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Bcl-XLONG Protein Expression and Phosphorylation in Granulosa Cells*

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ABSTRACT

Expression of Bcl-x protein was evaluated in hen ovarian follicles, relative to stage of development, and in cultured granulosa cells after treatment with various apoptosis-suppressing or -inducing agents. Using Western blot analysis, Bcl-XLONG was most frequently observed migrating as a doublet with a molecular mass of approximately 28 kDa; the apparent higher molecular mass band of this doublet was determined to represent a phosphorylated form. Consistent with previous findings reported for *bcl*-x messenger RNA, only the Bcl-XLONG (apoptosis-suppressing) form of protein was detected in the hen granulosa cells, and highest levels of Bcl-XLONG protein (sum of the protein doublet) expression were found in granulosa from preovulatory follicles together with tissues with immune function (*e.g.* spleen and bone marrow). Evidence for Bcl-XSHORT expression was found only in the theca and several nonovarian tissues. Immunocytochemical analysis of preovulatory *vs.* prehierarchal follicles confirmed the comparatively greater expression of cytoplasmic Bcl-XLONG, particularly in preovulatory follicle granulosa. Levels of Bcl-XLONG were significantly increased after 20 h of culture in the presence of 8-bromo-cAMP (8-br-cAMP; compared with culture in control medium) in granulosa cells from both stages of follicle development. Such results are correlated with the protein's proposed function to protect against cell death in apoptosis-resistant, preovulatory follicle granulosa cells and are consistent with the ability of this cAMP agonist to increase *bcl*-XLONG messenger RNA levels in cultured cells. Furthermore, several factors that have previously been demonstrated to suppress apoptosis in granulosa cells, *in vitro*, (*e.g.* 8-br-cAMP, LH, FSH) were found to rapidly (within 15 min) increase levels of phosphorylated Bcl-XLONG, compared with control cells, whereas an inhibitor of protein kinase A (H-89) blocked such phosphorylation. By comparison, transforming growth factor α, a factor previously found to attenuate apoptosis and apoptosis-inducing agents (*e.g.* paclitaxel, C8-ceramide, daunorubicin, UV irradiation) failed to phosphorylate Bcl-XLONG. From these studies, it is concluded that both the phosphorylation of Bcl-XLONG (a short-term response) and increased levels of Bcl-XLONG (a comparatively slower response) in hen granulosa cells are promoted by gonadotropins via the adenylyl cyclase/cAMP signaling pathway. Moreover, elevated levels of chicken Bcl-XLONG protein expression and its phosphorylated state are correlated with resistance to apoptotic cell death in granulosa cells *in vitro* and ultimately a resistance to ovarian follicle atresia *in vivo*. (*Endocrinology* **140:** 4521–4529, 1999)

THE PROCESS of apoptosis not only plays an important role in the development of pathological conditions within the ovary, including premature menopause and ovarian cancers, but also contributes to the regulation of normal ovarian follicle recruitment and atresia (1–3). Among the more important cellular regulators of apoptosis is the Bcl-2-related family of proteins, because members within this family have been demonstrated to express death-suppressing or death-inducing activity (4, 5). For instance, Bcl-2 (6) and Bcl-XLONG (7, 8) decrease the susceptibility of a cell to apoptosis, at least in part, by forming heterodimers with related, but proapoptotic, proteins such as Bax, Bad, and the alternatively-spliced Bcl-XSHORT (7, 9, 10). Consistent with these data is the proposal that the ratio of death-suppressing to death-inducing proteins can ultimately determine the viability of normal and cancerous cells within the ovary (11–13). Additionally, antiapoptotic Bcl-2 family proteins have been reported to function by associating with mitochondria and inhibiting the release of cytochrome c (14, 15) and preventing the processing and activation of downstream, executioner caspases such as caspase-3 (16, 17).

Both Bcl-2 and Bcl-XLONG proteins can be phosphorylated *in vivo*, and this posttranslational modification has been proposed to determine biological function. For example, in a variety of transformed cell lines, it has been concluded that phosphorylation of Bcl-2 blocks its antiapoptotic activity, possibly by inhibiting the formation of Bcl-2/Bax heterodimers and increasing intracellular levels of Bax monomers and homodimers (18–21). Alternatively, the effects of Bcl-2 phosphorylation have been reported to occur independent of Bax or Bad heterodimerization and to be associated with activation of Raf-1 kinase and Raf-1 phosphorylation (22). This latter mechanism is proposed to mediate apoptotic cell death induced by chemotherapeutics that disrupt microtubule architecture and prevent cell division (*e.g.* paclitaxel, vincristine; Refs. 21, 23, and 24). Recently, the phosphorylation of Bcl-XLONG has similarly been associated with proapoptotic cell death, but to date, these studies have been conducted only in transformed cell lines (18).

On the other hand, phosphorylation of Bcl-2 at serine/threonine residues is also found to correlate with increased antiapoptotic activity (25, 26). For example, Ras-induced apoptosis is blocked by Bcl-2 expression but not when Bcl-2 phosphorylation is prevented by serine/threonine kinase inhibitors (26). Although reasons for the apparent functional differences (anti- *vs.* proapoptotic actions) based upon phosphorylated state are currently not clear, it is possible that the effect(s) of phosphorylation is variable, depending on the location within the molecule and extent of phosphorylation,

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cell type (*e.g.* transformed *vs.* primary culture, and cell origin), and/or death-inducing signal. An alternative, although as yet unsubstantiated, possibility is that phosphorylation of Bcl-2 and/or Bcl-XLONG is not directly related to either cell death or survival but is instead a reflection of unrelated cellular processes (27).

We have previously reported that only the long, alternatively-spliced (death-suppressing) form of the *bcl*-x messenger RNA (mRNA) transcript (*bcl*-XLONG) is detected in hen granulosa cells, and that levels of *bcl*-XLONG mRNA expression in granulosa are positively correlated with resistance to apoptosis *in vitro* and follicle viability *in vivo* (8). Specifically, hen prehierarchal follicles (≤8 mm) are highly susceptible to undergoing atresia, and it has been estimated that fewer than 10% will survive to be recruited into the preovulatory hierarchy for final differentiation before ovulation. A significant percentage of granulosa cells from prehierarchal follicles undergo apoptosis after incubation in defined medium (estimated at 60–70% after only 6 h of incubation; Ref. 28), though a proportion of these cells is resistant to apoptosis, as evidenced by their ability to survive and slowly proliferate under conventional culture conditions. It has been speculated that cells from prehierarchal follicles that survive in culture may constitutively express higher levels of Bcl-XLONG protein. Moreover, follicles already recruited into the hierarchy (preovulatory follicles) do not become atretic under normal physiological conditions, and granulosa cells from such follicles are resistant to apoptosis when cultured *in vitro*. Preovulatory follicle granulosa cells express approximately 5-fold the levels of Bcl-XLONG mRNA, compared with granulosa from prehierarchal follicles (8).

Therefore, the present studies were conducted to characterize levels of Bcl-XLONG protein in hen ovarian follicles during development, to identify cellular mechanisms that regulate Bcl-XLONG expression, and to establish the relationship between the state of Bcl-XLONG phosphorylation and apoptosis in granulosa cells. Our results provide further support for a relationship between Bcl-XLONG expression and granulosa cell resistance to apoptosis, and they indicate that Bcl-XLONG phosphorylation in this nontransformed epithelial cell type may be associated with its antiapoptotic actions.

Materials and Methods

Animals and reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 weeks of age and laying regular sequences of five or more 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) and water and were exposed to a photoperiod of 15 h light, 9 h darkness, with lights on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed, approximately 16–18 h before a midsequence ovulation, by cervical dislocation. 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 transforming growth factor-α (TGFα) was obtained from Bachem California Inc. (Torrance, CA), and 8-bromo-cAMP (8-br-cAMP), phorbol 12-myristate 13-acetate (PMA), 3-isobutyl-1-methylxanthine (IBMX), and paclitaxel were acquired from Sigma Chemical Co. (St. Louis, MO). Recombinant human FSH (rhFSH, lot R1) and ovine LH (oLH, lot 26) were from the National Hormone and Pituitary Program. N-octanoylsphingosine (C8-ceramide) was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), and daunorubicin-HCl plus H-89 were from Calbiochem (San Diego, CA). The Bcl-x polyclonal antiserum was generated in a rabbit against a 101-amino acid (AA) chicken Bcl-x fusion protein [AAs 42–142 from the chicken Bcl-XLONG coding sequence (7); Upstate Biotechnology, Inc., Lake Placid, NY]. The fusion protein was produced by ligating the Bcl-XLONG complementary DNA into the pCAL-n vector, then transforming BL21(DE3)pLysS Competent Cells (Stratagene, La Jolla, CA). The overexpressed protein was gel-purified on a 10% SDS-polyacrylamide gel, then mixed 1:1 (vol:vol) with Complete Freund's adjuvant (first injection) or Incomplete Freund's adjuvant (booster injections) for immunization (100 μg fusion protein per injection).

Tissue collection

Tissues collected for analysis of Bcl-XLONG protein expression included cerebrum, oviduct, spleen, kidney, and bone marrow. In addition, granulosa and theca tissue from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles, follicles recently selected into the follicle hierarchy (9- to 12-mm diameter follicles), prehierarchal (6–8 mm and 3–5 mm) follicles, and stromal tissue was collected and prepared as previously described (29).

Granulosa cell cultures

Granulosa cells were collected from preovulatory (F1 and F2) or prehierarchal (6–8 mm) follicles and pooled within their respective group. Granulosa cell layers from each follicle group were immediately frozen at −70 C (T0 controls) or were dispersed with 0.3% collagenase (type 2; Worthington Biochemical Corp., Freehold, NJ) and plated in 6-well polystyrene culture plates (Falcon 3046; Fisher Scientific, Itasca, IL) at a density of approximately 1.5×10^6/well in 2 ml M199-HEPES supplemented with Hanks' salts (Life Technologies, Inc., Gaithersburg, MD; M199-HEPES) and 2.5% FBS (Life Technologies, Inc.). We have previously reported that the plating efficiency of preovulatory follicle granulosa cells is greater than 80%, whereas that for prehierarchal follicle granulosa is approximately 30%, with the remainder of cells determined to undergo apoptosis (28).

In the first set of culture experiments, granulosa cells from prehierarchal or preovulatory follicles were plated in the absence and presence of 1 mM 8-br-cAMP, 9 nM TGFα, or 167 nM PMA and cultured for 20 h. 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 (8, 29, 30). The following day, viable, adherent cells were washed once with fresh medium and collected directly into 125 μl protein lysis buffer (30). Proteins were stored frozen at −70 C until used.

In a second set of culture experiments, granulosa cells from prehierarchal follicles were precultured for 20 h, then treated for 15 min in the absence (Con) or presence of 1 mM 8-br-cAMP, 100 mIU/ml rhFSH, 100 ng/ml oLH, 167 nM PMA, or 9 nM TGFα. In addition, preovulatory follicle granulosa cells were precultured for 20 h, then treated for 15 min in the absence (Con) or presence of 1 mM 8-br-cAMP, 100 mIU/ml rhFSH, 100 ng/ml oLH, 50 μM C8-ceramide, 200 nM daunorubicin, or 200 nM paclitaxel (31). One additional well of cells was UV irradiated (120,000 $\mu J/cm^2$), then cultured for an additional 15 min. A 15-min treatment time was chosen to monitor rapid phosphorylation events that may be implicated in influencing upstream cell signaling. All cells in this experiment were then washed once with fresh medium and collected directly into 125 μl protein lysis buffer, as described above. Proteins were stored frozen at −70 C until used. The ability of C8-ceramide and daunorubicin treatments, plus UV irradiation, to induce apoptotic cell death in cultured granulosa cells was verified by evaluating oligonucleosome formation after 24 h of treatment. Although paclitaxel treatment did not induce apoptosis in cultured granulosa cells, the effectiveness of the paclitaxel preparation to initiate apoptosis was verified in HeLa cells (data not shown).

A final set of experiments was conducted to further evaluate the role of the adenylyl cyclase/cAMP signaling pathway in BclXLONG phosphorylation. Granulosa cells from prehierarchal follicles were precultured for 20 h, then treated for 15 min in the absence (Con) or presence of IBMX (10 mM), oLH (1, 10, or 100 ng/ml), or IBMX plus 1 ng oLH/ml.

Additional cells were pretreated for 15 min with the protein kinase A inhibitor, H-89 (33 or 100 μM; Ref. 32), then cultured an additional 15 min in the presence or absence of 10 ng oLH/ml. Samples were processed for protein, as described above.

Phosphatase treatment of cell lysates containing Bcl-XLONG

Preovulatory follicle granulosa cells were prepared fresh (T0 sample) or precultured for 20 h, then treated with 1 mM 8-br-cAMP or vehicle (M199/HEPES) for 15 min. Cells were subsequently collected and cell lysates prepared as described above. Ten micrograms of protein was incubated in the absence or presence of the serine/threonine/tyrosine phosphatase, lambda protein phosphatase (2000 U; New England Biolabs, Inc., Beverly, MA) at 30 C for 30 min. Protein samples were subsequently analyzed by Western blot, as described below.

Western blot analysis

Granulosa and other tissues were homogenized in lysis buffer, then centrifuged at 12,000 $\times$ *g* at room temperature (RT) for 10 min, as previously described (30). The supernatant was collected and protein quantified using the DC Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Protein samples were subsequently diluted with sample buffer, heated at 70 C for 10 min (with appropriate size markers), then centrifuged at 12,000 $\times$ *g* for 2 min. Samples were separated on a 12% SDS-PAGE gel before being transferred to a nitrocellulose membrane (Nitrobind, Fisher Scientific).

Membranes were blocked 1 h at RT in Western blocking solution [5% nonfat dry milk, TBST: 10 mM Tris (pH 8.0), 100 mM sodium chloride, 0.1% Tween 20]. The Bcl-XLONG antibody was diluted 1:10,000 in blocking solution and incubated with membranes at 4 C overnight, then membranes were washed (3 $\times$ 10 min) in blocking solution. Goat antirabbit serum : horseradish peroxidase (Pierce Chemical Co., Rockford, IL) was diluted 1:10,000 in blocking solution and incubated with membranes for 1 h at RT. Membranes were successively washed in TBST (3 $\times$ 10 min). Finally, blots were incubated with ECL Western blotting detection reagent (Amersham Pharmacia Biotech) for 1 min, then were wrapped and exposed to autoradiographic film for 1–5 min. The extent of antibody binding was standardized to β-tubulin (30) as quantitated by densitometry (UltraScan XL laser densitometer, Pharmacia LKB, Piscataway, NJ).

Immunocytochemistry

Tissues were fixed in Dietrick's fixative (4% formalin, 30% ethanol, 2% glacial acetic acid), dehydrated through graded ethanols, and paraffin embedded. Sections were cut at 6 μ and mounted on Plus slides (Fisher Scientific). Slides were deparaffinized in three changes of xylene (10 min each) and rehydrated through graded ethanol washes (100%, 90%, 70%, 50%, and distilled H_2O). Tissue sections were blocked for 1 h at RT in TBS [10 mM Tris (pH 8.0), 100 mM sodium chloride] containing 1.5% goat serum, followed by incubation in a humidified chamber with Bcl-x antibody (diluted 1:200 in blocking solution) for 1.5 h at RT or overnight at 4 C. Contol sections were incubated with preimmune serum. Slides were subsequently washed 3 $\times$ 5 min in TBS, followed by incubation in goat antirabbit IgG fluorescein isothiocyanate-conjugated secondary antibody (diluted 1:200 in blocking solution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min at RT. Finally, all slides were washed 3 $\times$ 5 min in PBS and for 30 sec in PBS + 1% Triton-X100 (Fisher Scientific), rinsed in H_2O, and mounted in an aqueous quencher (Aqua poly mount, Polysciences Inc., Warrington, PA). Images were collected with a MRC1024 laser confocal microscope (Bio-Rad Laboratories, Inc.) and analyzed using the Lasersharp software package (Bio-Rad Laboratories, Inc.).

Data analysis

Northern and Western blot experiments were repeated a minimum total of three times. Some of the data were analyzed by paired *t* test (*e.g.* T0 *vs.* T20 Con; see Figs. 5 and 8). Bcl-XLONG protein levels (total protein in doublet) and phosphorylated Bcl-XLONG (*upper band* of doublet) were also expressed as fold-difference (mean $\pm$ SEM) *vs.* a designated reference tissue (*e.g.* analysis of nonovarian and follicle hierarchy tissues), and cultured cells were compared with levels found in freshly collected (T0) cells (value for reference tissue arbitrarily set at 1). Before statistical analysis of culture experiments, it was established that only treatment groups, compared with the control cultured group, would be evaluated. Data were analyzed by one-way ANOVA (analysis of fold-difference data did not include the reference value) and the Fisher protected least-significant-difference multiple-range test (33).

Results

Bcl-XLONG protein expression and immunolocalization

Chicken Bcl-XLONG protein is most frequently detected, by Western blot analysis, as a doublet with a molecular mass of approximately 28 kDa. Protein levels (sum of the doublet protein) during follicle development are highest in granulosa cells from the two largest (F2 and F1) preovulatory follicles (Fig. 1). By contrast, Bcl-XLONG levels are not different during development within the theca layer (Fig. 2). Immunocytochemical analysis of preovulatory and prehierarchal follicles largely confirms the increased expression of cytoplasmic Bcl-XLONG within the preovulatory follicle granulosa layer, and it shows scattered regions of Bcl-XLONG expression within the theca of both prehierarchal and preovulatory follicles (Fig. 3). Outside of the ovary, the highest levels of Bcl-XLONG protein expression occur in the kidney and immune system tissues, bone marrow, and spleen, whereas comparatively lower levels are consistently detected in the oviduct and cerebrum (Fig. 4). Also of note was the detection of the alternatively spliced Bcl-XSHORT in theca, kidney, spleen, and cerebrum tissues, but not granulosa cells.

Regulation of Bcl-XLONG protein levels in granulosa cells

There is a significant increase in the amount of Bcl-XLONG protein in granulosa cells from 6- to 8-mm follicles that form a monolayer after 20 h of culture in control medium (Con), compared with freshly collected (T0) samples ($P < 0.01$; Fig. 5, *top*). However, a further increase in protein, above already

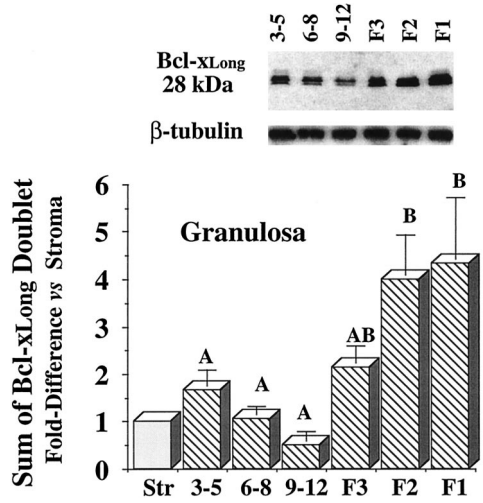

FIG. 1. Bcl-XLONG (sum of protein doublet) in granulosa tissue from ovarian follicles during development. Bcl-XLONG data are standardized to β-tubulin and are expressed as a fold-difference compared with a reference tissue, ovarian stroma (Str). F1, F2, and F3 indicate the largest through third largest preovulatory follicle, respectively; 9–12 mm, 6–8 mm, and 3–5 mm refer to diameter of follicles. n = 3–5 replicate experiments. A, B: mean $\pm$ SEM, $P \leq 0.05$.

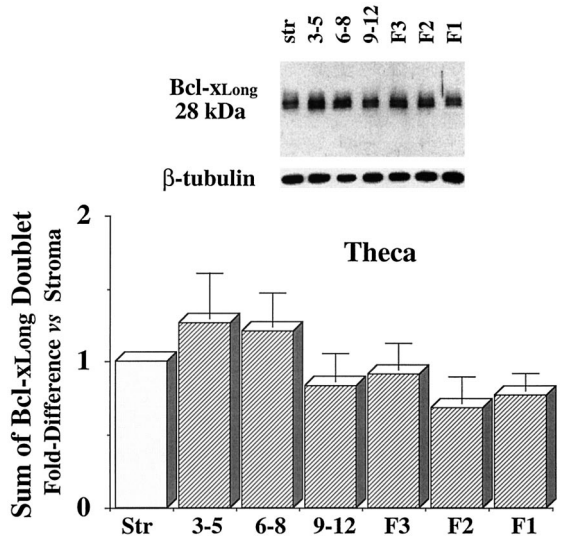

FIG. 2. Bcl-XLONG (sum of protein doublet) in theca tissue from ovarian follicles during development. Data are standardized to β-tubulin and are expressed as a fold-difference compared with a reference tissue, Str. Abbreviations are as in Fig. 1. Data represent mean ± SEM from three to five replicate experiments. $P > 0.05$, across all stages of development.

elevated levels, compared with prehierarchal follicles (see Fig. 1), is not observed after an overnight plating of granulosa cells from preovulatory follicles (T0 *vs.* Con; Fig. 5, *bottom*). Granulosa cells from follicles at both stages of development, cultured for 20 h in the presence of 8-br-cAMP, show a significant increase in Bcl-XLONG levels, compared with Con cells ($P < 0.01$), whereas TGFα has no effect; PMA treatment decreases Bcl-XLONG levels only in preovulatory follicle granulosa cells ($P < 0.05$).

Phosphorylation of Bcl-XLONG

Short-term treatment of cultured granulosa cells with 8-br-cAMP preferentially increases the proportion of a slower migrating (*upper*) band of Bcl-XLONG protein (Fig. 6). Treatment of cell lysate from 8-br-cAMP-treated cells with λ protein phosphatase virtually eliminates this *upper band* of Bcl-XLONG, and it results in accumulation of protein within the faster migrating, lower band. There is no evidence from Coomassie-stained gels (data not shown) or from the immunoreactive Bcl-XLONG or tubulin proteins (Fig. 6A) that the phosphatase treatment has any nonspecific proteolytic effects. It is concluded that, as recently reported for mammalian species (18), the upper band of the doublet represents an inducible, phosphorylated form of Bcl-XLONG.

In retrospect, an analysis of phosphorylated Bcl-XLONG from freshly collected follicle tissues (see Fig. 1) was conducted, and it was determined that levels within theca tissue during follicle development do not change ($P > 0.10$). By contrast, the amount of phosphorylated protein in the granulosa layer, expressed as a fold-difference *vs.* 3- to 5-mm follicles, is higher within each of the three largest preovulatory follicles, compared with 9- to 12-mm or 6- to 8-mm follicles ($P < 0.05$; Fig. 6B).

Short-term culture (15 min) in the absence or presence of various physiological and pharmacological agents did not alter the total amount of total Bcl-XLONG protein quantitated (*e.g.* the sum of the doublet protein) in granulosa cells from either prehierarchal or preovulatory follicles ($P > 0.01$; summarized data not shown). By contrast, 15 min of treatment with 8-br-cAMP, rhFSH, or oLH increased levels of phosphorylated Bcl-XLONG, compared with Con cells in granulosa from prehierarchal follicles (Fig. 7, *top*), whereas 8-br-cAMP and oLH (but not rhFSH) treatment significantly increased levels of phosphorylated Bcl-XLONG in preovulatory follicle granulosa (Fig. 7, *bottom*). Agents determined to induce apoptosis in hen preovulatory follicle granulosa cells (*e.g.* C8-ceramide, daunorubicin, and UV treatment; Ref. 28) or to promote Bcl-x phosphorylation in human malignant cells (*e.g.* paclitaxel; Ref. 18) did not increase levels of phosphorylated Bcl-XLONG, compared with control cells.

Levels of phosphorylated Bcl-XLONG decrease by approximately 60% after 20 h in culture (T0 *vs.* Con, $P < 0.05$; Fig. 8). Treatment with oLH, at doses of 10 and 100 (but not 1) ng/ml, significantly increases the level of phosphorylation, compared with Con. Although IBMX alone fails to alter levels of phosphorylation (compared with Con), IBMX plus 1 ng oLH/ml significantly increases phosphorylation. Finally, pretreatment of cultured preovulatory follicle granulosa, with the higher dose of the protein kinase A inhibitor, H-89 (100 μM), blocks the phosphorylation response after oLH treatment.

Discussion

The most significant findings from the present studies include the demonstration that: 1) Bcl-XLONG protein levels and the extent of its phosphorylation in cultured hen granulosa cells are increased by gonadotropins via a cAMP-mediated pathway; and 2) there exists a positive association between levels of induced phosphorylated Bcl-XLONG and agents that promote granulosa cell viability *in vitro*. Moreover, consistent with previously published *bcl*-XLONG mRNA data, higher levels of constitutively expressed Bcl-XLONG protein, such as those found in preovulatory (compared with prehierarchal) follicle granulosa cells, are associated with an inherent resistance to apoptosis normally found in cultured granulosa cells at this terminal stage of differentiation (8).

This is the first report to document patterns of chicken Bcl-XLONG (28 kDa) and Bcl-XSHORT (18 kDa) protein expression in avian cells, and the results presented generally parallel previously published profiles of *bcl*-XLONG mRNA from immune and ovarian tissues (7, 8). Specifically, there is no evidence for the expression of the alternatively spliced, proapoptotic Bcl-XSHORT form of the protein in granulosa cells, although the theca and several nonovarian tissues (kidney, spleen, and cerebrum) show varying levels of the protein. Bcl-XSHORT expression has previously been documented within tissues of the immune system (7), but the precise function and mechanisms by which it putatively acts in a proapoptotic fashion have not been fully elucidated. Expression of Bcl-XSHORT has also been documented within the rat ovary (11); however, these studies did not differentiate expression within the granulosa *vs.* theca layers. The present results suggest that the low levels of Bcl-XSHORT detected in the rat ovary may be of thecal origin. The highest

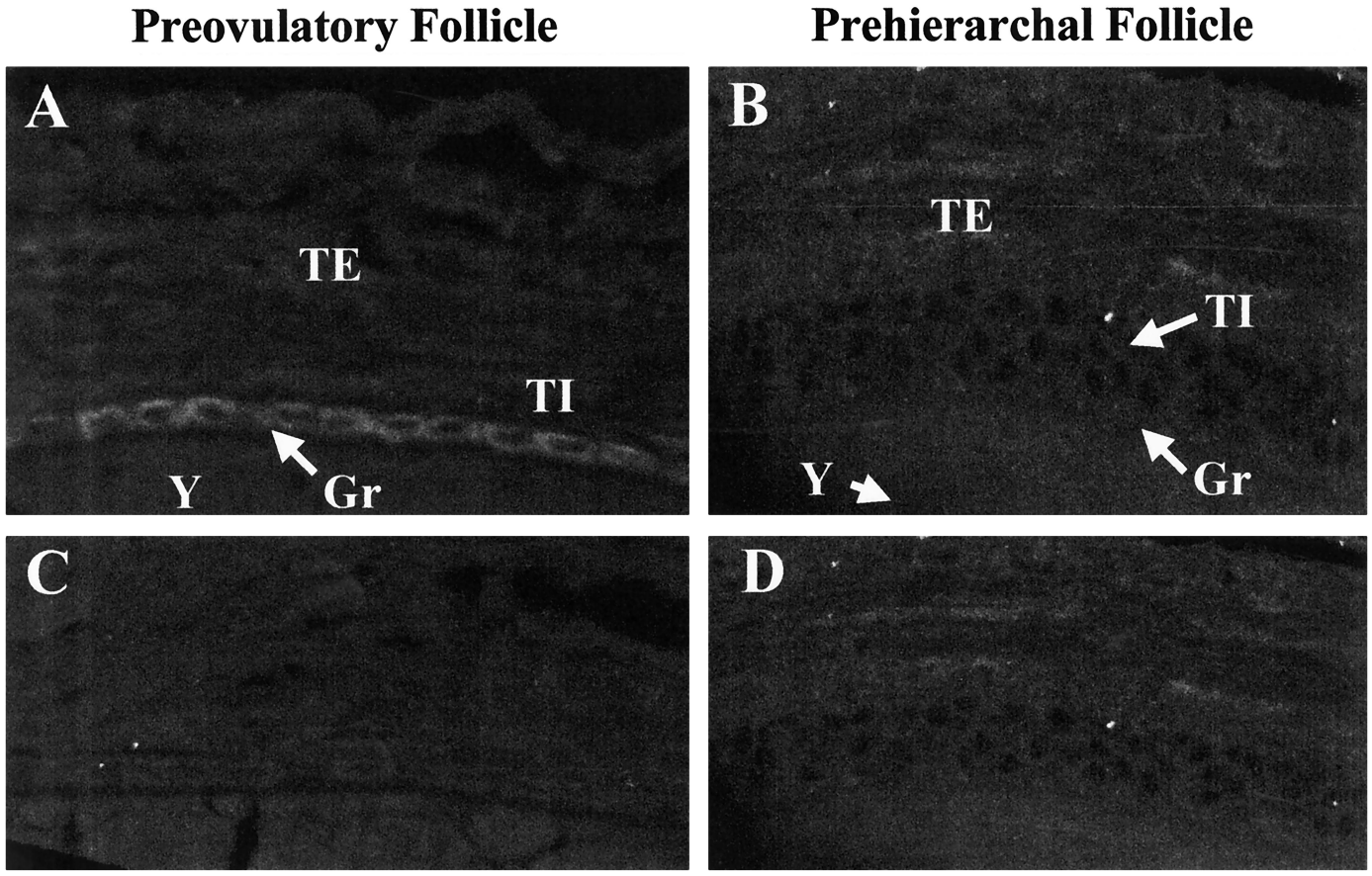

FIG. 3. Representative sections showing the distribution of Bcl-XLONG protein in preovulatory (A) and prehierarchal (B) follicles by immunocytochemical analysis. C and D, Controls after incubation with preimmune serum. Note the prominent presence of Bcl-XLONG protein (*arrowheads*) in cytoplasm of granulosa (Gr) from preovulatory, but not prehierarchal, follicles. By comparison, there is some degree of Bcl-XLONG staining in prehierarchal follicle theca interna (TI) and theca externa (TE). Y, Yolk. Magnification: ×600.

levels of Bcl-XLONG were consistently detected within the granulosa layer of preovulatory follicles, as determined by both immunoblot analysis and immunocytochemistry (Figs. 1–4). The pattern of granulosa cell cytoplasmic staining, in the absence of nuclear localization, is similar to that previously reported for a variety of mouse and human tissues (34) and is consistent with its well-documented cytoplasmic and mitochondrial sites of actions (5). Given the very low levels of Bcl-XSHORT detectable by Western blot analysis (Fig. 4), it is unlikely that this alternatively-spliced form contributes much, or any, signal detected by immunocytochemistry.

However, unlike *bcl*-XLONG mRNA, protein levels were not found to be increased in granulosa from 9- to 12-mm follicles (recruited within the previous 24 h from a cohort of prehierarchal follicles), compared with 6- to 8-mm follicle granulosa. Presumably, this is attributable to a lag in time from mRNA transcription to translation of the Bcl-XLONG protein. Though much, if not all, of the increased Bcl-XLONG protein is attributed to newly synthesized protein, we cannot entirely rule out the possibility of a small decrease in protein degradation. We have previously reported that follicle selection into the preovulatory hierarchy is associated with both increased *bcl*-XLONG mRNA expression and the acquisition of resistance to apoptosis, *in vitro*, in granulosa from 9- to 12-mm follicles. We speculated that the increased *bcl*-

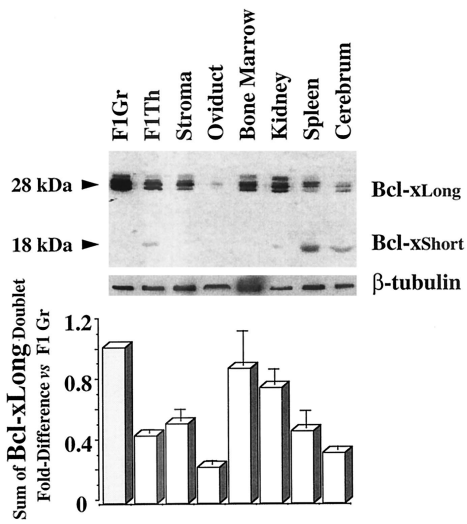

FIG. 4. Representative Western blot for Bcl-XLONG and Bcl-XSHORT proteins and summary of scanning data for Bcl-XLONG in various tissues from the domestic hen, expressed as a fold-difference compared with granulosa tissue from the largest preovulatory follicle (F1Gr). F1Th, Theca from the F1 follicle; Ovi, oviduct; BM, bone marrow; Kid, kidney; Spl, spleen; Cer, cerebrum. Mean ± SEM from four or five replicate observations.

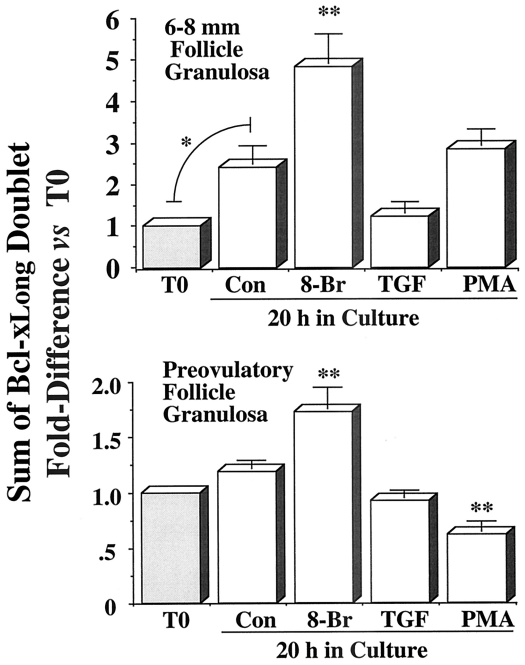

FIG. 5. Bcl-XLONG (sum of protein doublet) in granulosa cells from 6- to 8-mm follicles (*top*) or preovulatory follicles (*bottom*), collected immediately after dispersion (T0) or cultured for 20 h in the absence (Con) or presence of 1 mM 8-Br, 9 nM TGFα (TGF), or 167 nM PMA. Note difference in scale on ordinate between panels. Levels of Bcl-XLONG were standardized to β-tubulin and were expressed as a fold-difference (mean ± SEM from three to five replicate experiments) compared with T0. *, Paired *t* test showed a significant increase in Bcl-XLONG protein in granulosa from 6- to 8-mm follicles plated for 20 h, compared with T0 samples ($P < 0.05$). Additional statistical comparisons were performed on fold-difference data (excluding the T0 group) to compare treatment groups *vs.* the control (Con). **, $P < 0.01$.

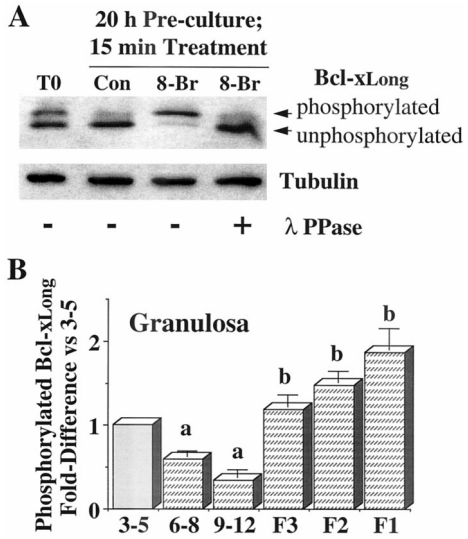

FIG. 6. A, cAMP-induced phosphorylation of Bcl-XLONG. Granulosa cells from preovulatory follicles were processed fresh (T0) or were precultured for 20 h. Treatment with 8-br (1 mM) for 15 min results in the accumulation of the Bcl-XLONG upper (phosphorylated) band. Subsequent incubation of cell lysate with λ protein phosphatase (+ PPase) restores the original mobility (compare *vs.* Con). Levels of tubulin show equal loading across treatments and indicate the lack of nonspecific proteolytic activity after PPase treatment. This experiment was repeated once with similar results. B, Analysis of phosphorylated Bcl-XLONG in granulosa cells from follicles during development, as determined from the scanning data presented in Fig. 1. Bcl-XLONG is presented as levels of phosphorylated Bcl-XLONG (*top band*) compared with that in 3- to 5-mm follicles. a and b, $P < 0.05$.

XLONG mRNA levels within the 9- to 12-mm follicle granulosa layer may be prerequisite for facilitating follicle selection (8, 35). Though data provided herein do not seem to support this initial proposal, the presence of elevated Bcl-XLONG protein in granulosa from the largest preovulatory follicles is consistent with a role for this antiapoptotic protein in protection against cell death, *in vitro*, and follicle atresia, *in vivo*. It remains possible that increased expression of other putative antiapoptotic proteins, such as the chicken inhibitor of apoptosis protein, ITA, may be more critical for the process of follicle selection into the preovulatory hierarchy (35).

Similar to the human Bcl-XLONG protein (18, 36), the approximate 28-kDa chicken homolog is commonly detected as a protein doublet, with the slower migrating band representing a phosphorylated form. Sequence analysis of the chicken Bcl-XLONG primary AA sequence reveals a total of 20 serine and 12 threonine residues that could potentially serve as phosphorylation sites throughout the protein (7). However, it has previously been reported that agonist-induced phosphorylation of the human Bcl-2 and Bcl-x proteins occurs on serine and threonine residues primarily within a highly variable loop region located between the BH4 and BH3 domains (19, 37, 38) and specifically at serine-70 (39). Significantly, there are 7 serine (including a conserved serine-70) and 3 threonine residues plus one cAMP consensus phosphorylation site (Arg-Thr-Asp-Thr) within this region in the chicken Bcl-XLONG protein. Though there generally occurs an increase in a single predominant band of phosphorylated protein after treatment with the gonadotropins, LH and FSH, occasionally there is evidence of additional phosphorylated forms (hyperphosphorylation) after treatment with the supraphysiological cAMP analog, 8-br-cAMP (*e.g.* Fig. 7, *bottom*). It is not yet clear whether such forms are of physiological relevance.

It is of significance to note that 8-br-cAMP and oLH, but not rhFSH, induce phosphorylation of Bcl-XLONG in preovulatory follicle granulosa. Furthermore, IBMX potentiates the effects of a noneffective dose of oLH, whereas inhibition of protein kinase A activity, using a pharmacologic blocker (H-89; Ref. 32), blocks the ability of oLH (10 ng/ml) to phosphorylate Bcl-XLONG. First, these findings indicate involvement of the adenylyl cyclase/cAMP pathway, perhaps acting at the cAMP consensus phosphorylation motif described above. Second, the differential phosphorylation in response to oLH, compared with rhFSH, is correlated to expression of the highest LH-receptor (R) mRNA levels during follicle development, and decreasing levels of FSH-R expression, in preovulatory follicle granulosa cells; moreover, rhFSH treatment fails to induce steroidogenesis in preovulatory follicle granulosa (40–42).

By comparison, both gonadotropins promote Bcl-XLONG phosphorylation in prehierarchal follicle granulosa cells that survive, after an overnight plating. We have previously reported that there exist high levels of the FSH-R transcript in 6- to 8-mm follicle granulosa, and that rhFSH treatment of

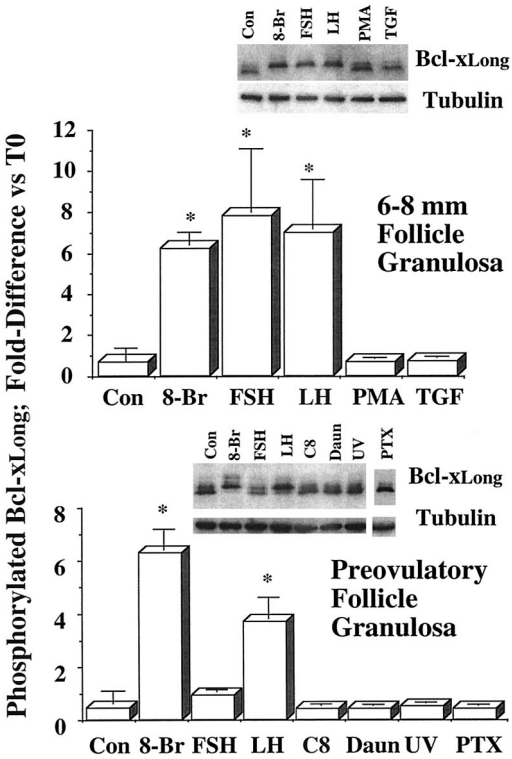

FIG. 7. Levels of phosphorylated Bcl-XLONG, standardized to β-tubulin and expressed as fold-difference compared with freshly collected (T0; data not shown) cells from 6- to 8-mm follicles (*top panel*) or preovulatory follicles (*bottom panel*). Cells were precultured for 20 h, then treated for 15 min in the absence (Con) or presence of 1 mM 8-Br, 100 mIU rhFSH, 100 ng/ml ovine LH, 167 nM phorbol ester (PMA), 9 nM TGFα (TGF), 50 μM C8-ceramide (C8), 200 nM daunorubicin (Daun), UV light (120,000 μJ/cm^2), or 200 nM paclitaxel (PTX). There is some evidence that a supraphysiological signal, such as 1 mM 8-br-cAMP, may induce a hyperphosphorylated state (additional slower migrating bands seen in *bottom panel*). Data were standardized to β-tubulin, and expressed as a mean ± SEM from three to five replicate experiments. *, $P < 0.05$ *vs.* Con.

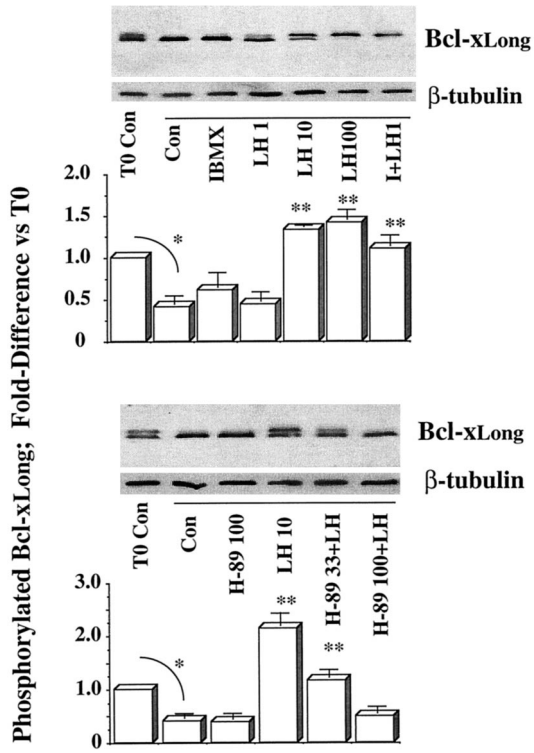

FIG. 8. Levels of phosphorylated Bcl-XLONG in cultured granulosa cells from preovulatory follicles, standardized to β-tubulin and expressed as fold-difference compared with freshly collected (T0) cells. *Top*, Cells were precultured for 20 h, then treated for 15 min in the absence (Con) or presence of IBMX (10 mM), LH (1, 10, or 100 ng/ml) or IBMX plus 1 ngLH/ml; *bottom*, cells were cultured for 20 h then pretreated for 15 min in the absence or presence of the protein kinase A inhibitor, H-89 (33 or 100 μM), then treated an additional 15 min in the absence or presence of 10 ng LH/ml. *, Paired *t* test showed a significant decrease in Bcl-XLONG phosphorylation after culture for 20 h, compared with T0 samples ($P < 0.05$). Additional statistical comparisons were performed on fold-difference data (excluding the T0 group) to compare treatment groups *vs.* Con. **, $P < 0.05$.

such cells, *in vitro*, induces cAMP accumulation (29). Similarly, oLH promotes Bcl-XLONG phosphorylation, even though levels of LH-R mRNA are comparatively low in cultured 6- to 8-mm granulosa cells. It is of significance to note that 8-br-cAMP treatment attenuates the progression of apoptosis in incubated prehierarchal follicle granulosa cells (8). Although TGFα is also known to attenuate the progression of apoptotic cell death in prehierarchal follicle granulosa cells, its actions are thought to be mediated via tyrosine kinase (not serine/threonine kinase) activity and thus predicted not to induce Bcl-XLONG phosphorylation. Finally, Bcl-XLONG phosphorylation also occurs in primary cultures of theca, ovarian stroma, and liver tissue after a 15-min treatment with 8-br-cAMP (data not shown), indicating that this phosphorylation event is not limited to cells of epithelial origin.

We have previously reported that a majority of prehierarchal follicle granulosa cells rapidly succumbs to apoptosis when cultured, *in vitro*, whereas a small proportion of these cells is resistant to cell death and survives to form a monolayer (8). Consistent with previously published results for *bcl*-XLONG mRNA (30) was the finding that Bcl-XLONG protein levels were similarly increased (by 2.4-fold) in the population of 6- to 8-mm follicle granulosa cells that successfully plated after culture for 20 h (Fig. 5, *top*). This result suggests that the survival of prehierarchal follicle granulosa cells, *in vitro*, may be directly or indirectly associated with elevated levels of Bcl-XLONG protein. Also consistent with *bcl*-XLONG mRNA (30) and levels of phosphorylated Bcl-XLONG (presented herein) was the finding that a 20-h treatment with 8-br-cAMP, but not TGFα or PMA, increased levels of Bcl-XLONG protein in both prehierarchal and preovulatory follicle granulosa cells (Fig. 5, *top* and *bottom*). Collectively, the implication of these findings is that both FSH and LH, acting via cAMP, function not only to promote granulosa cell proliferation and differentiation but also cell survival. The significance of the decrease in Bcl-XLONG levels in preovulatory follicle granulosa cells after treatment with PMA is less obvious, because this phorbol ester was previously found not to alter levels of *bcl*-x mRNA.

There are a number of recent reports documenting the relationship between agonist-induced phosphorylation of Bcl-2 and induction of apoptosis in a variety of transformed mammalian cell lines (*e.g.* 21, 22). Therefore, it was of interest

to evaluate the effects of several agents previously shown to induce apoptotic cell death in primary cultures of hen preovulatory follicle granulosa cells. Several agents used in the present experiments are proposed to initiate cell death via different cellular mechanims, although the complete pathway for any of these has yet to be defined. These include daunorubicin, which acts via the release of endogenous ceramide and activation of the stress-activated protein kinase pathway (28, 43, 44), and UV irradiation, which may target activation of the p53 tumor suppressor gene (28, 45). In addition, paclitaxel has previously been shown to promote Bcl-2 phosphorylation and induce apoptosis in several mammalian cell types, and its cell death-promoting effects are thought to be related to the ability of the drug to induce mitotic arrest (20–22, 31). Nevertheless, none of these agents was found to alter levels of phosphorylated Bcl-XLONG after short-term treatment of cultured preovulatory follicle granulosa (Fig. 7, *bottom*), thus providing no evidence for a relationship between Bcl-XLONG phosphorylation and the induction of apoptosis in this untransformed cell type. The inability of paclitaxel to induce apoptotic cell death in preovulatory follicle granulosa was not entirely unexpected, because the vast majority of preovulatory follicle granulosa cells are terminally differentiated and nonmitotic. More importantly, however, it has recently been questioned whether there is a direct relationship between bcl-2 phosphorylation and paclitaxel-induced cell death (27). For instance, phosphorylated Bcl-2 was found to remain complexed with Bax in transformed cells after paclitaxel treatment. Furthermore, phosphorylation of Bcl-2 was proposed to be more closely associated with the Cdc2 signaling cascade and mitotic activity than with events signaling apoptosis.

In conclusion, results from the present studies provide evidence for an association between Bcl-XLONG protein levels (total and phosphorylated) and its putative antiapoptotic function in hen ovarian granulosa cells. Though the only other report to date describing physiological consequence of Bcl-x phosphorylation concludes that its function is proapoptotic (18), it is noted that this study, as well as most studies of bcl-2 (*e.g.* 20–24), involved only transformed cell lines. Accordingly, it is difficult to directly relate results from transformed cells to primary cell cultures. It is possible that multiple serine/threonine kinases, differentially expressed during cell differentiation and/or regionally localized within the cell cytoplasm, may be involved in Bcl-XLONG phosphorylation. Such differences, including the number and location of phosphorylated residues within the primary sequence, may ultimately determine whether Bcl-XLONG exhibits an antiapoptotic or proapoptotic function. Alternatively, it remains a possibility that phosphorylation of Bcl-XLONG, as recently discussed for Bcl-2 (27), may not be directly related to facilitation or inhibition of apoptotic cell death but, rather, is a reflection of kinase activity involved in the regulation of mitosis or cell differentiation. Additional studies are required to differentiate among the above possibilities.

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References

1. **Ratts VS, Flaws JA, Kolp R, Sorenson CM, Tilly JL** 1995 Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. Endocrinology 136:3665–3668
2. **Hsueh AJ, Eisenhauer K, Chun SY, Hsu SY, Billig H** 1996 Gonadal cell apoptosis. Recent Prog Horm Res 51:433–455
3. **Tilly JL, Johnson AL** 1999 In: Hickman J, Dive C (eds) Chemotherapy and Apoptosis in the Ovary: Treatment Comes with a Price. Humana Press Inc., Totowa, NJ, pp 257–273
4. **Yang E, Korsmeyer SJ** 1996 Molecular thanotopsis: a discourse on the Bcl-2 family and cell death. Blood 88:386–401
5. **Reed JC** 1997 Double identity for proteins of the Bcl-2 family. Nature 387:773–776
6. **Hockenbery DM** 1995 bcl-2, a novel regulator of cell death. Bioessays 17:631–637
7. **Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB** 1993 bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597–560
8. **Johnson AL, Bridgham JT, Witty JP, Tilly JL** 1996 Susceptibility of avian granulosa cells to apoptosis is dependent upon stage of follicle development and is related to endogenous levels of *bcl*-xlong gene expression. Endocrinology 137:2059–2066
9. **Oltvai ZN, Milliman CL, Korsmeyer SJ** 1993 Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619
10. **Zha J, Harada H, Osipov K, Jockel J, Waksman G, Korsmeyer SJ** 1997 BH3 domain of Bad is required for heterodimerization with BCL-X_L and proapoptotic activity. J Biol Chem 272:24101–24104
11. **Tilly JL, Tilly KI, Kenton ML, Johnson AL** 1995 Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology 136:232–241
12. **Marx D, Binder C, Meden H, Lenthe T, Ziemek T, Hiddemann T, Kuhn W, Schauer A** 1997 Differential expression of apoptosis associated genes bax and bcl-2 in ovarian cancer. Anticancer Res 17:2233–2240
13. **Witty JP, Jensen RA, Johnson AL** 1998 Expression and localization of BCL-2 related proteins in human ovarian cancers. Anticancer Res 18:1223–1230
14. **Kluck RM, Bossey-Wetzel E, Green DR, Newmeyer DD** 1997 The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136
15. **Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng I-I, Jones DP, Wang X** 1997 Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275:1129–1132
16. **Monney L, Otter I, Olivier R, Ravn U, Mirzasaleh H, Fellay I, Poirier GG, Borner C** 1996 Bcl-2 overexpression blocks activation of the death protease CPP32/Yama/apopain. Biochem Biophys Res Commun 221:340–345
17. **Lindenboim L, Haviv R, Stein R** 1998 Bcl-xL inhibits different apoptotic pathways in rat PC12 cells. Neurosci Lett 253:37–40
18. **Poruchynsky MS, Wang EE, Rudin CM, Blagosklonny MV, Fojo T** 1998 Bcl-xL is phosphorylated in malignant cells following microtubule disruption. Cancer Res 58:3331–3338
19. **Chang BS, Minn AJ, Muchmore SW, Fesik SW, Thompson CB** 1997 Identification of a novel regulatory domain in Bcl-x(L) and Bcl-2. EMBO J 16:968–977
20. **Ibrado AM, Liu L, Bhalla K** 1997 Bcl-xL overexpression inhibits progression of molecular events leading to paclitaxel-induced apoptosis of human acute myeloid leukemia HL-60 cells. Cancer Res 57:1109–1115
21. **Srivastava RK, Srivastava AP, Korsmeyer SJ, Nesterova M, Cho-Chung YS, Longo DL** 1998 Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol Cell Biol 18:3509–3517
22. **Blagosklonny MV, Gianakakou P, El-Deiry WS, Kingston DGI, Higgs PI, Neckers L, Fojo T** 1997 Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. Cancer Res 57:130–135
23. **Haldar S, Jena N, Croce CM** 1994 Antiapoptosis potential of bcl-2 oncogene by dephosphorylation. Biochem Cell Biol 72:455–462
24. **Haldar S, Chintapalli J, Croce CM** 1996 Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res 56:1253–1255
25. **Chen C-Y, Faller DV** 1996 Phosphorylation of Bcl-2 protein and association with p21Ras in Ras-induced apoptosis. J Biol Chem 271:2376–2379
26. **Ito T, Deng X, Carr B, May WS** 1997 Bcl-2 phosphorylation required for anti-apoptotic function. J Biol Chem 272:11671–11673
27. **Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD, Pietenpol JA** 1998 Mitotic phosphorylation of bcl-2 during normal cell cycle progression and taxol-induced growth arrest. J Biol Chem 273:30777–30784
28. **Witty JP, Bridgham JT, Johnson AL** 1996 Induction of apoptotic cell death in hen granulosa cells by ceramide, *in vitro*. Endocrinology 137:5269–5277
29. **Tilly JL, Kowalski KI, Johnson AL** 1991 Stage of ovarian follicular development associated with the initiation of steroidogenic competence in avian granulosa cells. Biol Reprod 44:305–314

30. **Johnson AL, Bridgham JT, Witty JP, Tilly JL** 1997 Expression of bcl-2 and nr-13 in hen ovarian follicles during development. Biol Reprod 57:1096–1103
31. **Long BH, Fairchild CR** 1994 Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. Cancer Res 54:4355–4361
32. **Busch S, Wieland T, Esche H, Jakobs KH, Siffert W** 1995 G protein regulation of the Na+/H+ antiporter in Xenopus laevis oocytes. Involvement of protein kinases A and C. J Biol Chem 270:17898–17901
33. **Sokal RR, Rohlf FJ** 1995 Biometry. Freeman, New York, 887 pp
34. **Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L, Reed JC** 1994 Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. Cancer Res 54:5501–5507
35. **Johnson AL, Bridgham JT, Digby MR, Lowenthal JW** 1998 Expression of the inhibitor of T-cell apoptosis (*ita*) gene in hen ovarian follicles during development. Biol Reprod 58:414–420
36. **Broome HE, Dargan CM, Krajewski S, Reed JC** 1995 Expression of Bcl-2, Bcl-x, and Bax after T cell activation and IL-2 withdrawal. J Immunol 155:2311–2317
37. **Uhlmann EJ, D'Sa-Eipper C, Subramanian T, Wagner AJ, Hay N, Chinnadurai G** 1996 Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. Cancer Res 56:2506–2509
38. **Fang G, Chang BS, Kim CN, Perkins C, Thompson CB, Bhalla KN** 1998 "Loop" domain is necessary for taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting taxol-induced cytosolic accumulation of cytochrome c and apoptosis. Cancer Res 58:3202–3208
39. **Haldar S, Basu A, Croce CM** 1998 Serine-70 is one of the critical sites for drug-induced Bcl2 phosphorylation in cancer cells. Cancer Res 58:1609–1615
40. **Johnson AL, Bridgham JT, Wagner B** 1996 Characterization of a chicken luteinizing hormone receptor (cLH-R) cDNA, and expression of cLH-R mRNA in the ovary. Biol Reprod 55:304–309
41. **You S, Bridgham JT, Foster DN, Johnson AL** 1996 Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R), and expression of cFSH-R mRNA in the ovary. Biol Reprod 55:1055–1062
42. **Johnson AL** 1998 Ovarian cycles and follicle development in birds. In: Knobil E, Neill JD (eds) Encyclopedia of Reproduction. Academic Press, New York, vol 3:132–142
43. **Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R** 1995 Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. Cell 82:405–414
44. **Verheij M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Friedman A, Fuks Z, Kolesnick RN** 1996 Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380:75–79
45. **Wang Y, Rosenstein B, Goldwyn S, Zhang X, Lebwohl M, Wei H** 1998 Differential regulation of P53 and Bcl-2 expression by ultraviolet A and B. J Invest Dermatol 111:380–384