Expression and Function of a Proapoptotic Bcl-2 Family Member Bcl-XL/Bcl-2-Associated Death Promoter (BAD) in Rat Ovary*

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ABSTRACT
Bcl-2-related anti- and proapoptotic proteins are important in the decision step of the intracellular death program upstream from the caspase proteases. Targeted overexpression of Bcl-2 in ovarian somatic cells of transgenic mice leads to decreased apoptosis of granulosa cells and is associated with higher ovulation rate, increased litter size, and ovarian teratoma formation. The ability of exogenous Bcl-2 proteins to promote follicle cell survival suggests that the transgene can bind to endogenous ovarian Bcl-2 family members and modulate the intracellular apoptosis process in favor of cell survival. We used the yeast two-hybrid system to search for ovarian Bcl-2 interacting proteins. The screening of an ovarian fusion complementary DNA library yielded several clones encoding for the death agonist Bcl-XL/Bcl-2-associated death promoter (BAD). Dimerization of Bcl-2-related proteins mediated by the consensus Bcl-2 homology (BH) domains is essential for their apoptosis-regulating function. Consistent with these observations, yeast two-hybrid assays indicated that the interaction of Bcl-2 with BAD is dependent on both BH4 and BH2 domains of Bcl-2. Northern blot analysis showed a wide distribution of BAD messenger RNA (mRNA) in diverse tissues with highest levels in the lung, ovary, uterus, and brain. In situ hybridization analysis indicated BAD mRNA expression in granulosa cells of different sizes of follicles and also in the theca and interstitial cells. BAD mRNA was expressed in the ovaries between postnatal 15–27 days and did not alter during the developmentally occurring apoptosis found about postnatal day 18 when the first group of early antral follicles were formed. Similarly, BAD mRNA levels did not change during follicle atresia induced by estrogen withdrawal in immature rats. To study the role of BAD in the ovary, BAD complementary DNA was transfected into primary cultures of granulosa cells and in a gonadal tumor cell line. Overexpression of BAD induced apoptosis in both cell types, and the effect of BAD was reversed by a membrane-permeable caspase inhibitor, indicating that BAD induces apoptosis via the activation of caspase cysteine proteases. In summary, the death agonist BAD was identified as a Bcl-2-interacting protein in the ovary, and BAD mRNA is constitutively expressed in granulosa cells, suggesting that BAD is an essential part of the ovarian cell death process. Because BAD overexpression in granulosa cells leads to apoptosis, future studies on ovarian BAD binding proteins and hormonal regulation of the interactions among different Bcl-2 family members could provide a better understanding of the cellular mechanism of ovarian follicle atresia. (Endocrinology 138: 5497–5504, 1997)

In the mammalian ovary, only a small fraction of oocytes ovulate during the reproductive life, whereas the majority of ovarian follicles undergo atresia by a hormonally regulated apoptotic mechanism (1, 2). With a constant loss of follicles from the original pool, the ovary provides a unique model for studying the hormonal regulation of apoptosis. Gonadotropins, estrogens, several growth factors (epidermal growth factor, transforming growth factor-α, basic fibroblast growth factor, and insulin growth factor-I), and cytokines (interleukin-1β) can act as survival factors to rescue follicles from apoptotic demise (1–4). In contrast, androgens, GnRH, and tumor necrosis factor-α, are atretogenic factors (1, 5, 6). Although the extracellular signals regulating cell survival are often cell- and tissue-specific, a growing body of evidence suggests that the intracellular death program that is activated during apoptosis is similar in different cell types and conserved during evolution (2, 7).

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Recent investigations have shown that during apoptosis the activation of caspases, cysteine proteases related to the Caenorhabditis elegans protease ced (cell death)-3, results in cell death via proteolytic cleavage of substrate proteins vital to the maintenance of cellular homeostasis (7). In addition, the Bcl-2 family of proteins that consists of different anti- and proapoptotic members is important in the decision step of apoptosis (8). Bcl-2-related proteins act upstream from caspase proteases in the cell death pathway (9), and the balance between pro- and antiapoptotic Bcl-2-related proteins determines the fate of the cell (10). Recent studies further demonstrated that another C. elegans gene, ced-4, could bind to both Bcl-2 family members and caspases thus bridging the essential components of the cell death machinery (11).

The exact mechanism by which hormonal signals regulate the intracellular cell death process is not clear. A possible link between signal transduction initiated by extracellular survival factors and the cell death pathway is Bcl-XL/Bcl-2-associated death promoter (BAD), a proapoptotic member of the Bcl-2 family (12, 13) that lacks the transmembrane domain. In addition to heterodimerizing with other Bcl-2 family proteins, BAD was recently found to bind to 14–3–3, a group of proteins involved in intracellular signaling and cell cycle progression (13, 14).

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decreased apoptosis of granulosa cells on gonadotropin withdrawal and is associated with higher ovulation rate, increased litter size, and ovarian teratoma formation (15). These findings suggest that exogenous Bcl-2 protein can interact with the Bcl-2 system in ovarian somatic cells to modulate the cell’s susceptibility to undergo apoptosis. To characterize Bcl-2-interacting proteins in the ovary, we screened an ovarian fusion complementary DNA (cDNA) library by using the yeast two-hybrid system (16) and obtained several positive clones encoded for BAD, a proapoptotic Bcl-2 family member. To understand the potential role of BAD in the regulation of ovarian follicle atresia, we studied its messenger RNA (mRNA) expression and hormonal regulation in the rat ovarian cells and assessed its proapoptotic function in a primary culture of granulosa cells and in a gonadal cell line following the overexpression of BAD protein.

Materials and Methods
Screening of the ovarian hybrid cDNA library
We constructed an ovarian hybrid cDNA library by fusing the GAL4-activation domain (AD) with cDNAs prepared from 27-day-old female Sprague-Dawley rats treated 36 h earlier with a single dose of equine CG (Calbiochem, San Diego, CA) (14). pHT7 yeast reporter strain cells were initially transformed with GAL4-binding domain (BD) vector pGBT9 containing the human Bcl-2 cDNA fused in frame with the GAL4-BD cDNA following manufacturer’s instructions (Clontech, Palo Alto, CA). Transformants were selected on plates deficient for tryptophan, and the selected cells were transformed on the second step with DNA from the ovarian Matchmaker cDNA library (Clontech). The clones harboring Bcl-2 interacting proteins were then selected on plates lacking tryptophan, leucine, and histidine. Positive transformants were selected based on growth in media deficient in tryptophan, leucine, and histidine but supplemented with 30 mM 3-aminotriazole, and then based on Lac-Z reporter gene expression. Individual clones encoding AD-fusion cDNAs in positive yeast cells were obtained by transformation into HB101 strain Escherichia coli cells, followed by selection on an M9 growth medium. Among the positive clones sequenced, six independent clones encoding for near full-length rat BAD cDNAs were identified. After initial DNA sequencing, a full-length BAD cDNA was obtained using RT-PCR based on the rapid amplification of cDNA ends (RACE) protocol (Clontech).

To analyze interactions between BAD and different Bcl-2 mutants, truncated human Bcl-2 cDNAs were derived by using PCR amplification: the Bcl-2 homology (BH)4 domain mutant encompasses amino acids 31–239, the BH2 domain mutant amino acids 1–185, and the transmembrane domain mutant amino acids 1–218 of the human Bcl-2 (17). For the quantitation of protein-protein interactions, yeast cells were cotransformed with different pairs of fusion proteins and cultured in a medium containing 1% galactose and 1% galactose. After 48 h, cells were lysed, and β-galactosidase activity estimated by a colorimetric assay using 2-nitrophenyl-β-D-galactopyranoside as the substrate.

Preparation of nucleic acid probes
For the generation of hybridization probes, a clone encoding for the full-length rat BAD cDNA was excised from the pGADGH vector with restriction enzymes and subcloned into pgEMZ (Promega, Madison, WI) vector for the production of antisense and sense complementary RNAs (cRNAs) using the Riboprobe System (Promega). A glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was generated using the pTRI-GAPDH plasmid linearized with HindIII (Ambion, Austin, TX) as a template to derive a 404-bp cRNA.

Northern blot hybridization
Sprague-Dawley rats were obtained from Simonsen Labs. (Gilroy, CA). Ovaries from rats between 15–27 days of age were isolated and dissected free of adherent tissue under a stereomicroscope. Immature (24-day-old) rats were implanted with an estrogen [diethylstilbestrol (DES); Sigma, St Louis, MO] capsule to stimulate development of multiple early antral follicles. Ovaries were collected 3 days following the estrogen implantation. To induce follicle cell apoptosis, the DES capsules were removed at 3 days after implantation, and the ovaries were collected 1 day after the removal of the implant.

For the extraction of total RNA, ovaries and other tissues were homogenized using a Tissue-tearor (Bio-Spec Products, Bartlesville, OK) in Tri-Reagent solution (Molecular Research Center, Cincinnati, OH). At least two pools of tissues from each treatment group were used. Following RNA extraction, two aliquots of 250 µg RNA from each treatment were used for subsequent isolation of poly(A)⁺ RNA using the Oligotex oligo(dt) resin (Qiagen, Chatsworth, CA). For Northern blot hybridization, 1.0 µg each of poly(A)⁺ RNA was denatured and fractionated in 1% agarose gels containing formaldehyde, followed by capillary blotting onto Gene Screen nylon membranes (Du Pont, NEN Research Products, Boston, MA) and covalent cross-linking using a UV cross-linker (Stratagene, La Jolla, CA). Membranes were prehybridized for 4 h at 65°C in a solution containing 50% formamide, 5 × sodium phosphate buffer (SSPE), 5 × Denhardt’s solution, 0.5% SDS, and 500 µg/ml yeast transfer RNA, followed by overnight hybridization in the same conditions with 1 × 10⁶ cpm/ml of 32P-labeled BAD or a GAPDH cRNA probe. After hybridization, the membranes were washed twice in 2 × SSPE, 1% SDS at room temperature, followed by two washes in 1 × SSPE, 1% SDS at 65°C. Membranes were exposed to Kodak RX films (Eastman Kodak, Rochester, NY) for 3 days at ~70°C under intensifying screens. Each Northern blot experiment was repeated three times and one representative blot is shown.

In situ hybridization
Ovaries from immature, 26-day-old rats were isolated and fixed at 4°C for 4 h by immersion in 4% paraformaldehyde in PBS (pH 7.4), followed by dehydration in 0.5 m sucrose overnight. Tissue blocks were embedded in Tissue-Tek solution (Sakura Finetek USA, Torrence, CA) and snap frozen in liquid nitrogen. Twelve-micron thick cryosections were mounted on charged microscopic slides (Fisher Scientific, Pittsburgh, PA), postfixed in 4% paraformaldehyde, and stored at ~70°C up to 1 month. Hybridization and washes of cryosections were adapted from previously described methods (18, 19). After 2 weeks of exposure under NTB2 emulsion (Kodak, Rochester, NY), the slides were developed, counterstained, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ) for observation and photography.

Overexpression of BAD in cultured granulosa cells and a gonadal tumor (TT) cell line
To test the function of BAD in ovarian cell survival, full-length rat BAD cDNA was subcloned into the mammalian expression vector pCDNA3 (Invitrogen, San Diego, CA) under the control of the cytomegalovirus promoter. BAD cDNA subcloned in the reverse orientation (reverse BAD) or the same vector without an insert were used as control plasmids. Granulosa cells were obtained from estrogen-treated immature rats (DES implantation at 24 days of age) at 4 days after hormone treatment. Cells were isolated by needle puncture and plated at a density of 6 × 10⁵ cells/35 mm dish (Nunc, Roskilde, Denmark) in DMEM/F12 (Gibco BRL, Grand Island, NY) supplemented with 10% BSA (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 µM glutamine (Bio Whittaker, Walkersville, MD). Twenty hours later, granulosa cells were washed with the fresh medium four to six times to remove nonattached, dead cells. TT cells were plated at a density of 2 × 10⁵ cells/35 mm dish. Twenty four hours after plating, the cells were transfected using the lipofectamine reagent (Life Technologies, Gaithersburg, MD) with the expression plasmids, together with 1/10 of a reporter plasmid pCMV-β-gal to allow the identification of transfected cells. A 10-fold excess of the expression vectors was used to ensure that most of the cells expressing the β-galactosidase also expressed the protein of interest (20, 21). Cells were incubated with liposome-DNA complexes for 12 h in the serum-free medium, followed by the addition of FBS to a final concentration of 5%, and further incubation for 12 h. A cell-permeable caspase inhibitor z-Val-Ala-Asp-CH2F (z-VAD-fmk; Enzyme Systems Products, Dublin, CA) was added to the culture medium at 1.2 h after start of the transfection. After an additional culture in fresh medium containing 10% FBS for 12 h, the cells were fixed by 0.3% glutaraldehyde and stained.
with X-gal (4 mg/ml in a buffer containing 150 mM NaCl, 100 mM Na2HPO4, 3.3 mM K4Fe(CN)6·3H2O and 3.3 mM K3Fe(CN)6, pH 7.0) for 12 h to detect blue coloration as an indication of β-galactosidase expression. The number of blue cells was counted by microscopic examination at ×100 magnification. Transfection efficiency was 3–7% for granulosa cells and 10% for TT cells. Data are expressed as number of blue cells/35 mm dish (mean +/− SEM) based on counting six to nine independent samples of three separate experiments. Statistical differences between treatment groups were analyzed using one-way ANOVA and Tukey’s multiple range tests.

Results
BAD is a Bcl-2 binding protein in ovary: interactions between BAD and Bcl-2

To search for ovarian Bcl-2-interacting proteins, the human Bcl-2 cDNA was fused in frame with the yeast GAL4-BD in the pGBT9 expression vector. The Bcl-2-GAL4-BD fusion protein was used as a bait to screen a GAL4-AD-tagged ovarian cDNA library. Binding between the bait and interacting proteins was evaluated based on the activation of GAL1-HIS3 and GAL4-LacZ reporter genes (16). Library screening of 1.5 × 10⁶ yeast transformants with human Bcl-2 yielded ten positive clones; six of them encoded the death agonist protein BAD. Two of the remaining clones were members of the FK506 binding proteins, whereas the other two were due to auto-activation.

In yeast cells overexpressing the BAD-GAL4-AD fusion protein and the Bcl-2-GAL4-BD fusion protein, a strong interaction between these proteins was detected as assessed by yeast growth in media deficient in tryptophan, leucine, and histidine, and supplemented with 30 mM 3-aminotriazole [Fig. 1, Bcl-2 wild-type (WT)]. In addition, yeast cells cotransformed with BAD and truncated Bcl-2 mutants devoid of either BH domains 4 or 2, showed retarded growth, indicating the importance of these BH domains in the hetero-dimerization of BAD and Bcl-2. Likewise, yeast cells coexpressing a truncated Bcl-2 protein devoid of its transmembrane domain showed moderate growth, suggesting weaker interactions of this Bcl-2 mutant with BAD as compared with the WT Bcl-2. In contrast, BAD failed to dimerize with rat BAX or to homodimerize with itself as indicated by the lack of growth of yeast cells cotransformed with these vectors. No yeast cell growth was apparent when BAD was cotransformed with an empty vector or an unrelated protein (lamin). The observed protein-protein interactions were confirmed based on the analysis of the activities of the β-galactosidase reporter gene (OD value for yeast cells expressing BAD and different proteins: Bcl-2: 253 ± 8; Bcl-2 minus BH4: 28 ± 3; Bcl-2 minus BH2: 35 ± 9; Bcl-2 minus TM (transmembrane region): 50 ± 8; BAX: 33 ± 9; BAD: 37 ± 2; pGBT9: 33 ± 9; lamin: 33 ± 12; n = 3).

Tissue-specific expression of BAD: BAD mRNA is highly expressed in ovarian granulosa cells

Using Northern blot analysis, we hybridized poly(A)⁺ RNA from different tissues with a rat BAD cRNA probe. As shown in Fig. 2, varying levels of BAD mRNA of 1.2 and 1.7 kb in size were detected in all tissues examined. Corrected for GAPDH mRNA levels, the highest mRNA expression was seen in the lung, ovary, uterus, and brain (Fig. 2). Lower levels were found in the heart, small intestine, liver, spleen, and thymus. In addition, the lung and liver showed predominant expression of the 1.2-kb BAD

![BAD interactions with:](image-url)
Overexpression of BAD induces apoptosis in primary cultures of granulosa cells and in a gonadal tumor (TT) cell line via caspase activation

To study the possible function of BAD in the ovary, BAD was overexpressed in granulosa cells. BAD cDNA was subcloned into a mammalian expression vector pCDNA3, and the plasmid was transfected into primary cultures of granulosa cells and TT cells, a cell line derived from gonadal tumors of inhibin-α and p53 knock-out mice (24). A reporter plasmid encoding β-galactosidase was cotransfected with the BAD expression vector or control plasmids (BAD in reverse orientation or the empty vector) in a ratio of 1:10 to ensure that all cells expressing β-galactosidase also contained BAD-encoding or control plasmids. At 24 h posttransfection, the cells were fixed and stained with X-gal to identify transfected (blue) cells (20, 21).

Transfection of BAD cDNA into cultured granulosa cells resulted in a major loss of β-galactosidase-expressing (survival) cells, indicating that BAD exerts a proapoptotic effect on granulosa cells (Fig. 6A). In contrast, addition of a membrane-permeable caspase inhibitor VAD-fmk completely reversed the effect of BAD overexpression and restored the number of survival cells to levels comparable with those transfected with control plasmids (Fig. 6A). Likewise, transfection of the BAD-encoding vector into TT cells also showed a similar proapoptotic effect as seen in primary cultures of granulosa cells (Fig. 6B). Treatment of TT cells with a caspase inhibitor also reversed the proapoptotic effect of BAD, indicating that the action of BAD occurs by apoptosis via the activation of caspase cysteine proteases.

Discussion

The Bcl-2 family of proteins is important in the cell death pathway of a variety of species and cell types (25). However, evidence regarding the function and interactions of these proteins is mostly derived from studies using cell lines and less is known about their expression and regulation during physiologically occurring apoptosis. Targeted overexpression of Bcl-2 in ovarian somatic cells leads to decreased apoptosis of granulosa cells on survival factor withdrawal (14). The exact mechanism by which Bcl-2 overexpression leads to decreased apoptosis of the granulosa cells is not known. We hypothesize that the Bcl-2 transgene overexpressed in the ovary may dimerize with proapoptotic Bcl-2 family members thus rendering the cells less susceptible to undergo apoptosis. We used the yeast two-hybrid system to search for ovarian Bcl-2-interacting proteins. Screening of an ovarian fusion cDNA library yielded several clones encoding for the death agonist BAD.

Homo- and hetero-dimerization of Bcl-2-related proteins mediated by the consensus BH domains are important in the decision step of apoptosis (26, 27). Consistent with these observations, the present yeast two-hybrid assay indicated that the interaction of Bcl-2 with BAD is dependent on both BH2 and BH4 domains of Bcl-2. In contrast, deletion of the transmembrane domain of Bcl-2 still allowed weaker interaction between Bcl-2 and BAD. Furthermore, BAD neither homo-dimerized with itself nor hetero-dimerized with BAX

mRNA, whereas the thymus showed a mRNA of 1.7 kb. For other tissues tested, both forms of BAD messages could be found. These data suggested that BAD message is widely expressed in diverse tissues.

In situ hybridization analysis using BAD cRNA further showed BAD mRNA expression in the granulosa, theca, and interstitial cells (Fig. 3, A and B). BAD mRNA was detected in follicles of different developmental stages ranging from the secondary to the antral follicles. In contrast, hybridization with the sense BAD cRNA showed only low background signals (Fig. 3, C and D).

Northern blot hybridization of mRNA obtained from granulosa cells of estrogen-treated rats was performed to characterize and further substantiate BAD expression in granulosa cells, the ovarian cell type in which apoptosis takes place. As shown in Fig. 4, similar levels of BAD mRNA were found in isolated granulosa cells (lane 1) and the residual ovarian tissues (lane 2). These data verify the in situ findings of a wide distribution of BAD mRNA expression in different ovarian cell types.

Our earlier data showed an increase in follicle cell apoptosis during the first wave of follicle growth in prepubertal rats with internucleosomal DNA fragmentation coinciding with the formation of early antral follicles around day 18 of postnatal life (22). We isolated ovaries from rats between days 15–27 to investigate whether BAD mRNA expression changes during the developmentally occurring follicle cell apoptosis. As shown in Fig. 5, BAD mRNA was evenly expressed at all time points studied (lanes 1–5). Earlier studies also showed a major increase in follicle cell apoptosis following estrogen withdrawal in immature rats (23). Similarly, BAD mRNA levels were unaltered during apoptosis induction associated with estrogen withdrawal (Fig. 5, lanes 6–7).

FIG. 2. Northern blot analysis of BAD mRNA in multiple rat tissues. Northern blot hybridization analysis showed varying levels of BAD mRNA in different rat tissues. BAD mRNA levels were highest in lung, ovary, uterus, and brain. Most tissues showed expression of both 1.2- and 1.7-kb BAD mRNAs. In intestine and liver, 1.2-kb BAD mRNA was predominant, whereas in thymus, mainly 1.7-kb BAD was expressed. Hybridization with GAPDH cRNA reflected total amount and integrity of mRNA. One microgram poly(A)⁺ RNA/lane.

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in yeast cells consistent with previous findings in mammalian cells (12).

Relatively high levels of BAD mRNA were found in the ovary and uterus. These organs are characterized by dependency on trophic endocrine stimulation and exhibit cyclic apoptosis during the reproductive cycle (28, 29). *In situ* and Northern blot hybridization analyses showed BAD mRNA expression in all ovarian somatic cells. Expression in granulosa cells was detected during both preantral and antral stages of ovarian follicle development. At the mRNA level,

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**FIG. 3.** *In situ* hybridization of BAD mRNA in rat ovary. Brightfield (A and C) and darkfield (B and D) images of BAD mRNA hybridization signals in an ovary from a 27-day-old rat. A and B, An early antral follicle (af) showed BAD hybridization signals over granulosa cells (g). BAD mRNA was also detectable in theca (t) and interstitial (i) cells. In contrast, no signal was evident in follicle antrum (a). In an adjacent secondary follicle (2o), oocyte (O) showed lower signals, whereas granulosa cells were positive. C and D, No specific hybridization signal was detected with BAD sense cRNA probe. Magnification: ×250.
BAD expression was unaltered during the first wave of follicular cell apoptosis and remained constant during the onset of apoptosis induced by estrogen withdrawal. These data suggest that BAD is constitutively expressed as an essential part of the cell death process of the granulosa and other ovarian cells.

Although BAD mRNA levels do not vary under different physiological and experimental conditions, it is, however, possible that the function of BAD is regulated posttranslationally rather than at the transcriptional level. It was recently demonstrated that BAD can act as a molecular switch between signal transduction pathways and the cell death process, and that the proapoptotic activity of BAD is modulated by the degree of BAD phosphorylation (13). When BAD is in its hypophosphorylated form, it dimerizes preferably with Bcl-2 or Bcl-XL to prevent their antiapoptotic activity, possibly by liberating BAX, a proapoptotic Bcl-2-related protein (12). In contrast, when the cell is stimulated by survival factors, BAD is phosphorylated. BAD that is
phosphorylated at specific serine residues in two 14–3-3 consensus binding sites could be bound by the constitutively active protein 14–3-3, which also binds to key components of the signal transduction cascades, including Raf-1 kinase, protein kinase C isoforms, and phosphatidyl inositol 3 kinase (30). When BAD is phosphorylated and bound to 14–3-3, it no longer binds to Bcl-2 or Bcl-XL, thus hindering its proapoptotic potential. The exact kinase(s) responsible for the phosphorylation of BAD remain(s) to be identified (13). Because of the low affinity of the currently available BAD antibodies, possible modulation of BAD protein phosphorylation in the ovarian cells could not be addressed at the present time. Future analysis of the phosphorylation status of BAD in granulosa cells and the regulation of the putative BAD kinase(s) should provide information regarding the signal transduction pathways by which survival factors regulate the Bcl-2 cell death process in the mammalian ovary.

The potential proapoptotic effect of BAD in ovarian granulosa cells was examined by using a transfection assay. BAD exerted proapoptotic effects in both primary cultures of granulosa cells and in the tumorous gonadal TT cell line. The observed proapoptotic effects of BAD indicate that granulosa cells express a set of cell death pathway proteins to which BAD can bind and, when BAD is overexpressed, shift the balance of cell death/survival effectors to favor apoptotic demise of the cell. The exact proteins to which BAD binds in the granulosa cells are not known, but recent investigations have demonstrated the expression of both Bcl-2 and Bcl-X mRNAs in the ovary (31, 32). Future studies using the present ovarian fusion cDNA library and the yeast two-hybrid screening could allow identification of the exact heterodimerization partner(s) of BAD in ovarian cells.

Apoptosis of granulosa and TT cells, induced following BAD overexpression, was inhibited by a cell-permeable, specific caspase inhibitor, indicating that the effect of BAD is mediated by cysteine proteases of the caspase family. It appears that the Bcl-2 family of proteins exerts actions upstream from the caspases (9, 25). Recently, one of the bridging molecules between Bcl-2 family members and the caspases was identified in mammals (33). The C. elegans gene ced-4 can simultaneously bind to Bcl-XL and to caspase 1 or 8 (11). Ultimately, the activation of caspases will lead to proteolytic damage and the activation of endonucleases that are responsible for the internucleosomal DNA cleavage observed during granulosa cell apoptosis (34–36).

In addition to BAX (31), BAD is the second proapoptotic protein of the Bcl-2 family found to be expressed in ovarian granulosa cells, the cell type undergoing apoptosis during follicle atresia (23). Expression of BAD in the ovary is of special interest because it is a cytosolic protein of the Bcl-2 family, thus enabling it to regulate other Bcl-2 survival proteins, all of which have a transmembrane region and are presumably associated with the outer mitochondrial membrane and the nuclear envelope (12, 13). The development and maturation of ovarian follicles are dependent on constant trophic support by hormonal signals (1, 2). During follicle growth, the antral transition presents a critical stage for the developing follicles (37, 38). At this stage, FSH has been shown to be the most potent survival factor (38). BAD expression in these follicles may represent a molecular sensor for mediating survival support factor of the developing follicle. If the follicle is receiving sufficient FSH support, BAD would remain in its phosphorylated, inactive form rendering the cell less likely to undergo apoptosis.

In summary, the death agonist BAD was identified as a Bcl-2-interacting protein expressed in the rat ovary. BAD mRNA is constitutively expressed in different ovarian cells suggesting its potential role as an essential part of the cell death process. BAD overexpression in the ovarian granulosa cells and in an ovarian tumor cell line leads to apoptotic cell demise that can be inhibited by a caspase inhibitor. Future investigations focusing on the posttranslational modifications of BAD protein in the ovary and its regulation by follicle survival factors could provide insight into the molecular mechanisms regarding the hormonal regulation of ovarian follicle atresia.

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