described previously (4, 5). In brief, proteins separated by 12% SDS-PAGE were transferred to 0.2-μm nitrocellulose membranes (Sigma) using the Royal Genie electrophoretic blotter (Idea Scientific Co., Minneapolis, MN) at 350 mA for 5 h. Blots were preincubated in Tris-buffered saline containing 0.05% Tween-20 and 5% nonfat dried milk; after which, membranes were incubated overnight at 4 C with the following antibodies: mouse monoclonal antibody to prohibitin (1:2000; Neomarks, Fremont, CA), mouse monoclonal antibody to cytochrome c oxidase subunit II (COII) (1:500; Molecular Probes, Inc.), mouse anticytochrome c monoclonal antibody (1:500; PharMingen, San Diego, CA), polyclonal rabbit anti-caspase-3 (1:1000; PharMingen) monoclonal pantropic p53 antibody (1:1000; Oncogene Research Products, Cambridge, MA), polyclonal antibody to the StAR (1:2000), and polyclonal antibody to the PBR (1:1000). Membranes were incubated with the appropriate secondary antibody for 2 h at room temperature, and antibody binding was detected by chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

Northern blot analysis

Total RNA from RGA-1 cells was isolated as described by Ausubel *et al.* (19). RNA electrophoresis, transfer, probes labeling, and membrane hybridization were performed as previously described by Thompson *et al.* (5). The prohibitin cDNA probe was a generous gift from Dr. Keith McClung (Radford University, Radford, VA). Inhibin α- and β_A-probes were used as previously described by Woodruff *et al.* (20). RNA was normalized to ribosomal protein (RP)-S2 and β-actin.

Immunofluorescence confocal microscopy

The procedure used here is described, in detail, previously (4, 5). Anti-PCNA (antiproliferating cell nuclear antigen) and antiprohibitin antibodies were used at a dilution of 1:200.

Assessment of mitochondrial changes

Mitotracker Red. Cells were grown as described above and then were stained with 200 nM MitoTracker Red solution in RPMI-1640 medium at the permissive and nonpermissive temperatures for 30 min. After the incubation period, cells were rinsed three times in PBS and fixed with 3.7% paraformaldehyde for 15 min. For the reduction of aldehyde autofluorescence, coverslips were treated with 50 mM NH_4Cl in PBS for 10 min, washed three times in PBS, air-dried and mounted in Mowoil, and examined using a laser scanning confocal microscope imaging system (Olympus Corp., Melville, NY).

Transmission electron microscopy. RGA-1 cells, cultured at the permissive and nonpermissive temperatures, were fixed and processed for electron microscopy according to the method described by Anderson *et al.* (21).

JC-1 staining. In living cells, JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials or as an orange-red fluorescent J-aggregate at hyperpolarized membrane potential (22, 23). RGA-1 cells were grown on collagen-treated coverslips at 33 C, until 50% confluent, and shifted to 39 C for 24 h. Cells grown at both temperatures were stained with 10 μg/ml JC-1 solution in RPMI-1640 medium at the permissive and nonpermissive temperatures for 10 min. To dissipate plasma membrane potentials, cells were stained and maintained in high potassium buffer (137 mM KCl, 3.6 mM NaCl, 0.5 mM $MgCl_2$, 1.8 mM

$CaCl_2$, and 1× RPMI medium at pH 7.2 (22). Images were captured using a Carl Zeiss (Thornwood, NY) Axioscop microscope equipped with a cooled CCD camera.

Isolation of S-100 fractions and mitochondria

S-100 (cytosolic) fractions and mitochondria were prepared as described (24), with modifications. Briefly, permissive and nonpermissive cultured RGA-1 cells were harvested in PBS at 4 C. Cell pellets were resuspended in 5 vol buffer A [20 mM HEPES-KOH (pH7.5), 10 mM KCL, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1% aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, and 250 mM sucrose] and held at 4 C for 10 min. Cells were homogenized, and nuclei and cellular debris were removed by centrifugation at 500 × *g* for 10 min at 4 C. Mitochondria were collected from the supernatant by centrifugation at 10,000 × *g* for 15 min at 4 C, resuspended in buffer A, and held at −80 C. Cytosolic proteins were extracted by centrifugation of the mitochondrial supernatant at 100,000 × *g* for 1 h at 4 C. Nuclei isolation was performed as described by Gorski *et al.* (25). Protein levels of cellular fractions were analyzed by Western blot.

Protein assay

Total proteins were determined by a dye-binding assay (Bio-Rad Laboratories, Inc., Richmond, CA).

Data analysis

Experiments were repeated a least three times, and representative chemiluminescence and Northern autoradiograms were first scanned using a Power Macintosh computer (7600/132; Apple Computer Inc., Cupertino, CA) equipped with a ScanJet 6100C scanner (Hewlett-Packard Co., Greeley, CO). Quantification of the scanned images was performed according to the NIH Image Version 1.61 Software Program. Wilcoxon Mann-Whitney equal-variance and unequal-variance *t* tests were used to analyze changes in the levels of protein and RNA expression. Significance was considered at $P < 0.05$.

Results

Growth and differentiation of the established rat granulosa cell line

To demonstrate that RGA-1 cells were temperature-sensitive, cells were cultured at 33 C or 39 C for 8 d, harvested, and counted at the indicated time points (Fig. 1A). The RGA-1 cells divided faster and achieved a higher cell density at the permissive temperature (33 C) than at the nonpermissive temperature (39 C) (Fig. 1A). At the nonpermissive temperature, these cells displayed only limited replication, with progression toward a monolayer formation, during the first 2 d. This was followed by cessation of cell division. Growth inhibition at the nonpermissive temperature was reversible because cells shifted to the permissive temperature resumed the replication rate observed at 33 C (Fig. 1A).

As shown in Fig. 1B, RGA-1 cells exhibited marked morphological differences when grown at the permissive and nonpermissive temperatures. At 33 C, cells assumed an elongated shape and were stacked on each other, exhibiting a low cytoplasm-to-nucleus ratio. In contrast, cells were differentiated and assumed a monolayer appearance, with an increased cytoplasm-to-nucleus ratio and an appearance that resembled the primary granulosa cell cultures when grown at 39 C (Fig. 1B).

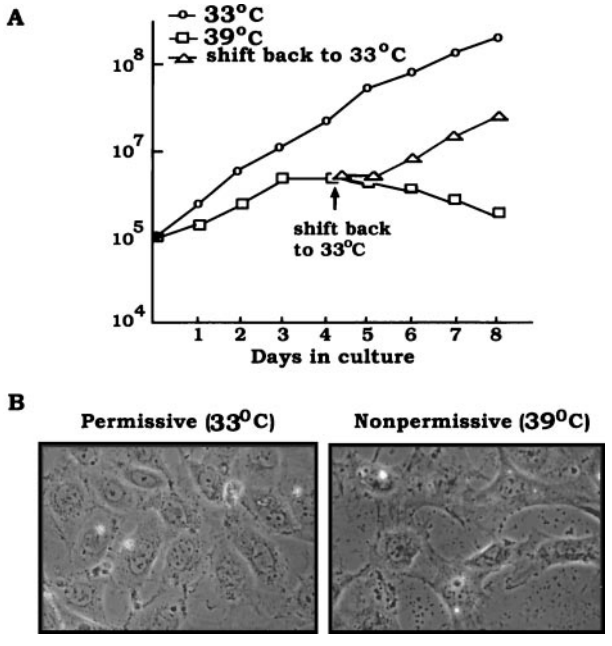

FIG. 1. Growth curve and phase contrast light microscopy images of RGA-1 cells cultured at the permissive (33 C) and nonpermissive (39 C) temperatures. Cells (10^5) were plated in RPMI 1640 medium containing 2.5% FCS and cultured at 33 C and 39 C for 8 d (A). On d 4 (*arrow*), a group of cells were shifted from 39 C to 33 C. Cells were harvested in triplicate for each time point, by trypsinization, and counted (n = 3). Cells cultured at 33 C, near confluence, appeared overlapped and binucleated (B, *left panel*). Cells cultured at 39 C appeared to change shape and be differentiated and mononucleated (B, *right panel*). Data are mean ± SD of triplicate cultures. *Bar*, 10 μm.

Expression of inhibin α and β_A, StAR, and PBR in RGA-1 granulosa cells

It has been previously shown that inhibin α and β_A are expressed in primary rat granulosa cells and in an immortalized granulosa cell line, GRM02 (20, 26). Moreover, these gene products have been shown to modulate folliculogenesis and can be regulated by agents that modulate cAMP and protein kinase C activities (27, 28). We therefore sought to examine whether RGA-1 cells were able to express inhibin α and β_A. As shown in Fig. 2A, when RGA-1 cells were cultured at 33 C (proliferating cells), both inhibin α- and β_A-transcripts were undetectable, whereas cells induced to differentiate at 39 C resulted in marked expression of both 1.5- and 5.7-kb transcripts for inhibin α and β_A, respectively. Moreover, when RGA-1 cells were stimulated with forskolin (an activator of adenylate cyclase) at the permissive temperature (33 C), for 24 h, a marked increase in the expression of inhibin α transcript was observed (Fig. 2B, lane 2), whereas the phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) had no effect on its expression (Fig. 2B, lane 3). In contrast, TPA stimulation of RGA-1 cells for 24 h resulted in elevated expression of inhibin β_A level (Fig. 2C, lane 3). No detectable expression of inhibin β_A was observed after forskolin stimulation (Fig. 2C, lane 2).

In addition to inhibin gene products, we also examined RGA-1 cell cultures for the expression of both PBR and the StAR. These gene products play key roles in cholesterol transport, leading to steroid production (29, 30). Western blot

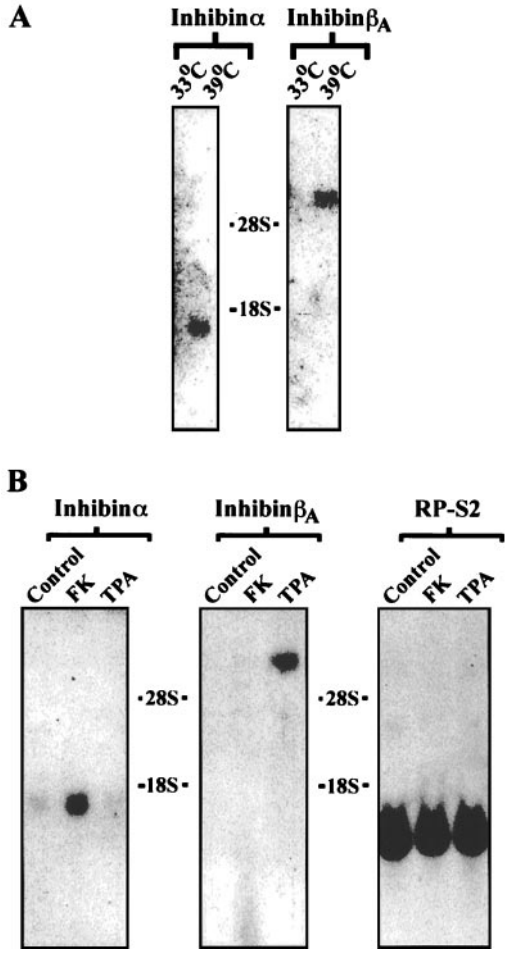

FIG. 2. Northern blot analysis of mRNA levels for inhibin α and β_A, and the effect of forskolin (FK) and phorbol ester (TPA) on these mRNAs in RGA-1 cells. Five micrograms of RNA from RGA-1 cells, cultured either at 33 C and 39 C (A) or treated with FK (10 μM) and TPA (30 nM) for 24 h at 33 C, were applied to each lane and analyzed for mRNAs for inhibin α and β_A by Northern blotting. RP-S2 was used as an internal control.

analyses, using antibodies specific to each protein, revealed that both StAR (30 kDa) and PBR (15 kDa) were constitutively expressed in RGA-1 cells cultured at both temperatures (Fig. 3A, lanes 1 and 2). Expression of StAR and PBR were lower at 33 C, compared with a 3-fold elevation of both protein levels, when these cells were grown at the nonpermissive temperature (Fig. 3B).

Expression of prohibitin in RGA-1 granulosa cells

Because prohibitin expression correlates with primary granulosa cell differentiation and steroidogenesis (4, 5), the expression of prohibitin was examined in RGA-1 cells during cellular growth and differentiation. Using Western blotting techniques, the antiprohibitin antibody recognized a 30-kDa protein expressed at both permissive and nonpermissive temperatures (Fig. 4A). Subsequent two-dimensional SDS-PAGE analysis revealed that three isoforms of this protein were expressed at the nonpermissive temperature condition (Fig. 4B). Prohibitin protein expression increased at 39 C when the cells were in a differentiated state and exhibited

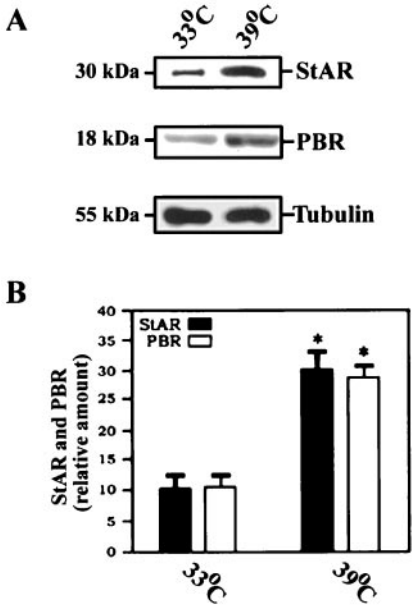

FIG. 3. Western blot analysis of protein levels for StAR and PBR in RGA-1 cells. Fifty micrograms of protein from RGA-1 cells, cultured at 33 C and 39 C, were applied to each lane and analyzed for protein levels for StAR and PBR by Western blotting (A). Representative blots (A) were scanned using the NIH Image software program computer-assisted analysis system for quantitative assessment of changes in protein levels (B). The bar graph represents the mean ± SEM of results from three replicate experiments after normalization of data against tubulin protein. *, Significant difference is at $P < 0.05$.

limited replication (Fig. 4, A and B). Densitometric analysis of prohibitin levels revealed an increase greater than 2-fold in expression at 39 C (Fig. 4D). Using a prohibitin cDNA probe, Northern blot analyses identified mRNAs encoding major and minor transcripts with molecular sizes of 1.2 and 1.9, respectively (Fig. 4C). A 1.5-fold increase in the major transcript (1.2 kb) was observed at 39 C, when compared with expression levels at 33 C (Fig. 4F). Because prohibitin is known to be associated with mitochondria; correlation between mitochondrial function and prohibitin expression were studied using a well-characterized mitochondrial gene product. Accordingly, the expression of COII was evaluated as an index of mitochondrial energy function. The 15-kDa protein COII (Fig. 4A) was found to be expressed at both temperature conditions. However, a 2-fold reduction in COII protein expression was evident under differentiated conditions (Fig. 4E). The tumor suppressor protein, p53, plays a key role in the control of cell proliferation through its induction of $p21^{WAF1/Cip1}$, which causes cells to arrest in G_o/G_1 and G_2-M, by binding to and inhibiting cyclin-dependent kinases as well as PCNA. These studies indicated that p53 protein levels were significantly increased when the RGA-1 cells were cultured at 39 C (Fig. 4A). Taken together, our studies indicate some degree of differentiation.

Localization of prohibitin to mitochondria in RGA-1 granulosa cells

To confirm and extend the analyses of prohibitin expression, indirect immunofluorescence staining, to localize the intracellular distribution of the protein, was performed in

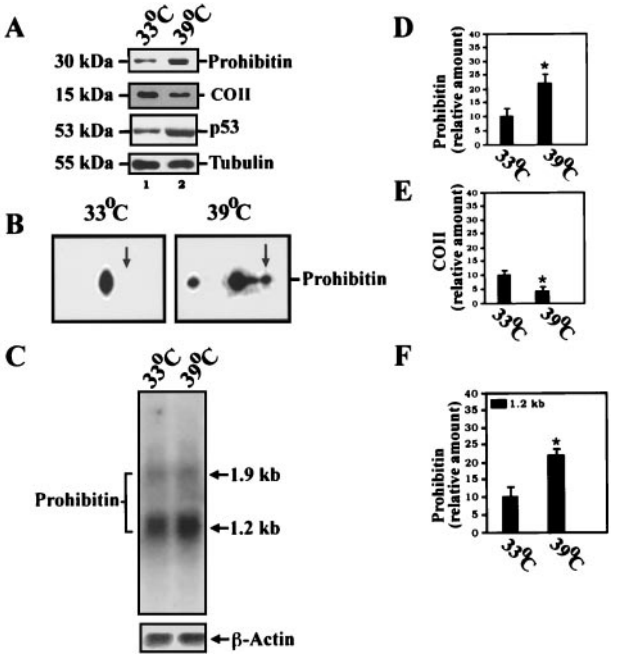

FIG. 4. Western and Northern blot analyses of protein and mRNA levels for prohibitin, COII, and p53 in RGA-1 cells. Fifty micrograms of protein and 5 μg of RNA were applied to each lane from RGA-1 cells cultured at 33 C and 39 C and subjected to Western and Northern blot analyses (A and E). Samples of protein were further focused for 16,000 volt-hours, with a mixture of pH 3–10 and pH 5–7 ampholyte and following the second dimension Western blotting procedure detected spots for prohibitin (B). The bar graphs represent the mean ± SEM of results from three replicate experiments after normalization of data for prohibitin and COII against tubulin protein and β-actin mRNA (C, D, and F). *, Significant difference is at $P < 0.05$; *arrow*, acidic prohibitin isoform.

cultured RGA-1 cells. These studies were designed to examine whether the cellular localization patterns of prohibitin correlated with the proliferation status of RGA-1 cells by using PCNA, a cofactor of DNA polymerase δ and cyclin-cdk complexes, as a marker for this process (31). PCNA is expressed mainly in proliferating cells (32) and has been shown, particularly in the rat ovary, to be a sensitive marker of granulosa cell proliferation (33). Cells grown at the permissive temperature exhibited an abundance of PCNA that was localized primarily to the nucleus (Fig. 5, B and C), whereas weak punctate cytoplasmic prohibitin immunoreactivity seemed to be associated with the mitochondria (Fig. 5, A and C) of some cells. An abundance of PCNA is indicative of cellular proliferation. Interestingly, cells that were undergoing cytokinesis at the permissive temperature showed an increase in prohibitin immunoreactivity when compared with other cell populations (Fig. 5B, *arrow*). When the cells were cultured at the nonpermissive temperature for 48 h, an intense mitochondrial immunoreactive staining was observed both in the perinuclear region and in cell processes (Fig. 5, D and F). Patches of fluorescence could be observed over some nuclei; but at higher focal planes, this could be distinctly identified as mitochondria. A higher magnification image (presented in Fig. 5, G–I) allows better visualization of prohibitin. An inverse PCNA immunoreactivity was observed with prohibitin expression when these cells were differentiated (Fig. 5, E and F, H and I). There was no detectable immunostaining of nuclei and other organelles that could be seen by both fluorescence and phase contrast microscopy. For the first time, these results clearly delineate the localization and association of prohibitin within the mitochondria of ovarian granulosa cells. Surprisingly, we observed the apparent localization of PCNA to the mitochondria of nonproliferating cells. This result seems consistent with the recent finding that nuclear factors can translocate to the mitochondria (34, 35).

Analysis of mitochondrial morphology and function in RGA-1 granulosa cells

To ascertain whether prohibitin is specifically localized to mitochondria, and in an effort to gain insight into the morphological and functional status of this organelle within proliferating and differentiated RGA-1 cells, we employed the use of two well-known lipophilic cationic probes, Mitotracker red and JC-1, coupled with confocal and electron microscopy. Transmission electron microscopy was used to demonstrate mitochondrial morphological characteristics in the RGA-1 cells. Moreover, these organelles are well known to play a significant role during the process of steroid metabolism and apoptosis within granulosa cells (36). Confocal microscopy, coupled with the lipophilic dye, mitotracker red, was used to detect specific mitochondrial binding independent of the energetic state ($\Delta\Psi_m$). Similar to the observed appearance of the mitochondria after prohibitin immunostaining (Fig. 5), both confocal and electron microscopy revealed primarily rounded mitochondrial structures when cells were cultured at the permissive temperature (Fig. 6, A and C). However, at the nonpermissive temperature, extended reticular networks (Fig. 6B), as well as enlarged and elongated (Fig. 6D) mitochondrial structures, were observed. In addition, the appearance of the smooth endoplasmic reticulum was evident, and prohibitin was located to the inner mitochondrial membrane of granulosa cells (data not shown).

Mitochondria accumulate lipophilic cations because of their negative membrane potential (22, 23). The extent of dye uptake accurately reflects the redox potential across the mitochondrial membrane (37, 38). The J-aggregate-forming lipophilic cation JC-1, which normally exists in solution as a monomer emitting green fluorescence, assumes a dimeric configuration emitting red fluorescence in a reaction driven by mitochondrial transmembrane potential (22, 23, 37, 38). Examination by epifluorescence microscopy revealed an increase in J-aggregates at 33 C when cells were proliferating (red fluorescence signal) (Fig. 7, A and B). We observed an increase in the monomeric form of JC-1 at 39 C when cells were fully differentiated (Fig. 7, D and F) (green fluorescence signal).

Expression of prohibitin in subcellular fractions of RGA-1 granulosa cells

Although immunocytochemical studies revealed the association of prohibitin with mitochondria distributed throughout the cytoplasm of RGA-1 cells, it was still necessary to determine whether prohibitin was exclusively asso-

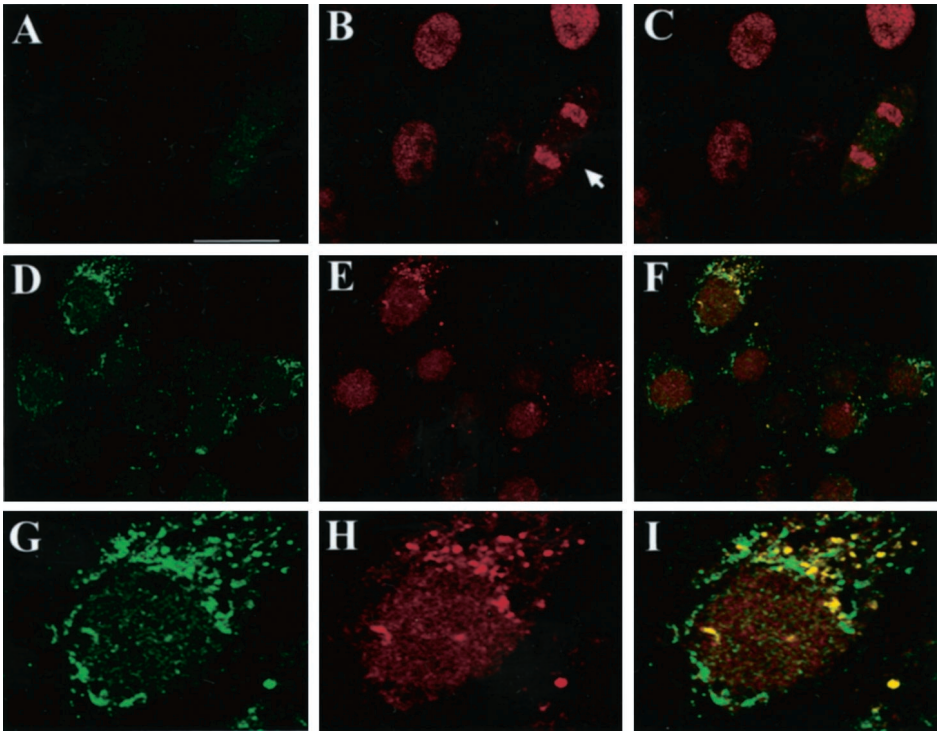

FIG. 5. Localization of prohibitin to the mitochondria in RGA-1 cells. Cells were cultured at the permissive (A, B, and C) and nonpermissive temperatures (D–I) as described in *Materials and Methods*. Cells were fixed with 3% paraformaldehyde and stained with prohibitin (A, D, and G) or PCNA (B, E, and H) antibodies and double-visualized for both proteins in (C, F, and I). Images G, H, and I were higher magnification, demonstrating staining specificity for prohibitin (G, *green*) or PCNA (H, *red*), with a double exposure for the prohibitin and PCNA proteins (I). *Arrow*, Cell-undergoing cytokinesis; *bar*, 20 μm.

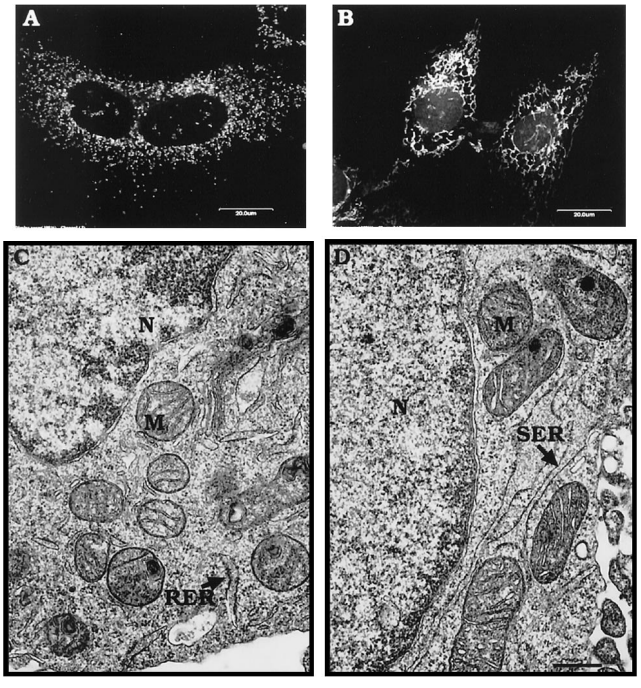

FIG. 6. Visualization of the mitochondria morphology in RGA-1 cells cultured at the permissive and nonpermissive temperatures. Cells were either cultured at 33 C (A and C) or at 39 C for 48 h (B and D) and stained with the lipophilic dye Mitotracker Red (A and B) or fixed for electron microscopy (C and D). In A and C, the majority of the mitochondria are rounded; whereas in B and D, they are elongated. *Bar*, 20 μm for A and B, and 200 nm for C and D.

ciated with the mitochondria. Fractionated RGA-1 cells were separated into three subcellular compartments (cytosolic, mitochondria, and nuclear), and equal amounts of protein extracts from these fractions were subjected to Western blot analyses to assess relative prohibitin and cytochrome c levels. Fig. 8, B and C, revealed that prohibitin expression was primarily associated with the mitochondrial fraction from both cultures grown at 33 C and 39 C, respectively. Moreover, prohibitin expression increased quantitatively at 39 C. Overexposure of the membrane showed only trace amounts of prohibitin in the cytosolic fraction when compared with the mitochondrial fraction (data not shown). Interestingly, doublet bands could also be identified at 110–107 and 35 kDa in the nuclear fractions isolated from both temperature conditions, with no observable difference in expression. Cytochrome c was used as a marker for mitochondrial identification, and no expression of this protein was revealed in the nuclear isolated extracts.

Prohibitin is associated with the early stages of apoptosis in RGA-1 cells

To assess whether prohibitin is associated with apoptosis in RGA-1 cells, we examined the release of cytochrome c from the mitochondria and the processing of procaspase-3 to the active enzyme, because these events have been described as early markers for apoptosis in granulosa cells (39–41). As shown in Fig. 9, when RGA-1 cells were cultured at the nonpermissive temperature, an initial increase in prohibitin protein level was observed at 48 h and further increased at 72 h. Concomitant with an increase in prohibitin expression, a reduction in mitochondrial cytochrome c occurred in parallel with the processing of procaspase-3 to the active enzyme (48–96 h, Fig. 9).

Discussion

The goal of this study was to develop a rat granulosa cell line that could serve as a model system for the expression and

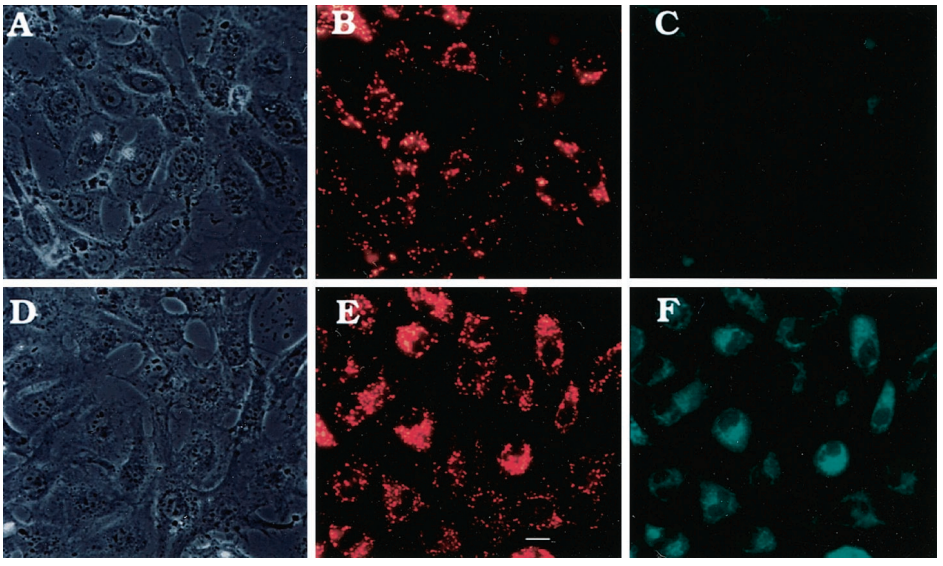

FIG. 7. Mitochondrial membrane potential in RGA-1 cells cultured at the permissive and nonpermissive temperatures. Phase contrast (A and D) and epifluorescene (B, C, E, and F) of RGA-1 cells stained with JC-1, demonstrating a reduction in mitochondrial membrane potential during differentiation. Cells were grown at the permissive (A, B, and C) and the nonpermissive (D, E, and F) temperatures. *Bar*, 20 μm.

regulation of prohibitin during cell growth, differentiation, and apoptosis. A conditionally immortalized cell line, which retains many of the cell-type specific characteristics observed in primary cells, was developed by transfecting granulosa cells with a temperature-sensitive mutant (tsA209) of the SV40 virus. The *ts*A mutants of SV40 viruses, are defective in the A gene required for the maintenance of the transformed phenotype in mammalian cells (42, 43). Therefore, the *ts*A mutant-infected granulosa cells are conditionally immortalized only at a permissive temperature of 33 C. At a nonpermissive temperature of 39 C, these cells differentiate to a morphological phenotype reminiscent of primary granulosa cells (4). Long-term culturing of RGA-1 cells at the nonpermissive temperature subsequently leads to cell death.

RGA-1 cells, like other reported granulosa cell lines, express a subset of genes that are known to be specifically up-regulated during granulosa cell development and differentiation *in vivo*. Our study clearly shows that RGA-1 cells retained the capacity to express cytokines and steroidogenic factors involved in follicular development. Moreover, RGA-1 cells selectively express both inhibin α- and β_A-mRNA during differentiation. These gene products have been shown to play key roles in the regulation of the pituitary gonadotropin, FSH, steroidogenesis, and folliculogenesis (44, 45). Previous studies demonstrate that cAMP regulates the expression of both inhibin α- and β_A-mRNAs in cultured granulosa cells and that the phorbol ester TPA selectively induces β_A-mRNA expression (27, 28). In this study, treatment of RGA-1 cells with cAMP and TPA induced expression of inhibin α- and β_A-mRNA subunits, respectively, at the permissive temperature. Though we did not observe an induction of the inhibin β_A-mRNA subunit by cAMP, it is likely that the levels of β_A expression in RGA-1 cells were attenuated at the 24-h time point examined. This is consistent with reports indicating that cAMP induction of inhibin β_A is fairly rapid and transient in primary cells (28).

Differentiated granulosa cells characteristically express high levels of steroidogenic enzymes and steroidogenic proteins, such as StAR and PBR proteins (29, 30). When induced to differentiate at the nonpermissive temperature, RGA-1 cells significantly up-regulate both StAR and PBR proteins, when compared with expression at the permissive temperature. It is plausible that the mechanisms mediating StAR- and PBR- induction may have been initiated in primary cells before SV40 immortalization, because they originated from diethylstilbestrol treated females.

The successful establishment of conditionally immortalized RGA-1 granulosa cells provides a potentially versatile tool for functional analysis of prohibitin. The prohibitin gene has been implicated in development (5, 46, 47), growth arrest (10–12), differentiation (4, 5, 48), and apoptosis (5, 49) and may also function as a potential tumor suppressor (50–53). Our laboratory has previously identified and characterized prohibitin gene products in granulosa cells both *in vitro* and *in vivo* (4, 5). Moreover, we have shown a relationship between granulosa cell differentiation and increased prohibitin expression, even during the early stages of apoptosis (5). In the current study, RGA-1 cells seem to express prohibitin in a manner indicative of a role in regulating cell growth. For example, RGA-1 cells, cultured at the nonpermissive temperature, were induced to differentiate; and they demonstrated increased prohibitin protein and mRNA levels. In addition, two-dimensional electrophoretic analyses revealed that the more acidic isoform of prohibitin was expressed during differentiation. These results support a direct correlation between increased prohibitin expression and primary granulosa cell differentiation and steroidogenesis (4, 5). Furthermore, because prohibitin has known association with the mitochondrion (6–9), the relationship between prohibitin and COII expression was assessed. It was revealed that increased prohibitin expression is associated with a down-regulation in the COII protein, which plays a central role in cellular energy provision during oxidative phosphorylation (54, 55). The observed decrease in COII expression is likely a reflection of the transition from a higher energy demand state in proliferating cells to one of lower energy demand in differentiated cells. Indirect immunocytochemistry was used to confirm the above by localizing prohibitin to the mitochondria. The distribution patterns for prohibitin revealed a punctate cytoplasmic localization in RGA-1 cells cultured at

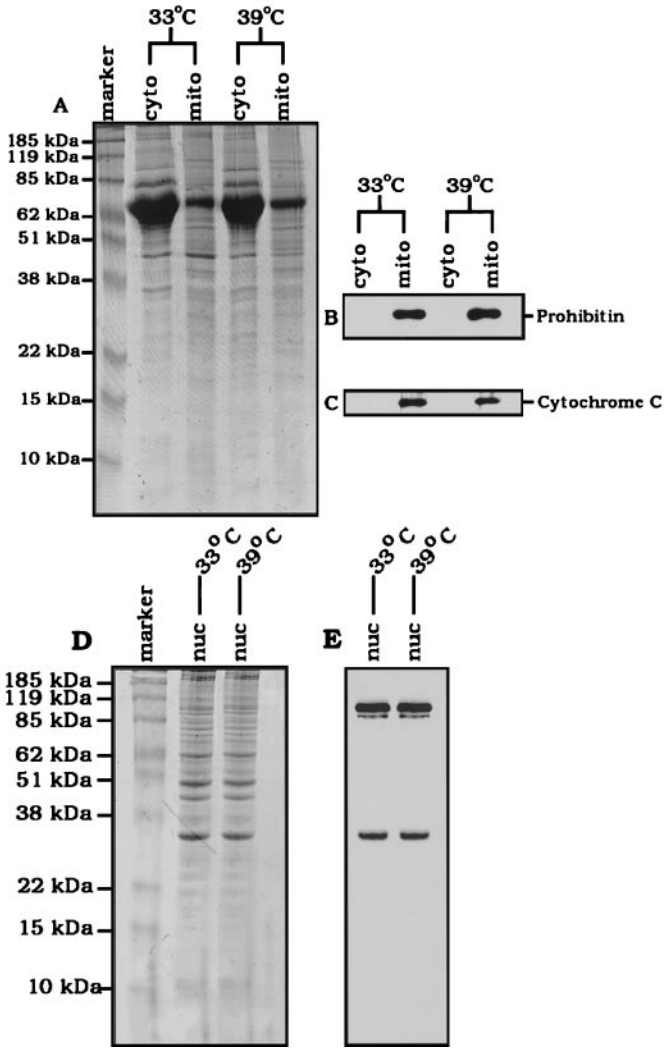

FIG. 8. Location of prohibitin in subcellular fractions of proliferating and differentiated RGA-1 cells. Fifty micrograms of protein (A and D) from the cytosol (cyto), mitochondria (mito), and nuclei (nuc) of cells cultured at permissive and nonpermissive temperatures were applied to each lane and subjected to Western blot analysis (B, C, and E) as described in *Materials and Methods*. Blots were probe with antibodies to prohibitin and cytochrome c.

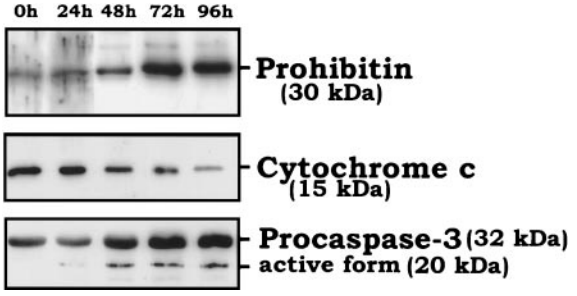

FIG. 9. Activation of apoptosis in RGA-1 cells. Prohibitin, mitochondrial cytochrome c release, and the processing of procaspase-3 to the active enzyme in RGA-1 cells. RGA-1 cells were cultured at the nonpermissive temperature for 24, 48, 72, and 96 h and cells processed for the isolation of mitochondria and whole-cell extracts for Western blot analysis. Cytochrome c immunostaining was on isolated mitochondria samples.

both permissive and nonpermissive temperatures. The majority of this expression was associated with the mitochondria, as revealed by the mitochondrial-specific marker, Mitotracker red. During cellular differentiation, ultrastructural examination revealed changes in mitochondrial size and shape in association with increased prohibitin expression. This increase in prohibitin expression also correlated well with changes in mitochondrial transmembrane potential, as indicated by J-aggregate-forming lipophillic cationic dye assay. Similar results have been observed with the prohibitin homologue Phb1p in yeast mitochondria (56). The yeast studies demonstrated that increases in Phb1p protein and mRNA levels occurred when there was an imbalance in mitochondrially translated products.

A determination of the precise subcellular distribution of prohibitin in granulosa cells is important for complete characterization of prohibitin function in these cells. To validate the localization of prohibitin expression to the mitochondria, RGA-1 cell extracts were partitioned into subcellular fractions. Analyses of immunoblots of extracts from cells cultured under permissive and nonpermissive conditions confirmed that prohibitin is predominantly localized to the mitochondria. Interestingly, the prohibitin antibody identified a doublet at 110 and 107 kDa, as well as a 35-kDa protein in the nucleus. These results are intriguing, in light of recent reports that prohibitin is associated with the membrane-bound IgM receptor of B-lymphocytes (52, 57) and may physically interact with the retinoblastoma tumor suppressor protein and its family members p107 and p130 to repress all transcriptionally active members of the E2F family in breast cancer cells (52, 53). Moreover, deletion mutant studies of the NH-terminal amino acids support the notion that the NH-terminal sequence of prohibitin may, in part, be responsible for the directionality and binding of this protein to mitochondrial membranes (6, 53). A majority of published reports have demonstrated close associations between prohibitin and the mitochondria. Along these lines, Nijtmans *et al.* (56) suggested prohibitin may act as a membrane-bound chaperone, which functions to stabilize misfolding of mitochondria-associated proteins.

In studies of events related to apoptosis in RGA-1 cells, we observed an increase in prohibitin protein levels, corresponding decreases in cytochrome c, and increased processing of procaspase-3 to the active enzyme. These results concur with previous findings indicating that the release of cytochrome c from the mitochondria and the processing of procaspase-3 are early markers for the demise of granulosa cells (39–41). Mitochondrial cytochrome c has been shown to exhibit dual functions, in controlling both cellular energetics and apoptosis. Through interactions with apoptotic protease-activating factors, cytochrome c was shown to play an integral role in initiating the activation of a cascade of caspases on release into the cytosol. These findings indicate the possibility that prohibitin up-regulation occurs in response to destabilization in mitochondrial function resulting from initiation of apoptosis.

Altogether, our results confirm the localization of prohibitin to the mitochondria and reveal that the regulation of prohibitin expression in RGA-1 granulosa cells is associated with changes in mitochondrial structure and function. Up-

regulation of prohibitin in granulosa cells may also reflect the respiratory status of the mitochondria during cellular differentiation and apoptosis. We have also provided evidence that prohibitin is associated with other cellular compartments. These observations suggest multifunctional roles for prohibitin in granulosa cells during follicular development. Prohibitin's specific role may likely depend on cellular localization, cell type, oligomeric state, and the cellular concentration of a ligand, substrate, cofactor, or product (58).

In summary, we have successfully established a conditionally immortalized rat granulosa cell line that expresses important granulosa cell genes. These cells exhibit a temperature-dependent phenotype, with respect to morphology, growth, and the expression of prohibitin. RGA-1 cells will serve as a useful model in studies related to the functional relevance of prohibitin during cell differentiation and apoptosis.

Acknowledgments

We thank Drs. James Lillard and Jonathan Stiles for their critique and valuable comments. Special thanks are extended to Drs. Keith McClung, Douglas Stocco, and Vassili Papadopoulos for their generous gifts of prohibitin cDNA and antibodies to StAR and PBR, respectively. We also thank Patrick Abramson, from the Department of Information Technology, for photographic and computer imaging assistance.

Received January 19, 2001. Accepted May 3, 2001.

Address all correspondence and requests for reprints to: Dr. Winston E. Thompson, Department of Obstetrics and Gynecology, Morehouse School of Medicine, 720 Westview Drive Southwest, Atlanta, Georgia 30310. E-mail: thompsw@msm.edu.

This investigation was supported, in part, by NIH Grants GM-08248, RR-03034 (to W.E.T.), NS34194, and HD-21921 (to K.E.M.).

References

1. **Hirshfield AN** 1991 Development of follicles in the mammalian ovary. Int Rev Cytol 124:43–101
2. **Richards JS** 1980 Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 60:51–89
3. **Richards JS, Hedin L** 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. Annu Rev Physiol 50: 441–463
4. **Thompson WE, Sanbuissho A, Lee GY, Anderson E** 1997 Steroidogenic acute regulatory (StAR) protein (p25) and prohibitin (p28) from cultured rat ovarian granulosa cells. J Reprod Fertil 109:337–348
5. **Thompson WE, Powell JM, Whittaker JA, Sridaran R, Thomas KH** 1999 Immunolocalization and expression of prohibitin, a mitochondrial associated protein within the rat ovaries. Anat Rec 256:40–48
6. **Ikonen E, Fiedler K, Parton RG, Simons K** 1995 Prohibitin, an antiproliferative protein, is localized to mitochondria. FEBS Lett 358:273–277
7. **Coates PJ, Jamieson DJ, Smart K, Prescott AR, Hall PA** 1997 The prohibitin family of mitochondrial proteins regulate replicative life span. Curr Biol 7:607–610
8. **Berger KH, Yaffe MP** 1998 Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae*. Mol Cell Biol 18:4043–4052
9. **Steglich G, Neupert W, Langer T** 1999 Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Mol Cell Biol 19:3435–3442
10. **McClung JK, Danner DB, Stewart DA, et al.** 1989 Isolation of a cDNA that hybrid selects antiproliferative mRNA from rat liver. Biochem Biophys Res Commun 164:1316–1322
11. **Nuell MJ, Stewart DA, Walker L, et al.** 1991 Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol Cell Biol 11:1372–1381
12. **Roskams AJ, Friedman V, Wood CM, et al.** 1993 Cell cycle activity and expression of prohibitin mRNA. J Cell Physiol 157:289–295
13. **Zeleznik AG, Hillier SG, Knazek RA, Ross GT, Coon HG** 1979 Production of long term steroid-producing granulosa cell cultures by cell hybridization. Endocrinology 105:156–162
14. **Amsterdam A, Zauberman A, Meir G, Pinhasi-Kimhi O, Suh BS, Oren M** 1988 Cotransfection of granulosa cells with simian virus 40 and Ha-RAS oncogene generates stable lines capable of induced steroidogenesis. Proc Natl Acad Sci USA 85:7582–7586
15. **Fitz TA, Wah RM, Schmidt WA, Winkel CA** 1989 Physiologic characterization of transformed and cloned rat granulosa cells. Biol Reprod 40:250–258
16. **Zilberstein M, Chou JY, Lowe Jr WL, et al.** 1989 Expression of insulin-like growth factor-I and its receptor by SV40-transformed rat granulosa cells. Mol Endocrinol 3:1488–1497
17. **Baum G, Suh BS, Amsterdam A, Ben-Ze'ev A** 1990 Regulation of tropomyosin in transformed granulosa cell lines with steroidogenic ability. Dev Biol 142: 115–128
18. **Rao IM, Gadson Jr PF, Anderson E, Hornsby PJ, Mahesh VB** 1993 Characterization of progesterone biosynthesis in a transformed granulosa cell line. Mol Cell Endocrinol 94:121–128
19. **Ausubel FM, Brent R, Kingston RE, et al.** 1994 Current protocols in molecular biology. New York: John Wiley and Son Inc.; vol 1–3
20. **Woodruff TK, Meunier H, Jones PB, Hsueh AJ, Mayo KE** 1987 Rat inhibin: molecular cloning of alpha- and beta-subunit complementary deoxyribonucleic acids and expression in the ovary. Mol Endocrinol 1:561–568
21. **Anderson E, Selig M, Lee GY, Little B** 1988 An *in vitro* study of the effects of androgens on the cytoskeleton of ovarian granulosa with special reference to actin. Tissue Cell 20:855–874
22. **Smiley ST, Reers M, Mottola-Hartshorn C, et al.** 1991 Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation C-1. Proc Natl Acad Sci USA 88:3671–3675
23. **Reers M, Smith TW, Chen LB** 1991 J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 30:4480–4486
24. **Yang J, Liu X, Bhalla K, et al.** 1997 Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria is blocked. Science 275:1129–1132
25. **Gorski K, Carneiro M, Scheibler U** 1986 Tissue-specific *in vitro* transcription from the mouse albumin promoter. Cell 47:767–776
26. **Vanderstichele H, Delaey B, de Winter J, et al.** 1994 Secretion of steroids, growth factors, and cytokines by immortalized mouse granulosa cell lines. Biol Reprod 50:1190–1202
27. **Pei L, Dodson R, Schoderbek WE, Maurer RA, Mayo KE** 1991 Regulation of the alpha inhibin gene by cyclic adenosine 3′,5′-monophosphate after transfection into rat granulosa cells. Mol Endocrinol 5:521–534
28. **Tuuri T, Eramaa M, Van Schaik RH, Ritvos O** 1996 Differential regulation of inhibin/activin alpha- and beta A-subunit and follistin mRNAs by cyclic AMP and phorbol ester in cultured human granulosa-luteal cells. Mol Cell Endocrinol 121:1–10
29. **Papadopoulos V, Mukhim AG, Costa E, Krueger KE** 1990 The peripheral-type benzodiazepine receptor is functionally linked to Leydig cell steroidogenesis. J Biol Chem 265:3772–3779
30. **Stocco DM** 1997 The steroidogenic acute regulatory (StAR) protein two years later. Endocr Rev 6:99–109
31. **Xiong Y, Zhang H, Beach D** 1992 D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505–514
32. **Hall PA, Levison DA** 1990 Review: assessment of cell proliferation in histological material. J Clin Pathol 43:184–192
33. **Oktay K, Schenken RS, Nelson JF** 1995 Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. Biol Reprod 53:295–301
34. **Brenner C, Kroemer G** 2000 Mitochondria-the death signal integrators. Science 289:1150–1151
35. **Li H, Kolluri SK, Gu J, et al.** 2000 Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. Science 289:1159–1164
36. **Strauss 3rd JF, Golos TG, Silavin SL, Soto EA, Takagi K** 1988 Involvement of cyclic AMP in the functions of granulosa and luteal cells: regulation of steroidogenesis. Prog Clin Biol Res 267:177–200
37. **Johnson LV, Walsh ML, Bockus BJ, Chen LB** 1981 Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J Cell Biol 88:526–535
38. **Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A** 1997 JC-1, but not DiOC6 (3) or rhodamine 123, is a reliable fluorescent probe to assess $\Delta\Psi$ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett 411:77–82
39. **Boone DL, Tsang BK** 1998 Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. Biol Reprod 58:1533–1539
40. **Izawa M, Nguyen PH, Kim HH, Yeh J** 1998 Expression of the apoptosis-related genes, caspase-1, caspase-3, DNA fragmentation factor, and apoptotic protease activating factor-1, in human granulosa cells. Fertil Steril 70:549–552
41. **Robles R, Tao X-J, Trbovich AM, et al.** 1999 Localization, regulation and possible consequences of apoptotic protease-activating factor-1 (apaf-1) expression in granulosa cells of the mouse ovary. Endocrinology 140:2641–2644
42. **Martin RG, Chou JY** 1975 Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J Virol 15:599–612
43. **Chou JY** 1985 Establishment of rat liver lines and characterization of their metabolic and hormonal properties: use of temperature-sensitive SV-40 virus. Methods Enzymol 109:385–396

44. **Ardekani AM, Romanelli JC, Mayo KE** 1998 Structure of the rat inhibin and activin betaA-subunit gene and regulation in an ovarian granulosa cell line. Endocrinology 139:3271–3279
45. **Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP** 1990 Inhibin and activin locally regulate rat ovarian folliculogenesis. Endocrinology 127:3196–3205
46. **Eveleth DD, Marsh JL** 1986 Sequence and expression of the Cc gene, a member of the dopa decarboxylase gene cluster of Drosophila: possible translational regulation. Nucleic Acids Res 15:6169–6183
47. **Choongkittaworn NM, Kim KH, Danner DB, Griswold MD** 1993 Expression of prohibitin in rat seminiferous epithelium. Biol Reprod 49:300–310
48. **Tanno S, Fukuda I, Saito Y, Ogawa K** 1997 Prohibitin expression is decreased in the regenerating liver but not in chemically induced hepatic tumors in rats. Jpn J Cancer Res 88:1155–1164
49. **Welburn SC, Murphy NB** 1998 Prohibitin and RACK homologues are up-regulated in trypanosomes induced to undergo apoptosis and in naturally occurring terminally differentiated forms. Cell Death Differ 5:615–622
50. **Sato T, Saito H, Swensen J, et al.** 1992 The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. Cancer Res 52:1643–1646
51. **Sato T, Sakamoto T, Takita K, Saito H, Okui K, Nakamura Y** 1993 The human prohibitin (PHB) gene family and its somatic mutations in human tumors. Genomics 17:762–764
52. **Wang S, Nath N, Adlam M, Chellappan S** 1999 Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. Oncogene 18:3501–3510
53. **Wang S, Nath N, Fusaro G, Chellappan S** 1999 Rb and prohibitin target distinct regions of E2F1 for repression and respond to different upstream signals. Mol Cell Biol 19:7447–7460
54. **Taanman JW, Burton MD, Marusich MF, Kennaway NG, Capaldi RA** 1996 Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome-c oxidase subunits in chronic progressive external ophthalmoplegia. Biochim Biophys Acta 1315:199–207
55. **Saraste M,** 1999 Oxidative phosphorylation at the fin de siecle. Science 283: 1488–1492
56. **Nijtmans LG, de Jong L, Artal Sanz M, et al.** 2000 Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J 19:2444–2451
57. **Terashima M, Kim KM, Adachi T, et al.** 1994 The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. EMBO J 13:3782–3792
58. **Jeffery CJ** 1999 Moonlighting proteins. Trends Biochem Sci 24:8–11