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Expression of the Inhibitor of T-Cell Apoptosis (*ita*) Gene in Hen Ovarian Follicles during Development[1]

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ABSTRACT

It is now well established that within the hen ovary, preovulatory follicles rarely become atretic and that granulosa cells from preovulatory follicles are relatively resistant to undergoing apoptosis in vitro. By comparison, prehierarchal (≤ 8-mm diameter) follicles are highly susceptible to becoming atretic in vivo, and approximately 70% of granulosa cells collected from 3- to 8-mm-diameter follicles rapidly undergo apoptosis when incubated for as little as 6 h in vitro in defined medium. The present studies were conducted to characterize expression of an inhibitor of apoptosis (*iap*) gene, inhibitor of T-cell apoptosis (*ita*), within hen follicle tissues at various stages of follicle development. The *ita* gene product has recently been shown to share homology within both the baculovirus repeat sequences of the N-terminus and the zinc ring-finger motif from the C-terminus and was originally determined to be expressed in chicken cells of T-lymphocyte lineage. In the present studies, highest levels of *ita* mRNA within the granulosa cell layer were found in preovulatory (atresia-resistant) follicles, with significantly lower levels detected in prehierarchal follicles. After 24 h of primary culture, *ita* mRNA levels increased in granulosa cells from preovulatory follicles by 3.2-fold as compared to those in freshly collected cells and were elevated by 8.9-fold in those granulosa cells from 6- to 8-mm follicles that successfully formed a primary culture monolayer. Moreover, *ita* mRNA levels were significantly increased in 6- to 8-mm-follicle granulosa cells after only 2 h of suspension culture, and this increase could be prevented by actinomycin D. This spontaneous increase in *ita* expression may serve to protect from cell death the relatively small population of prehierarchal follicle granulosa cells that survive in vitro. It is concluded from these data, taken together, that patterns of *ita* mRNA expression during follicle development are consistent with a potential role for this gene in protecting granulosa cells from apoptosis and thus maintaining follicle viability.

INTRODUCTION

Follicle atresia in mammals and birds occurs via apoptosis, and this process is first evidenced, and subsequently observed predominantly, within the granulosa cell layer [1–3]. The hen ovary provides a unique and useful model system in which to study cellular and molecular mechanisms mediating granulosa cell apoptosis because characteristics of two populations of follicles, differing in susceptibility to atresia, can be directly compared within follicles from the single left ovary of the hen. These include slow-growing, prehierarchal (3- to 8-mm diameter) follicles that are highly susceptible to undergoing atresia, and hierarchal preovulatory (9-mm diameter and larger) follicles that are in the process of final differentiation prior to ovulation and that under normal physiological conditions do not become atretic. Furthermore, it has previously been documented that a large proportion of granulosa cells from prehierarchal follicles rapidly undergo apoptosis under culture conditions (within 3–6 h after plating or in suspension culture), while granulosa cells from preovulatory follicles are highly resistant to apoptosis, in vitro, even after several days as a primary culture [3–6].

Although the precise molecular mechanisms and sequence of events that lead to apoptotic cell death have yet to be elucidated in any cell type, there is considerable evidence that activation of intracellular proteases is prerequisite for initiation of apoptosis. Initial support for enzyme-mediated apoptosis originated from genetic studies on the nematode, *Caenorhabditis elegans*, in which the expression of the *ced-3* gene was determined to be obligatory for apoptotic cell death [7]. Sequence analysis of the gene and the crystallized structure of the encoded protein revealed structural homology to mammalian interleukin-1β converting enzyme. It is now evident that there exists an ever-expanding family of related proteases within mammalian cells that play key roles in both apoptosis and the inflammatory process (for review, see [8, 9]). On the basis of two common catalytic properties within this group of enzymes, a cysteine protease mechanism and the ability to cleave after an aspartic acid, the term "caspase" has been proposed as the trivial name for all related family members [10]. Caspase activation occurs following ligand interaction with CD95 (or Fas), tumor necrosis factor receptor, or death receptor-3 via a series of receptor-mediated signal transduction pathways [11]. However, it is currently proposed that caspase activation is a "distal" event in the initiation of apoptosis, as overexpression of Bcl-2-related proteins blocks caspase-induced proteolysis and apoptotic cell death [12–14].

Additional inhibitors of apoptosis, unrelated to the Bcl-2 family of proteins, were initially found to be expressed by the baculovirus [15, 16]. These inhibitor of apoptosis proteins (IAPs) prevent viral-induced apoptotic cell death in a variety of cells, presumably as a mechanism to enhance infectivity and facilitate viral replication. Several mammalian homologues containing a C-terminal zinc ring-finger motif and N-terminal repeat elements common to baculoviral IAPs have now been identified [17–19]. Though the exact mechanisms by which they act have yet to be determined, one possible mechanism is that they inhibit apoptosis by regulating intracellular signals required for activation of caspases.

A novel avian *iap* gene, inhibitor of T-cell apoptosis (*ita*), has recently been characterized and was originally reported to be selectively expressed in cells of lymphocyte origin (e.g., thymus and spleen) but not detected by Northern blot analysis in the adult liver, brain, kidney, heart, or bone marrow [20]. Two human *ita* homologues were sub-

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sequently identified, and at least one maps to 11q22 from the human genome [20, 21]. Results reported herein extend the initial findings in the chicken by documenting the expression of *ita* mRNA in hen ovarian granulosa and theca tissues. The data presented demonstrate that differential patterns of expression within the granulosa cell layer during follicle development are consistent with a potential role for this gene in supporting granulosa cell (and thus follicle) viability by regulating processes that initiate apoptosis.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 wk of age and laying regular sequences of at least 8–10 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and exposed to a photoperiod of 15L:9D with lights-on at 0000 h. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed by cervical dislocation approximately 16–18 h prior to a midsequence ovulation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Recombinant human transforming growth factor α (TGFα) was purchased from Bachem (Torrance, CA), and actinomycin D was from Gibco-BRL (Grand Island, NY); additional reagents were acquired from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

Tissue Collection and Preparation

Granulosa and theca tissues from the largest (F1), second-largest (F2), and third-largest (F3) preovulatory follicles, from follicles recently selected into the follicle hierarchy (9- to 12-mm follicles), from prehierarchal (6- to 8-mm and 3- to 5-mm) follicles, and ovarian stromal tissue were collected and prepared as previously described [22, 23]. Samples of immune system (spleen, bursa, thymus) and nonovarian endocrine (pituitary) tissues were processed for comparative purposes. In addition, all follicles measuring 6–8 mm in diameter were collected from ovaries of four different hens and arranged by size from largest to smallest, and the granulosa layer from each follicle was isolated. The number of follicles within this size category totaled 8, 10, 11, and 14 for the four hens, respectively, and each granulosa layer was stored separately. Finally, morphologically normal and early atretic follicles (3- to 5-mm diameter) were processed without separating granulosa and theca layers. Early atretic follicles were identified based on the presence of follicle haemorrhagia, collapsed morphology, and opaque appearance [4]. All tissues were immediately frozen at −70°C until further processed.

Granulosa Cell Cultures

Granulosa cells were collected from preovulatory (F1 and F2) or prehierarchal (6- to 8-mm) follicles and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) as previously described [22]. It should be noted that such granulosa cell preparations are pure populations of cells uncontaminated with cells of lymphocyte origin. Granulosa cells were frozen at −70°C immediately after dispersion (T0 controls) or were plated on 60-mm polystyrene culture plates (Falcon 3004; Fisher Scientific, Pittsburgh, PA) at a density of approximately 1.5×10^6/ml in 2 ml medium 199 supplemented with Hanks' salts (Gibco-BRL; M199-Hepes) and containing 2.5% fetal bovine serum (Gibco-BRL). It has previously been reported that the plating efficiency of such prehierarchal follicle granulosa cells is approximately 30% with the remaining cells determined to undergo apoptosis, while the plating efficiency of preovulatory follicle granulosa cells is > 80% [5]. The following day, adherent cells were washed once with fresh medium and removed via trypsinization. Cells were pelleted at 200 × *g* for 5 min, then stored at −70°C until processed further. In addition, granulosa cells from 6- to 8-mm follicles were frozen immediately after dispersion (T0 controls) or placed into suspension culture for 0.5–3 h in serum-free M199-Hepes or for 3 or 6 h in serum-free medium containing 1 μg/ml actinomycin D. Following suspension culture, cells were gently pelleted by centrifugation (200 × *g*; 20°C; 5 min), media were discarded, and cells were frozen (−70°C) until further processed.

In addition, preovulatory (F1 and F2) and prehierarchal (6- to 8-mm diameter) follicle granulosa cells were dispersed separately, and aliquots were immediately frozen (T0 controls) or were incubated as suspension cultures (2.5×10^6 per 12 × 75-mm polypropylene culture tube) in the absence or presence of treatments in 2 ml M199-Hepes for 20 h at 37°C in a shaking water bath. Treatments included medium alone (control), 1 mM 8-bromo-cAMP (8-br-cAMP), 3.3 nM TGFα, or 162 nM phorbol 12-myristate 13-acetate (PMA). These agents and doses were selected for use based upon their previously established actions to affect cell viability and/or cell differentiation in granulosa cells [4, 5]. After suspension culture, cells were gently pelleted by centrifugation, collected, and frozen as described above.

Northern Blot Analysis

Total cellular RNA from each tissue and stage of follicle development was isolated using Trizol Reagent (Gibco-BRL), and purity and quantity were assessed by measuring the optical density of each sample at 260 nm versus 280 nm. Total cellular RNA (10 to 15 μg) was electrophoresed on 1% agarose gels in the presence of formaldehyde and then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight. Analysis of *ita* and *bcl*-x mRNA was conducted using cDNA probes labeled by the random-prime labeling method using the Megaprime DNA labeling system (Amersham, Arlington Heights, IL) and [α^{32}P]dCTP (3000 Ci/mmol; Amersham). The chicken *ita* probe consisted of an approximate 2-kilobase (kb) cDNA derived from the *ita* coding region [20]. Messenger RNA for *bcl-x*$_{Long}$ was analyzed using a 485-base pair (bp) cDNA probe as previously described, and it is noted that these studies failed to identify a *bcl-x*$_{Short}$ transcript expressed in the hen ovary [4]. Blots were prehybridized for 30 min at 60°C and subsequently hybridized overnight at 60°C, according to previously described methods [24–27]. Membranes were exposed to autoradiographic film at −70°C for 1–3 days and subsequently analyzed by densitometric scanning (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), or they were exposed to phosphorimaging plates (1–2 days) and analyzed using a Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). When appropriate (see data anal-

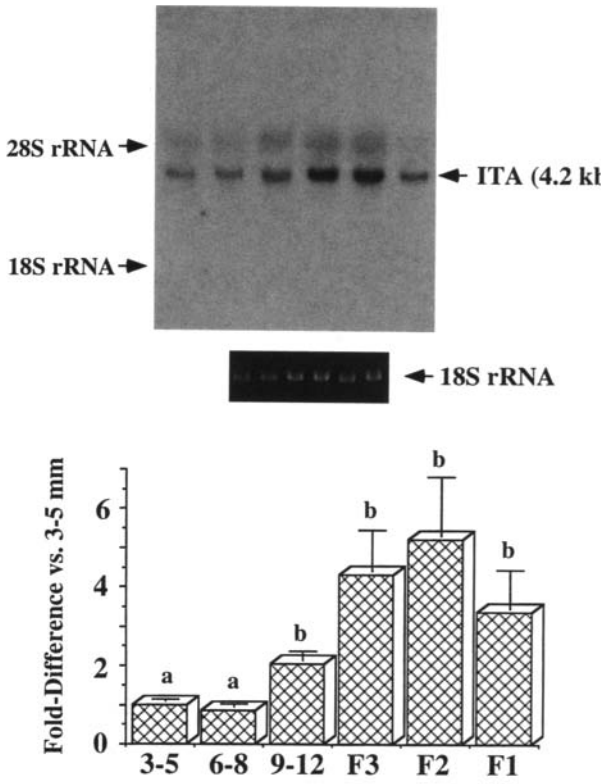

FIG. 1. Representative Northern blot of *ita* mRNA from granulosa cells during follicle development and ethidium bromide-stained gel for 18S ribosomal (r) RNA (upper panels), and summary of densitometric scanning data (bottom panel; mean densitometric units expressed as fold difference compared to 3- to 5-mm follicles; n = 3–4 replicate experiments). F1 and F2, largest and second-largest preovulatory follicle, respectively; 6–8 mm and 3–5 mm refer to diameter of follicles. Data represent mean ± SEM, a,b: $p < 0.05$.

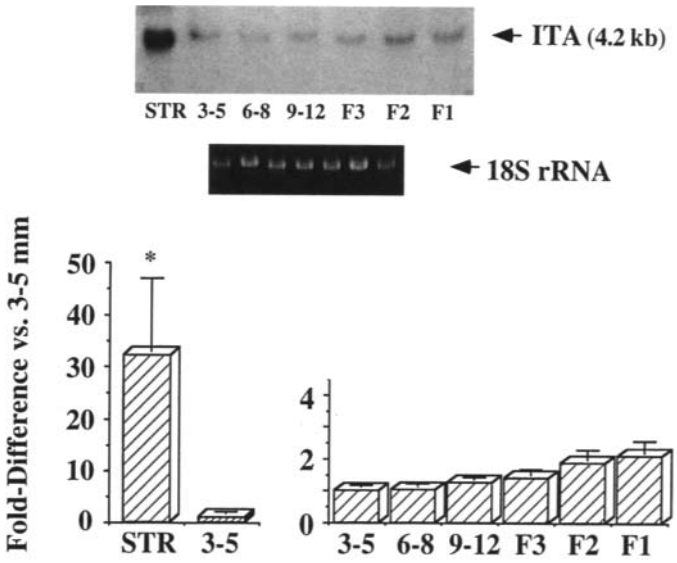

FIG. 2. Representative Northern blot of *ita* mRNA and ethidium bromide-stained gel for 18S ribosomal (r) RNA from theca tissue during follicle development (upper panels), and summary of densitometric scanning data for *ita* mRNA (lower panels; mean densitometric units expressed as fold difference compared to 3- to 5-mm follicles; n = 3–4 replicate experiments). F1 and F2, largest and second largest preovulatory follicle, respectively; 3–5, 6–8, and 9–12 refer to diameter of follicles (mm); STR, stromal tissue. Data represent mean ± SEM; * $p = 0.007$.

ysis), blots were rehybridized to a random-primed chicken 18S ribosomal RNA cDNA probe (a 225-bp product amplified by polymerase chain reaction) to standardize for equal loading of RNA samples.

Analysis of Oligonucleosome Formation

Genomic DNA was prepared from all treated samples and corresponding controls as previously reported [3, 4]. After isolation and quantitation of DNA, 1 μg per sample was 3′ end-labeled with [α^{32}P]dideoxy-ATP (3000 Ci/mmol; Amersham) using the terminal transferase reaction, and the degree of low-molecular-weight (LMW; < 20 kb) DNA was analyzed by autoradiography as previously described [4].

Data Analysis

Northern blot analyses were repeated on a minimum of three independent occasions with different pools of cells or tissues. Equal loading of RNA from granulosa and theca tissues during follicle development was verified following the evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA. This method of analysis has proven reliable for the measurement of a variety of genes expressed during hen follicle development when standard housekeeping genes are not expressed at constant levels [24–27]. By comparison, because of the potential for treatment-related differences in cell viability following granulosa cell suspension culture, mRNA data from such studies were standardized to 18S ribosomal RNA. Messenger RNA levels or protein levels (fold difference versus the appropriate control) are presented as the mean ± SEM of data from replicate experiments. Data were analyzed by Student's *t*-test or with the use of one-way analysis of variance with significant interactions determined using the Fisher protected least-significant difference multiple range test. In addition, regression analysis was conducted to evaluate expression levels of granulosa layer *ita* mRNA versus follicle size within the group of 6- to 8-mm prehierarchal follicles, and versus *bcl*-x mRNA.

RESULTS

Tissue Expression of ita mRNA

Levels of granulosa cell *ita* mRNA were first increased during follicle development at the stage associated with follicle selection (9–12 mm; $p < 0.05$ versus prehierarchal follicle granulosa cells) and remained elevated in all preovulatory stages (Fig. 1). While there were no significant differences in *ita* mRNA levels among follicle groups in the theca layer (Fig. 2, right graph), there was a trend for increased levels through the progression of follicle development ($p = 0.005$, by regression analysis). Ovarian stromal tissue contained considerably higher levels of transcript than theca from 3- to 5-mm follicles ($p = 0.007$; Fig. 2, left graph). Also of note is the finding that levels of *ita* mRNA were increased by 2.9 ± 0.7-fold in atretic compared to normal 3- to 5-mm follicles ($p = 0.02$; data not shown). While *ita* mRNA was detected in all tissues investigated, levels were highest in ovarian stroma and the spleen and lowest in the pituitary (Fig. 3).

Levels of ita mRNA in Cultured Granulosa Cells

Prehierarchal (6- to 8-mm diameter) and preovulatory (F1 and F2) follicle granulosa cells cultured for 24 h expressed 8.9 ± 1.8-fold and 3.2 ± 0.8-fold higher levels of

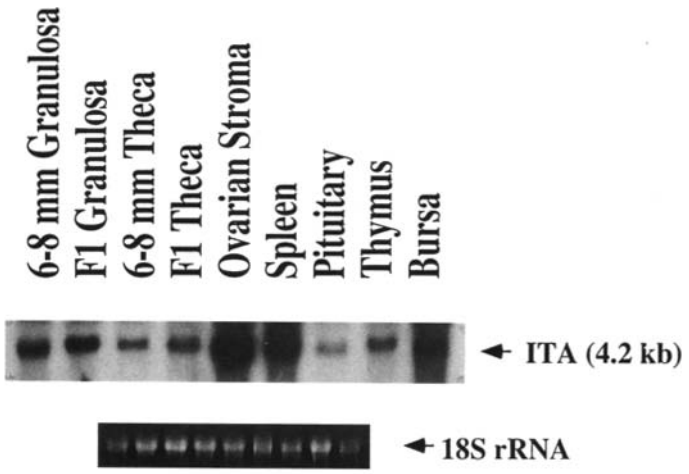

FIG. 3. Comparison of *ita* mRNA levels in ovarian tissues (granulosa, theca, stroma), immune tissues (spleen, thymus, bursa), and a nonovarian endocrine (pituitary) tissue. This experiment was repeated once with similar results. For abbreviations, see figure legends 1 and 2.

ita mRNA, respectively, than their respective freshly collected (T0) cells ($p < 0.05$; Fig. 4). Moreover, increased levels of *ita* mRNA were observed in prehierarchal follicle granulosa cells after 2 h of suspension culture, and the increase after 3 h was blocked by treatment with actinomycin D (Fig. 5, upper graph). The actions of actinomycin D were not associated with a change in the level of oligonucleosome formation, as the degree of LMW labeling was not different between untreated and actinomycin-treated cells after 6 h of suspension culture (Fig. 5, lower graph).

Levels of *ita* mRNA were significantly increased in prehierarchal follicle granulosa cells cultured in the presence of 1 mM 8-br-cAMP, but not TGFα or PMA, as compared to levels in 20-h-cultured control cells ($p < 0.05$; Fig. 6, left graph). By comparison, there was no difference in *ita* mRNA levels following a 20-h suspension culture with F1 and F2 follicle granulosa cells in any treatment as compared to controls ($p > 0.1$; Fig. 6, right graph).

Relationship between Expression of ita and bcl-x$_{Long}$ in 6- to 8-mm Prehierarchal Follicles

Within the pool of follicles increasing in size from 6 to 8 mm in diameter (from which a single follicle per day is

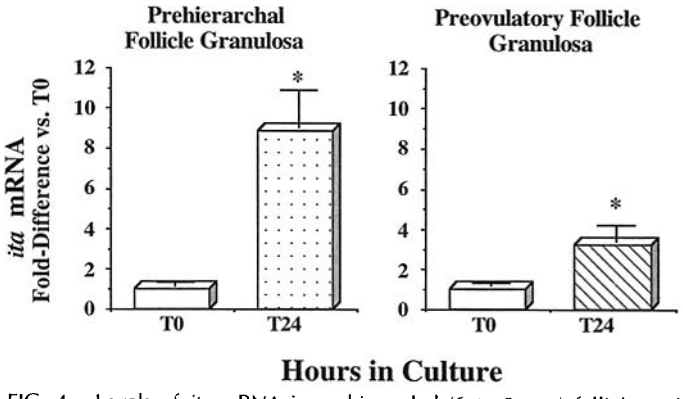

FIG. 4. Levels of *ita* mRNA in prehierarchal (6 to 8 mm) follicle and preovulatory (F1 and F2) follicle granulosa cells that form a primary culture 24 h after plating. Data represent the mean fold increase (± SEM) compared to freshly collected (T0) cells. * $p < 0.05$; n = 4–6 replicate experiments.

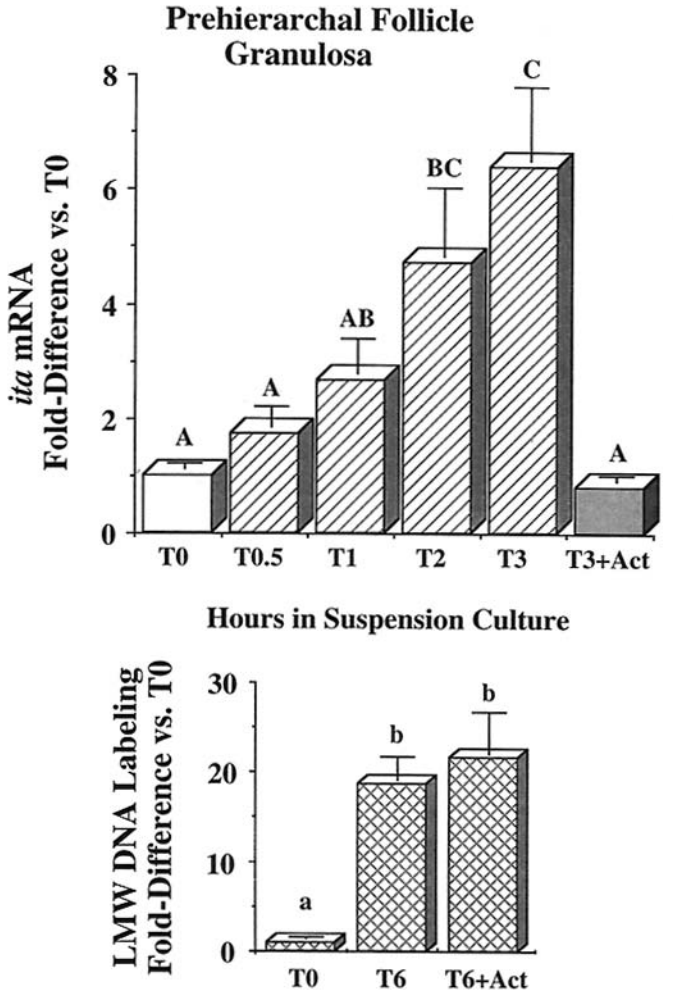

FIG. 5. **Upper**) Levels of *ita* mRNA in 6- to 8-mm follicle granulosa cells after suspension culture for 0.5–3 h in serum-free medium in the absence (T0.5-T3) or presence (T3+Act) of 1 μg/ml actinomycin D. **Lower**) Oligonucleosome formation following suspension culture for 6 h in the absence or presence of 1 μg/ml actinomycin D. Data represent the mean fold increase (± SEM) compared to freshly collected cells (T0). A, B, C; a, b: $p < 0.05$; n = 3–5 replicate experiments.

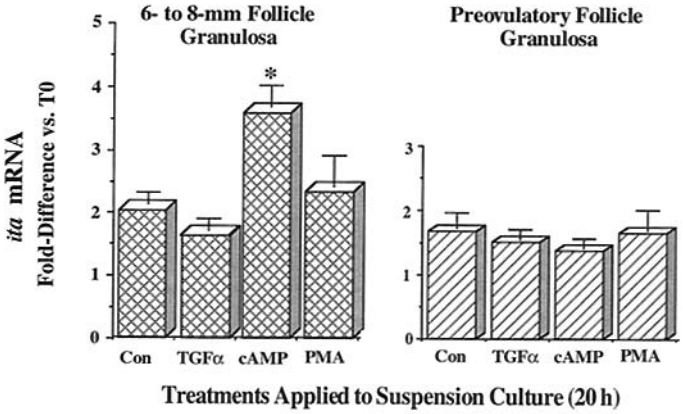

FIG. 6. Levels of *ita* mRNA in granulosa cells from prehierarchal (6 to 8 mm) and preovulatory follicles in the absence (Con) or presence of TGFα (3.3 nM), 8-bromo-cAMP (cAMP; 1 mM), or PMA (167 nM) after 20 h of suspension culture. Data represent the mean fold difference (± SEM) versus freshly collected cells (T0). * $p < 0.05$ versus Con; n = 3–5 replicate experiments.

TABLE 1. Regression analysis of granulosa cell *ita* and *bcl-x* mRNA levels versus size of follicle and versus one another within the pool of prehierarchal follicles measuring 6 to 8 mm in diameter.

Number of 6- to 8-mm follicles[a]	*ita*	*bcl-x*	*ita* vs. *bcl-x*[b]
10	R = −0.51	r = −0.75	R = +0.96
	p = 0.13	p = 0.01	$p < 0.001$
8	R = −0.71	r = −0.69	R = +0.77
	p = 0.05	p = 0.06	$p = 0.001$
14	R = +0.49	r = +0.48	R = +0.54
	p = 0.08	p = 0.09	$p < 0.04$
11	R = +0.35	r = +0.41	R = +0.86
	p = −0.29	p = 0.21	$p < 0.001$

[a] Number of follicles represents the total within this size range collected from each of four hens; this cohort of follicles represents the group from which a single follicle will be selected per day into the preovulatory hierarchy.
[b] R = regression coefficient, positive (+) or negative (−).

selected for final differentiation), neither granulosa cell *ita* nor *bcl*-x mRNA levels were found to be positively correlated to follicle size in any of the four hens ($p > 0.08$, by regression analysis for individual hens). On the other hand, expression of *ita* was positively correlated with expression of *bcl*-x mRNA in each of the four hens ($p < 0.05$; Table 1).

DISCUSSION

Expression of chicken *ita* mRNA has previously been described in several tissues of T-lymphocyte origin, including the spleen and thymus [20]. Results from the present studies extend these findings by documenting *ita* mRNA expression in ovarian follicle tissues as well as the pituitary. Given that immune tissues and ovarian follicles are routinely subject to selection or deletion at various times during their life span, it is perhaps not surprising to find relatively high expression of this and other putative cell death-regulating genes (e.g., *bcl-x*$_{Long}$ [4]; *ich*-1 [27]).

Changes in *ita* mRNA levels in granulosa cells throughout follicle development generally parallel those previously reported for *bcl-x*$_{Long}$ [4]. Given the previous suggestion that ITA and mammalian IAPs play a role in suppressing apoptosis in T lymphocytes at the level of some, as yet undetermined, early signaling event in the process of apoptosis [20, 28], it is reasonable to propose that the *ita* gene plays a similar role in hen ovarian granulosa cells. For instance, during follicle development, highest levels of *ita* mRNA are found in granulosa from follicles that have been selected into the preovulatory hierarchy (e.g., follicles 9 mm in diameter and larger). These follicles are not normally subject to becoming atretic, and granulosa cells from such follicles are highly resistant to undergoing apoptosis, in vitro [4]. The *ita* gene is also expressed in the theca layer, and similar to what is seen with *bcl-x*$_{Long}$, there is a lack of obvious change in mRNA levels with regard to follicle development. Significantly greater levels of *ita* mRNA were detected within the stroma layer of follicles, but given that ovarian thecal and stromal tissues contain significant numbers of cells of immune tissue origin [29, 30], it cannot be determined from the present data whether *ita* expression is specifically related to ovarian follicle viability.

It has previously been determined that approximately 30% of granulosa cells from prehierarchal (6- to 8-mm) follicle granulosa cells remain viable and form a primary monolayer after 24 h of culture [5]. These adhering cells express significantly higher levels of *bcl-x*$_{Long}$ (but not *bcl*-2) mRNA as compared to the entire population of cells prior to plating [31, 32]. It has been speculated that such surviving cells originate primarily from follicles that were destined for selection into the preovulatory hierarchy [31]. In the present study, *ita* mRNA levels were 8.9-fold higher in granulosa cells that successfully form a monolayer in culture as compared to the population of freshly collected (T0) cells (Fig. 4). This increase is significantly greater than the increase that could be attributed solely to an enrichment for apoptosis-resistant granulosa cells following culture. Moreover, the spontaneous increase in *ita* mRNA is evident within 2 h of suspension culture (Fig. 5) and occurs prior to the time when significant oligonucleosome formation (a hallmark of apoptosis) can first be detected [25]. Induced expression of *ita* mRNA in granulosa cells following suspension culture is consistent with results from incubated spleen cells stimulated by concanavalin for 4–24 h as previously reported by Digby et al. [20]. The observation that cotreatment with actinomycin D completely blocks the spontaneous increase in granulosa cell *ita* mRNA indicates the requirement for active transcription. On the other hand, actinomycin D treatment does not alter the extent of LMW DNA labeling, indicating the absence of nonspecific effects on cell viability. Taken together, these data suggest that increased *ita* mRNA transcription in cultured prehierarchal follicle granulosa cells represents an early event that may aid in protecting a select group of granulosa cells from undergoing apoptosis.

Also of note is the observation that *ita* mRNA levels are increased by almost 3-fold in whole atretic compared to morphologically normal follicles. The observation of increased *ita* mRNA during atresia is similar to that previously reported for urokinase plasminogen activator ([25]) and *bcl*-2 [32] mRNA, but is in direct contrast to the marked decrease observed for several other mRNA transcripts that have been evaluated (e.g., FSH receptor [24], LH receptor mRNA [26], *bcl-x*$_{Long}$ [4], *ich*-1 [27]]. While increased levels of urokinase plasminogen activator during early apoptosis are perhaps predictable given this enzyme's function in cellular remodeling, increased levels of the putative anti-apoptotic genes, *bcl*-2 and *ita*, are somewhat unexpected. However, without knowing the source of increased *ita* (i.e., granulosa or theca tissue) it is difficult to speculate on the physiological implication of this finding. For instance, it is possible that the approximate 3-fold increase in *ita* may in large part be due to the increased presence of lymphocytic tissues (e.g., macrophages [29]) during the process of atresia. Additional studies will be required to determine the relationship of increased *ita* mRNA to the initiation or progression of follicle atresia.

Similar to what is seen in prehierarchal follicles, *ita* mRNA levels in preovulatory follicle granulosa cells increase after 24 h of culture, although the extent of increase is not as dramatic. While it remains possible that the relatively smaller change in static mRNA levels is due to both an increase in *ita* mRNA transcription and degradation, it is considered more likely that the rate of *ita* transcription is already near maximal in preovulatory follicle granulosa cells. This speculation may be supported by the finding that suspension culture of preovulatory (F1 and F2) follicle granulosa cells with general activators of the protein kinase A intracellular signaling pathway (8-br-cAMP), of the protein kinase C pathway (PMA), or TGFα (an activator of protein tyrosine kinase) failed to stimulate increased *ita* expression (Fig. 6, right graph). By comparison, 8-br-cAMP stimulated increased levels of *ita* mRNA in prehierarchal follicle granulosa cells (Fig. 6, left graph). This latter finding is consistent with the ability of 8-br-cAMP and FSH, presumably acting via the adenylyl cyclase/cAMP second messenger pathway, to attenuate the progression of apoptosis in prehierarchal follicle granulosa cells [4]. It is also of interest that 8-br-cAMP has recently been shown to increase levels of *bcl-x*$_{Long}$ mRNA in both prehierarchal and preovulatory follicle granulosa cells [32].

Selection of a single follicle per day into the preovulatory hierarchy, in vivo, occurs from a small pool of 6- to 8-mm follicles; and although the fate of follicles not selected on any given day has yet to be determined, it is thought that the majority are destined to undergo atresia and are continually replaced by smaller, growing follicles. Moreover, it has not been established in this or any mammalian species what factors predispose a follicle for recruitment into the final stages of growth and differentiation prior to ovulation. Given that granulosa cells from 6- to 8-mm (prehierarchal) follicles are highly susceptible to undergoing apoptosis [4, 25], it is possible that one prerequisite for follicle selection is the appropriate expression of "death-suppressing" genes, including *bcl-x*$_{Long}$ [4] and possibly *ita*. We have previously reported that, similar to findings for *ita*, *bcl*-x mRNA levels first increase in granulosa cells at the stage of development corresponding to those follicles that have most recently been recruited into the follicle hierarchy (9- to 12-mm-diameter follicles [4]). Although it has been proposed that the expression of both Bcl-2 and Bcl-*x*$_{Long}$ proteins provides a protective effect against the initiation of apoptosis by forming dimers with the death-promoting Bcl-2-related protein Bax [33] in a variety of tissues, we have recently determined that constitutive expression of *bcl*-x$_{Long}$ mRNA within hen granulosa cells is considerably greater than that of *bcl*-2 mRNA. Thus, we have concluded that, as proposed for several mammalian cell types, *bcl-x*$_{Long}$, and not *bcl*-2, is the more physiologically relevant death-suppressing gene expressed in hen granulosa cells [32].

In the present studies, of particular interest was the observation that highest levels of *bcl-x*$_{Long}$ and *ita* mRNA are not necessarily expressed in granulosa cells from the largest follicle(s) within the cohort of 6- to 8-mm prehierarchal follicles, but that follicles with highest levels of granulosa cell *bcl*-x mRNA also expressed the highest levels of *ita* mRNA (Table 1). From these data it is speculated that the largest follicle(s) within this pool may not necessarily be destined to become recruited into the preovulatory hierarchy; alternatively, these data suggest that elevated expression of death-suppressing genes within the granulosa layer may be prerequisite for follicle selection. This speculation is supported by the observations (discussed above) that 1) both *bcl-x*$_{Long}$ and *ita* mRNA levels are significantly increased within granulosa cells from follicles most recently selected into the preovulatory hierarchy (e.g., 9- to 12-mm follicles) as compared to pooled granulosa cell layers from all follicles 6–8 mm in diameter ([4]; Fig. 1) and 2) granulosa cells that form a primary culture monolayer have significantly increased levels of *bcl-x*$_{Long}$ and *ita* mRNA compared to freshly collected, noncultured cells ([4]; Fig. 4).

In summary, the present studies of *ita* mRNA expression in the ovary suggest that this *iap* family member may play a role in maintaining granulosa cell, and thus follicle, viability. Moreover, based upon the positive correlation between *ita* and *bcl-x*$_{Long}$ mRNA expression in granulosa layers of 6- to 8-mm follicles, it is proposed that follicle recruitment into the preovulatory hierarchy may selectively occur from those prehierarchal follicles in which the granulosa cell layer expresses elevated levels of apoptosis-suppressing factors and are thus most resistant to undergoing apoptosis. Currently, experiments are being conducted to overexpress an *ita* antisense cDNA construct in cultured granulosa cells in an effort to evaluate the relationship between ITA protein expression and granulosa cell viability.

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